Molecular Epidemiology of Begomoviruses That Infect Vegetable Crops in Southwestern Cameroon

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Cover: The crops; okra (*Abelmoschus esculentus*), pepper (*Capsicum annuum*), and tomato (*Solanum lycopersicum*) as well as the weeds; *Clerodendrum umbellatum* and *Emilia coccinea*, from which some begomoviruses and satellite molecules were isolated in this study.

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Abstract

Begomoviruses are plant-infecting viruses, which are transmitted by the whitefly vector *Bemisia tabaci*. They have a genome of single-stranded DNA that consists of either a single (monopartite) or two components (bipartite) with a component size of approximately 2.8 kb. Many monopartite begomoviruses in the Old World have been found to be associated with betasatellite and alphasatellite molecules, which are about half the size of their helper begomovirus genome. Betasatellites have been shown to be necessary for inducing severe disease symptoms. In Cameroon, *B. tabaci* has been associated with suspected begomovirus infections in many crop and weed species. Despite their growing importance, only begomoviruses infecting cassava have been studied in Cameroon in any detail. Thus, there was a need for additional information on diversity and distribution of begomoviruses and satellites in vegetable crops and dictyledonous weeds, which likely serve as virus reservoirs. In field studies carried out in this study, a high incidence of okra leaf curl disease was found in Cameroon. Sequencing of viral genomes showed that the okra plants were infected by viruses of two previously known begomovirus species (*Cotton leaf curl Gezira virus* and *Okra yellow crinkle virus*) as well as a new recombinant begomovirus species (Okra leaf curl Cameroon virus). In addition, a betasatellite (*Cotton leaf curl Gezira betasatellite*) and two alphasatellites (Okra leaf curl Mali alphasatellite and Okra yellow crinkle Cameroon alphasatellite) were identified. Tomato plants with leaf curling were shown to contain isolates of a new begomovirus, Tomato leaf curl Cameroon virus, and an alphasatellite, Tomato leaf curl Cameroon alphasatellite (ToLCCMA). To study the potential begomovirus complexes infecting weeds, begomoviruses and satellites in plants of the weed *Ageratum conyzoides* with leaf curl symptoms were characterized. Sequence analyses showed that they were infected by isolates of a new begomovirus (Ageratum leaf curl Cameroon virus), two new betasatellites (Ageratum leaf curl Cameroon betasatellite and Ageratum leaf curl Buea betasatellite), an alphasatellite (ToLCCMA) and two types of defective recombinants between a begomovirus and ToLCCMA. Putative recombinations were detected in begomovirus genomes for all three plant species studied, indicating that recombination is an important mechanism for their evolution. A close relationship between the begomoviruses infecting tomato and *A. conyzoides*, and the detection of the same alphasatellite in them support the idea that weeds are important reservoirs for begomoviruses and their satellites. This study has revealed a huge complexity of begomoviruses and DNA satellites previously largely unknown in West and Central Africa. With this high diversity, recombination potential and transmission by *B. tabaci*, begomoviruses and their associated DNA satellites pose a serious threat to crop production in the region.

Keywords: plant disease, mixed infection, *Abelmoschus esculentus*, *Ageratum conyziodes*, satellite DNAs, begomovirus diversity, native species, *Solanum lycopersium*

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Dedication

To my beloved grandmother **Mami Lucia Zilefac Nkemnkia** (*Abrehzi*) of blessed memory, who though not educated, did all she could that I be educated and whom because of this work, I did not have the opportunity to see the last moment of her life before her transition into glory. As the legend of biology, **Linnaenus** puts it "Thus speak the stones …."I say Thus speak the plants… yes they will call your name each time I pick their leaves for analyses. To my children: **Sally**, **Queen** and **Pio** whom I had to abandon in search of this knowledge. Many times you asked me daddy when are you coming back home? This day (September 30 2010) is the answer.

The more I know and strife to know more, the more I realize I know very little and he who claims to be master of knowledge in any level and dimension of thinking, is ignorant and a liar to universal and/or fundamental truth. **W.N. Leke**, Uppsala, Sweden.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Leke, W. N.**, Ramsell, J. N. E., Njualem, D. K., Titanji, V. P. K., Legg, J. P., Fondong, V. N., Brown, J. K., and Kvarnheden, A. 2007. FTA technology facilitates detection and identification of begomoviruses from okra plants in Cameroon. African Crop Science Society Conference Proceedings 8: 655-660.

II **Leke, W. N**., Brown, J. K., Ngane, E.B., Ngeve, J.M., and Kvarnheden, A. (2010). Discovery of a diverse group of helper begomoviruses and satellite DNAs infecting okra in Cameroon. (Muscript)

III **Leke, W. N**., Kvarnheden. A., Ngane, E.B., Titanji, V. P. K., and Brown, J. K. 2010**.** A new begomovirus and an associated alphasatellite infecting tomato in Cameroon. (Manuscript)

IV **Leke, W.N**., Brown, J.K., Ligthart, M.E., Naeem, S., Njualem, D.K. and Kvarnheden, A. 2010. *Ageratum conyzoides*: a host to a previously uncharacterized begomovirus disease complex in Cameroon. (Manuscript)

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Additional publication

I. **Leke, W.N**., Njualem, D.K., Nchinda, V.P., Ngoko, Z., Ngeve, J.M., Brown, J.K. and Kvarnheden, A. 2009. Molecular identification of Maize streak virus reveals the first evidence for a subtype A1 isolate infecting maize in Cameroon. Plant Pathology. 58: 782-782/ New Disease Reports [http://www.bspp.org.uk/ndr/] Volume 19.

Abbreviations

AbMV	*Abutilon mosaic virus*
ADK	Adenosine kinase
ACMD	African cassava mosaic disease
ACMV	*African cassava mosaic virus*
ACMV-[CM]	*African cassava mosaic virus*-[Cameroon]
ACMV-[KE]	*African cassava mosaic virus*-[Kenya]
ALCCMA	Ageratum leaf curl Cameroon alphasatellie
ALCCMB	Ageratum leaf curl Cameroon betasatellite
ALCCMV	Ageratum leaf curl Cameroon virus
ALCD	Ageratum leaf curl disease
AA	Amino acid
BCTV	*Beat curly top virus*
BDMV	*Bean dwarf mosaic virus*
BGMV	*Bean golden mosaic virus*
βC1	Beta C1 protein
CP	Coat protein
CR	Common region
CLCuGB-[CM]	Cotton leaf curl Gezira betasatellite-[Cameroon]
CLCuGV	*Cotton leaf curl Gezira virus*
CLCuMV	*Cotton leaf curl Multan virus*
ClLCCMV	Clerodendrum leaf curl Cameroon virus
ClGMV	Clerodendrum golden mosaic virus
CPGMV	*Cow pea golden mosaic virus*
dsDNA	Double-stranded DNA
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EACMCV	*East African cassava mosaic Cameroon virus*
EACMV	*East African cassava mosaic virus*
EpYVB	Eupatorium yellow vein betasatellite

EpYVV	*Eupatorium yellow vein virus*
EYVV	Emilia yellow vein virus
FBNYV	*Faba bean necrotic yellow virus*
HIV	Human immune deficiency virus
HoLCrV	*Hollyhock crumple virus*
ICMV	*Indian cassava mosaic virus*
IR	Intergenic region
ICTV	International Committee on Taxonomy of Viruses
LC	Leaf curl
LCD	Leaf curl disease
LIR	Long intergenic region
MaYMCMV	Malvastrum yellow mosaic Cameroon virus
MSD	Maize streak disease
MSV	*Maize streak virus*
mRNA	Messenger RNA
miRNA	MicroRNA
MP	Movement protein
MPB	Movement protein B
NW	New world
NES	Nuclear export signal
NLSs	Nuclear localization signals
NSP	Nuclear shuttle protein
OLCMLA-[CM]	Okra leaf curl Mali alphasatellite-[Cameroon]
OLCD	Okra leaf curl disease
OLCBFA	Okra leaf curl Burkina Faso alphasatellite
OLCuCMV	Okra leaf curl Cameroon virus
ORF	Open reading frame
OW	Old world
OYCrCMA	Okra yellow crinkle Cameroon alphasatellie
OYCrV	*Okra yellow crinkle virus*
OYVMV	*Okra yellow vein mosaic virus*
PepYVMLV	*Pepper yellow vein Mali virus*
PCR	Polymerase chain reaction
PVX	*Potato virus X*
PTGS	Post-transcriptional gene silencing
PreCP	Pre-coat protein
RDP3	Recombination detection programe3
RDR	Recombination-dependent-replication
Rep	Replication-associated protein
RepA	Replication-associated protein A
RepB	Replication-associated protein B
REn	Replication enhancer protein

RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RCA	Rolling cycle amplification
RCM	Rolling cycle mechanism
RISC	RNA-induced silencing complex
RNAi	RNA interference
SIR	Short intergenic region
ssDNA	Single-stranded DNA
SiGMCoV	*Sida golden mosaic Colombia virus*
siRNA	Small interfering RNA
SWC	Southwestern Cameroon
SLCMV	*Sri Lanka cassava mosaic virus*
TGMV	*Tomato golden mosaic virus*
TMV	*Tobacco mosaic virus*
ToLCD	Tomato leaf curl disease
ToLCCMA	Tomato leaf curl Cameroon alphasatellite
ToLCCMV	Tomato leaf curl Cameroon virus
ToLCGHV	*Tomato leaf curl Ghana virus*
ToLCJAV	*Tomato leaf curl Java virus*
ToLCPV	*Tomato leaf curl Philippines virus*
ToLCNIV	*Tomato leaf curl Nigeria virus*
ToLCTGV	*Tomato leaf curl Togo virus*
ToLC	Tomato leaf curl
ToLCV	*Tomato leaf curl virus*
ToMoV	*Tomato mottle virus*
TPCTV	*Tomato pseudo-curl top virus*
TYLC	Tomato yellow leaf curl
TYLCCNV	*Tomato yellow leaf curl China virus*
TYLCV	*Tomato yellow leaf curl virus*
TYLCV-IL	*Tomato yellow leaf curl virus*-Israel
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
TrAP	Transcriptional activator protein
TGS	Transcriptional gene silencing
YV	Yellow vein

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Introduction

In developing countries, including Cameroon, a greater portion of the population depends on small-scale farming for their income and subsistence. Crops are frequently affected by a wide array of diseases showing varying degree and kind of symptoms. Most of the causal agents of the diseases are biotic, not ruling out the involvement of abiotic factors too. Amongst the biotic factors, virus diseases constitute a bulk of the diseases observed in all plant types, with variable symptoms including leaf curling and distortion, green or yellow foliar mosaic, stunting of plants, and reduced yields. The diseases caused by plant viruses can be devastating both on crops (yield reduction) and hence man. Some virus diseases have been very devastating for African agriculture, for example, the famous African cassava mosaic disease (ACMD) (Harrison & Robinson, 1999; Ngeve et al., 2003; Fargette et al., 2006), okra leaf curl disease (OLCD) currently associated with a complex of the monopartite begomoviruses *Cotton leaf curl Gezira virus* (CLCuGV), *Okra yellow crinkle virus* (OYCrV) and Okra leaf curl Cameroon virus (OLCuCMV) and a diverse group of alphasatellites and betasatellites (Leke et al., 2007; Shih et al., 2007; Kon et al., 2009; Shih et al., 2009; Tiendrébéogo et al., 2010; **II**) in West and Central Africa and tomato yellow leaf curl /tomato leaf curl (TYLC/ToLC) disease (Osei et al., 2008; Zhou et al., 2008; Chen et al., 2009; Lett et al., 2009) in Africa. The viruses associated with the diseases of cassava, okra and tomato above belong to the genus *Begomovirus*, family *Geminiviridae*, and are vectored by the whitefly *Bemisia tabaci* (Brown et al., 1995; Brown and Czosnek, 2002; Brown, 2007; 2010). Another virus disease that has been very devastating for African agriculture is the maize streak disease (MSD) (Varsani et al., 2008; Leke et al., 2009), caused by *Maize streak virus* (MSV) (genus *Mastrevirus*, family *Geminiviridae*) and vectored by leafhoppers (Alegbejo et al., 2002; Stanley et

al., 2005). Even with this knowledge, information at the molecular level on the prevalence of geminiviruses in West and Central Africa is very limited.

The earliest known written record describing what was almost certainly a plant virus disease is a poem in Japanese written by the Empress Koken in 752 AD and translated by T. Inouye as follows:

> In this village
> It looks as if frosting continuously
> For, the plant I saw
> In the field of summer
> The color of the leaves were yellowing

The plant this poem was referring to has now been identified as *Eupatorium lindleyanum*, with yellowing symptoms (Hull, 2002). The yellowing symptoms have been shown to be caused by a begomovirus/betasatellite complex: *Eupatorium yellow vein virus* (EpYVV) and *Eupatorium yellow vein betasatellite* (EpYVB) (Saunders et al., 2003; Briddon et al., 2008). It took about 1100 years from empress Koken's observation of the yellowing *Eupatorium* plants until the birth of plant virology. Martinus Willem Beijerinck, a Dutch microbiologist, published a paper in 1889 titled: Über ein contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblatter (Beijerinck, 1889; English translation Johnson, 1942). In this work, he demonstrated that the mosaic disease of tobacco was not caused by bacteria, but by a liquid or soluble agent (*Contagium vivum fluidum*) that reproduced in the living plant and that was smaller than bacteria. Beijerinck's *contagium vivum fluidum* is now known to be *Tobacco mosaic virus* (TMV), which causes mosaic symptoms in infected tobacco plants.

There are many definitions of a virus currently in use, but the one by Hull (2002) reads: "A virus is a set of one or more nucleic acid template molecules, normally encapsidated in a protective coat or coats of protein or lipoprotein that is able to organize its own replication only within suitable host cells. It can usually be horizontally transmitted between hosts. Within such cells, virus replication is (1) dependent on the host's protein-syntheszing machinery, (2) organized from pools of required materials rather than by binary fission, (3) located at sites that are not separated from host cell contents by lipropotein bilayer membrane, and (4) continually giving rise to variants through various kinds of change in the viral nucleic acids". The viral particle is a nucleoproteic structure having a single type of nucleic acid, either DNA or RNA (Lwoff, 1957). Its nucleic acid contains up to 12 genes in plant viruses and encodes proteins that, in a living cell, fulfill the functions required for its survival, replication and spread. The nucleic acid codes for at least one protein indispensable for its replication. Apart from replication,

there are other functions depending on the virus type: (1) protective function, within and outside of the cell and also against the host proteins, the capsid protein (2) movement from cell to cell and long distance within the plant (3) transmission from plant to plant by vectors. Van Regenmortel & Fauquet (2000) made the important distinction between the entities called virus and the viral particle or virion as follows: "A virion has intrinsic physicochemical and structural properties that suffice to characterize it exhaustively. A virus on the other hand, has in addition relational properties that exist only by virtue of its relation with other entities such as host or vector. These relational and emergent properties are revealed only when a virus infects a cell and is integrated into its metabolic activities during the replication cycle".

There are 81 different virus genera recognized by the International Committee on Taxonomy of Viruses (ICTV) that contain viruses infecting plants (http://phene.spmc.columbia.edu/ictv/index.htm).

Family *Geminiviridae*

Although the diseases caused by geminiviruses represent a serious constraint to agriculture, little was known about the causal agents of the diseases until the isolation of virus particles with unique twinned quasi-isomeric morphology associated with maize streak and beet curly top diseases (Bock et al., 1974; Mumford, 1974). This attribute provided the name geminivirus, from Gemini, the sign of the zodiac symbolized by twins (Harrison et al., 1977), and has remained a unifying feature of this family of viruses. Structural analysis demonstrated that the 22 x 38 nm particles associated with MSV consist of two incomplete $T = 1$ icosahedra (Zhang et al., 2001), and a similar structure was subsequently observed for *African cassava mosaic virus* (ACMV) (Böttcher et al., 2004). In groundbreaking research, Harrison et al., (1977) and Goodman (1977a) demonstrated that the so-called geminate particles associated with ACMV, MSV and *Bean golden mosaic virus* (BGMV) contained circular ssDNA, and this genomic DNA was infectious when re-introduced to plants by mechanical inoculation (Goodman, 1977b), setting geminiviruses apart from all other plant viruses that had been characterized at that time. Evidence was provided for BGMV and *Tomato golden mosaic virus* (TGMV) to suggest that at least some genimiviruses had divided genomes (Haber at al., 1981; Bisaro et al., 1982; Hamilton et al., 1982). Shortly afterwards, the nucleotide sequence of cassava latent virus (subsequently renamed ACMV) was established, and infectious clones were used to demonstrate a bipartite genomic structure (Stanley, 1983; Stanley & Gay, 1983). Subsequently, the monopartite geminiviruses MSV, BCTV and *Tomato pseudo-curly top virus* (TPCTV) were similarly characterized (Howell, 1984; Mullineaux et al., 1984; Stanley et al., 1986; Grimsley et al., 1987; Briddon et al., 1996), resulting in the present-day recognition of four genera (*Mastrevirus*, *Begomovirus*, *Curtovirus* and *Topocuvirus*) in the family *Geminiviridae* by ICTV (Stanley et al., 2005). Viruses of the *Geminiviridae*

family are characterized by having either one or two small, circular, ssDNA components of ~2.6-3.0 kb encapsidated in paired icosahedral or 'geminate' particles (22 x 38 nm) (Zhang et al., 2001). The viruses in the family *Geminiviridae* replicate in the host cell nucleus and are transmitted in a non-propagative, persistent and circular manner by insect vectors and have the propensity to infect phloem cells (Harrison, 1985; Lazarowitz, 1992; Arguello-Astorga et al., 1994; Sunter et al., 1994; Harrison and Robinson, 1999; Varma & Malathi, 2003). The components share a common region that contains motifs required for the control of gene expression and replication, notably conserved reiterated motifs and a putative stem-loop structure containing the highly conserved nonanucleotide TAATATTAC that functions in the initiation of rolling circle replication (Hanley-Bowdoin et al., 1999). There are now more than 200 officially recognized geminivirus species (Fauquet et al., 2008). Members of the family *Geminiviridae* are classified into four genera based on their genome organization, hosts and insect vectors (Stanley et al., 2005; Fauquet et al., 2008). Mastreviruses (type species MSV) are transmitted by leafhoppers and have one genome component. They have been found only in the Eastern Hemisphere (Europe, Africa, Asia and Australia) and have been associated with many diseases of monocot plants though some mastreviruses have also been associated with diseases of dicot plants (Stanley et al., 2005). Their genome has four genes: *V1* and *V2* encode the coat protein (CP) and the movement protein (MP), respectively, on the virion strand. The replication-associated proteins: Rep (C1:C2) and RepA (C1) are encoded by the complementary sense strand. The genes on the virion and complementary strands are separated by the long intergenic region (LIR) and the short intergenic region (SIR). Topocuviruses (type species TPCTV) have a single genome component of ~3 kb and are transmitted by treehoppers. Topocuviruses have only been reported in the Americas (New World, NW) and are thought to have emerged as a recombination between a mastrevirus and a begomovirus (Briddon et al., 1996). Curtoviruses (type species *Beet curly top virus*, BCTV) are similar to topocuviruses in terms of the genome size of ~3 kb, but are transmitted by leafhoppers and infect exclusively dicot plants (Stanley et al., 2005). The economically most important, geographically wide-spread and numerous geminiviruses are within the genus *Begomovirus* (type species BGMV), which contains more than 200 species (Fauquet et al., 2008). Begomoviruses are vectored by the whitefly *B. tabaci* (Brown et al., 1995; Brown & Czosnek, 2002; Jones, 2003; Varma & Malathi, 2003; Brown, 2007; Brown, 2010) and infect only dicot plants. The genome organization of begomoviruses is illustrated below (Fig. 1).

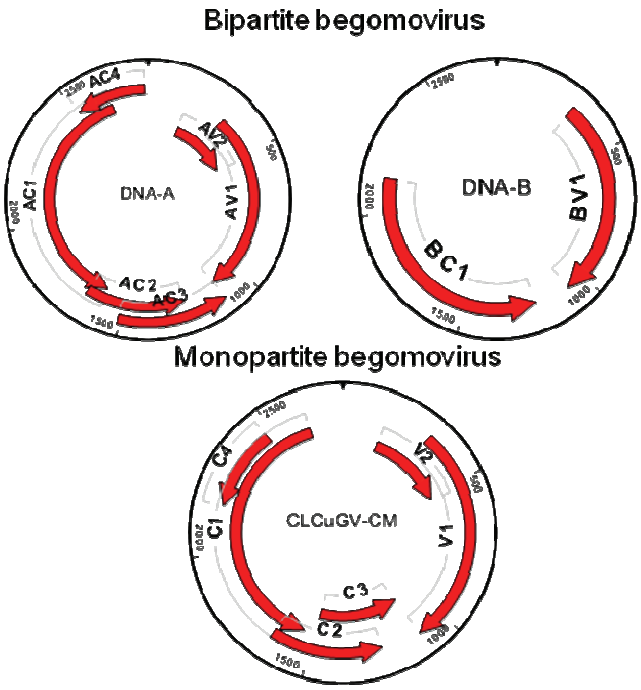


Figure 1. Genome organisation of members of the genus *Begomovirus*. Arrows represent open reading frames. *V1*/(*AV1*) coat protein gene, *V2*/*AV2* precoat prorein gene, *C1* (*AC1*) replication initiator protein gene, *C2*(*AC2*) transcription activator protein gene, *C3* (*AC3*) replication enhancer protein gene, *C4* (*AC4*) symptom determinant protein gene, *BV1* nuclear shuttle protein gene and *BC1* movement protein gene.

Genus *Begomovirus*

Begomoviruses have genomes consisting of either two genomic components, bipartite (known as DNA-A and DNA-B) of about equal size (~2.8 kb), or a single component, monopartite, homologous to the DNA-A component of bipartite viruses (Rojas et al., 2005; Stanley et al., 2005) (Fig. 1). Begomoviruses cause many diseases of dicotyledonous crops and wild plants. The symptoms typically consist of leaf-curling, mosaic, vein yellowing or more generalized leaf yellowing, often accompanied by stunting of plant growth (Fig. 2). Some of these diseases are among the world's most economically important plant virus diseases, for example, mosaic diseases of cassava in Sub-Saharan Africa probably cause annual yield

losses exceeding $2 billion in value of a staple food for millions of poor people (Thresh et al., 1997).

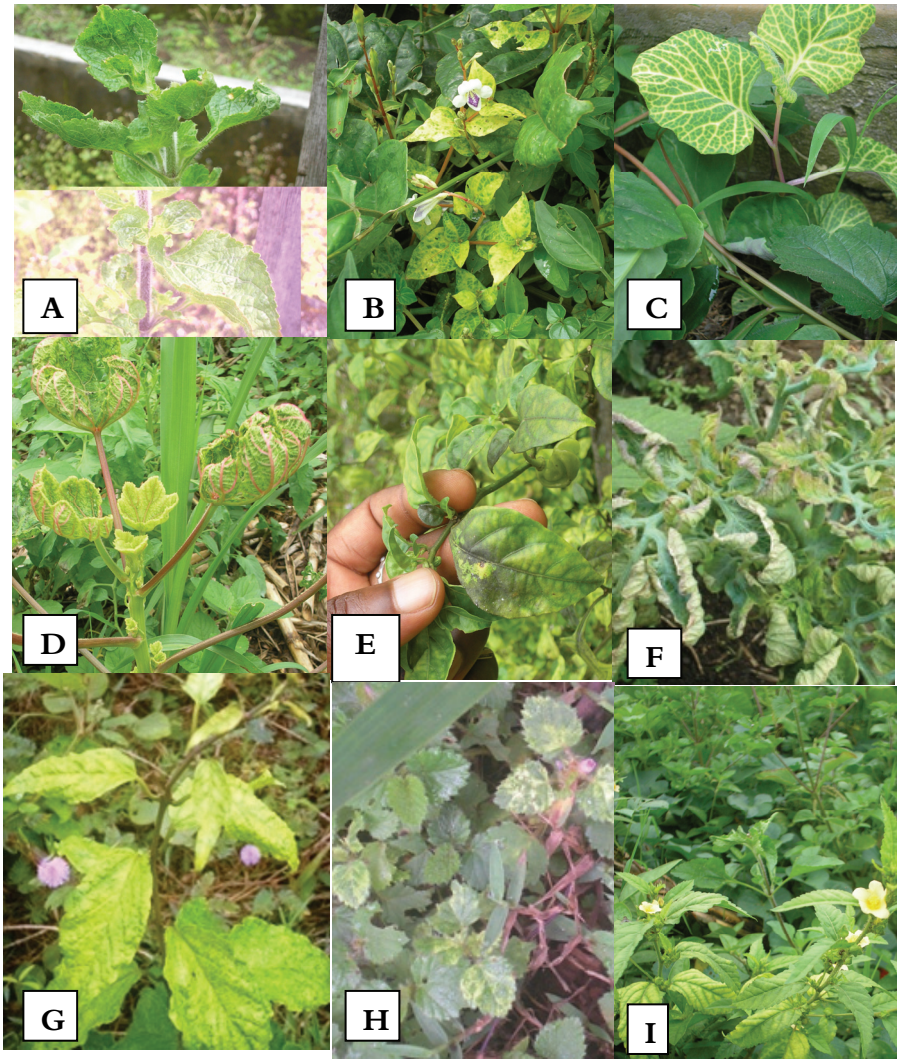


Figure 2. Some disease symptoms associated with tentative begomovirus infection in crops and weeds in Cameroon: A, *Ageratum conyzoides*; B, *Asystasia gangetica*; C, *Emilia cocinea*; D, Okra, *Abelmoschus esculentus*; E, Pepper, *Capsicum annuum*; F, Tomato, *Solanum lycopersicum*; G, *Clerodendrum umbellatum*; H, *Malvastrum* spp and I, *Sida corymbosa*.

Genome organization of begomoviruses

A. Bipartite begomoviruses

Except for a short sequence of ~200 nts, referred to as the "common region" (CR), the DNA-A and DNA-B components share no sequence similarity. The CR contains the nonanucleotide TAATATTAC sequence, where rolling circle replication is initiated and that is conserved amongst members of the family *Geminiviridae* (Eagle et al., 1994; Padidam et al., 1996; Harrison & Robinson, 2002). Both genomic components contain protein-coding sequences on the viral sense strand and on the complementary strand. Six genes seem to be universally present in all bipartite begomoviruses. The DNA-A component contains one gene (*AV1*) on the viral sense strand and three genes (*AC1*, *AC2*, *AC3*) on the complementary strand for the New World bipartite begomoviruses (Harrison & Robinson, 1999), and an additional gene *AV2* in the viral sense strand and *C4* on the complementary strand for the OW bipartite begomoviruses (Hanley-Bowdoin, 1999). The sense and complementary strands of the DNA-B component each contains one gene, *BV1* and *BC1*, respectively (Sanderfoot & Lazarowitz, 1996) (Fig. 1).

B. Monopartite begomoviruses

The genome of monopartite begomoviruses contains six open reading frames (ORFs). The coat protein gene (*CP* or *V1*), and *V2,* are expressed from the viral sense strand, and *C1* (Rep), *C2*, *C3*, and *C4*, are expressed from the complementary strand (Navot et al., 1991) (Fig. 1).

Some known functions of begomovirus-encoded proteins

CP (AV1/V1): Coat protein. Encapsidates the viral DNA and is implicated in viral movement within the plant as well as in whitefly transmission (Wartig et al., 1997; Hanley-Bowdoin et al., 1999; Harrison & Robinson, 1999; Sharma & Ikegami, 2009). It might also play a role in limiting the viral DNA copy number by down-regulating Rep activity, specifically nicking (Yadava et al., 2010).

PreCP (AV2/V2): Pre-coat protein. Implicated in virus movement (Wartig et al., 1997; Hanley-Bowdoin et al., 1999; Harrison & Robinson, 1999; Sharma & Ikegami, 2009) and also as a suppressor of RNA-silencing (Avi et al., 2007; Yadava et al., 2010).

Rep (AC1/C1): Replication-associated protein. Is a multifunctional, oligomeric protein, which possesses site-specific DNA-binding to the reiterated motifs (iterons) at the intergenic region (IR) and initiates DNA replication by introducing a nick and ligation at the conserved nonanucleotide sequence, executes ATP-dependent topoisomerase I, ATPase and helicase activities and also binding of retinoblastoma-related proteins (Hanley-Bowdoin et al., 1999; Harrison & Robinson, 1999; Pant et al., 2001; Choudhury et al., 2006).

TrAP (AC2/C2): Transcriptional activator protein. Transactivates expression of virion-sense genes from both DNA-A and DNA-B (Hanley-Bowdoin et al., 1999; Harrison & Robinson, 1999; Wang et al., 2003; Trinks et al., 2005; Gopel et al., 2007; Pandey et al., 2009), inactivates adenosine kinase (ADK) (Wang et al., 2003), binds siRNA (Vanitharani et al., 2004) and interacts with tomato karyopherin α (Gopel et al., 2007). It has also been reported as a suppressor of RNA silencing in bipartite begomoviruses (Voinnet et al., 1999; Wang et al., 2003; Vanitharani et al., 2004; Trinks et al., 2005) as well as monopartite begomoviruses (Dong et al., 2003; Gopel et al., 2007; Kon et al., 2007).

REn (AC3/C3). Replication enhancer protein. Increases viral replication (Hanley-Bowdoin et al., 1999; Harrison & Robinson, 1999).

AC4/C4. Symptom determinant implicated in the control of cell-to-cell movement, and may counter a host response to Rep expression and suppression of RNA-silencing by binding of siRNAs (Wartig et al., 1997; Rojas et al., 2001; Vanitharani et al., 2004; Fondong et al., 2007; Gopal et al., 2007; Pandey et al., 2009).

BV1 (NSP). Nuclear shuttle protein. Transport of viral DNA between the nucleus and the cytoplasm and host range properties of the virus (Noueiry et al., 1994; Sanderfoot & Lazarowitz, 1995; Hanley-Bowdoin et al., 1999; Harrison & Robinson, 1999).

BC1 (MPB). Movement protein. Mediates cell-to-cell movement and viral pathogenic properties (Schaeffer et al., 1995; Jeffrey et al., 1996; Lazarowitz & Beachy, 1999).

Begomovirus infection cycle

The begomovirus infection cycle begins when a viruliferous whitefly vector, *B. tabaci*, when feeding on a suitable host, inoculates the phloem cells with virus particles. Once in the plant cells, the viral particles are uncoated and the viral nucleic acids enter the nucleus where replication and transcription occur. The virus movement is dependent on the CP and interactions with

the host transport network, where a complex is established between the viral ssDNA and CP that enters the nucleus (Gafni & Epel, 2002). Recently, it has been shown that the CP of *Tomato leaf curl Java virus* (ToLCJAV) has two putative nuclear localization signals (NLSs), ^{16}KVRRR^{20} and ^{52}RKPR^{55}, located at the N-terminal part, responsible for the transport of viral ssDNA in and out of the plant cell nucleus (Sharma & Ikegami, 2009). Begomovirus replication follows a rolling circle mechanism (RCM) strategy (Fig. 3) (Saunders et al., 1991; Stenger et al., 1991). Initially, before the rolling circle (RC) step, the ssDNA is converted into a double-stranded DNA (dsDNA) intermediate product (Fig. 3) (Kammann et al., 1991; Saunders et al., 1992), solely by plant cellular factors. The RC step requires the concerted action of the viral Rep protein (AC1/C1), REn (AC3/C3) and cellular factors, such as *RAD54* (Yadava et al., 2010), with Rep being the initiator of the process that leads to the production of new ssDNA products (Fig. 3). Recombination-dependent replication (RDR) (Jeske et al., 2001) is another strategy where the host factors alone or in combination with the Rep protein are necessary for replication. The newly synthesized ssDNA can (i) re-enter the DNA replication pool, (ii) encapsidate (coating) or (iii) be transported outside the nucleus to the neighboring cell, through plasmodesmata, with the help of viral movement proteins (MPs) (Stenger et al., 1991; Heyraud et al., 1993; Stanley, 1995). The viral DNA replication origin (Ori) has a modular architecture (Fontes et al., 1994; Sanz-Burgos & Gutierrez, 1998) and binding sites for Rep (Fontes et al., 1992; Lazarowitz et al., 1992; Orozco & Hanley-Bowdoin, 1998). Transcription also takes place inside the nucleus and is bidirectional from promoter sequences located in the IR. Transcription can be quite complex, frequently giving rise to multiple polycistronic mRNAs. The convergent transcripts overlap for several nucleotides at the 3' ends (Hanley-Bowdoin et al., 1989; Sunter & Bisaro, 1989; Hanley-Bowdoin et al., 1999). The next step in the infection cycle is the cell-to-cell and systemic spread of the viral ssDNAs produced and exported out of the plant cell nucleus. This movement is entirely dependent on the CP and V2 in the case of monopartite begomoviruses and in the case of bipartite begomoviruses, on the two MPs, the NSP and MPB, encoded by the B component (Sanderfoot & Lazarowitz, 1996; Sanderfoot et al., 1996; Lazarowitz, 1999; Lazarowitz & Beachy, 1999; Gafni & Epel, 2002). For the viral ssDNA to be exported out of the nucleus, a nuclear export signal (NES), ^{245}LKRIY^{250}, located at the C-terminal region of ToLCJAV CP amino acid (AA) sequence has recently been implicated (Sharma & Ikegami, 2009). Begomovirus infection often results in changes in plant cells and organelles and the appearance of virus-associated structures.

Some begomoviruses are restricted to the vascular system while others can invade the mesophyll (Morra & Petty, 2000). The loss of tissue specificity could be attributed to infection by many begomoviruses or co-infection with other viruses (Brown, 1997).

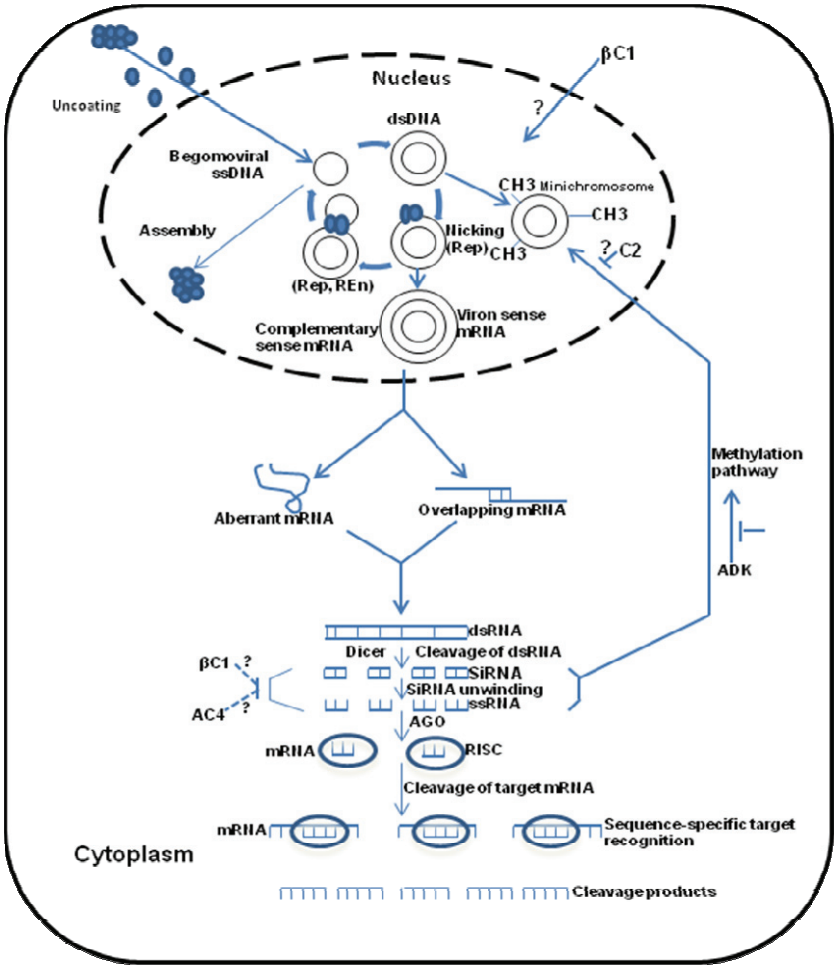


Figure 3. Begomovirus DNA replication cycle and intracellular movement of viral DNAand RNA silencing defence of plants. ADK: adenosine kinase, AC2/C2: transcription activator protein, AC4: symptom determinant protein, βC1: beta C1 protein, Rep: replication-associated protein, REn: replication enhancer protein (Adopted from Sharma & Ikegami, 2008).

DNA satellite molecules associated with monopartite begomoviruses

Satellites are defined as viruses or nucleic acids that depend on the helper virus for their replication but lack extensive nucleotide sequence homology to the helper virus and are dispensable for its proliferation (Mayo et al., 2005). Satellite viruses encode a structural protein that encapsidates its own nucleic acid while satellite nucleic acids rely on the helper virus structural protein for encapsidation and do not necessarily encode additional non-structural proteins. A third type of agent, referred to as satellite-like nucleic acid, also depends on the helper virus for its replication but provides a function that is necessary for the biological success of the helper virus and is therefore considered as part of the helper virus genome. The first satellite RNA was identified in 1969 in association with the nepovirus *Tobacco ringspot virus* (Schneider, 1969), and since then a large number of satellite RNAs, associated with several groups of plant viruses have been reported (Mayo et al., 2005), with the majority of the satellites interfering with the replication of the helper viruses, resulting in attenuated symptoms. Some satellites exacerbate disease symptoms induced by the helper virus or produce novel symptoms which are usually not associated with the helper virus infections (Roossinck et al., 1992).

The first begomovirus satellite DNA to be discovered was found to be associated with the monopartite begomovirus *Tomato leaf curl virus* (ToLCV) from Australia (Dry et al., 1997). This 682 nt circular ssDNA depends on ToLCV for its replication and encapsidation, but its replication can also be supported by other begomoviruses. It has no proven effects on the viral replication or on symptoms caused by ToLCV. It has no extensive ORFs and has little sequence similarity to its helper virus (ToLCV) except for the nonanucleotide TAATATTAC sequence present in the stem loop of all geminiviruses (Behjatnia et al., 1998). Failure to reproduce yellow vein symptoms in *Ageratum conyzoides* (goat weed) by re-introduction of *Ageratum yellow vein virus* (AYVV) (Saunders & Stanley, 1999; Saunders et al., 2000), another monopartite begomovirus isolated from goat weed showing yellow vein symptoms, which was otherwise shown to be infectious in *Nicotiana benthamiana* (Tan et al., 1995), suggested that another factor was required to restore pathogenicity in the natural host. In a search for additional viral components, a number of small circular recombinant components, each containing the AYVV origin of replication together with sequences of unknown origin, were isolated from infected goat weed (Stanley et al., 1997). Similar recombinants were also identified for the begomoviruses associated with cotton leaf curl disease (CLCuD) (Liu et al., 1998; Briddon

et al., 2000, 2001). The significance of the unidentified sequences within the recombinants was not appreciated at the time, but they were to provide a vital clue in the discovery of a new class of satellites. As was observed for AYVV, the cloned genomic component of Cotton leaf curl virus (renamed *Cotton leaf curl Multan virus* (CLCuMV)) failed to produce typical CLCuD symptoms, suggesting the presence of another factor (Briddon et al., 2000), whose search resulted in the isolation of a small circular ssDNA, referred to as DNA-1 (Mansoor et al., 1999), that is a representative of a new class of components associated with monopartite begomoviruses (Briddon et al., 2004).

Recently, many monopartite begomoviruses have been identified that associate with a type of satellite molecule referred to as betasatellite, composed of ssDNA, ~1.3 kb in size and approximately half the size of the helper begomoviruses. Many of the betasatellites are required for typical disease symptom development (Saunders et al., 2000; Briddon et al., 2001; Jose & Usha, 2003; Saunders et al., 2003; Zhou et al., 2003; Briddon et al., 2003). Despite their recent discovery, betasatellites may have existed for many centuries, e.g., EpYVB in association with EpYVV were later on found to cause EpYVD that was described about 1250 years ago (Saunders et al., 2003). Betasatellites all require a helper begomovirus for replication, local and systemic spread, and whitefly vector-mediated transmission, and some have been shown to modulate symptom severity (Briddon et al., 2003). The betasatellite molecule contains one ORF (*βC1*) (Fig. 4), an A-rich region ~240 nts long and a satellite-conserved region (SCR), of ~220 nts. The *βC1* has in one instance been shown to be responsible for the suppression of jasmonic acid signaling involved in at least one gene silencing pathway (Yang et al., 2008). The transgenic *Arabidopsis thaliana* expressing βC1 of *Tomato yellow leaf curl China virus* (TYLCCNV) was shown to develop disease symptoms like that observed in begomovirus-infected tobacco plants, in that plants exhibited upward leaf curling, foliar enations, and sterile flowers (Tao & Zhou, 2004). Apart from the nonanucleotide sequence, betasatellites do not share any significant sequence similarity with the helper begomoviruses.

The begomovirus/betasatellite complexes are often associated with a second type of circular ssDNA satellite, initially referred to as DNA-1 (Mansoor et al., 1999, 2001; Saunders & Stanley, 1999; Briddon et al., 2004), but now called alphasatellites (Mubin et al., 2009b). Alphasatellites encode a single protein that shares high nt identity with the replication-associated (activator) protein (Rep) (Fig. 4), a rolling-circle replication initiator protein encoded by viruses in the genus *Nanovirus*, family

Nanoviridae that also have a genome of circular ssDNA (Gronenborn, 2004). Consequently, alphasatellites are capable of autonomous replication, but require a helper begomovirus for spread in plants and for whitefly vector transmission. In addition to Rep, alphasatellites also have an A-rich region, ~200 nts long, down stream of the Rep-encoding region. Recently, it has been demonstrated that the Rep of the alphasatellite associated with *Tobacco curly shoot virus* (TbCSV) can be used as a virus-induced gene silencing vector (VIGS) (Huang et al., 2009). In contrast to betasatellites, alphasatellites possess in their stem loop the nonanucleotide sequence, TAGTATTTAC also found in the stem loop of viruses in the family *Nanoviridae*. Alphasatellites can affect both begomovirus titer and symptom development in host plants (Saunders and Stanley, 1999; Patil and Fauquet, 2010). Initially it was thought satellite molecules were limited to the OW, but recently, alphasatellites have been found associated with NW begomoviruses (Paprotka et al., 2010; Romay et al., 2010), thus expanding the geographical distribution of satellite molecules associated with begomoviruses.

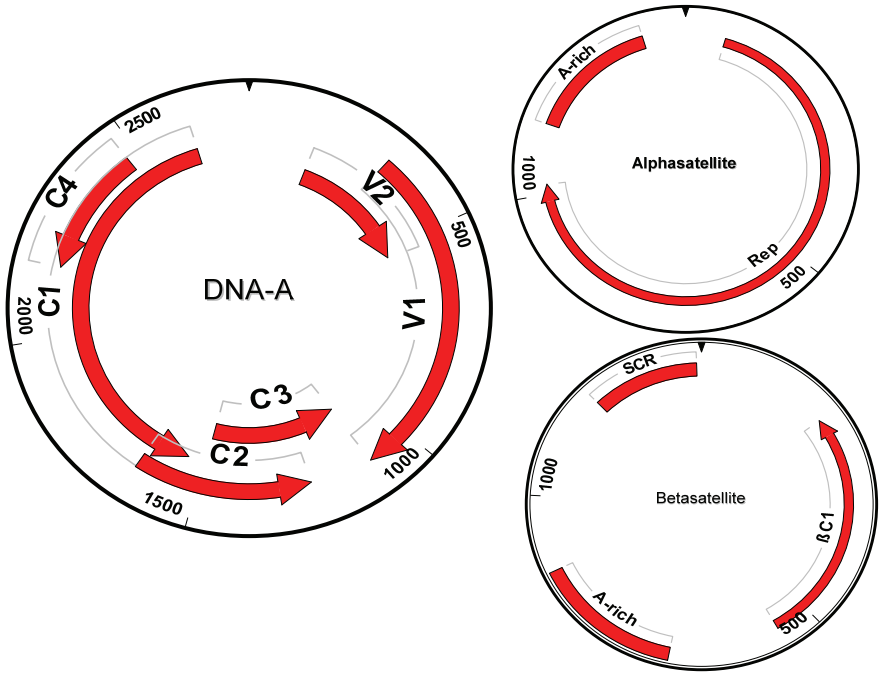


Figure 4. The genome organization of a monopartite begomovirus associated with an alphasatellite and a betasatellite. A-rich: adenosine-rich region of the genome; βC1: the gene encoding the betaC1 protein; SCR: satellite-conserved region.

Plant defense against begomovirus infections

Plants like any other living systems are in constant interaction with a wide array of pathogens and therefore must develop a surveillance system that safe guards their existence or they may otherwise perish. Plants can accomplish this in many ways. RNA silencing is an evolutionary conserved surveillance system that occurs in eukaryotes, including animals (RNA interference), fungi (quelling) and plants (Napoli et al., 1990; Cogoni & Macino, 1997, 2000; Fire et al., 1998; Baulcombe, 1999; Fire, 1999; Li et al., 2002). RNA silencing is a sequence-specific defense mechanism that can target both cellular and viral mRNA for degradation. It is a widely used mechanism for inactivating gene expression (Seal et al., 2006). The methylation of DNA virus genomes inhibits virus transcription thereby blocking replication. RNA-directed methylation is a recently identified form of defense against DNA viruses (Wang et al., 2003). The hypersensitive response is another form of defense whereby, upon infection of a plant cell by the pathogen (virus), the plant releases chemical compounds that results in the death of the infected cell, thereby stopping further spread of the pathogen.

Begomovirus defense mechanism against RNA-silencing strategy of plants

Viruses have evolved or acquired functional proteins (suppressors) that suppress RNA silencing by targeting different steps of the silencing pathways (Voinnet, 2001; Roth et al., 2004; Sharma & Ikegami, 2008). The recent development of molecular techniques has led to significant advances in our knowledge of begomoviruses, their genomes and roles in disease etiology (Sharma & Ikegami, 2008). Begomoviral encoded suppressor proteins are believed to act at different points in the silencing pathways (Fig. 3). Considering that begomoviruses replicate in the nucleus and their genomes are made up of DNA and do not possess a dsRNA phase in their replication cycle, how do they then trigger RNA silencing in plants? The begomovirus dsDNA serves as the template for both replication and transcription, with the transcription occurring bidirectionally with two major polycistronic transcripts in opposite orientation from the CR (bipartite begomoviruses) or IR (monopartite begomoviruses) that contains the bidirectional promoter sequences. The *AV2-AV1* (bipartite begomoviruses)/*V2-V1* (monopartite begomoviruses) transcripts and the complementary sense *AC1-AC3* (bipartite begomoviruses)/*C1-C3* (monopartite begomoviruses) transcripts overlap by 4 nts at their 3' ends (Chellappan et al., 2004). It is therefore thought that the overlapping transcripts in opposite polarity at the 3'end may

generate double-stranded mRNA (dsmRNA), which could induce RNA silencing (Voinnet, 2001) and are therefore targets of RNA-silencing in plants (Muangsan et al., 2004).

In bipartite begomoviruses, the AC2 (TrAP) has been reported to suppress RNA-silencing by controlling the expression of host genes coding for positive or negative effectors of RNA-silencing. It is believed to inactivate adenosine kinase (ADK), whose function has been implicated in the methylation of the replicative form of begomoviruses and therefore suppresses local silencing (Trinks et al., 2005; Vanitharani et al., 2005; Wang et al., 2005)(Fig. 3). The C4 protein of bipartite begomoviruses such as ACMV, EACMV, *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) can suppress RNA silencing due to their ability to bind to micro RNA (miRNA) and small interfering RNA (siRNA) (Vanitharani et al., 2004; Chellanppan et al., 2005; Fondong et al., 2007).

For the monopartite begomoviruses, the V2 protein of TYLCV-IL has recently been identified as an RNA-silencing suppressor and may exert its suppressor effect by targeting a step in the RNA-silencing pathway that occurs after siRNA production (Avi et al., 2007) (Fig. 3). It is therefore thought that TYLCV-IL encodes two types of RNA-silencing suppressors: the V2 protein for earlier silencing events and C2 protein for the later silencing events (Sharma & Ikegami, 2008).

Betasatellites that associate with monopartite begomoviruses have been shown to induce typical disease symptoms in plants and suppress gene silencing (Saunders et al., 2000; Briddon et al., 2001; Mansoor et al., 2003; Briddon et al., 2003; Cui et al., 2005; Kon et al., 2006). It has been demonstrated by mutational analysis that the betasatellites single ORF encodes the pathogenicity determinant βC1, and transgenic expression of the 14 kDa βC1 protein or expression from a *Potato virus* X (PVX) vector results in severe developmental abnormalities (Zhou et al., 2003, Cui et al., 2004; Saunders et al., 2004; Kon et al., 2007; Tao and Zhou, 2008; Yang et al., 2008). The precise mechanism of action of the βC1 protein is presently unknown.

The evolution and diversity of begomoviruses

Today, evolution can be observed in real time by scientists, with the fastest evolution occurring in viruses within months, resulting, for example, in rapid development of drug resistance in human immunodeficiency virus (HIV). The phenotype of living organisms is always a result of the genetic information that they carry and pass on to the next generation and its

interaction with the environment. Thus, if we want to study the driving force of evolution, we have to investigate the changes in the genetic information (Vandamme, 2009).

During duplication of the genetic information, the DNA or RNA polymerase can occasionally incorporate a non-complementary nucleotide. In addition, bases in a DNA strand can be chemically modified due to environmental factors such as UV light or chemical substances. These modified bases can potentially interfere with the synthesis of the complementary strand and thereby also result in a nucleotide incorporation that is not complementary to the original nucleotide. When these changes escape the cellular repair mechanisms, the genetic information is altered, resulting in what is called a 'point mutation' (Vandamme, 2009). The genetic code has evolved in such a way that a point mutation at the third codon position rarely results in an amino acid change (only in 30% of the possible changes). A change at the second codon position always, and at the first codon position mostly (96%), results in an amino acid change. Mutations that do not result in amino acid changes are called silent or 'synonymous mutations'. When a mutation results in the incorporation of a different amino acid, it is called non-silent or 'non-synonymous'.

The fact that all begomoviruses irrespective of their origin, OW or NW, have a similar genomic organization, with the lack of *AV2* ORF in the NW begomoviruses, shows that they all have a common origin irrespective of their geographical distribution. What then is responsible for the emergence of the more than 200 species that have been identified so far? Originally, it was considered that plant viruses evolve more slowly than animal viruses (Blok et al., 1987). However, emerging reports indicate that some (+) ssRNA and ssDNA viruses (*Geminiviridae* and *Nanoviridae*) infecting plants have substitutions rates that are very similar to those observed for mammalian RNA viruses (Jenkins et al., 2002; Fargette et al., 2008; Gibbs et al., 2008; Simmons et al., 2008; Duffy & Holmes, 2008, 2009). The following factors have been identified as the driving force behind the evolution and emergence of new begomoviral species.

A. Mutation

Different naturally occurring or laboratory isolates of a begomovirus may differ by either single or multiple point mutations, which may alter the sequence of encoded proteins, or by small indels. These differences can affect symptom type and severity (Stanley, 1995), host range (Lazarowitz, 1992) and whitefly transmission (Noris et al., 1998). In naturally occurring isolates, silent (synonymous) nucleotide changes, such as many of those in the third position in codons, are more numerous and may considerably

outnumber coding (nonsynonymous) changes (Ooi et al., 1997; Sanz et al., 1999) as seen in the *CP* genes of field isolates of CLCuMV and *Okra yellow vein mosaic virus* (OYVMV) (Zhou et al., 1998), presumably because random sequence mutations have been accompanied by strong selection pressure for optimal function of the *CP*. A similar pattern of mutations occurs in the *AC1* gene, but changes in *AV2* and *AC4* are constrained by their effects on the *CP* and *AC1* genes that overlap them (Sanz et al., 1999). The rate of point mutations has been studied for three different begomoviruses, TYLCV, TYLCCNV and EACMV and shown to be similar as for RNA viruses ~10^{-4} substitutions/site/year (Ge et al., 2007; Duffy & Holmes, 2008, 2009), despite expectations that it should be lower for DNA viruses due to DNA polymerase proof-reading activity. It was concluded that the most commonly observed substitution mutation is the transition mutation type rather than deletions or insertions. The studies demonstrated that the mutation rate in the genome of begomoviruses is dependent on the type of virus, host plant, age of the inoculated plant and inoculum homogeneity. However, it appears that begomoviruses do not utilize the normal host mechanism for mismatch repair involving DNA methylation, which allows nondeleterious mutations to be maintained (Roossinck, 1997).

B. Recombination

Recombination is a process by which segments from one DNA or RNA strand become incorporated into that of a different individual strand during replication. Although the first reports of recombination in begomoviruses under field conditions were relatively recent (Harrison et al., 1997; Zhou et al., 1997; Padidam et al., 1999), it has become clear from many reports since then that recombination represents a normal rather than exceptional evolutionary mechanism of these ssDNA viruses (Padidam et al., 1999), with the amount of material they exchange ranging from small portions of a few nucleotides to very large fragments. The rate of recombination is therefore contributing to the recent emergence of new begomoviruses across the world (Seal et al., 2006). The frequency of recombination in begomoiruses can be partly explained by a recombination-dependent replication mechanism that occurs in several begomoviruses (Jeske et al., 2001; Preiss and Jeske, 2003) and the satellite molecules they support (Alberter et al., 2005). The primary function of recombination could be to repair ssDNA defects that have arisen through mutation. Resulting recombinants would subsequently only become prevalent in field populations where the recombination resulted in a selective advantage (Seal et al., 2006). Evidence of recombination being involved in the evolution of begomoviruses emanates from the identification of contiguous regions of nucleotide

sequences that are of different origins. In the well established cases, the presence of chimeric sequences is obvious from the sequence analyses of the different genome regions as in the case of OLCuCMV identified in this study (**II**). The sequences of the putative progenitors, CLCuGV and OYCrV are known and the *V1/V2* region of OLCuCMV is almost identical to the corresponding *V1/V2* of OYCrV and distinctly different from that of CLCuGV. Similarly, the *C1/C4* region of OLCuCMV is almost identical to the corresponding region of CLCuGV, but distinctly different from that of OYCrV. There have been many reports of incongruent gene phylogenies suggesting the involvement of recombinant begomoviruses, but for which the parents and recombination junctions remain unknown (Zhou et al., 1998; Berrie et al., 2001; Galvão et al., 2003; Nawaz-ul-Rehman & Fauquet, 2009; this study, **IV**). It is believed that recombination between different DNA-A components is the main source of molecular variation among begomoviruses (Padidam et al., 1999) and results in the gain of virulence for the recombinants (Nawaz-ul-Rehman & Fauquet, 2009). The rapidly increasing number of published sequences of begomoviruses and improvements to software for predicting recombination events (Martin et al., 2005) have greatly improved our understanding in the area of molecular virology.

Recombination events have also been reported between begomoviruses and satellite DNAs, and between different betasatellite molecules (Briddon et al. 2001, 2003; Saunders et al., 2001; Nawaz-ul-Rehman & Fauquet, 2009; this study, **IV**).

C. Reassortment (Pseudo-Recombination)

Reassortment is the exchange of DNA-A and DNA-B genomic components (Seal et al., 2006). This phenomenon is frequently observed in bipartite begomoviruses and can be achieved between isolates of the same begomovirus (Stanley et al., 1985), but not between distinct begomovirus species (Lazarowitz et al., 1992; Frischmuth et al., 1993), simply because the heterologous DNA-B components are not replicated and not because their gene products are inactive. The ability of begomoviruses to exchange components was first shown experimentally in the 1980s (Stanley et al., 1985). It has been reported that some DNA-B gene products (BC1) can mediate movement within the plant of a heterologous DNA-A (Frischmuth et al., 1993). Reassortment may also occur where the parental begomoviruses differ in properties, for example, like the reassortment that was observed between *Tomato mottle virus* (ToMoV) and *Bean dwarf mosaic virus* (BDMV) (Gilbertson et al., 1993). Both possible reassortants were

viable, but induced attenuated symptoms, produced relatively small amounts of DNA-B and one was difficult to subculture. In another reassortment involving the two closely related begomoviruses S*ida golden mosaic Colombia virus* (SiGMCoV) and *Abutilon mosaic virus* (AbMV), one was viable while the other was not and although the viable reassortant infected *Nicotiana* species, it did not infect neither *Sida rhombifolia* nor *Malva parviflora*, which are natural hosts of both parental viruses (Höfer et al., 1997). Reassortants derived from closely related begomoviral species therefore, (i) tend to produce low levels of one B component or both A and B components in inoculated cells, (ii) fail to invade systemically some or all the plant species infected by their parental viruses and where systemic infection occurs, (iii) to induce attenuated symptoms. For these reasons, secondary genetic changes will often be needed if interspecific reassortants are to acquire sufficient biological fitness, for example, host adaptation to survive under natural conditions (Harrison & Robinson, 1999). Some monopartite begomoviruses are also thought to have acquired a DNA-B component permanently under some field conditions changing to bipartite begomoviruses; these begomoviruses have been described as "mono-bipartites" (Saunders et al., 2002; Chakraborty et al., 2003).

Thus, understanding processes driving the evolution of any given plant virus provides a framework for monitoring changes in the virus populations and subsequently designing of appropriate control measures (García-Arenal et al., 2001; Elena et al., 2008). Analysis of plant virus evolution often involves making inferences of their phylogenetic relationships, population genetic structures, past demographic histories, molecular clocks, and adaptive evolution events (Kuhner et al., 1998; Nei & Kumar, 2000; García-Arenal et al., 2001; Holmes, 2003; Drummond et al., 2005; Pérez-Losada et al., 2008; Gibbs et al., 2010). As such, the use of an appropriate nucleotide substitution model - a model of evolution - is necessary to describe changes in character state, i.e, the rate of change from one nucleotide to another (Liò & Goldman, 1998; Posada & Krandal, 1998, 2001; Posada & Buckley, 2004; Sullivan & Joyce, 2005; Shapiro et al., 2006; Som, 2006). To study these changes, the programme MEGA4 (Tamura et al., 2007) that contains the said models, have been widely used in this study.

D. Selection pressure

Although mutations, reassortment and particularly recombination are known as the mechanisms that generate variation in begomoviruses, little is known about the selection pressures that operate and drive begomovirus evolution. In general, viruses appear to be selected for according to Darwin's concept of "survival of the fittest" (Roossinck, 1997). Sequence analyses of

virus genes show that selection is often negative, presumably due to numerous mutations being deleterious and many virus proteins being multifunctional (García-Arenal et al., 2003).

Selection pressures will differ depending on the vector population and plant host. It has been reported that the major factor influencing the evolution of begomoviral CP is the need to interact with the insect vector (Briddon et al., 1997; Simon et al., 2003) and possibly also GroEL proteins of endosymbiotic bacteria thereof (Morin et al., 2000). Thus very different evolutionary selection pressures are operating on some of the virion-sense genes (*CP*) versus complementary-sense genes (*Rep*) (Seal et al., 2006). It is therefore not surprising that different evolutionary origins have been proposed in several recombinant begomoviruses for the virion-sense genes (*V1* and *V2*) (Zhou et al., 1998; **II, IV**). The plant hosts or varieties grown, and timing thereof, are important factors that can select for particular vector populations and hence, virus strains co-evolving with these populations. In the absence of vector selection, the most variable selection pressure on the virus would be on the genes *C1* to *C4* that interact predominantly with host factors and this would explain the very frequent reports of recombinants in the *C1-C4* region of both monopartite (Fauquet et al., 2005; **II**, **III**) and bipartite begomoviruses (Idris and Brown, 2004). Recombination at some overlapping junctions between genes might also have been selected due to such genetic changes enabling some host plant defences to be avoided; the junction between the *AC1* and *AC2* genes for ACMV-[CM] has been found to be a predominant target for host-plant gene silencing (Chellapan et al., 2004). In India, the existence of distinct cassava and sweet potato whiteflies has been reported (Lisha et al., 2003). The cassava whiteflies reproduce on cassava, eggplant, tomato and tobacco, but not on cotton or sweet potato, whereas, the sweet potato whiteflies reproduce on sweet potato, cotton, eggplant, tomato and tobacco, but not on cassava. Large-scale cultivation of cassava, cotton or sweet potato will therefore alter markedly the prevalence of different biotypes and any co-adapted begomoviruses.

Begomovirus genetic diversity appears to be influenced by a complex interaction of selection pressures exerted by both the genetic diversity of the vector and by that of plant hosts on both virus and vector. Complex plant-virus-vector interactions can result in devastating epidemics as seen for the cassava mosaic pandemic in East Africa (Colvin et al., 1999, 2004). Begomoviruses appear to have a more specific interaction with their vector than their hosts and seem to adapt to new hosts fairly readily with frequent reports of new strains infecting previously unaffected hosts (Usharani et al., 2004). A better understanding of the relationship between vector and virus

population diversity would be achievable if future studies concentrate on determining both the genetic diversity of *B. tabaci* and begomoviruses present on the same individual host plants.

Transmission of begomoviruses

The whitefly *B. tabaci* (Fig. 5) is a widespread phloem-feeding pest of dicotyledonous crops in the tropical and subtropical regions of Africa, America, Asia, Europe and Oceania and can transmit more than 200 different plant viruses responsible for the loss of millions of hectares of valuable food and industrial crops (Brown, 1994; Morales & Anderson, 2001; Jones, 2003; Morales & Jones, 2004; Legg & Fauquet, 2004). The *B. tabaci* complex colonizes more than 600 host plant species (Cock, 1993), belonging to 77 families (Basu, 1995). Almost all of the plant viruses transmitted by *B. tabaci* are begomoviruses. *B. tabaci* is the only known vector species for begomoviruses, and hence the global distrinbution of begomoviruses is closely related to that of this species. The prevailing *B. tabaci* biotypes present in a region will affect the begomoviruses present. Most biotypes can transmit a range of begomoviruses, but they do so with very differing efficiencies depending on both virus species and bioype (Bedford et al., 1994; Maruthi et al., 2002). Increase in populations of *B. tabaci* is a general characteristic of begomovirus disease epidemics, and facilitates the spread of begomoviruses into and within crops, and their transmission to and from weed hosts. Increased populations of *B. tabaci* are associated with a range of factors including conducive climatic conditions (Morales & Jones, 2004), the spread of the more fecund B-biotype (Polston & Anderson, 1997; Perring, 2001), the cultivation of particular crops or varieties (Costa, 1975; Morales & Anderson, 2001; Varma & Malathi, 2003), and virus infection of the host (Colvin et al., 2004). Begomoviruses are transmitted by *B. tabaci* in a circulative and persistent manner. Except for *Tomato yellow leaf curl Sardinia virus* (TYLCSV) whose DNA was detected in eggs, nymphs, and adults, of the first generation progeny (Bosco et al., 2004), no replication of begomoviruses has been found in *B. tabaci*. Apart from causing damage to plants by the begomoviruses that they transmit, *B. tabaci* also causes direct damage to crops by inducing noticeable physiological disorders (irregular ripening, silver leaf, and severe chlorosis) (Costa & Brown, 1991; Legg et al., 2004; Brown, 2010). Also, the large populations of *B. tabaci* observed on suitable hosts induce the growth of superficial fungi (sooty molds) on the honeydew excreted by immature and adult whiteflies.

These fungi greatly reduce the photosynthetic capacity of the plants and may eventually lead to the death of the plants and total crop failure.

Figure 5. The infestation of a tomato plant in Cameroon by the *Bemisia tabaci* which is insect vector of begomoviruses.

Begomoviruses and satellite DNAs infecting crops in West and Central Africa

Until recently, knowledge on the prevalence of begomoviruses in West and Central Africa has been rather scanty. Initially, information on the existence of begomoviruses infecting crops in the region was based on serology and hybridization, with emphasis on ACMD, OLCD and tomato leaf curl disease (ToLCD) (N'Guessan et al., 1992; Fargette et al., 1993; Swanson & Harrison, 1994; Konate et al., 1995; Czosnek & Laterrot, 1997). With the advent of more advanced molecular techniques in the study of begomoviruses, such as polymerase chain reaction (PCR), rolling cycle amplification (RCA)/restriction fragment length polymorphism (RFLP) and sequencing, the situation is gradually improving, leading to the identification of previously unknown begomovirus/satellite complexes (Fondong et al., 2000; Leke et al., 2007; Zhou et al., 2008; Chen et al., 2009; Kon et al., 2009; Lett et al., 2009; this study). The concentration of previous research in the region on ACMD, OLCD and ToLCD, clearly underscores the importance of these diseases and that they have been a long standing problem. This problem therefore calls for urgent attention, which has been somewhat neglected probably due to the lack of trained personnel in advanced molecular techniques.

Until recently, information on the existence and diversity of DNA satellite molecules associated with monopartite begomoviruses has been mainly from Asia (Bull et al., 2004; Nawaz-ul-Rehman & Fauquet, 2009; Sivalingam et al., 2010), though there has been a report on the identification of some DNA satellites associated with bipartite begomoviruses of cassava from Tanzania and alphasatellites from Kenya, in Africa (Briddon et al., 2004; Briddon & Stanley, 2006). Recently, one betasatellite; Cotton leaf curl Gezira betasatellite (CLCuGB), initially identified in the Nile basin has been identified in West Africa, associated with diseased okra and tomato

(Chen et al., 2009; Kon et al., 2009; Shih et al., 2009; Tiendrébéogo et al., 2010; this study). Also, two types of alphasatellites, Okra leaf curl Mali alphasatellite (OLCuMA) and a novel type of alphasatellite from okra, Okra leaf curl Burkina Faso alphasatellite (OLCBFA) (Kon et al., 2009; Tiendrebeogo et al., 2010; **II**) have been identified in West Africa, with those from Cameroon being the first report from the Central African region.

In Cameroon, before the initiation of this project, *B. tabaci* has been associated with suspected begomovirus infections in many crop species, including cassava, bean, cotton, eggplant, pepper, squash, tomato, okra and watermelon as well as weeds of the genera *Ageratum*, *Asystasia*, *Clerodendrum*, *Emilia* and *Malvastrum*. This observation was based on the consistent presence of *B. tabaci* on plants exhibiting characteristic symptoms of begomovirus infection (leaf curling and distortion, green or yellow foliar mosaic, stunting, reduced yields). Despite their growing importance, only two begomoviruses have been studied in Cameroon in any detail at the molecular level, EACMCV and ACMV (Fondong et al., 2000). TYLCV (Czosnek and Laterrot, 1997) has been detected by hybridization, but sequencing and sequence analyses were necessary for specific identification. Thus, there was a pressing need for additional information on the diversity and distribution of begomoviruses and satellites in vegetable crops and/or dicotyledonous weeds, which likely serve as virus reservoirs. This study thus presents a tip of the iceberg of the diversity of begomoviruses and associated satellite DNAs infecting crops and weeds in SWC and the Central African region.

Aims of the study

This thesis is focused on begomoviruses and satellite molecules infecting the vegetable crops okra (*Abelmoschus esculentus*), tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*) and watermelon (*Citrullus lanatus*) as well as the weeds *Ageratum conyzoides*, *Asystasia gangetica*, *Clerodendrum umbellatum*, *Emilia cocinea* and *Malvastrum* spp in SWC. Prior to this study, knowledge on the existence and prevalence of begomoviruses in Cameroon at the molecular level has been limited to those infecting cassava. Therefore, before any appropriate control measures for the spread of begomoviral diseases can be implemented, large scale sequencing of begomovirus genomes and satellite molecules from crops and weed host plants in localized areas are needed to improve our understanding of the relative effects of the prevailing host and weed genotypes on virus diversity.

The specific objectives were:

- To determine the identity, genetic variability and distribution of suspected begomoviruses causing leaf curl (begomovirus-like) symptoms in cultivated and uncultivated hosts in southwestern Cameroon.
- To determine the incidence and severity of OLCD and characterize at the molecular level the unique begomoviruses and DNA satellites associated with OLCD.
- Characterize at the molecular level the begomoviruses and associated DNA satellites infecting tomato in SWC.
- Characterize at the molecular level the begomoviruses and associated DNA satellites infecting Ageratum in SWC.
- Contribute to the general understanding of the existence and evolution of begomoviruses and DNA satellites in Cameroon and the West and Central African regions as a whole.

Results and discussions

Provisional identification and classification of begomoviruses infecting crops and weeds in SW Cameroon based on the core coat protein gene

The core region of the *CP* gene is highly conserved amongst begomoviruses and has been proposed for the provisional identification and classification of members of the genus *Begomovirus* (Brown et al., 2001). It has since then been widely used (Harrison et al., 2002; Delatte et al., 2002; Ala-Poikela et al., 2005; Hernandez-Zepeda et al., 2007).

Symptomatic weeds and crops displaying different symptom phenotypes (Fig. 2) were collected from different locations in SWC from 2006 to 2009 (Tables 1 and 2) and were tested for infection of begomoviruses by PCR using the universal primers AV494 and AC1048 that amplify a ~580 nucleotide fragment of the core region of the *CP* gene (Wyatt & Brown, 1996) and/or RCA (Inoue-Nagata et al., 2004) (**I**, **II** and **III**). All the cultivated plant species tested, including okra, pepper, tomato and watermelon, as well as weeds of the genera *Ageratum*, *Asystasia*, *Clerodendrum*, *Emilia* and *Malvastrum* were positive for begomoviruses.

Analysis of the core *CP* gene sequence revealed ten provisional SWC begomovirus species, based on the recently proposed guidelines for the classification of begomoviruses (Fauquet et al., 2008). Those sharing > 89% nt identities with virus sequences available in GenBank were considered isolates of the same species or later re-classified based on analyses using full-length sequence data (Table 2).

Begomoviruses of cultivated crop species previously reported from Africa and also provisionally identified from SWC were (1) CLCuGV (**I** and **II**)

from okra, (2) OYCrV (**I** and **II**) was identified from okra and also from *A. conyzoides*, *Asystasia gangetica,* pepper and watermelon. Therefore, pepper and watermelon as well as *A. conyzoides*, and *A. gangetica* may be possible alternative hosts for OYCrV (Fig. 6 isolate names in purple), but full-length sequences and infection experiments are needed to confirm them as such.

Seven potentially new begomovirus species were identified from one cultivated and four wild plant species. The provisional species names herein proposed (Table 2) were based on the convention of employing the symptoms occurring in the host plant (Fig. 2), together with the common or genus name of the plant host and geographic origin (Fauquet et al., 2008). ALCCMV was identified from *A. conyzoides*, displaying leaf curl symptoms (Figs. 1 & 9); Clerodendrun golden mosaic virus (ClGMV) (Fig. 6a) and Clerodendrum leaf curl Cameroon virus (ClLCCMV) (Fig. 6b) from two different plants of *C. umbellatum*, displaying golden mosaic symptoms (Fig. 1) and leaf curl symptoms (not shown), respectively; Emilia yellow vein virus (EYVV) from *E. cocinea*, displaying yellow vein symptoms (Fig. 1); Malvastrum yellow mosaic Cameroon virus (MaYMCMV) from a *Malvastrum* spp, with yellow mosaic symptoms (Fig. 1); Tomato leaf curl Cameroon virus (ToLCCMV) and Tomato leaf curl Fontem virus (ToLCFmV) from tomato showing yellow leaf /leaf curl symptoms (Figs. 1 & 8).

Phylogenetic analysis of core *CP* (Fig. 6a) and a partial sequence obtained by RCA (Fig. 6b), and sequences of begomoviruses from Africa, Asia and Mediterranean placed the isolates herein identified into five well-supported clades, (1) the West African tomato leaf curl begomovirus clade, containing *Tomato leaf curl Ghana virus* (ToLCGHV) (EU350585), *Tomato leaf curl Kumasi virus* (ToLCKuV) (EU847739), *Tomato leaf curl Nigeria virus* (ToLCNGV) (FJ685621) and *Tomato leaf curl Togo virus* (ToLCTGV) (FJ685620) and the isolates herein identified (ALCCMV, EYVV, ToLCCMV and ToLCFmV) from *A. conyzoides*, *E. cocinea* and tomato at a bootstrap value of 97%. This therefore suggests a possible movement of the begomoviruses identified from the weeds *A. conyzoides* and *E. cocinea* to tomato and vice versa and thereby expanding the host range for the viruses. (2) a clade containing isolates of MaYMCMV (bootstrap value 100%), (3) the CLCuGV/HoLCrV clade, containing isolates of CLCuGV and HoLCrV identified from the Nile Basin and West Africa and two CLCuGV isolates herein identified, with the Cameroonian isolates grouping more closely with the Egyptian CLCuGV isolates (bootstrap value 99%), (4) the OYCrV clade, grouping the OYCrV isolates from Mali and those herein identified in okra, pepper and watermelon as well as the weed, *A. conyzoides* (bootstrap value

100%) and (5) a clade containing *Cowpea golden mosaic virus* (CPGMV) from Nigeria and the isolate ClGMV, herein identified in *C. umbellatum*, (bootstrap value 83%).

Since recombination is so frequent amongst begomoviruses, and have been detected in this study (**II**, **III** and **IV**), it is possible that some of the isolates which showed > 89% nt identities based on the core *CP* sequences with those in the database, may be reclassified upon obtaining full-length sequences, therefore increasing or reducing the number of putative new species from SWC.

Provisional identification, herein based on the core *CP* sequence, indicated that at least six begomoviral species were infecting five native plant species in SWC, together with five additional begomoviral species of four cultivated hosts (Table 1 and 2). Altogether, this report and the previous reports by Fondong et al. (2000) and Lett et al. (2009) indicate that at least 14 different begomovirus species are present in Cameroon with 13 in SWC alone. Collectively, the data suggests that the region supports a highly diverse group of begomoviruses.

For establishing new begomovirus species and for definite classification, sequencing of at least the complete DNA-A component is necessary (Fauquet et al., 2008). Full-length sequence data obtained in this study nessecitated the reclassification of ALCCMV, ToLCCMV and OLCuCMV which shared > 89% nt identities with other known begomoviruses as new begomoviruses (**II**, **III** and **IV**) thus increasing the number of identified begomoviruses species in SWC.

It has been shown in Asia that plants of the genera *Ageratum* and *Malvastrum* are native hosts to begomoviruses that can subsequently be transmitted to cultivated crop species (Stanley et al., 1997; Saunders and Stanley, 1999; Saunders et al., 2001; Mansoor et al., 2003; Stanley, 2004; Huang & Zhou, 2006; Graham et al., 2010). It is therefore anticipated that as more surveys are conducted in Cameroon, cultivated crops may be found infected by the begomoviruses ALCCMV, EYVV, ClGMV, ClLCCMV and MaYMCMV, also suggested by the phylogenetic groupings (Fig. 6).

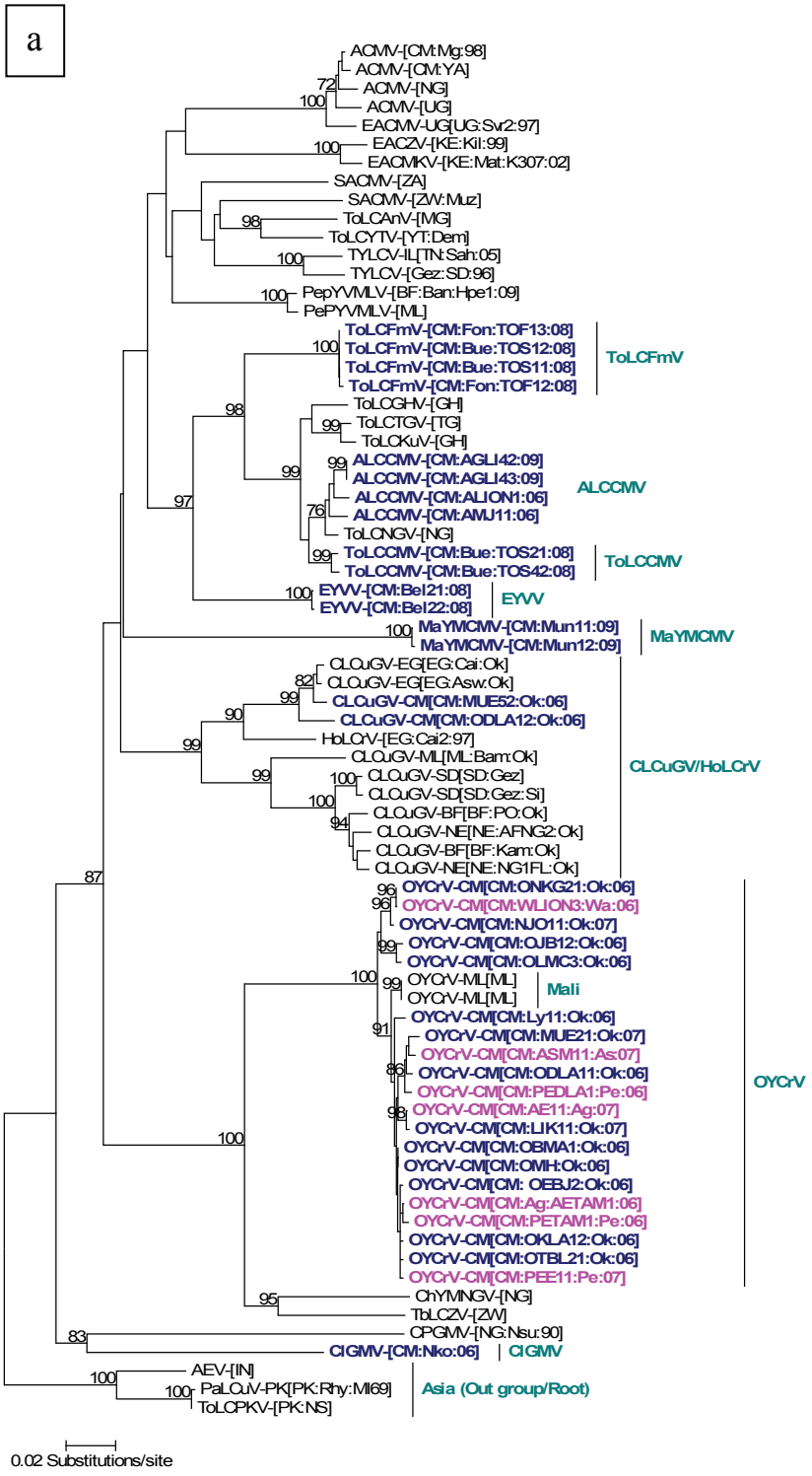
Table 1. *Plant species and geographic distribution of field samples collected for molecular characterization of begomoviruses. Only a representative of the total number of samples tested for begomovirus infection is listed.*

Sample code	Plant species	Field location	Region	Year	Number of clones sequenced
AE	*Ageratum conyzoides*	Ekona	South West	2007	3
AETAM	*A. conyzoides*	Etam	South West	2006	3
AGLI	*A. conyzoides*	Liongo	South West	2009	3
ALION	*A. conyzoides*	Liongo	South West	2006	3
AMJ	*A. conyzoides*	Manjo	Littoral	2006	3
ASM	*Asystasia gangetica*	Melong	Littoral	2007	3
Bel	*Emilia cocinea*	Belluah	South West	2008	3
CNBB	*Clerodendrum*	Ndop	North West	2008	3
LIK	*Abelmoschus esculentus* (okra)	Likomba	South West	2007	3
MUE	Okra	Muea	South West	2007	3
Mun	*Malvastrum*	Mundemba	South West	2009	3
NJO	Okra	Njombe	Littoral	2007	3
NKO	*Clerodendrum*	Nkongsamba	Littorale	2006	3
OBMA	Okra	Kumba	South West	2006	3
ODLA	Okra	Douala	Littorale	2006	3
OEBJ	Okra	Ebonji	South West	2006	3
OJB	Okra	Njombe	South West	2006	3
OKLA	Okra	Kolla	Littoral	2006	3
OLMC	Okra	Loum Chattier	Littoral	2006	3
OMH	Okra	Buea	South West	2006	3
ONKG	Okra	Nkongsamba	Littoral	2006	3
OTBL	Okra	Tomble	South West	2006	3
PEDLA	*Capsicum annuum* (pepper)	Douala	Littoral	2006	3
PEE	Pepper	Ekona	South West	2007	3
PETAM	Pepper	Etam	South West	2006	3
TOF	*Solanum lycopersicum* (tomato)	Fontem	South West	2008	3
TOS	Tomato	Buea	South West	2007	3
WLION	*Citrullus lanatus* (watermelon)	Liongo	South West	2006	3

Table 2. *Provisional begomovirus species identified from native and cultivated eudicots in SWC (based on the core CP sequence), established or proposed nomenclature. Nucleotide identity was used for putative species classification.*

Clone	Species with highest identity	Nt identity (%)	Final classification[a]
AE11	OYCrV	97	
AETAM1	OYCrV	97.4	
AGLI42	ToLCNGV	95.9	ALCCMV⋆
ALION1	ToLCNGV	96.1	ALCCMV⋆
AMJ11	ToLCNGV	95.8	ALCCMV⋆
ASM11	OYCrV	96.5	
Bel21	ToLCGHV(EYVV)⋆	81.7	
CNBB21	WmCSV (ClLCCMV)⋆	61.3	
LIK11	OYCrV	96.9	OLCuCMV⋆
LY11	OYCrV	97.2	
MUE21	OYCrV	96.1	
MUE52	CLCuGV	97.6	
Mun11	CLCuGV (MaYMCMV)⋆	68.8	
NJO11	OYCrV	95.6	
Nko1	PaLCuV (ClGMV)⋆	66.6	
OBMA1	OYCrV	97.8	
ODLA11	OYCrV	96.7	
ODLA12	CLCuGV	94.5	
OEBJ2	OYCrV	97.8	
OJB12	OYCrV	95.6	
OKLA12	OYCrV	97.0	
OLMC3	OYCrV	95.6	
OMH	OYCrV	97.8	
ONKG21	OYCrV	95.4	
OTBL21	OYCrV	97.2	
PEDLA1	OYCrV	96.7	
TOF12	ToLCNGV (ToLCFmV)⋆	85.1	
TOS11	ToLCNGV (ToLCFmV)⋆	85.1	
TOS21	ToLCNGV	94.3	ToLCCMV⋆
TOS42	ToLCNGV	93.9	ToLCCMV⋆
WLION3	OYCrV	95.4	

[a] Reclassified following full-length sequence analyses, ⋆Proposed nomenclature for potentially new begomovirus species from SWC


a
ACMV-[CM:Mg:98]
ACMV-[CM:YA]
ACMV-[NG]
ACMV-[UG]
EACMV-UG[UG:Svr2:97]
EACZV-[KE:Kil:99]
EACMKV-[KE:Mat:K307:02]
SACMV-[ZA]
SACMV-[ZW:Muz]
ToLCAnV-[MG]
ToLCYTV-[YT:Dem]
TYLCV-IL[TN:Sah:05]
TYLCV-[Gez:SD:96]
PepYVMLV-[BF:Ban:Hpe1:09]
PePYVMLV-[ML]
ToLCFmV-[CM:Fon:TOF13:08]
ToLCFmV-[CM:Bue:TOS12:08]
ToLCFmV-[CM:Bue:TOS11:08]
ToLCFmV-[CM:Fon:TOF12:08]
ToLCFmV
ToLCGHV-[GH]
ToLCTGV-[TG]
ToLCKuV-[GH]
ALCCMV-[CM:AGLI42:09]
ALCCMV-[CM:AGLI43:09]
ALCCMV-[CM:ALION1:06]
ALCCMV-[CM:AMJ11:06]
ALCCMV
ToLCNGV-[NG]
ToLCCMV-[CM:Bue:TOS21:08]
ToLCCMV-[CM:Bue:TOS42:08]
ToLCCMV
EYVV-[CM:Bel21:08]
EYVV-[CM:Bel22:08]
EYVV
MaYMCMV-[CM:Mun11:09]
MaYMCMV-[CM:Mun12:09]
MaYMCMV
CLCuGV-EG[EG:Cai:Ok]
CLCuGV-EG[EG:Asw:Ok]
CLCuGV-CM[CM:MUE52:Ok:06]
CLCuGV-CM[CM:ODLA12:Ok:06]
HoLCrV-[EG:Cai2:97]
CLCuGV-ML[ML:Bam:Ok]
CLCuGV-SD[SD:Gez]
CLCuGV-SD[SD:Gez:Si]
CLCuGV-BF[BF:PO:Ok]
CLCuGV-NE[NE:AFNG2:Ok]
CLCuGV-BF[BF:Kam:Ok]
CLCuGV-NE[NE:NG1FL:Ok]
CLCuGV/HoLCrV
OYCrV-CM[CM:ONKG21:Ok:06]
OYCrV-CM[CM:WLION3:Wa:06]
OYCrV-CM[CM:NJO11:Ok:07]
OYCrV-CM[CM:OJB12:Ok:06]
OYCrV-CM[CM:OLMC3:Ok:06]
OYCrV-ML[ML]
OYCrV-ML[ML]
Mali
OYCrV-CM[CM:Ly11:Ok:06]
OYCrV-CM[CM:MUE21:Ok:07]
OYCrV-CM[CM:ASM11:As:07]
OYCrV-CM[CM:ODLA11:Ok:06]
OYCrV-CM[CM:PEDLA1:Pe:06]
OYCrV-CM[CM:AE11:Ag:07]
OYCrV-CM[CM:LIK11:Ok:07]
OYCrV-CM[CM:OBMA1:Ok:06]
OYCrV-CM[CM:OMH:Ok:06]
OYCrV-CM[CM: OEBJ2:Ok:06]
OYCrV-CM[CM:Ag:AETAM1:06]
OYCrV-CM[CM:PETAM1:Pe:06]
OYCrV-CM[CM:OKLA12:Ok:06]
OYCrV-CM[CM:OTBL21:Ok:06]
OYCrV-CM[CM:PEE11:Pe:07]
OYCrV
ChYMNGV-[NG]
TbLCZV-[ZW]
CPGMV-[NG:Nsu:90]
ClGMV-[CM:Nko:06]
ClGMV
AEV-[IN]
PaLCuV-PK[PK:Rhy:MI69]
ToLCPKV-[PK:NS]
Asia (Out group/Root)
0.02 Substitutions/site

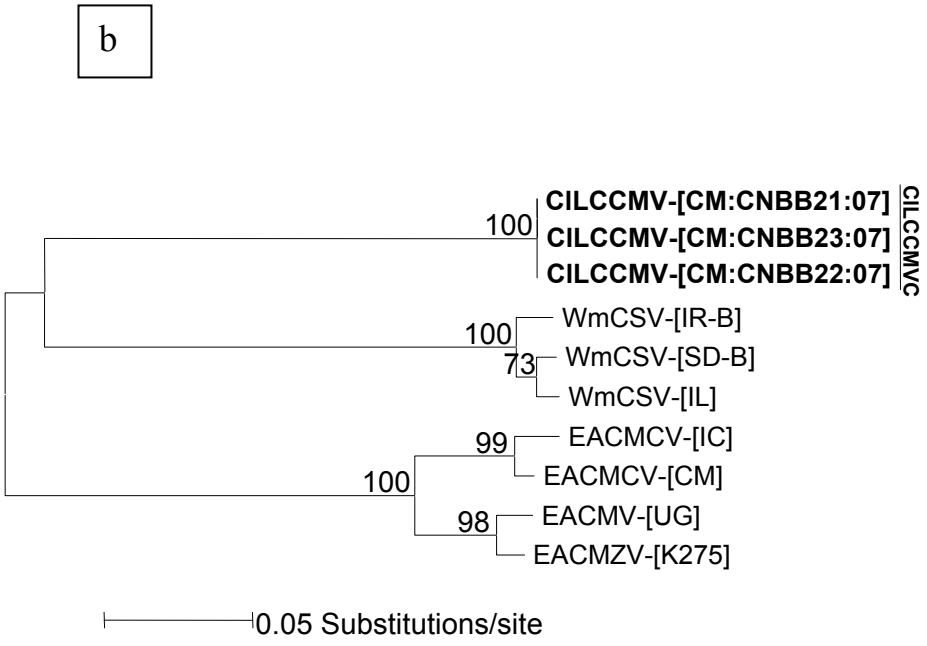


Figure 6. Neighbor-joining analysis using MEGA 4.0, showing the predicted relationships between begomovirus isolates based on the alignment of core *CP* (a) and sequences obtained following rolling cycle amplication (partial B-component) (b). The begomovirus isolates from SWC are in bold and coloured. Isolates in purple are tentative alternative hosts for *Okra yellow crinkle virus* (OYCrV). Numbers represent bootstrap values higher than 70 percent.

Begomovirus disease complex associated with OLCD (I and II)

Why okra?

Okra is a widely cultivated vegetable and can be found in almost every market in Africa. In Ghana, it is the fourth most popular vegetable after tomato, pepper and eggplant. In Sudan, the common okra is the third or fourth most popular vegetable; whereas in Cameroon the two okra species, *Abelmoschus caillei* (West African okra) and *A. esculentus* (common okra) combined represent the second most important vegetable in the market after tomato (Schippers, 2000). Most okras are eaten in cooked or processed form, whereas young fruits may also be eaten fresh (Schippers, 2000). Okra is also used as a good source of gum and its fibers are traditionally used to make rope. The ground pulp of *A. caillei* stems is used as a stabilizer when making Pita beer in northern Ghana (Schippers, 2000). Besides its usefulness, okra is particularly prone to many pest and diseases and amongst them, OLCD (Fig. 7) and the infestation by *B. tabaci* take the lead. This is directly

linked to the ability of most okras to withstand long dry seasons when the whitefly populations peak.

Figure 7. Okra (*Abelmoschus. esculentus)* in southwestern Cameroon with leaf curl disease symptoms.

OLCD and begomovirus/satellite sequence properties

Initially okra samples were randomly collected from 10 different locations in SWC in 2006 and tested for the presence of begomoviruses by PCR using the universal primers (Wyatt & Brown, 1996). All samples from the 10 locations were positive for begomoviruses, using the FTA card technology (**I**). Cloning, sequencing and sequence analysis revealed that the okra samples were infected by CLCuGV (Idris & Brown, 2002; Idris et al., 2002; Idris et al., 2005; Shih et al., 2009) and OYCrV (Shih et al., 2007 **I**) and in a single case as a mixed infection of both (**I**). Based on these results, a more detail survey was conducted in 2008 to document disease incidence and to determine whether other begomoviruses than CLCuGV and OYCrV were associated with the disease. In addition, full-length sequences were needed to confirm that the identified viruses were really CLCuGV and OYCrV. The survey of three okra fields in SWC showed a relatively high incidence of OLCD ranging from 20-27% and with a mean of 22.7%, consistent with what had previously been established in two West African countries: Burkina Faso and the Ivory Coast (Konate et al., 1995; N'Guessan et al.,

1992; **II**). This result therefore underscores the importance of the disease in the region. The samples collected in 2008 were also screened for begomovirus infection following the procedure described above; with an infection rate of 67% in both symptomatic and non-symptomatic plants (12 and 3 plants tested, respectively). This therefore demonstrates the importance of latent infections in begomovirus disease epidemiology (**II**). Some samples with severe disease symptoms (disease severity 2-4) were negative for PCR. Given the many problems associated with PCR detection of begomoviruses in okra (Fang et al., 1992; Jose & Usha, 2000; **I**), the conclusion that could be drawn from this is that begomoviruses were present but undetectable (**II**). Sequence analyses of the cloned PCR products for the core *CP* confirmed that okra in SWC is indeed infected by CLCuGV and OYCrV and in one instance, with mixed infection of both CLCuGV and OYCrV identified in the same field. Using two specific abutting primer pairs, MUE5F/MUE5R and OKSF/OKSR, designed from the core *CP* sequences of CLCuGV and OYCrV, respectively, it was possible to amplify the expected ~2.8 kb full-length begomoviral genomes from four okra plant samples. Sequence analyses of the cloned products revealed that they had structural features typical of monopartite begomoviruses (**II**). Pair-wise nucleotide sequence comparison as well as phylogenetic and recombination analyses revealed that okra in SWC is infected by at least three distinct begomoviruses. Two of the full-length sequences were identified as two new strains of the previously identified CLCuGV and OYCrV: CLCuGV-CM and OYCrV-CM. One was identified as a putative new species, Okra leaf curl Cameroon virus (OLCuCMV), being a recombinant of CLCuGV and OYCrV, with recombination detected to have occurred in the *V1/V2* region, which was well supported by six recombination detection program 3 (RDP3) methods (**II**). The results of the phylogenetic analysis showed that the four okra-infecting begomovirus isolates from SWC grouped into one of two major clades: (i) the CLCuGV/*Hollyhock leaf crumple virus* (HoLCrV) clade that contains all CLCuGV and HoLCrV isolates previously identified in the Nile Basin and West Africa, as well as OLCuCMV-[CM:Lys11:08] and CLCuGV-CM[CM:Mue5:Ok:08] (100% bootstrap), and (ii) the OYCrV clade that contains the previously identified OYCrV isolates from Mali, OYCrV-CM[CM:Mue1:08], and OYCrV-CM[CM:Njo52:08] (100% bootstrap). OLCuCMV-[CM:Lys11:08] formed a unique branch within the CLCuGV/HoLCrV clade, whereas CLCuGV-CM[CM:Mue5:Ok:08] was most closely related to CLCuGV isolates described from Egypt. The results suggest that these begomoviruses are not likely to have been recently introduced into SWC and that they are indigenous strains (**II**). OLCuCMV

shared less than 89% sequence identity with known isolates of CLCuGV and so was considered an isolate of a new distinct species. A mixed infection is a prerequisite for recombination to occur and in this case it is consistent with the previous identification of CLCuGV and OYCrV in the same okra plant (**I**) and from two plants in the same field (**II**).

Using the universal abutting betasatellite primers Beta01 and Beta02 (Briddon et al., 2002), the expected ~1.3 kb fragment was amplified from the four okra plant samples from which CLCuGV, OYCrV and OLCuCMV were obtained (**II**). The betasatellite sequences shared 92% nucleotide identity with the betasatellite isolates: Cotton leaf curl Gezira betasatellite-Mali (CLCuGB-[ML:Ok:06] and CLCuGB-[ML:To:06]) and Cotton leaf curl Gezira betasatellite-Niger (CLCuGB-[NE:Ok:08] and CLCuGB-[NE:Ok:08]), from Mali and Niger, respectively (Kon et al, 2009; Shih et al., 2009) (**II**). Based on the phylogenetic grouping and since the nt identity exceeds the species suggested cutoff for betasatellite demarcation of 79% (Briddon et al., 2008), the Cameroonian betasatellite isolates are considered isolates of CLCuGB and are designated CLCuGB-[CM:Ok:08] (**II**). As was observed for the phylogenetic groupings of their helper begomoviruses (CLCuGV, OYCrV and OLCuCMV), CLCuGB isolates from West Africa also grouped into well-supported clades according to geographic origin. This thus suggests that CLCuGB and the helper viruses have a similar ancient evolutionary history and therefore a long standing association.

Using the universal abutting alphasatellite primers DNA101 and DNA102 and specific primers (Bull et al., 2003; **II**), alphasatellites of the expected ~1.3 kb size were amplified from two okra plants as CLCuGB-[CM] and they represented two genotypes herein designated Alpha-1 and Alpha-2 (**II**). The genome organization of Alpha-1 was similar to that of other alphasatellites described thus far and has as its closest relative Okra leaf curl Mali alphasatellite (OLCuMLA-[ML]) identified from okra in Mali (Kon et al., 2009; **II**). A phylogenetic analysis with alphasatellite and nanovirus nucleotide sequences placed Alpha-1 and Alpha-2 into two separate well-supported clades: (i) all previously identified alphasatellites from Africa and Asia (alphasatellite clade), as well as Alpha-1 (99% bootstrap), and (ii) the recently identified Gossypium mustilinum symptomless Pakistan alphasatellite (GsmSPKA) from Pakistan, two isolates of the nanovirus *Faba bean necrotic yellows virus* (FBNYV) (Katul et al., 1998) as well as Alpha-2 in what could be termed the novel alphasatellite/nanovirus clade (85% bootstrap). Within the alphasatellite clade, three sub-clades were resolved: (a) the Asian sub-clade, grouping all

isolates from Asia at a bootstrap value of 94%, (b) the Cameroon/Mali sub-clade, including isolates of Alpha-1 and OLCuMLA-[ML] at a bootstrap value of 98% and (c) the Egypt/Kenya sub-clade, including the isolates from Egypt and Kenya (98% bootstrap). Within the novel alphasatellite/nanovirus clade, two sub-clades were also resolved: (a) the novel alphasatellite sub-clade, including the isolates of GsmSPKA from Pakistan and isolates of Alpha-2 from Cameroon at a bootstrap value of 100% and (b) the nanovirus sub-clade, including two isolates of FBNYV (99% bootstrap). Thus, Alpha-2 and its closest relatives are divergent DNA alphasatellites in relation to those described to date, that may eventually be found to represent a biologically and/or genetically distinctive alphasatellite type. Based on pair-wise nt sequence identity, phylogenetic analysis, and a suggested species demarcation threshold of >83% for alphasatellite species (Mubin et al., 2009b), Alpha-1 and Alpha-2 are herein designated Okra leaf curl Mali alphasatellite-[Cameroon] (OLCuMLA-[CM]) and Okra yellow crinkle Cameroon alphasatellite (OYCrCMA), respectively (**II**).

Identification of a new begomovirus and alphasatellite in tomato in SWC (III)

The importance of tomato (*Solanum lycopersicum*) as a vegetable crop worldwide cannot be overemphasized and as already mentioned above, it is the number one vegetable found in the Cameroonian market. Despite its importance, tomato plants frequently display TYLC/ToLC disease symptoms, the most devastating disease of cultivated tomato worldwide, which causes huge yield losses and this can have a significant economic impact, causing a reduction in income for farmers and distributors. Many species of begomoviruses have been associated with the TYLC/ToLC disease of cultivated tomato worldwide, and also recently from West Africa (Osei et al., 2008; Zhou et al., 2008; Chen et al., 2009; Lett et al., 2009). Before the initiation of this study, TYLC/ToLC disease of tomato in Cameroon had been associated with TYLCV based on hybridization studies (Czosnek & Laterrot, 1997) but sequencing and sequence analyses were necessary for specific begomovirus identification. TYLC/ToLC disease symptoms (Figure 8) of cultivated tomato have been observed for a very long time in SWC.

Figure 8. Plants of tomato (*Solanum lycopersicum*) in Southwestern Cameroon showing tomato yellow leaf curl /tomat leaf curl disease symptoms

In 2008, a survey was conducted in three farmers' fields where a total of 15 tomato leaf samples were collected and pressed onto FTA classic cards (**I**, **II**) and the viral nucleic acid was recovered as described (**III**). The samples were tested for the presence of begomoviruses by PCR using the universal primers as described (**I**, **II**, **III**). A single begomovirus species was identified that showed 86% nt identity to *Tomato leaf curl Ghana virus* (ToLCGHV) (Osei et al., 2008). Based on the sequences of the *CP* gene, the specific abutting primer pair TOS2F/TOS2R was designed and used to amplify the expected ~2.8 kb fragment from two samples. The complete genome sequences were determined to be 2797 nts. They shared the highest nt identity at 85.2% with Tomato leaf curl Nigeria virus (ToLCNGV). Their intergenic region had 326 nts and contained several features of monopartite begomoviruses: a typical 33-nucleotide potential loop region which includes the conserved nonanucleotide sequence (TAATATTAC) present in all geminiviruses (Hanley-Bowdoin et al., 1999). RDP3 analysis provided evidence of recombination in one event within the *C1* gene of both isolates, between ToLCNGV and *Pepper yellow vein Mali virus* (PepYVMLV), with ToLCNGV as the major parent and PepYVMLV as the minor parent. The results of a phylogenetic analysis (Fig. 10 isolates in puple) showed that the two tomato-infecting begomovirus isolates from southwestern Cameroon grouped in a well supported clade (bootstrap 100%) with other tomato leaf curl begomoviruses from West Africa in what could be termed the West African tomato leaf curl begomovirus clade. Within the clade, two subclades were further resolved: (i) the Cameroon/Nigeria subclade comprising the isolates herein identified and ToLCNGV-[NG:06], and (ii)

the Ghana/Togo subclade, comprising the isolates of ToLCGHV, ToLCKuV and ToLCTGV from Ghana and Togo. The exclusion of the recombinant region identified in the two isolates of the Cameroonian tomato-infecting begomovirus did not alter the position of the taxa in the tree (not shown), indicating that the recombined region did not greatly influence the phylogenetic status of these isolates. Based on the attributes advanced above and the current begomovirus species demarcation and nomenclature (Fauquet et al., 2008), the isolates herein identified from SWC were considered isolates of a distinct begomovirus species: Tomato leaf curl Cameroon virus (ToLCCMV) (III). Given that tomato is not indigenous to Africa, the 'West African ToLC'- inducing begomoviruses may possibly either have been introduced recently and/or emerged from an endemic eudicot.

The identification of different species of West African tomato leaf curl begomoviruses in Ghana [Osei et al., 2008], Nigeria, and Togo, all in West Africa, and in Central Africa (**IV**) suggests that West African tomato leaf curl viral species are prevalent and are perhaps more widely distributed in sub-Saharan Africa than previously expected.

Using the universal abutting alphasatellite primers DNA101 and DNA102 (Bull et al., 2003; **III**), alphasatellites of size (1.4 kb) were amplified from the same tomato plants as ToLCCMV, herein designated TOS2D1 and TOS2D2. The genome organization of the tomato-infecting alphasatellite isolates was similar to that of other alphasatellites described thus far. It shared the highest nt sequence identity of 74.7 and 74.2% with two isolates of Ageratum yellow vein alphasatellite (AYKA-[EG:SB45:95], AJ512960; AYKA-[KE:SB32:01], AJ512963), respectively, identified from Egypt and Kenya (Briddon et al., 2004). The second highest identity at 68.8% nt identity, was an isolate of Okra leaf curl Mali alphasatellite (OLCuMLA-[ML:06], EU589450) from Mali (Kon et al., 2009), also from West Africa.

The two satellites had features characteristic of other nanovirus-like alphasatellites discovered thus far [Briddon et al., 2004], including a stem-loop structure with the conserved nanovirus nonanucleotide sequence (TAGTATTAC) and a single viral-sense ORF, which encoded a predicted Rep protein of 317 AA. The alphasatellite shared no significant sequence similarity with its prospective helper ToLCCMV.

A phylogenetic analysis placed the TOS2D1 and TOS2D2 sequences as a subgroup within an African clade of alphasatellites. The *Rep* nt sequences were aligned for selected members of the 'old begomovirus alphasatellite' group, including those of TOS2D1 and TOS2D2. Based on the alignment, an insertion of six nucleotides, CTGCAG, was identified in the central

region of the TOS2D1 and TOS2D2 *Rep* gene, resulting in a putative Rep protein that was 317 instead of 315 AA, and that was longer by two residues. Subsequent phylogenetic analysis of the aligned *Rep* nt sequences produced a tree structure similar to the complete alphasatellite sequence tree (**III**).

RDP3 analysis did not predict any recombination among these alphasatellites. Based on the pair-wise nt sequence identities, the results of the phylogenetic analysis, and the proposed species demarcation threshold at >83% for alphasatellites (Mubin et al., 2009b), TOS2D1 and TOS2D2 are sufficiently divergent to be considered as members of a new species of alphasatellite, for which the name Tomato leaf curl Cameroon alphasatellite (ToLCCMA) is proposed.

Begomovirus disease complex associated with ageratum leaf curl disease (ALCD) in SWC (IV)

Ageratum conyzoides (goat weed) is a common and widespread weed species that is found in almost all agro-ecological zones of Cameroon. It is frequently infested by *B. tabaci* with characteristic leaf curl (LC) begomovirus disease symptoms (Figure 9), but it has never been tested for begomovirus infections. In Asia, both yellow vein (YV) and LC disease symptoms are commonly observed in *A. conyzoides* (Stanley et al., 1997; Saunders & Stanley, 1999; Saunders et al., 2001; Mansoor et al., 2003; Stanley, 2004; Huang & Zhou, 2006), and has been frequently reported to be a host to many begomovirus disease complexes (Stanley et al., 1997; Saunders & Stanley, 1999; Saunders et al., 2001; Mansoor et al., 2003; Stanley, 2004; Huang & Zhou, 2006). Using the begomovirus universal primers as previously described (**I**, **II**, **III**), the core *CP* region was amplified from three goat weed plants from two locations in SWC. The amplified sequences were 90% identical to ToLCCMV. The specific primers TOS2F/TOS2R were used to amplify an expect ~2.8 kb fragment from one ageratum plant sample, AGLI4. Three clones, AGFG14, AGFG23 and AGFG24, of an isolate of a new tentative begomovirus species were obtained after cloning of the PCR products. Two clones (AGFG14 and AGFG24) were more closely related and shared 98% nt identity and only 96% nt identity with AGFG23. AGFG14 and AGFG24 shared the highest nt identity (85.5%) with ToLCNGV (**III**), while AGFG23 shared the highest nt identity (86.9%) with ToLCTGV. Based on the sequence of AGFG14, the primer pair (Agl1-F/Agl1-R) was designed and used to obtain an additional clone (pBAL) from the same goat weed plant that shared 98% nt identity with AGFG14 and AGFG24. Further analyses revealed two

recombination break points in AGFG14, AGFG24 and pBAL at nucleotide coordinates 732-803 involving AGFG23 and ToLCNGV and the result was well supported by five RDP3 recombination detection methods. These recombination break points were not detected in AGFG23 (**V**). They were <86% identical to other begomovirus genomes in GenBank. Thus, based on the ICTV-approved rules for begomovirus strain or species demarcation and nomenclature (Fauquet et al., 2008), AGFG14, AGFG23, AGFG24 and pBAL are members of a new tentative begomovirus species for which the name Ageratum leaf curl Cameroon virus (ALCCMV) is proposed. The phylogenetic analysis grouped ALCCMV with recently identified tomato-infecting begomoviruses from West Africa (100% bootstrap). Within this clade, two sub-clades were resolved: (i) one clade with ALCCMV and ToLCNGV (99% bootstrap) and (ii) another clade grouping Tomato leaf curl Ghana virus (ToLCGHV) [EU350585], Tomato leaf curl Kumashi virus (ToLCKuV) [EU847739] and ToLCTGV (99% bootstrap) (Fig. 10 isolates in red).

Figure 9. Ageratum conyzoides with leaf curl disease symptoms in Southwestern Cameroon.

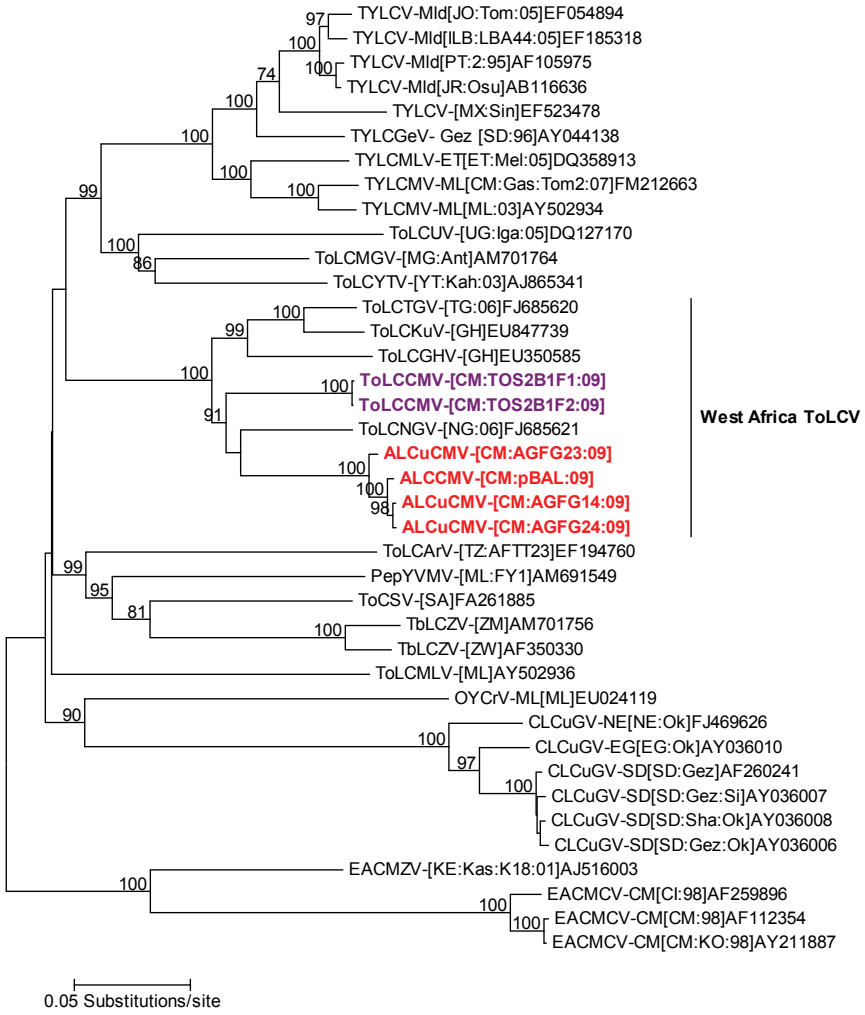


Figure 10. Neighbor-joining analyses using MEGA 4.0 of the complete genome of isolates of Ageratum leaf curl Cameroon virus (ALCCMV) and Tomato leaf curl Cameroon virus (ToLCCMV) and other selected begomoviruses from GenBank. Isolates of ALCCMV are in red while isolates of ToLCCMV are in puple. Horizontal lines are in proportion to the number nuscloetide substitutions per site. Numbers represent bootstrap values of out 2000 replicates. Only bootstrap values with greater 70% are shown. The accession numbers are shown on the tree. Abbreviations are according to Fauquet at al. (2008).

In addition, two betasatellites (1258-1389 nts) and two alphasatellites were cloned and sequenced from the same goat weed plant. The betasatellites were named SatB-1 and SatB-2. SatB-1 was 1258 nts in length and shared 72.1-74.7% nt identity with SatB-2. SatB-1 shared its highest nt identity at 49.9% with two isolates of CLCuGB, from Sudan. SatB-2 shared its highest

nt identity at 51.4-52.6% with an isolate of CLCuGB from Sudan. Based on the current established nomenclature and classification of betasatellites Briddon et al., 2008), the names Ageratum leaf curl Buea betasatellite (ALCBuB) (SatB-1) and Ageratum leaf curl Cameroon betasatellite (ALCCMB) (SatB-2) were proposed (**IV**). The search for alphasatellites yeilded two genotypes, SatA-1 and SatA-2, which were only 56.4% identical to each other. SatA-1 shared its highest nt identity at 86% with an isolate of ToLCCMA (**III**). SatA-2 shared its highest nt identity at 53% with ToLCCMA (**III**). The SatA-2 clones were of two types, herein referred to as SatA-2a and SatA-2b (**IV**). The two SatA-2a clones, SatA7 and SatA19 were 1383 and 1393 nt in length, respectively. Sequence comparisons showed that they likely are recombinants between ALCCMV and ToLCCMA, with nt 1-357 and nt 1032-1383 of SatA7 showing 99% and 100% identity to ALCCMV, respectively, and nt 359-1031 showing 97% identity to ToLCCMA. The recombinants contained the complete IR of ALCCMV, the partial ALCCMV genes *CP*, *V2* and *Rep* as well as a partial ToLCCMA *Rep* gene. The organization of SatA-2a is very similar to that of the chimeric defective virus components detected previously for begomoviruses and alphasatellites infecting *A. conyzoides* and cotton in Asia (Liu et al., 1998; Saunders and Stanley, 1999; Stanley et al., 1997) which were referred to as DIDNA. The two clones of SatA-2b, AGLD2 and AGLD3, were 1413 nt in length and were structurally very similar to SatA-2a. However, while nt 387-1045 of AGLD2 shared 100% identity with ToLCCMA, nt 1-384 showed the highest identity at 95% to ALCCMV and nt 1031-1413 the highest identity at 93% to ToLCTGV. This suggests that SatA-2b emerged as a result of recombination between ToLCCMA and an as yet uncharacterized begomovirus. The alphasatellite neighbor-joining tree grouped SatA-1 and SatA-2 into two separate, well-supported clades: (i) one containing clones of the SatA-1 (100% bootstrap) and (ii) the other clade containing clones of SatA-2 (100% bootstrap). The phylogenetic analysis including alphasatellites and the defective virus components (**IV**) grouped SatA-1 and SatA-2 together with the ToLCCMA isolate from tomato (**III**). Based on the pairwise nt sequence identity, phylogenetic analysis, and a species demarcation threshold of >83% suggested for alphasatellites (Mubin et al., 2009b), the SatA-1 clones were considered isolates of ToLCCMA: ToLCCMA-[CM:AGLD1:Ag:09] and ToLCCMA-[CM:SatA8:Ag:09].

Although knowledge of begomovirus/satellite complexes infecting wild species in Africa is still lacking, these results show that *A. conyzoides* in Cameroon harbors a complex comprising a new species of begomovirus (ALCCMV), two new betasatellite species (ALCBuB and ALCCMB), an

alphasatellite (ToLCCMA), and recombinant begomovirus/alphasatellites analogous to but divergent from those described from Asia. Understanding the dynamics and function of viral and non-viral molecules in begomovirus ecology in wild and cultivated plant hosts represents an exciting new challenge.

Since begomoviruses easily adapt to new hosts, the transmission of the newly identified begomovirus/satellite/DIDNA complex in ageratum to cultivated crops is fairly readily possible should the ageratum *B. tabaci* biotype has a wider host range. This therefore poses a serious threat to crop production in Sub-Sharan Africa as crop production is constantly under expansion, coupled with the rapidly increasing temperatures worldwide, a big contributing factor that favors the spread of *B. tabaci* and hence the begomoviruses they transmit.

Conclusions

The main conclusions from the results presented in this thesis are:

1. FTA card technology is an effective means of collecting, storing and isolating DNA for samples of okra and other crops. The DNA extracts can subsequently be used for PCR detection of begomoviruses, which otherwise can be problematic for some crops.
2. Okra in Cameroon is a host to at least three begomoviruses; CLCuGV also found infecting okra in Burkina Faso, Egypt, Mali, Niger and Sudan; OYCrV also found in Mali and OLCuCMV from Cameroon as well as CLCuGB also found in the above mentioned countries and two alphasatellites; (i) OLCuMLA also found in Burkina Faso and Mali and OYCrCMA also found in Burkina Faso. This suggests okra in Africa is a host to many begomovirus complexes, with possible yet un-identified variants and/or species.
3. Tomato in SWC is infected by at least one new begomovirus species, ToLCCMV, and one new alphasatellite species, ToLCCMA.
4. *Ageratum conyzoides* (goat weed) in SWC is a host to a previously undescribed begomovirus complex comprising at least one new begomovirus species, ALCCMV, two new betasatellites species, ALCBuB and ALCCMB as well as the alphasatellite ToLCCMA and a begomovirus/alphasatellite recombinant, otherwise referred to as a defective begomovirus.
5. At least three tentative species of alphasatellites are associated with monopartite begomoviruses in Cameroon.
6. Recombination was identified in CLCuGV-CM, OLCuCMV, ALCCMV and the defective begomovirus. This therefore suggests that

recombination is a strong driving force for the evolution of begomoviruses in West and Central Africa.

7. There are still possibly many species of un-identified begomoviruses in West and Central Africa.
8. Begomoviruses and DNA satellites infecting crops in Africa and Asia have evolved separately.
9. From the virus disease management standpoint, results generated by this study indicate that it is unlikely that smallholders in West and Central Africa can introduce much disruption to the begomovirus annual cycles, due to the many potential alternative native and crop hosts for the viruses and /or *B. tabaci* vector.

Future perspectives

- From the survey conducted in SWC, it was clearly evident that the OLCD incidence is high, thus suggesting that the disease poses a serious threat to okra production in the region. However, to understand the real impact of OLCD in Cameroon, clearly, more extensive surveys are required, including a wider geographical area as well as different agro-ecological zones and times of the year.

- From this study, there is a strong indication that all the begomoviruses and satellites identified have evolved in Africa. Many alternative hosts of CLCuGV and CLCuGB have been identified in the Nile Basin and West Africa and in this study, we have identified OYCrV from *A. gangetica* and *A. conyzoides*, both wide-spread weeds, as well as from pepper and watermelon, based on the core *CP* gene. This therefore suggests they may be alternative hosts to OYCrV. However, full-length begomovirus sequences are needed to confirm them as such. This therefore underscores the need for more extensive surveys to identify additional native species, which are acting as begomovirus reservoirs in West and Central Africa. From a practical stand point, identifying such native species will go a long way in controlling the diseases. Farmers will be well informed and able to manage the weeds possibly by rouging and thereby disrupting the *B. tabaci* haplotype populations, which are known to be host specific.

- Resistant crops seem to be necessary for slowing down the begomovirus epidemics, but breeding of such varieties will be difficult due to the high diversity of begomoviruses infecting crops in West and Central Africa.

- There is an urgent need for scientists involved in begomovirus research in West and Central Africa to collaborate and highlight the urgent need for funding to target long-term interdisciplinary projects on cropping systems holistically rather than on individual plant hosts and/or countries.

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