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MOLECULAR VARIABILITY OF CASSAVA *Bemisia tabaci* AND ITS EFFECTS ON THE SPREAD OF CASSAVA MOSAIC BEGOMOVIRUSES IN EAST AFRICA

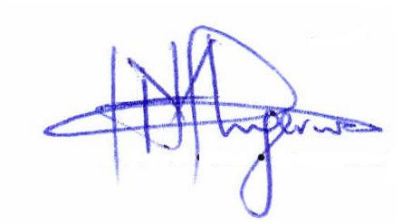
By

HABIBU MUGERWA

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Signed……..Date……29/08/2013……

H. Mugerwa

Abstract

Bemisia tabaci is the vector of cassava mosaic begomoviruses and cassava brown streak viruses which are main production constraints to cassava in sub-Saharan Africa. Current vector dynamics involved in the spread of both viruses in the region was established through comparison of the mitochondria cytochrome oxidase I DNA. Two distinct species were obtained: sub-Saharan Africa clade 1 (SSA1), comprising of two sub-clades (I & II), and a South West Indian Ocean Islands (SWIO) species. SSA1 sub-clade I whiteflies were widely distributed in East Africa. SSA1 sub-clade II whiteflies predominated the coast regions of Kenya, southern & coast regions of Tanzania and widespread in Uganda. SWIO whiteflies occurred in the coastal region of Kenya. This study also revealed that SSA1 sub-clade I haplotypes performed significantly better than SSA1 sub-clade II haplotypes with respect to mean number of eggs laid, developing instars and hatched adults on healthy, *African cassava mosaic virus-[Tanzania:2001]* and *East African cassava mosaic Kenya virus*-infected plants. There was no boost in whitefly numbers by the CMB-infected plants. The fecundity and development differences observed between SSA1 sub-clade I and II haplotypes have major epidemiology implications on the CMGs in the region

Dedication

To my dear wife, Moreen Nakalungi Mugerwa, together with the family of my dad Mr. and Mrs. Hussein Kamya and above all the Almighty God who has led me this far.

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Publications

1. H. Mugerwa, M. E. C. Rey, T. Alicai, E. Ateka, H. Atuncha, J. Ndunguru & P. Sseruwagi, Genetic diversity and geographical distribution of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) genotypes associated with cassava in East Africa. **Ecology and Evolution 2012; 2(11): 2749-2762**

2. H. Mugerwa, M. E. C. Rey, F. Tairo, J. Ndunguru & P. Sseruwagi, Two haplotypes of sub Saharan Africa 1 *Bemisia tabaci* associated with cassava exhibit distinct biological differences. **Submitted in the Journal of Applied Entomology 2013.**

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1. H. Mugerwa, M. E. C. Rey, T. Alicai, E. Ateka, H. Atuncha, J. Ndunguru & P. Sseruwagi, Molecular characterization and geographical distribution of cassava *Bemisia tabaci* genotypes in East Africa. Global Cassava Partnership Second Scientific Conference, June 18th – 22th, 2012, Kampala, Uganda.

2. H. Mugerwa, M. E. C. Rey, F. Tairo, J. Ndunguru & P. Sseruwagi, Two haplotypes of sub-Saharan Africa clade 1 *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) associated with cassava in East Africa exhibit distinct biological differences in fecundity and development. 12th international Plant Virus Epidemiology Symposium meeting, Jan 27th – 31st, 2013, Arusha, Tanzania.

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RATIONALE

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a phloem-feeding insect composed of a complex of more than 24 morphologically indistinguishable cryptic species (Dinsdale *et al*., 2010; De Barro *et al*., 2011; Shu-sheng *et al*., 2012). It is believed to have originated in southeast Asia/Indian subcontinent (Mound and Halsey, 1978; Gill, 1990) or possibly Africa (Gill, 1990; Campbell *et al*., 1996; De Barro *et al*., 2011). *B. tabaci* is widely distributed and economically important as a pest and a vector of plant viruses (Brown *et al.,* 1995a; Brown *et al*., 2000) in the tropical and subtropical regions of the world (Brown and Bird, 1992; Bedford *et al*., 1994; Fishpool and Burban, 1994; Hill, 1994).

B. tabaci causes extensive damage to plants through heavy phloem feeding which can result to above 50% yield reduction, induction of phytotoxic disorders, irregular fruit ripening, chlorosis of leaves, honey dew excretes which encourage growth and development of sooty mold fungi that darkens leaves and fruits, and transmission of plant viruses (Byrne and Bellows, 1991; Costa and Brown, 1991).

B. tabaci transmits cassava mosaic begomoviruses (CMBs) and cassava brown streak viruses (CBSVs), the causative agents of cassava mosaic disease (CMD) and cassava brown streak disease (CBSD), respectively responsible for devastating yield losses in cassava (Maruthi *et al*., 2005; Legg *et al*., 2011), a major staple food crop for over 200 million people in Africa (Horton, 1988; IITA, 1990; Dahniya, 1994). Annual estimated economic losses attributed to the CMD alone in the East and Central Africa were US $ 1.9-2.7 billion (Legg *et al*., 2006).

CMD is characterized of various leaf symptoms. These include mosaic, misshapen, mottling and twisted leaflets, were several affected plants have reduced lamina, leaf size and chlorosis of leaves. Stunted plant and reduced tuberous production is attained due to the reduced photosynthetic efficiency (Agrios, 1988). CBSD is characterized of dry brown-black necrotic rot of the tuberous roots. Other symptoms include yellow blotchy chlorosis or feathery chlorosis in the secondary and tertiary veins, as well as brown necrotic streaks on the green portions of stems and seed capsules. Leaf symptoms are most prominent on the lower parts of the plant, which can make them hard to recognize unless plants are examined carefully and before leaves have senesced (Nichols, 1950).

In the recent research work carried out in Uganda, two cassava associated genotypes of *B. tabaci* designated as Ug1 and Ug2 were identified as the vectors associated with the spread of the severe CMD (Legg *et al*., 2002; Sseruwagi *et al*., 2005). The current distribution of *B. tabaci* genotypes in the region is yet to be established in East Africa (Kenya, Tanzania and Uganda). The whiteflies at the CMD epidemic front were referred to as the invaders (Ug2) and the ones ahead were referred to as the indigenous species (Ug1) (Legg *et al*., 2002). But Sseruwagi *et al*., (2005) reported that the populations of the indigenous species had increased and occupied most cassava growing area in Uganda. Whether the continued spread of the severe CMD is still associated with the 'invader' or not in the region, it is not yet known. This will be investigated in this study. The severe CMD epidemic disease 'front' was characterized of severe unusually disease phenotypes and high whitefly populations. An appearance of a new whitefly genotype or a mutual beneficial interaction between the vector and infected plant was suggested as the possible cause of the raised whitefly population (Omongo, 2003; Colvin *et al*., 2004). Colvin et al. (2004) further suggested that the increased fecundity and development rate of *B. tabaci* was as a result of change in plant amino acids.

Further investigation about the increased whitefly population due to host-pathogen-vector interactions need to be established.

As a result of the devastating yield losses caused by cassava mosaic disease whose causative agents are vectored by the *B. tabaci* on cassava, which provides food security in East and Southern Africa, it is important to understand the effect of begomoviruses on the growth and development of the whitefly. This study seeks also to give the current status of the genotype distribution of the *B. tabaci* and more insight on the spread of the disease in East Africa hence aiding develop appropriate control measures.

Objectives of study

This study sought to establish the genetic variability of cassava *B. tabaci* species and its effects on the spread of cassava mosaic begomoviruses in East Africa following the 'post epidemic' time.

The specific aims of the study were;

1) To establish the genetic variability of the principal *B. tabaci* species on cassava through comparison of sequences of the portion of the Mitochondria DNA cytochrome oxidase I (mtCOI) gene in the East Africa (Kenya, Tanzania and Uganda).
2) To establish the effect of cassava mosaic begomoviruses on the growth and development of the whitefly and its importance on the spread of cassava mosaic disease in the region.

CHAPTER ONE

Literature review

1.1 Whitefly (*Bemisia tabaci*)

1.1.1 Whitefly biology

Normally, *B. tabaci* feed on the under surface of tender young leaves where they lay eggs. The eggs are ovoid and posses an extension of a chorion called pedicel which is believed to be a guide for spermatozoa during fertilization (Byrne & Bellows, 1991) and also primary conduit through which water is absorbed from a plant (Buckner *et al*., 2002). The eggs hatch to release crawlers (first instar) which posses functional legs able to move quickly in search of available minor veins and upon reaching the appropriate phloem they remain sessile till adult stage (Byrne & Bellows, 1991). The second and third instars resemble each other and differ in size (Gill, 1990; Fishpool and Burban, 1994) while the fourth instar or 'pupa' (Lopez-Avila, 1986; Byrne and Bellows, 1991) is shield shaped, broadly elliptical (Gill, 1990) with two red eye spots at the anterior end visible beneath the translucent integument (Von Arx *et al*., 1983, 1984). Adult whiteflies after emergence from the pupa case differ in size with females being larger about 1mm and males about 0.8mm (Azab *et al*., 1970; Gill, 1990).

B. tabaci developmental time from egg to adult is significantly different according to the host plant it feeds on (Coudriet *et al.* 1985). Twelve generations are attained annually under field condition on average (Husain and Trehan, 1933; Butler *et al*., 1983; Fishpool and Burban,

1994). From the egg to the adult, developmental times were 107 days on cotton in India (Husain and Trehan, 1933), 14.5 days on aubergine in Israel (Avidov, 1956), 18.6 days on sweet potato, 29.8 days on carrot in the laboratory (Coudriet *et al*., 1985), and averaged 21 and 28 days for dry and rainy season respectively on cassava in Ivory Coast (Fishpool *et al*., 1995). Estimated number of eggs laid per female range from 28 to 394 eggs (Byrne and Bellows, 1991; Fishpool and Burban, 1994; Palaniswami *et al*., 1996).

Different studies have been conducted on the mating behaviour of whiteflies (Maruthi *et al*., 2004; Maruthi *et al*., 2001; Xu *et al*., 2010). *B. tabaci* reproduce parthenogenetically with the unmated females producing haploid males while mated females producing both male and females (Byrne and Bellows, 1991). The ratio of male to female usually is 1:2 under field conditions and depends on host and host species, temperature and time of the year (Pruthi and Samuel, 1942; Sharaf and Batta, 1985). On average, the longevity of the adults differs with females having longer life span (35 days) than the males (20 days) (Azab *et al*., 1972; Butler *et al*., 1983).

1.1.2 Colonisation and population dynamics

Varying populations of whitefly depending on the stage of plant growth have been observed to occur throughout the growing period of cassava (Fishpool *et al*., 1995; Otim-Nape *et al*., 1996). Adults invade slowly and establish within a sufficiently grown crop and a small population appears after 3 weeks of the initial colonization followed by rapid build up in 3 to 4 months after planting (Fishpool and Burban, 1994; Otim-Nape *et al*., 1996). Rapid whitefly multiplication occurs during this period due to appropriate foliage support. A steady population growth follows for a short period, followed by a rapid decline to low residual level

maintained throughout the rest of the crop's growth period (Fishpool and Burban, 1994, Fishpool *et al*., 1995).

B. tabaci population dynamics and activity are deemed to depend on nutritional quality of the host-plant, changes in the climatic factors (temperature, rain, wind and relative humidity), and natural enemy populations (Fishpool *et al*., 1987; Fishpool and Burban, 1994; Legg, 1995). Food resources are devoted to aerial growth during the early growth period (1 to 3 months) and declines are observed after 4 to 5 months when the process of root tuberisation begins. A greater whitefly population during the first 3 months is usually observed than when the plants are more mature (Silvestre and Arraudeau, 1983). Boost in whitefly population is favoured by high temperatures and radiation, and low rainfall and relative humidity (Fargette *et al*., 1992; Fishpool and Burban, 1994). However, Legg *et al*. (1994) observed population growth greatest when the rapid leaf growth occurred, which was associated to both high temperatures and rainfall. The adults disperse mainly by the aid of wind moving short and long distances (Cohen and Ben-Joseph, 1986; Blackmer and Byrne, 1993), and also by the aid of humans who move immature and adult stages on planting material (Joyce, 1981; Mound, 1983; Byrne and Bellows, 1991). Cropping practices such as planting date (Robertson, 1987; Fargette *et al*., 1990), crop disposition (Thresh *et al*., 1994) and intercropping (Fargette and Fauquet, 1988) further influence whitefly population dynamics and hence aiding the spread of the whitefly-transmitted viruses.

1.1.3 Whitefly systematics

B. tabaci's genetic complexity was first recognised in 1950's when the morphologically indistinguishable populations were reported to differ in host plant adaptability, host range,

and capabilities of plant virus transmission (Bird *et al*., 1957; Mound *et al*., 1963; Costa and Russell, 1975; Bird and Maramorsch, 1978). This led to the development of a concept that *B. tabaci* composed of series of biotypes (Costa and Brown, 1991; Bedford *et al*., 1994). Since then, over 20 biotypes have been described (Perring, 2001).

Among the most commonly studied molecular techniques in whitefly systematic is: protein polymorphism involving isozyme variation in esterases (Wool *et al*., 1989; Brown *et al*., 1995) and DNA-based molecular techniques, like random amplified polymorphic DNA (RAPD) PCR fingerprinting (Gawel and Barlett, 1993; De Barro and Driver, 1997; Guirao *et al*., 1997), amplified fragment length polymorphism (AFLP) markers (Cervera *et al*., 2000), the mitochondrial DNA marker genes, mitochondrial cytochrome oxidase I- mtCOI (Simon *et al*., 1994; Frohlich *et al*., 1999; Brown *et al*., 2000), the ribosomal RNAs, 16SrDNA (Prokaryotes) (Clark *et al.*, 1992; Frohlich *et al*., 1999) and 18SrDNA (eukaryotes) (Campbell *et al*., 1993, 1994), and a ribosomal nuclear marker of the internal transcribed spacer I (ITSI) region sequences (De Barro *et al*., 2000, 2005).

First endeavours made to distinguish *B. tabaci* biotypes using a 16S rDNA mitochondrial gene found in the eubacterial endosymbionts of whiteflies were of a limited use, as the endosymbionts were indistinguishable in different insect hosts because the 16S rDNA is highly conserved (Simon *et al.*, 1991; Clark *et al*., 1992). Using the 18S rDNA however, two base differences between the A and B biotypes was detected by Campell *et al.* (1993, 1994) and concluded that the two biotypes were not separate species. Further evidence permitting a clear distinction between the A and B biotypes of *B. tabaci* was attained when DNA-based RAPD-PCR was used to study the genetic similarity between the two biotypes (Gawel and

Barlett, 1993; Perring *et al*., 1993), and between the B biotype and other biotypes of *B. tabaci* (De Barro and Driver, 1997; Moya *et al*., 2001).

The mtCOI marker (Frohlich *et al*., 1999) has been used extensively to study the genetic variability and evolutionary relationships among *B. tabaci* from different geographical locations and host-plant species (Sseruwagi, 2005). Legg *et al*. (2002) identified two distinct cassava-associated *B. tabaci* genotype clusters in Uganda using mtCOI marker, designated as Uganda 1 (Ug1) and Uganda 2 (Ug2), which at the time of the study in 1997/8 occurred in area 'ahead' and 'behind' the CMD epidemic 'front,' respectively. Ug1 was suggested to be the indigenous or local genotypes, while the Ug2 genotypes as an 'invasive' population with the closest relative from Cameroon in West Africa. The mtCOI marker has been used also to detect five distinct geographic populations of cassava *B. tabaci* forming a distinct southern African clade (Berry *et al*. 2004). The genotype distribution of *B. tabaci* in the post epidemic areas of Uganda was also described by Sseruwagi *et al*. (2005a) using the mtCOI marker.

More recently using the 3.5% pairwise genetic divergence identified by Dinsdale *et al*. (2010) as being the boundary separating different species among the *B. tabaci*, twenty four well-defined high level phylogeographical groups (names of associated biotypes are placed in parentheses where applicable): Mediterranean (Q, J, L, Sub-Saharan Africa Silverleaf); Middle East-Asia Minor 1 (B, B2); Middle East-Asia Minor 2; Indian Ocean (MS); Asia I (H, M, NA); Australia/Indonesia; Australia (AN); China 1 (ZHJ3); China 2; Asia II 1 (K, P, ZHJ2); Asia II 2 (ZHJ1); Asia II 3; Asia II 4; Asia II 5 (G); Asia II 6; Asia II 7 (Cv); Asia II 8; Italy (T); Sub-Saharan Africa 1; Sub-Saharan Africa 2 (S); Sub-Saharan Africa 3; Sub-Saharan Africa 4; New World (A, C, D, F, Jatropha, N, R, Sida); and Uganda, have been identified.

1.2 Cassava mosaic disease

1.2.1 General background

Cassava mosaic disease (CMD) was reported first in Tanzania by Warburg in 1894 and was first proposed to be a viral disease by Zimmermann in 1906, and serious yield losses caused by the disease were not observed until 1920's (Storey, 1936). The virus was initially called cassava latent virus (Bock *et al*., 1981) until the first sequence was published by Stanley and Gay (1983) satisfying Koch's postulate (Bock and Woods, 1981) hence renamed to ACMV. Estimated yield losses believed to have been caused by the disease were between US$ 1.9-2.7 billion in Africa (Legg *et al*., 2006).

1.2.2 Genome organization, gene function and DNA replication of CMGs

Cassava mosaic geminiviruses (CMGs), family *Geminiviridae*; genus *Begomovirus* are bipartite, single-stranded, closed circular DNA viruses (Bock *et al*., 1978; Bock and Woods, 1983; Robinson *et al*., 1984) encapsidated within a geminate protein coat which measures approximately 35 × 22 nm (Harrison *et al*., 1977; Sequeira and Harrison, 1982). The bipartite genomic DNA of CMGs compromised of DNA-A and DNA-B molecules have similar sizes with 2800 nucleotides long (Harrison *et al*., 1977; Hong *et al*., 1993). DNA-A has two virion-sense and four complementary-sense open reading frames whose primary functions are: to produce protein coat, mediate virus replication inside the nucleus of the host cell and modulate interactions with host genes involved with post-transcriptional gene silencing (PTGS) (Hanley-Bowdoin *et al*., 1999). DNA-B has single virion-sense and complementary-

sense genes controlling nuclear transport and both intra-cellular and long distance movements (Hanley-Bowdoin *et al*., 1999).

1.2.3 Genetic variability and geographical distribution

First attempt made to detect ACMV using nucleic acid hybridization was by Robinson *et al*. (1984). Sequeira and Harrison (1982) and Thomas *et al*. (1986) using polyclonal antibodies and murine monoclonal antibodies (mAbs) raised against ACMV particles respectively, detected geminivirus infection in cassava mosaic affected plants. Subsequently, three CMG isolates (A, B and C) produced distinct characteristic epitope profiles when then a panel of 17 mAbs to ACMV were used on the mosaic infected cassava (Harrison and Robinson, 1988; Swanson, 1992; Swanson *et al*., 1992). The three isolates were reported to be of different non-overlapping geographical distributions, with group A isolates mainly occurring in Africa west of the rift valley and in South Africa, group B isolates occurring east of the rift valley and along the east coast of Africa, while group C isolates occurred in India and Sri Lanka (Swanson and Harrison, 1994). The A, B and C isolates were ascribed; *African cassava mosaic virus* (ACMC), *East African cassava mosaic virus* (EACMV) and *India cassava mosaic virus* (ICMV), under the genus *Begomovirus,* family *Geminiviridae*.

Seven CMGs have now been described from sub Saharan Africa (Fauquet *et al*., 2007) including: ACMV (Bock and Woods, 1983) the most widely distributed species and occurs in most areas in the cassava growing belt of Africa, *South Africa cassava mosaic virus* (SACMV) mainly occurring in South Africa and Swaziland (Rey and Thompson, 1998; Berrie *et al*., 2001), *East African cassava mosaic Malawi virus* (EACMV-[MW]) (Zhou *et al*., 1998), *East Africa cassava mosaic Cameroon virus* (EACMCV) (Fondong *et al*., 2000),

East Africa cassava mosaic Zanzibar virus (EACMZV) (Maruthi *et al*., 2004) and *East Africa cassava mosaic Kenya virus* (EACMKV) (Bull *et al*., 2006). Due to the huge number of CMBs recorded in the east African countries, Ndunguru *et al*. (2005) proposed this region as a centre of African CMBs diversity. On the Asian subcontinent, *Indian cassava mosaic virus* (ICMV) (Swanson and Harrison, 1994) and *Sri lanka cassava mosaic virus* (SLCMV) (Saunders *et al*., 2002) occurring in India and Sri Lanka have been reported.

CHAPTER TWO

Genetic diversity and geographical distribution of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) genotypes associated with cassava in East Africa

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Key words: Cytochrome oxidase I (mtCOI), genetic diversity, geographic distribution mitochondria, Whitefly

Abstract

The genetic variability of whitefly (*B. tabaci*) species, the vectors of cassava mosaic begomoviruses (CMBs) in cassava growing areas of Kenya, Tanzania and Uganda was investigated through comparison of partial sequences of the mitochondria cytochrome oxidase I (mtCOI) DNA in 2010/11. Two distinct species were obtained including sub-Saharan Africa 1 (SSA1), comprising of two sub-clades (I & II), and a South West Indian Ocean Islands (SWIO) species. Among the SSA1, sub-clade I sequences shared a similarity of 97.8-99.7% with the published Uganda 1 genotypes, and diverged by 0.3-2.2%. A pairwise comparison of SSA1 sub-clade II sequences revealed a similarity of 97.2-99.5% with reference southern Africa genotypes, and diverged by 0.5-2.8%. The SSA1 sub-clade I whiteflies were widely distributed in East Africa (EA). In comparison, the SSA1 sub-clade II whiteflies were detected for the first time in the EA region, and occurred predominantly in the coast regions

of Kenya, southern and coast Tanzania. They occurred in low abundance in the Lake Victoria Basin of Tanzania and were widespread in all four regions in Uganda. The SWIO species had a sequence similarity of 97.2-97.7% with the published Reunion sequence and diverged by 2.3-2.8%. The SWIO whiteflies occurred in coast Kenya only. The sub-Saharan Africa 2 whitefly species (Ug2) that was associated with the severe CMD pandemic in Uganda was not detected in our study.

2.1 Introduction

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is widely distributed worldwide and is composed of a complex of more than 24 morphologically indistinguishable cryptic species (Dinsdale *et al*., 2010; De Barro *et al*., 2011; Shu-sheng *et al*., 2012). The 3.5% pairwise genetic divergence identified by Dinsdale *et al*. (2010) as being the boundary separating different species, is further supported by evidence for either complete or partial mating isolation between a number of the putative *B. tabaci* 'species' (Xu *et al*., 2010; Wang *et al*., 2011). *B. tabaci* has gained increased importance as a crop pest and a vector of plant viruses, particularly geminiviruses in the genus *Begomovirus* (family, *Geminiviridae*) in tropical and subtropical regions of the world (Poulston and Anderson, 1997). In sub-Saharan Africa, *B. tabaci* is a major vector of cassava mosaic begomoviruses (CMBs) and cassava brown streak viruses (CBSVs), the causative agents of cassava mosaic disease (CMD) and cassava brown streak disease (CBSD), respectively. The two diseases cause devastating yield losses in cassava (Maruthi *et al*., 2005; Legg *et al*., 2011).

An epidemic of severe CMD was first reported to cause devastating effects (100% yield loss) to cassava crops in Uganda in the 1990s (Otim-Nape *et al*., 1997). Subsequent studies

associated the spread of the epidemic with unusually high whitefly populations (Legg, 1999; Otim-Nape *et al*., 2000; Colvin *et al*., 2004). The high whitefly populations were believed to be a result of several factors including an invasive whitefly (Legg *et al*., 2002), whiteflies feeding on plants infected with the severe EACMV-UG2 (Colvin *et al*., 2004, 2006) and the widespread occurrence of whitefly-susceptible cassava varieties (Omongo *et al*., 2004; 2012) in the severe CMD pandemic affected areas.

Using the mitochondria cytochrome oxidase I (mtCOI) marker (Frohlich *et al*., 1999), Legg *et al*. (2002) identified two distinct cassava-associated *B. tabaci* genotype clusters, designated as Uganda 1 (Ug1) and Uganda 2 (Ug2), which currently fall in genetic groups designated as sub-Saharan Africa 1 (SSA1) and sub-Saharan Africa 2 (SSA2), respectively (Dinsdale *et al*., 2010). The Ug1 occurred in areas 'ahead' of the epidemic front, while Ug2 was the predominant population at the 'front'. It was suggested that the Ug1 was the indigenous or local population, while the Ug2 could be an 'invader' population with its closest relatives from Cameroon in West Africa. The occurrence of the two *B. tabaci* species on cassava in Uganda was further confirmed by Maruthi *et al*. (2004), although with diminishing proportions of the Ug2 species in 2003 (Sseruwagi, 2005).

Cassava mosaic disease continues to devastate cassava crops in East and Central Africa threatening the lives of over 200 million people (Legg *et al*., 2006). As a consequence, a number of programs have been instituted by African governments through the national agricultural research systems (NARS), and different local and international stakeholders to monitor the spread of the disease and enforce mitigation measures. However, limited research has been conducted to establish the current situation of the *B. tabaci* species associated with the disease. A clear understanding of whitefly species associated with the spread of the

CMBs in the region would be invaluable to assist the development of durable integrated pest and disease management (IPDM) strategies.

This study sought to establish the genetic diversity and geographical distribution of *B. tabaci* associated with CMD and CBSD on cassava in East Africa (Kenya, Tanzania and Uganda). We use the term 'genotypes' or 'haplotypes' to refer to genetically distinct sequences within the species boundary diverging by less than 3.5%, while 'species' refers to a genetic group of closely related sequences exhibiting more than 3.5% divergence with other species as described by Dinsdale *et al*. (2010).

2.2 Methods and materials

2.2.1 Study area

The study was conducted in three East African countries, Kenya, Tanzania and Uganda. In each country, major cassava producing areas were demarcated as follows: Western, Nyanza and Coast provinces (Kenya); Lake Victoria Basin, Southern zone and Coast zone (Tanzania); and central, northern, eastern and western regions (Uganda).

Kenya: Western and Nyanza provinces share a similar agro-ecology, which is characterised by: bimodal rainfall ranging 950mm-1500mm annually, temperature ranges between 18.4-25.4°C, altitude of 900m-1800m and a savannah grass land. The coast province has rainfall ranging 500mm-1000mm annually, temperature ranges between 22.4-30.3°C, altitude of 900m-1800m and a savannah grass land (http://www.infonet-biovision.org/default/ct/690/agrozones).

Tanzania: Lake Victoria Basin is characterized by: bimodal rainfall ranging 1000mm-2000mm annually, temperature ranges between 17-28°C, altitude of 1000-1800m and has savannah vegetation with scattered tall trees. The southern zone has unimodal rainfall ranging 600mm-800mm annually, temperature ranges between 18-28°C, altitude of 200m-600m and is composed of woodland, bush land thickets and grassland. Coast zone has a bimodal rainfall ranging 750mm-1200mm annually, temperature ranges between 22-30°C, altitude of under 300m and a savannah grass land (http://www.fas.usda.gov/pecad/highlights/ 2005/09/ tanzania_2005/images/TZ_AEZ.htm).

Uganda: The cassava growing regions are characterized by: temperatures ranging 25-31°C and altitude ranging between 900-1500mm. Central region has bimodal rainfall averaging 1000mm annually and vegetation is savannah grassland with moderate biomass. Northern region has both unimodal and bimodal rainfall averaging 800mm annually and covered with short savannah grassland. Eastern region has a bimodal rainfall ranging 750mm-1200mm annually with short savannah grassland. The western region has bimodal rainfall ranging 1000-1500mm annually and is a forest savannah area. (http://www.fao.org /ag/AGP /AGPC/doc/Counprof/ uganda/uganda.htm).

2.2.2 Whitefly collection

Adult whiteflies (Fig. 1) were collected using an aspirator from 3 to 5 months old cassava plants from different regions in Kenya, Tanzania and Uganda (Table 1) and stored in 70% ethanol. Geo-coordinates (latitude and longitude) were recorded using a Geographical Positioning System (GPS) for each sampled location.



Figure 2 Adult whitefly (*B. tabaci*)

Table 1 Whitefly mitochondria cytochrome oxidase I sequences used in the study

Genotype name	Name in data base	Country	Region/Province	GenBank accession no.
Ke-Nyz23a	KeNyanza23a	Kenya	Nyanza	JQ286408
Ke-Nyz23b	KeNyanza23b	Kenya	Nyanza	JQ286409
Ke-C1	KeCoastC1	Kenya	Coast	JQ286410
Ke-C2	KeCoastC2	Kenya	Coast	JQ286411
Ke-C3	KeCoastC3	Kenya	Coast	JQ286412
Ke-C4	KeCoastC4	Kenya	Coast	JQ286413
Ke-C5	KeCoastC5	Kenya	Coast	JQ286414
Ke-C6	KeCoastC6	Kenya	Coast	JQ286415
Ke-C7	KeCoastC7	Kenya	Coast	JQ286416
Ke-C8	KeCoastC8	Kenya	Coast	JQ286417
Ke-N1	KeNyanzaN1	Kenya	Nyanza	JQ286418
Ke-N2	KeNyanzaN2	Kenya	Nyanza	JQ286419
Ke-N3	KeNyanzaN3	Kenya	Nyanza	JQ286420
Ke-N4	KeNyanzaN4	Kenya	Nyanza	JQ286421
Ke-N5	KeNyanzaN5	Kenya	Nyanza	JQ286422
Ke-N6	KeNyanzaN6	Kenya	Nyanza	JQ286423
Ke-N7	KeNyanzaN7	Kenya	Nyanza	JQ286424
Ke-W1	KeWesternW1	Kenya	Western	JQ286425
Ke-W2	KeWesternW2	Kenya	Western	JQ286426
Ke-W3	KeWesternW3	Kenya	Western	JQ286427
Ke-W5	KeWesternW5	Kenya	Western	JQ286429
Ug-2a	UgArua2a	Uganda	Northern	JQ286430
Ug-2b	UgArua2a	Uganda	Northern	JQ286431
Ug-3a	UgSoroti3a	Uganda	Eastern	JQ286432
Ug-3b	UgSoroti3b	Uganda	Eastern	JQ286433
Ug-24a	UgKatakwi24a	Uganda	Eastern	JQ286434
Ug-43a	UgMasindi43a	Uganda	Western	JQ286435
Ug-43b	UgMasindi43b	Uganda	Western	JQ286436
Ug-43c	UgMasindi43c	Uganda	Western	JQ286437
Ug-43d	UgMasindi43d	Uganda	Western	JQ286438
Ug-43e	UgMasindi43e	Uganda	Western	JQ286439
Ug-43f	UgMasindi43f	Uganda	Western	JQ286440
Ug-92a	UgAdjumani92a	Uganda	Northern	JQ286441
Ug-101a	UgKitgum101a	Uganda	Northern	JQ286442
Ug-101b	UgKitgum101b	Uganda	Northern	JQ286443
Ug-113a	UgMpigi113a	Uganda	Central	JQ286444
Ug-113b	UgMpigi113b	Uganda	Central	JQ286445
Ug-157a	UgNakaseke113a	Uganda	Central	JQ286446
Tz-8b	TzBunazi8b	Tanzania	Kagera, Lake zone	JQ286447
Tz-10b	TzRugera10b	Tanzania	Kagera, Lake zone	JQ286448
Tz-16a	TzOukasole16a	Tanzania	Kagera, Lake zone	JQ286449
Tz-16b	TzOukasole16b	Tanzania	Kagera, Lake zone	JQ286450
Tz-18a	TzChamugisha18a	Tanzania	Kagera, Lake zone	JQ286451

Genotype name	Name in data base	Country	Region	GenBank accession no.
Tz-23a	TzNyakanzi23a	Tanzania	Kagera, Lake zone	JQ286452
Tz-23b	TzNyakanzi23b	Tanzania	Kagera, Lake zone	JQ286453
Tz-28a	TzMpomvu28a	Tanzania	Mwanza, Lake zone	JQ286454
Tz-49a	TzKwasunga49a	Tanzania	Tanga, Coastal zone	JQ286455
Tz-53a	TzMabukweni53a	Tanzania	Tanga, Coastal zone	JQ286456
Tz-57a	TzMkata57a	Tanzania	Tanga, Coastal zone	JQ286457
Tz-57b	TzMkata57b	Tanzania	Tanga, Coastal zone	JQ286458
Tz-58a	TzMbwewe58a	Tanzania	Tanga, Coastal zone	JQ286459
Tz-58b	TzMbwewe58b	Tanzania	Tanga, Coastal zone	JQ286460
Tz-62a	TzMbinga62a	Tanzania	Ruvuma, Southern zone	JQ286461
Tz-62b	TzMbinga62b	Tanzania	Ruvuma, Southern zone	JQ286462
Tz-65a	TzMkinga65a	Tanzania	Ruvuma, Southern zone	JQ286463
Tz-68a	TzMkoha68a	Tanzania	Ruvuma, Southern zone	JQ286464
Tz-70a	TzMatomondo70a	Tanzania	Ruvuma, Southern zone	JQ286465
Tz-70b	TzMatomondo70b	Tanzania	Ruvuma, Southern zone	JQ286466
Tz-75a	TzLikalangilo75a	Tanzania	Tanga, Coastal zone	JQ286467
Tz-77a	TzRwengu77a	Tanzania	Ruvuma, Southern zone	JQ286468
Tz-81a	TzMajala81a	Tanzania	Ruvuma, Southern zone	JQ286469
Tz-83a	TzMasaiyaleo83a	Tanzania	Mtwara, Southern zone	JQ286470
Tz-90a	TzMasaiyaleo90a	Tanzania	Mtwara, Southern zone	JQ286471
Tz-90b	TzMasaiyaleo90b	Tanzania	Mtwara, Southern zone	JQ286472
Tz-91a	TzTikule91a	Tanzania	Lindi, Southern zone	JQ286473
Tz-93a	TzWangurukuru93a	Tanzania	Lindi, Southern zone	JQ286474
Tz-93b	TzWangurukuru93b	Tanzania	Lindi, Southern zone	JQ286475
Tz-95b	TzIkwiriri95b	Tanzania	Coast, Coastal zone	JQ286476
Tz-97a	TzNgunja97a	Tanzania	Coast, Coastal zone	JQ286477
Tz-H1	TzMwanzaH1	Tanzania	Mwanza, Lake zone	JQ286478
Tz-H4	TzTangaH4	Tanzania	Tanga, Coastal zone	JQ286479
Tz-H9	TzKageraH9	Tanzania	Kagera, Lake zone	JQ286480
Tz-H12	TzKageraH12	Tanzania	Kagera, Lake zone	JQ286481
Tz-H15	TzKageraH15	Tanzania	Kagera, Lake zone	JQ286482
Tz-H18	TzMwanzaH18	Tanzania	Mwanza, Lake zone	JQ286483
Tz-H20	TzMwanzaH20	Tanzania	Mwanza, Lake zone	JQ286484
Tz-H24	TzTangaH24	Tanzania	Tanga, Coastal zone	JQ286485
Tz-H27	TzMwanzaH27	Tanzania	Mwanza, Lake zone	JQ286486
Tz-H30	TzMwanzaH30	Tanzania	Mwanza, Lake zone	JQ286487

2.2.3 Extraction of whitefly DNA

Three adult female whiteflies were randomly selected from each collection site. Each individual insect was ground in 10µl of lysis buffer (5mM Tris-HCl, Ph8.0, 0.5 mM EDTA, 0.5% Nonidet P-40, 1mg/ml proteinase K) using the tips of 0.2µl PCR tubes. The lysis product was incubated for 15min at 65°C and further 10min at 95°C. Subsequently, it was centrifuged (~60 sec) briefly and placed immediately on ice prior to PCR amplification. Lysis was carried out as described by Frohlich *et al*. (1999).

2.2.4 PCR amplification of mtCOI DNA and Sequencing

A total of 79 (Kenya - 21, Tanzania - 41 and Uganda - 17) whiteflies were used to study the genetic variability and distribution of cassava-associated *B. tabaci* genotypes in cassava growing areas of Kenya, Tanzania and Uganda in 2010/11. Amplification of mtCOI DNA was achieved by the use of a primer pair MT10/C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and MT12/L2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA) as per Simon *et al*. (1994). A DNA template of 5µl was used in a PCR reaction mixture of 25µl, containing 1× *Taq* buffer with Mg^{2+}, 0.2mM deoxynucleotide triphosphate (dNTPs), 0.32mM each of primers MT10 and MT12 and 0.625U *Taq* DNA polymerase.

Initial denaturation of template DNA was conducted for 3 min followed by 30 cycles of denaturation at 94°C for 30s, primer annealing at 52 °C for 30s and extension at 72 °C for 1 min. The final extension of 10 min was run at 72 °C and the reaction held at 4 °C in a Perkin Elmer DNA thermal cycler. Electrophoresis of PCR products was run in 1% agarose gel

stained in ethidium bromide in 1× TAE buffer in a submarine gel unit and visualized using UV light. PCR products of the expected 850 bp size were obtained. Bands were excised from the agarose gel and purified for DNA cloning using a Qiagen gel Purification Kit as per the manufacturer's procedure. Purified PCR products were cloned using the pGEM-T easy vector as per the manufacturer's instructions and sent to Bioscience Centre for Eastern and Central Africa (BecA), Nairobi for sequencing.

2.2.5 Phylogenetic analysis of mtCOI sequence

Whitefly mtCOI sequences were edited manually to produce a consensus sequence of 817 bp for each individual whitefly using the Editseq programme of DNAStar computer package (Version 5.03, DNASTAR Inc.). The edited sequences were aligned together with reference whitefly sequences obtained in the GenBank using Cluster W (weighted) (Thompson *et al*., 1994) algorithm option available in the MEGA 5.02 program (Tamura *et al*., 2011).

Aligned sequences were trimmed to about 650 bp and subjected to a heuristic search and subtree-pruning-regrafting branch swapping using maximum parsimony method available in MEGA 5.02. The ML tree was reconstructed using maximum parsimony optimality criterion with among-site rate variation corresponding with gamma distribution and a general-time – reversible substitution model with the rate matrix set to 1. For parsimony analysis, bootstrapping (Felsenstein, 1985) was performed with Phylogenetic Analysis Using Parsimony (PAUP) using the heuristic option for 1000 replication at a 70% confident limit (Swofford *et al*., 1998).

The following reference mtCOI sequences and their genbank accession numbers (indicated in brackets) were used for the analysis: Asia1 Thailand [AF164671]; Asia II China [AJ784261]; Asia II China [AJ783706]; Asia II China [AY686083]; Asia II China [AY686088]; Asia II China [AF418666]; Asia II 9 China Hunan [HM137313]; Asia II 10 China Guangdong [HM137356]; Asia II India [AJ748374]; Asia II Pakistan [AJ510065]; Asia III Taiwan [DQ174528]; Australia Bundaberg [GU086328]; Australia Indonesia [AB248263]; China [AY686085]; China [AY686091]; China 3 Yunnan [EU192050]; Italy [AY827596]; MedAmAF Pakistan [AJ510075]; MedAmAf BioB Reunion [AJ550177]; Med Syria [AB297897]; Ms Reunion [AJ550178]; New World Colombia [AJ550168]; SubsahAf1 Ug [AY057185]; SubSahAf1 Ug[AY057181]; SubsahAf1 Moz [AF344278]; SubsahAf1 SA [AF344264]; SubsahAf2 Ug [AY057194]; SubsahAF3 Cameroon [AF344257]; SubsahAf4 Cameroon [AF344247]; Uganda [AF418665]. The species used in the analysis as out group were *Bemisia afer* [GU220055] and *Bemisia subdecipens* [GU220056] (Dinsdale *et al*., 2010).

2.3 Results

2.3.1 Phylogenetic analysis of whitefly mtCOI sequences

A PCR fragment of the *mtCOI* gene (~850 bp) was obtained for each adult whitefly using the primer pair: MT10/C1-J-2195 and MT12/L2-N-3014 (Fig. 2). A consensus sequence was obtained for each mtCOI 850 bp nucleotide sequence for the 79 whiteflies. The sequences have been deposited in the GenBank database as accession numbers JQ286408 to JQ286487 (Table 1).

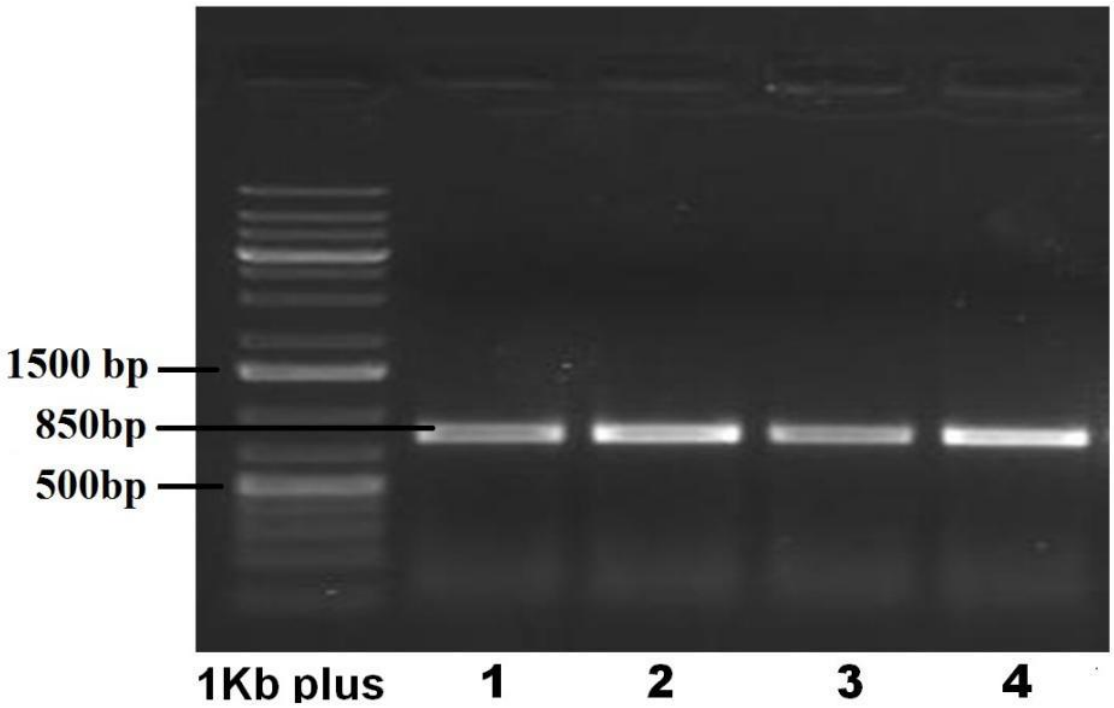


Figure 3 Agarose gel of PCR-amplified products of the expected 850 bp size. Lanes 1, 2, 3 and 4 are individual whitefly (*B. tabaci*) insects

Based on the phylogenetic analysis of reference mtCOI sequences, members of *B. tabaci* generally group into distinct species clusters in the New World or Old World (Dinsdale *et al*., 2010). The New World *B. tabaci* from Colombia is genetically distinct from the Old World members from Australia, Mediterranean/North Africa/Middle East, Southeast Asia/Far East/India and sub-Saharan Africa. Cassava-associated *B. tabaci* genotypes from Kenya grouped into the New World sub-Saharan Africa 1 (SSA-1) genetic clade with the exception of one genotype, which clustered with the SWIO genetic group (Delatte *et al*., 2011). Within the SSA-1 genetic group, the Kenyan genotypes clustered further in two sub-clades hereafter named sub-clade I and sub-clade II. Sub-clade I contained Kenyan genotypes with a sequence similarity of 97.9-99.7% with the published Ug1 genotypes (Fig. 3) that occurred ahead of the severe CMD epidemic-affected areas in the 1990s (Legg *et al.*, 2002). The sequences were 0.3-2.2% divergent (Table 2). Sub-clade II comprised of genotypes with 97.1-99.4% sequence similarity to the southern Africa (SA) genotypes from Mozambique and South Africa (Berry *et al*., 2004; Esterhuizen *et al*., 2012) (Fig. 3) and 0.6-2.9% sequence divergence (Table 2). Only one sequence clustered with the SWIO genetic group (Fig. 3) with 97.2-97.7% sequence similarity and 2.3-2.8% divergence (Table 2).

Phylogenetic analysis of the mtCOI *B. tabaci* from Tanzania grouped all the sequences into the SSA-1 genetic group with two sub-clades as was the case for the Kenyan sequences (Fig. 4). A pairwise comparison of mtCOI sequences of the sub-clade I Tanzanian *B. tabaci* genotypes revealed a sequence similarity of 97.9-99.7% with reference Ug1 genotypes, and a divergence of 0.3-2.2% amongst the sequences (Table 2). Sub-clade II genotypes shared 98-99.2% sequence similarity with SA genotypes (Fig. 4). The sequences diverged by 0.6-2% (Table 2). Similar to the Tanzanian grouping, results obtained for Ugandan *B. tabaci* grouped the genotypes into the SSA-1 genetic group with the sub-clades I & II (Fig. 5). Sub-clades I

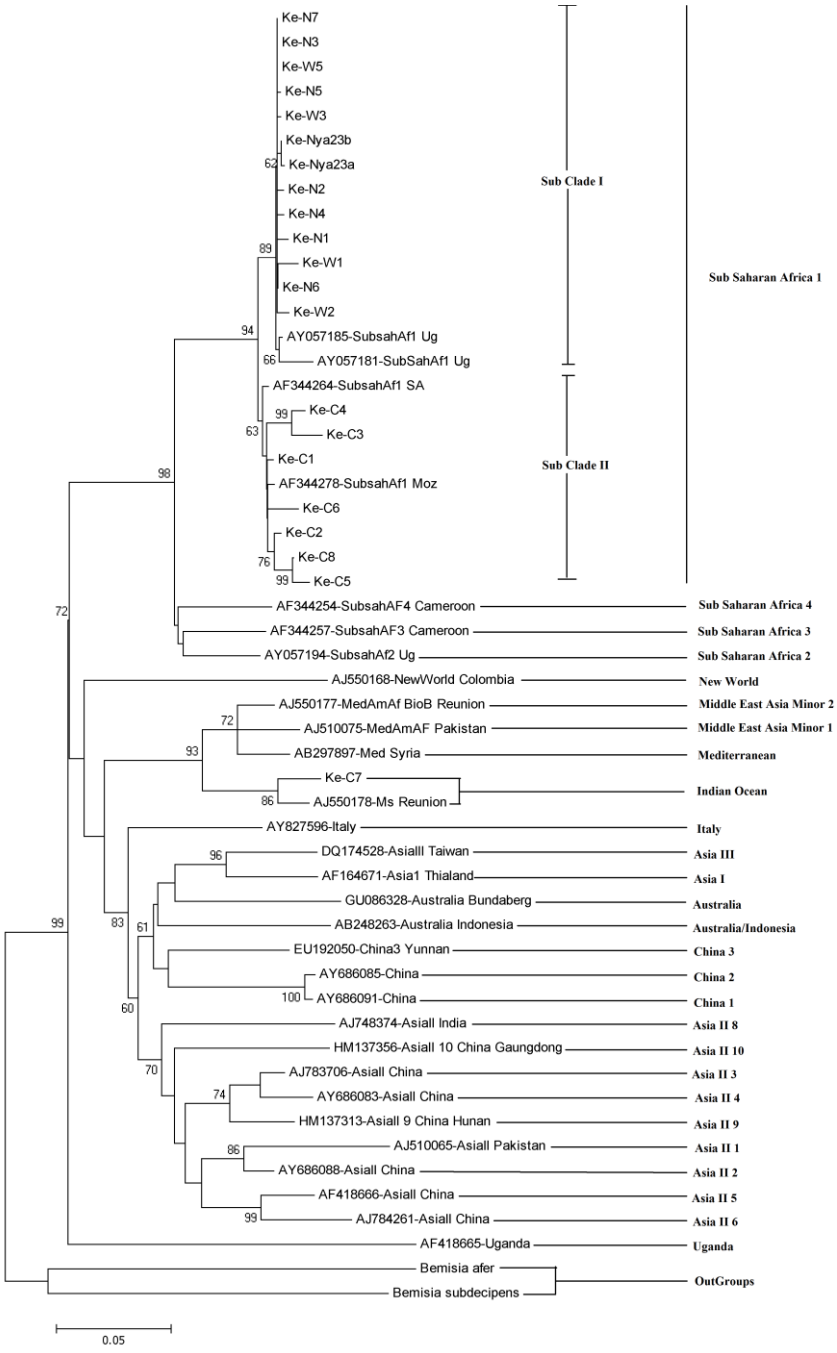


Figure 4 Phylogenetic tree based on the mitochondrial cytochrome oxidase I sequence for *B. tabaci* collected in Kenya generated using maximum likelihood available in MEGA5 software program. *B. subdecipens* and *B. afer* are included as outgroups. Whiteflies used in the study are labelled: Ke-N1, Ke-N2, Ke-N3, Ke-N4, Ke-N5, Ke-N6, Ke-N7, Ke-Nyz23a,KeNyz23b (Nyanza region); Ke-W1, Ke-W2, Ke-W3, Ke-W5 (Western region); Ke-C1, Ke-C2, Ke-C3, Ke-C4, Ke-C5, Ke-C6, Ke-C7, Ke-C8 (Coastal region).

Table 2 A pairwise comparison of the mitochondrial cytochrome oxidase I (mtCOI) nucleotide sequence (representatives), expressed as percent nucleotide divergence between adult *B. tabaci* populations identified on cassava in East Africa (Kenya, Tanzania and Uganda) as calculated by Clustal algorithm (Thompson *et al*., 1994) in 2010/11.

	Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	Ke-W2	-	0.8	0.5	3.3	2.0	3.7	19.3	0.9	0.6	0.5	2.0	1.7	1.5	0.6	1.1	2.0	1.9	2.5	2.5	2.2	0.8	1.5	2.0	20.3	20.9	9.5	24.6
2	Ke-N2		-	0.3	3.1	1.9	3.9	19.1	0.8	0.5	0.3	1.9	1.5	1.4	0.5	0.9	1.9	1.7	2.3	2.3	2.0	0.6	1.4	1.8	20.1	20.7	9.3	24.2
3	Ke-N3			-	2.8	1.5	3.6	18.7	0.5	0.2	0.0	1.5	1.2	1.1	0.2	0.6	1.5	1.4	2.0	2.0	1.7	0.3	1.1	1.5	19.7	20.3	9.0	24.0
4	Ke-C5				-	1.8	4.1	20.0	3.3	3.0	2.8	1.8	2.5	3.3	2.9	3.4	3.7	1.7	2.3	2.3	4.4	3.1	2.0	1.8	21.1	21.7	10.2	25.9
5	Ke-C1					-	2.8	18.5	2.0	1.7	1.5	0.6	1.2	2.0	1.7	2.2	2.5	0.5	1.1	1.1	3.1	1.9	0.8	0.6	19.5	20.1	9.0	24.7
6	Ke-C3						-	20.3	4.1	3.7	3.6	2.8	3.4	4.2	3.8	4.2	4.6	2.6	2.9	3.3	5.2	3.9	2.9	2.8	21.3	21.9	11.1	26.4
7	Ke-C7							-	19.3	18.9	18.7	18.1	18.6	19.1	18.9	19.3	19.3	18.3	17.7	18.4	20.5	18.7	18.5	18.4	2.3	2.8	18.3	25.2
8	Tz-10b								-	0.6	0.5	2.0	1.7	1.5	0.6	1.1	2.0	1.9	2.5	2.5	2.2	0.8	1.5	2.0	20.3	20.9	9.5	24.4
9	Tz-H9									-	0.2	1.7	1.4	1.2	0.3	0.8	1.7	1.5	2.2	2.2	1.8	0.5	1.2	1.7	19.9	20.5	9.2	24.2
10	Tz-H4										-	1.5	1.2	1.1	0.2	0.6	1.5	1.4	2.0	2.0	1.7	0.3	1.1	1.5	19.7	20.3	9.0	24.0
11	Tz-58b											-	1.2	1.7	1.7	2.2	2.5	0.5	1.1	1.1	3.1	1.9	0.8	0.6	19.1	19.7	9.0	23.8
12	Tz-70b												-	1.7	1.4	1.8	2.5	1.1	1.7	1.7	2.8	1.5	1.1	1.2	19.7	20.3	8.8	24.9
13	Tz-65a													-	1.2	1.7	2.6	1.9	2.5	2.5	2.6	1.4	1.5	2.0	20.1	20.7	9.7	24.0
14	Tz-43a														-	0.5	1.7	1.5	2.2	2.2	1.8	0.5	1.2	1.7	19.9	20.5	9.2	24.2
15	Ug-43d															-	2.2	2.0	2.6	2.3	2.3	0.9	1.7	2.2	20.3	21.0	9.5	24.5
16	Ug-43e																-	2.3	3.0	2.6	3.0	1.9	2.3	2.5	20.3	20.9	9.9	24.9
17	Ug-113b																	-	0.9	0.9	3.0	1.7	0.6	0.5	19.3	19.9	8.8	24.5
18	Ug-92a																		-	1.2	3.6	2.3	1.2	1.1	18.9	19.5	9.2	24.5
19	Ug-43b																			-	3.4	2.3	1.2	1.1	19.4	20.1	9.1	24.6
20	Sub Saharan Africa 1 Ug																				-	1.7	2.6	3.1	21.5	22.2	10.2	26.0
21	Sub Saharan Africa 1 Ug																					-	1.4	1.9	19.7	20.3	8.6	24.5
22	Sub Saharan Africa 1 SA																						-	0.8	19.5	20.1	9.0	24.7
23	Sub Saharan Africa 1 Moz																							-	19.4	20.1	9.0	24.6
24	Ms Reunion																								-	0.8	19.1	24.0
25	Ms Reunion																									-	19.7	24.2
26	Sub Saharan Africa 2 Ug																										-	24.9
27.	*Bemisia afer*																											-

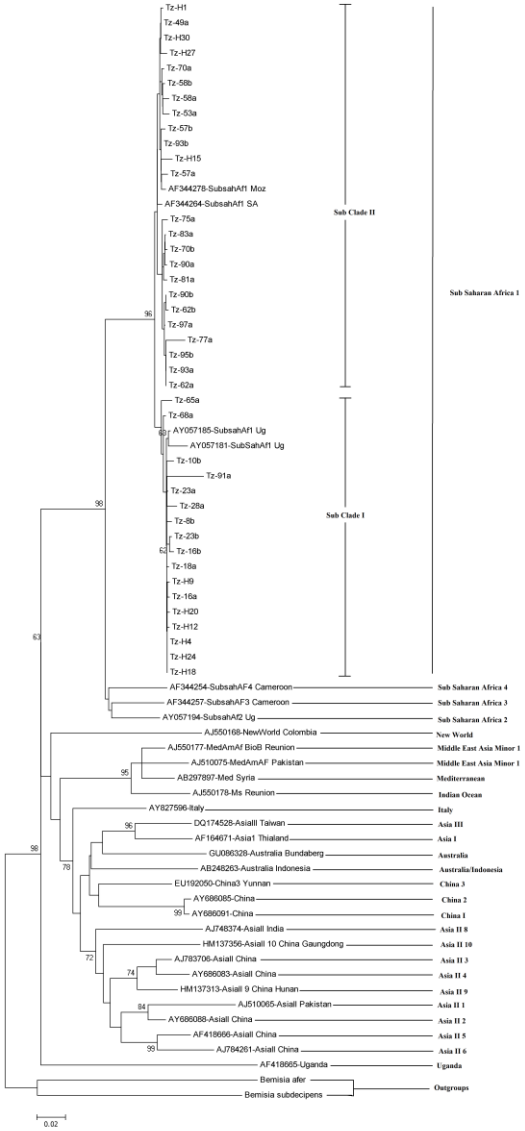


Figure 5 Phylogenetic tree based on the mitochondrial cytochrome oxidase I sequence for *B .tabaci* collected in Tanzania generated using maximum likelihood available in MEGA5 software program. *B. subdecipens* and *B. afer* are included as outgroups. Whiteflies used in the study are labelled: Tz8b, Tz10a, Tz16a, Tz16b, Tz18a, Tz23a, Tz23b, Tz28b, TzH9, TzH12, TzH15, TzH18, TzH20, TzH27, TzH30 (Lake Victoria Basin), Tz49a, Tz53b, Tz57a ,Tz57b, Tz58a, Tz58b, Tz75b, Tz95a, Tz95b, Tz97a, TzH4, TzH24 (Coastal region) and Tz62a, Tz62b, Tz65a, Tz68a, Tz70a, Tz70b, Tz75b, Tz77a, Tz81a, Tz83a, Tz90a, Tz91a, Tz93a, Tz93b (southern zone).

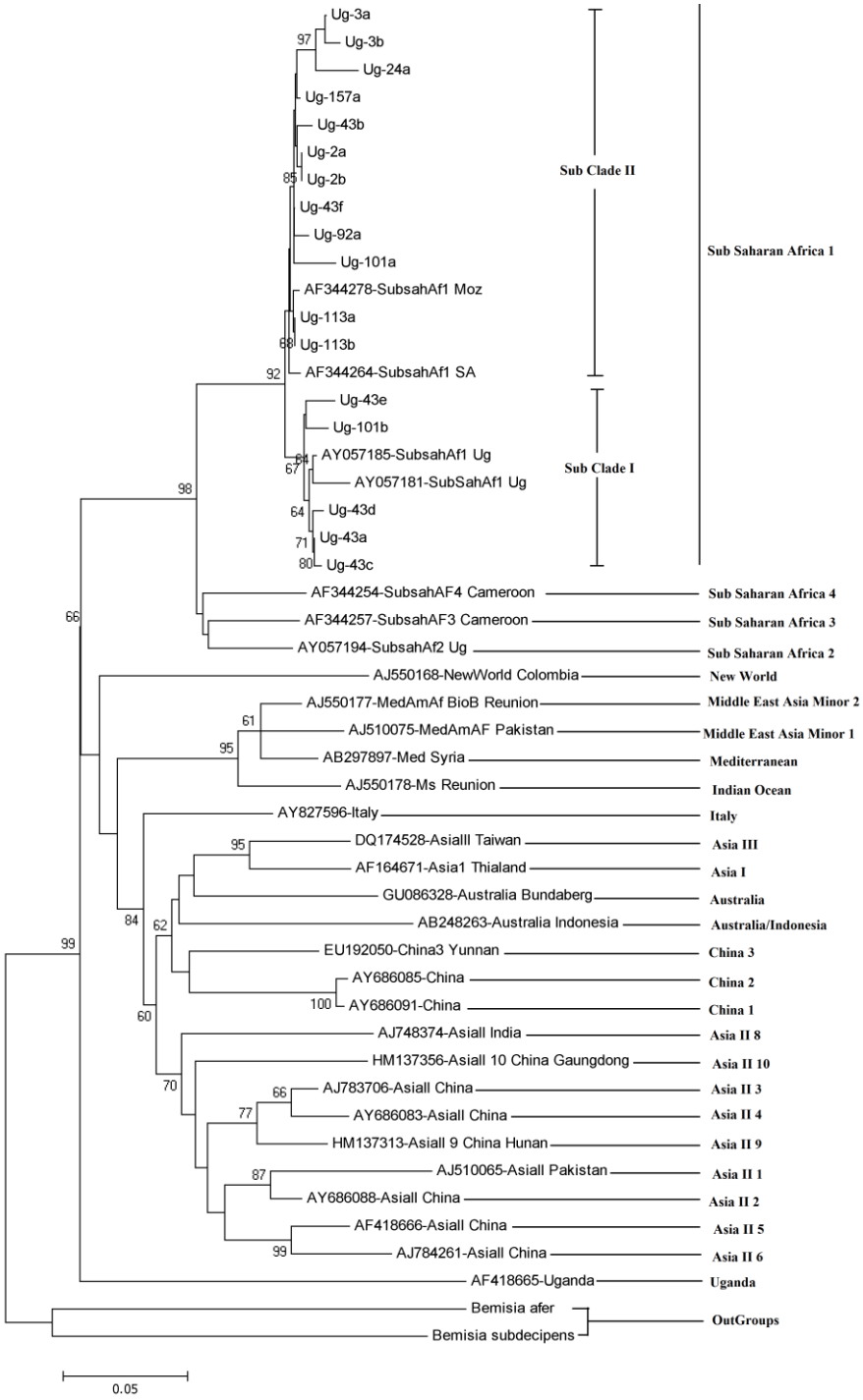


Figure 6 Phylogenetic tree based on the mitochondrial cytochrome oxidase I sequence for *B .tabaci* collected in Uganda generated using maximum likeilihood available in MEGA5 software program. *B. subdecipens* and *B. afer* are included as outgroup. Samples are labelled: Ug3a, Ug3b, Ug24a (East); Ug43a, Ug43b, Ug43c, Ug43d, Ug43e, Ug43f (West); Ug2a, Ug2b, Ug92a, Ug101a, Ug101b (North); Ug113a, Ug113b, Ug157a (Central) Uganda.



Figure 7 Phylogenetic tree based on the mitochondrial cytochrome oxidase I sequence for *B. tabaci* collected in East Africa (Kenya, Tanzania and Uganda) generated using maximum likelihood available in MEGA5 software program. *B. subdecipens* and *B. afer* are included as outgroup.

and II shared sequence similarities of 97.1-99.5% and 98.8-99.5% with Ug1 and SA genotypes, respectively. Within sub-clade I and II, the sequences diverged by 0.5-2.9% and 0.5-1.2%, respectively (Table 2).

A combined phylogenetic analysis of mtCOI sequences of *B. tabaci* from all three countries (Kenya, Tanzania and Uganda) grouped all the genotypes into SSA-1 genetic clade with exception of one genotype, which clustered with the SWIO genetic group (Fig. 6), confirming the results obtained for the individual countries. Sequences for *B. tabaci* in the two sub-clades (I & II) within SSA-1 genetic group were 0.3-2.8% divergent (Table 2). As expected, sequences of the Reunion *B. tabaci* from Kenyan, which grouped with SWIO genetic group diverged by 2.3-2.8% (Table 2).

2.3.2 Analysis of Molecular Variance (AMOVA)

A hierarchical analysis of molecular variance (ANOVA) (Excoffier *et al*., 2005) was conducted to assess the genetic differentiation of the *B. tabaci* populations in East Africa (Table 3). The two populations were grouped into the SSA-1 clade, with the two sub-clades (I & II), and SWIO with Reunion whitefly group. Comparative results from this study revealed significant differences among groups/clades ($P < 0.001$, FCT = 0.71529), among populations within groups ($P < 0.001$, FSC = 0.20534) and within populations ($P = 0.008$, FST = 0.77375). The highest contribution to the total variance was the differences among groups (71.53%). A similar result was obtained with the Tajima and Nei distance method (data not shown).

2.3.3 Geographical distribution of *B. tabaci* genotypes in East Africa

Results demonstrated a clear geographical distribution of two *B. tabaci* species belonging to the SSA-1 (sub-clade I and sub-clade II) and SWIO (Reunion) clades, in each country. The distribution within each region in the three countries was also evaluated. In Kenya, whiteflies were obtained in Western, Nyanza and Coast provinces. The predominant species in all three provinces was the SSA-1, which comprised of 61.9% (13/21) sub-clade I (Ug1-like) and 33.3% (7/21) sub-clade II (SA-like) genotypes. The SWIO (Reunion) species comprised only 4.8% (1/21) (Table 4). Between regions, the sub-clade I genotypes were predominant in Nyanza (69.2%) and lowest in Western province (30.8%). Sub-clade I genotypes were conspicuously absent in the Coast province. Interestingly, the sub-clade II genotypes occurred in the Coast province only. The SWIO genotype occurred only in the Coast province (Table 4), while sub-clade I was only detected in Western and Nyanza provinces and sub-clade II predominated in the Coast province.

Tanzanian whiteflies were obtained in three zones, including Lake Victoria Basin, Coast and Southern zones. The SSA-1 sub-clade I and II genotypes comprised 41.5% (17/41) and 58.5% (24/41) of the whiteflies from Tanzania, respectively (Table 4). Between region comparisons revealed that the sub-clade I (Ug1-like) genotypes were most abundant in the Lake Victoria Basin (88.2%). The sub-clade II (SA-like) genotypes were predominant in the southern zone (54.2%). Sub-clade I dominated in the Lake Victoria Basin (83.3%), whereas the sub-clade II genotypes dominated in the Coast and southern zones (Table 4).

Table 3 Hierarchical analysis of molecular variance and F-statistics of genetic differentiation for East African *B. tabaci* populations grouped according to species (groups), among populations within groups and within populations. The population structure was obtained using a pairwise difference distance method in ARLEQUIN version 3.1 (Excoffier et al. 2005).

Source of variation	**d.f.**	**Sum of Squares**	**Variance components**	**% of variation**	***F*-Statistics**	***P*-value[a]**
Among groups	1	98.812	40.95471 Va	71.53	0.71529	<0.0001
Among populations within group	1	141.357	4.34738 Vb	5.85	0.20534	<0.0001
Within populations	76	984.528	12.95432 Vc	22.63	0.77375	<0.0001
Total	78	1224.696	57.25641			

Va, variation due to differences among groups (Sub-clade I, Sub-clade II and Reunion); Vb, variation due to population differences within groups; Vc, variation due to population differences; [a]$P < 0.05$.

Table 4 Geographical distribution of *B. tabaci* species in Kenya, Tanzania and Uganda, 2010/2011. Without parenthesis is the comparison across regions within each country well as with parenthesis is the comparison between species/genotypes obtained within a region.

Country	**Region**	**Species occurrence (%)**			**No. of samples (n)**
		Sub Saharan Africa 1		**South West Indian Ocean**	
		Sub-clade I	**Sub-clade II**		
Kenya	Western	30.8(100)	0(0)	0(0)	4
	Nyanza	**69.2(100)**	0(0)	0(0)	9
	Coast	0(0)	**100(87.5)**	100(12.5)	8
		13	**7**	**1**	**21**
Tanzania	Lake zone	**88.2(83.3)**	12.5(16.7)	0(0)	18
	Coastal zone	0(0)	33.3(100)	0(0)	8
	Southern zone	11.8(13.3)	**54.2(86.7)**	0(0)	15
		17	**24**	**0**	**41**
Uganda	Western	**80(66.7)**	16.7(33.3)	0(0)	6
	Central	0(0)	**33.3(100)**	0(0)	3
	Eastern	0(0)	25.5(100)	**0(0)**	3
	Northern	20(25)	25.5(75)	0(20)	5
		5	**12**	**0**	**17**

In Uganda, whiteflies were obtained in four geographic regions: western, central, eastern and northern regions. The SSA1 sub-clade II genotypes were the most abundant with 70.6% (12/17) occurrence (Table 4). Between regions sub-clade I genotypes were most abundant in the western region (80%). Interestingly, no sub-clade I genotypes were detected in the central and eastern regions in this study. The sub-clade II genotypes occurred highest in central (33.3%) region and occurred in equal proportions in eastern and northern regions. Sub-clade I dominated in western region with 66.7% occurrence. On the other hand, sub-clade II was the dominant type in central, eastern and northern regions (Table 4).

2.4 Discussion

Using the mtCOI gene (Frohlich *et al*., 1999) as the molecular marker, our study reports the occurrence of two *B. tabaci* species belonging to two distinct clades/groups of whiteflies, namely sub-Saharan Africa 1 (SSA-1) and South West Indian Ocean Islands (SWIO), on cassava crops in Kenya, Tanzania and Uganda. Phylogenetic trees were predicted using both maximum parsimony and the maximum likelihood methods with similar results, but only the maximum likelihood results are discussed. Genetic differentiation of the cassava-associated East African *B. tabaci* populations using AMOVA had the highest contribution to the total variance as differences among groups, which corroborates the results obtained with mtCOI sequence phylogenetic analysis. The SSA-1 species had two closely related sub-clades (I and II), which were earlier reported on cassava as Uganda 1 (Ug1) and southern Africa (SA) genotypes in Uganda (Legg *et al*., 2002) and southern Africa (Berry *et al*., 2004; Esterhuizen *et al*., 2012), respectively.

We reported here for the first time the occurrence of a Reunion whitefly species that clustered among the SWIO genetic group on cassava in Kenya. It is not clear whether this whitefly can reproduce on cassava as only the adults were used for the mtCOI analysis in this study. A Reunion (Ms) whitefly was also reported to colonise a number of non-cassava plants species, including *Commelina benghalensis*, *Gossypium hirsutum* and *Phaseolus vulgaris* in Uganda (Sseruwagi *et al*., 2005a). It is possible that the Reunion whitefly was just 'visiting' or 'feeding' at the time of sampling cassava. More definitive studies should be carried out to ascertain the colonisation status of cassava by these whiteflies.

There was a clear geographical distribution of the cassava *B. tabaci* species in the East African region (Kenya, Tanzania and Uganda). Previous studies reported the sub-clade I genotypes to occur widely in areas ahead of the severe CMD pandemic 'front' indigenous populations, and were associated with very low numbers (Legg *et al*., 2002; Maruthi *et al*., 2004). However, in the current study, the SSA-1 sub-clade I genotypes were not only widespread in Western and Nyanza provinces (Kenya), the Lake Victoria Basin and Southern zone (Tanzania), and western and northern regions (Uganda), but they also occurred in high population abundance (data not presented).

Previously the super abundant whitefly populations were a characteristic of the severe CMD pandemic (Legg, 1999; Otim-Nape *et al*., 2000; Colvin *et al*., 2004), which was attributed in part due to entry into Uganda of an invasive whitefly species of the sub-Saharan Africa 2 (SSA-2) genetic group with closest relatives in Cameroon, commonly referred to as the 'invader/Ug2' (Legg *et al*., 2002). Interestingly, we did not detect the SSA-2 whitefly species on cassava in any of the three countries in the current study. The diminishing occurrence of the SSA-2 whiteflies in the severe CMD-affected areas in Uganda was reported

(Sseruwagi, 2005). A likely explanation for their complete absence in the current study and the resurgence of the SSA-1 (sub-clade 1) whitefly species in high populations in EA could be the result of backcrosses between the indigenous whitefly population (SSA-1 sub-clade I) and the SSA-2 invasive population, that resulted in a hybrid population with SSA-1 (sub-clade I) mtCOI and the invasive traits of the SSA-2 species (J.K. Brown, personal communication). Further studies are required to affirm the hybrid hypothesis.

On the other hand, the complete absence of the SSA-1 (sub-clade I) genotypes in Coast Kenya and Tanzania, and central and eastern Uganda could possibly be due to displacement by the SSA-1 (sub-clade II) genotypes, which comprised a majority of the whiteflies in these areas. Elsewhere, population increase of *B. tabaci*, particularly in areas where whiteflies were previously unimportant, was attributed to the appearance of new 'biotypes'/species complexes (Bedford *et al.*, 1994; Brown, 2001). For example, in south-western United States of America (USA), the B-biotype/Middle East-Asia Minor species (De Barro *et al*., 2011) was introduced in the region through ornamental plants (Brown *et al*., 1995b; Frohlich *et al*., 1999), where it increased steadily in distribution and abundance, ultimately displacing the 'local' A-biotype/New World species (Costa *et al*., 1993; De Barro *et al*., 2011). On the other hand, the Middle East-Asia Minor species (B-biotype), which invaded southern Spain failed to, displace the Q-biotype/Mediterranean indigenous species (Moya *et al*., 2001; De Barro *et al*., 2011). Displacement of indigenous *B. tabaci* species by the invasive Middle East-Asia Minor (B-biotype) and Mediterranean (Q-biotype) species has also been reported recently in China (Xu, 2009; Chu *et al*., 2010; Crowder *et al*., 2010; Wang *et al*., 2011), and the Q-biotype has recently been reported in South Africa (SA) (Esterhuizen *et al*., 2012).

We report for the first time the occurrence of SSA-1 (sub-clade II) whitefly in East Africa. These whiteflies predominated in Coast Kenya, Southern and coast Tanzania, and were low in the Lake Victoria Basin of Tanzania, and widespread in all four regions in Uganda. The SSA-1 (sub-clade II) may be the indigenous whitefly species in Coast Kenya, Southern and Coast Tanzania. It is generally believed to be the indigenous whitefly in southern Africa, including SA, Malawi and Mozambique (Berry *et al*., 2004; Esterhuizen *et al*., 2012); countries that share common boundaries and climate with Tanzania. However, the occurrence of the SSA-1 (sub-clade II) whiteflies in the Lake Victoria Basin of Tanzania and in Uganda was unexpected and requires further investigation.

In conclusion, our results indicate that the two SSA-1 sub-clades (I & II), which group together as a single species in the SSA-1 *B. tabaci* genetic group due to their less than 3.5% divergence in the mtCOI (Dinsdale *et al*., 2010), could differ in important aspects of their biology, such as fecundity, virus transmission, and mating ability. This requires further investigation.

CHAPTER THREE

Two haplotypes of sub-Saharan Africa 1 *Bemisia tabaci* associated with cassava exhibit distinct biological differences

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Key words: Whitefly, eggs, instars, fecundity, development

Abstract

A study was conducted to determine the number of eggs laid and instar development of two cassava *Bemisia tabaci* haplotypes belonging to sub-Saharan Africa 1 (SSA1) genetic group in East Africa. Healthy and cassava mosaic begomovirus (CMB)-infected [*African cassava mosaic virus-[Tanzania: 2001]* (ACMV-[TZ: 01]) & *East African cassava mosaic virus-Kenya* (EACMV-KE)] plants were used under screenhouse conditions at Mikocheni Agricultural Research Institute (MARI), Tanzania in 2011-12. Two haplotypes, sub-clade I and II of SSA1 were obtained from Lake Victoria Basin and Coast Region, respectively, in Tanzania and their colonies established at MARI. Single female 1-5 day old adults of sub-clade I and II haplotypes were allowed to feed and oviposit on healthy and CMB-infected cassava for 3 days. Sub-clade I performed significantly better than sub-clade II haplotypes in mean number of eggs laid, developing instars and hatched adults on Healthy and CMB-infected plants in the first and repeat trials. Sub-clade I and II haplotypes produced 33.52 & 23.59 (eggs), 27.02 & 16.74 (1^{st} instars), 24.44 & 14.66 (2^{nd} & 3^{rd} instars), 23.83 & 13.62 (4^{th}

instars) and 22.29 & 13.13 (hatched adults), and 30.94 & 23.63 (eggs), 22.40 & 17.32 (1^{st} instars), 19.91 & 15.21 (2^{nd} & 3^{rd} instars), 19.11 & 14.12 (4^{th} instars) and 18.05 & 13.55 (hatched adults) in the first and repeat trials, respectively. Generally, number of eggs laid, developing instars and hatched adults were not significantly different on healthy and CMB-infected cassava in both trials. Highest mortality for sub-clade I and II haplotypes occurred on EACMV-KE and ACMV-[TZ: 01]-infected plants, respectively. Survivorship was higher on healthy than CMB-infected cassava plants for both haplotypes. There was no boost in whitefly numbers by the CMB-infected plants. These results clearly show that the SSA1 sub-clade I and II haplotypes differ in fecundity and development.

3.1 Introduction

Bemisia tabaci, (Gennadius) (Hemiptera: *Aleyrodidae*) is among the most important agricultural pests feeding on over 700 host plant species (Mann *et al*., 2009). It is a key vector for plant viruses, including cassava mosaic begomoviruses (CMBs) (Storey and Nichols 1938) and cassava brown streak viruses (CBSV) (Maruthi *et al*., 2005), two viral diseases devastating cassava in Africa (Legg *et al*., 2011).

First reports of the severe form of CMD, which affected major cassava growing areas of central Uganda, occurred in 1988 (Otim-Nape *et al*., 2001). Subsequently, the severe disease spread southwards ravaging cassava fields. It was discovered that the severe disease was caused by *East Africa cassava mosaic virus-Uganda* (EACMV-UG) (Zhou *et al*., 1997), a recombinant of *East African cassava mosaic virus* (EACMV) and *African cassava mosaic virus* (ACMV). The 'severe CMD epidemic' was characterized by unusually severe disease

symptoms (Gibson *et al*., 1996), high whitefly populations (Otim-Nape *et al*., 1997; Colvin *et al*., 1999) and rapid disease spread (Legg & Ogwal 1998).

The unusually high whitefly populations associated with the 'severe CMD pandemic' in Uganda were attributed to: (1) appearance of an 'invasive' whitefly referred to as Uganda 2 (Ug2) (Legg *et al*., 2002) or currently as 'sub Saharan Africa 2' (SSA2) (Dinsdale *et al*., 2010) believed to be more fecund than the 'local' population, Uganda 1 (Ug1) (Legg *et al*., 2002); (2) changes in amino acid (asparagines, glutamine, tryptophan and tyrosine) levels in EACMV-UG-infected cassava plants (Colvin *et al*., 2004); (3) yellowing symptoms in CMD-symptomatic leaves which attract whiteflies (Omongo, 2003); and (4) an increase in the area (acreage) of whitefly-susceptible cassava varieties in Uganda reported to attract huge populations (Omongo *et al*., 2004, 2012).

Elsewhere, other studies on plant-pathogen-vector interactions, biology of whitefly super-abundance and displacement of indigenous by invasive species demonstrated positive, negative or neutral effects of viruses on their insect vectors. Guo *et al*. (2010) reported a significant increase in eggs laid by invasive B biotype whiteflies feeding on tobacco infected with *Tomato yellow leaf curl China virus* (TYLCCV) but not on tomato. The B/Middle East-Asia Mirror 1 and Q/Mediterranean whitefly performed better on virus-infected than on uninfected tobacco plants in contrast to the indigenous ZHJ1/Asia II 2 and ZHJ2/Asia II 1 whiteflies (Liu *et al*., 2009). However, Thompson (2002) found no significant increase in the population of cassava *B. tabaci* fed and let to develop on healthy and EACMV-infected plants at Namulonge; a hot spot of severe CMD in Uganda in the 1990s.

A key feature of the severe CMD-affected areas remains the high whitefly populations on cassava plants. For example, the Lake Victoria Basin (LVB) in Tanzania is still the hot spot for the severe CMD caused by EACMV-UG and dual infections of ACMV-[TZ:01] & EACMV-UG, while the Coast Region (CR) is exclusively affected by a single CMB species; EACMV-KE (Ndunguru & Tairo, 2010). Recently, data obtained from country-wide surveys on CMD in Tanzania indicated high whitefly populations on cassava plants in LVB and very low populations in CR (Ndunguru & Tairo, 2010). Further, two genetically distinct *B. tabaci* haplotypes (sub-clade I and II) of SSA1 were reported on cassava in the LVB and CR in Tanzania, respectively (Mugerwa *et al*., 2012). These results suggest the geographic distribution of cassava *B. tabaci* haplotypes with particular CMB species.

The current study whose results are reported here, tested the hypothesis that the two cassava *B. tabaci* haplotypes (sub-clades I and II) of SSA1 have distinct biological traits (fecundity and development). The findings add to the body of knowledge explaining the possible causes of superabundant whitefly populations on cassava in East Africa.

3.2 Methods and materials

3.2.1 Study area

Two experiments were conducted in a screenhouse at Mikocheni Agricultural Research Institute (MARI), Dar es Salaam, Tanzania in 2011-12. MARI is 70m above sea level and screenhouse day temperature and relative humidity (RH) between April and June 2012 ranged from between 25 & 27°C and 74 & 82%, respectively (Table 5).

3.2.2 Raising of CMD-free cassava plants

Twenty eight days old cassava tissue culture plants of a local Tanzanian cultivar 'Kibandameno' were acclimatized in sterile soil media for 14 d in a humid chamber. Thereafter, they were transferred to larger buckets (5 cm in diameter) and maintained in a screenhouse at 25 & 27°C and 74 & 82%, respectively (Table 5).

3.2.3 Raising of CMD-affected cassava plants

Mature stem cuttings (10 cm) were collected from CMD-symptomatic cassava plants of a local susceptible cultivar in Bukoba, Lake Victoria Basin (LVB) and Bagamoyo, Coast Region (CR) in Tanzania. The predominant CMBs were identified as ACMV-[TZ: 01] and EACMV-UG in LVB and EACMV-KE in CR. The stem cuttings were grown in soil in buckets and were maintained in separate screenhouses until use in the experiments

3.2.4 Establishing *B. tabaci* colonies

Fourth instar nymphs of the SSA1 sub-clade I and II haplotypes were collected from cassava plants in LVB and CR, respectively in 2011. The nymphs were kept on moistened tissue in petri-dishes during transportation from field to MARI until they emerged. One day old whitefly adults were allowed to feed, oviposit and establish separately on virus-free cassava plants of Kibandameno in confined cages. The whitefly colonies were maintained regularly and the temperature and RH recorded daily for the period of the study (Table 5).

Table 5 Day temperature and Relative Humidity in screenhouses used for the fecundity study at MARI, Dar es salaam, Tanzania between March to April and May to June, 2012

Temp/RH	**First trial**			**Repeat trial**		
	April	**May**	**Mean**	**May**	**June**	**Mean**
Temp (°C)	26.1	26.3	**26.2**	26.5	25.2	**25.9**
RH (%)	81.0	81.8	**81.4**	76.4	72.8	**74.6**

3.2.5 Determining virus identity in cassava plants and whiteflies

In order to confirm the virus identity in the CMD-symptomatic cassava plants raised from collections in LVB and CR, DNA was extracted from leaves of 14 d old plants using the method of Dellaporta *et al*., (1983). Further, to ensure that the whiteflies ingested the viruses during the 3 d they fed and oviposited on CMB-infected plants, 10 randomly selected whiteflies per treatment were tested for virus presence using specific PCR.

PCR amplification of cassava begomoviral DNA was conducted with ACMV-[TZ: 01] (JSP001 & JSP002), EACMV-KE (EAB555-F & EAB555-R) and EACMV-UG (UV-AL1/R & ACMV-CP/R3) specific primers amplifying products of 776, 506, and 1700 base pairs (bp), respectively (Fondong *et al*., 2000). A DNA template of 2µl was used in a PCR reaction mixture of 25 µl, containing 1× *Taq* buffer with Mg^{2+}, 0.2 mM deoxynucleotide triphosphate (dNTPs), 0.2 mM each of primers and 0.625U *Taq* DNA polymerase. Initial denaturation of template DNA was conducted for 3 min followed by 30 cycles of denaturation at 94^{o}C for 1 min, primer annealing at 56 ^{o}C for 1 min and extension at 72 ^{o}C for 2 min. The final extension of 10 min was run at 72 ^{o}C and the reaction held at 4 ^{o}C in a Perkin Elmer DNA thermal cycler. Electrophoresis of PCR products was run in 1% agarose gel stained in ethidium bromide in 1× TAE buffer in a submarine gel unit and visualized using UV light.

3.2.6 Analyzing mtCOI to confirm whitefly colony purity

Five adult whiteflies were picked randomly from each colony and lyzed to release total DNA following the method of Frohlich *et al*. (1999). Polymerase chain reaction was performed with universal primers: MT10/CI-J-2195 and MT12/L2-N-3014 (Simon *et al*., 1994) for

amplification of a fragment (~850bp) of the mtCOI gene as described in Mugerwa et al. (2012). Bands were excised from the agarose gel and purified for DNA cloning using a Qiagen gel Purification Kit (QIAGEN, Venlo, the Netherlands) as per the manufacturer's procedure. PCR products were cloned and sequenced at BecA, Nairobi, Kenya.

Whitefly mtCOI sequences were manually edited using the Editseq programme available in the DNASTAR software package (DNASTAR, Madison, Wisconsin) to produce a consensus sequence (~780-800) for each individual adult insect. Sequences were aligned using the Clustal W together with *B. tabaci* references available in the EMBL/DDBJ/GenBank databases. A heuristic search and subtree-pruning-regrafting branch swapping was conducted using maximum likelihood (ML) and parsimony methods (Swofford, 1998).

3.2.7 Experiment procedure

Treatments included: healthy (CMB-free), ACMV-[TZ: 01] and EACMV-KE-infections planted out in ten plants per treatment. Single infections of each virus were used for the study after indexing of the CMB-infected stems. The experiment was repeated once.

Newly emerged 1-4 d old adult whiteflies were picked from established pure colonies for sub-clade I and II using an aspirator. In order to select females, the whiteflies were treated with carbon dioxide gas for 3 to 5 sec and placed on a green tissue paper on ice in a petri-dish to keep them unconscious. Single females (individuals with larger abdomen) were introduced separately into clip cages (3cm in diameter and depth) attached on the underside of fully-formed young leaves on healthy, ACMV-[TZ: 01] and EACMV-KE-infected plants. Care was taken to attach the clip cages on CMD-symptomatic leaves on the virus-infected plants.

Each whitefly was allowed to feed and oviposit for 3 d after which it was removed and stored in 70% ethanol until PCR analysis to confirm ingestion of the viruses using 10 randomly selected whiteflies per treatment.

3.2.8 Collecting and analyzing data

Data were collected on fecundity and whitefly development. Fecundity was determined by counting the number of eggs oviposited by each female over 3 d using a hand-held lens. Conversely, development was determined by counting the number of hatched 1^{st} instars, combined 2^{nd} & 3^{rd} instars, 4^{th} instars and hatched adults at 9-11 d, 17 d, 20 d and 21-26 d, respectively.

Data were tested for variation using a two-way analysis of variance (ANOVA) at 5% significance using GenStat discovery edition 4 statistical package (version 10.3, VSN International Ltd). Mortality (K-value) was calculated as the difference in the mean values of the successive developmental stages: eggs, 1^{st} instars, 2^{nd} &3^{rd} instars and 4^{th} instars as K_1, K_2, K_3, K_4, respectively.

3.3 Results

3.3.1 Colony establishment and analysis of *B. tabaci* mtCOI sequences

Whiteflies collected from the LVB took two generations (~52 d) to establish. In contrast, it took several attempts to establish the CR colony. PCR was conducted to confirm the genetic identities of the two colonies and the sequences deposited in the GenBank database as accession numbers KC207857 to KC207861 (LVB) and KC207862 to KC207866 (CR).

Phylogenetic analysis of the mtCOI sequences grouped the LVB and CR colonies within SSA1 with two sub-clades I and II as expected. Sub-clade I comprised of haplotypes from LVB and shared a nucleotide (nt) sequence similarity of 98.8-99.7% and divergence of 0.3-1.2% with the published Uganda 1 (Ug1) haplotypes (Fig. 7). On the other hand, sub-clade II comprised haplotypes from CR and shared nt sequence similarity of 99-100% and divergence of 1% with the published southern Africa haplotypes (Fig. 7).

3.3.2 PCR analysis of CMBs in cassava plants and whiteflies

Using the virus species-specific JSP001/2 and EAB555F&R PCR primers, ACMV-[TZ: 01] (776 bp) and EACMV-KE (506 bp) were confirmed in the CMD-symptomatic plants. Further, ACMV was detected in 40% each of sub-clade I and II haplotypes. On the other hand, EACMV-KE was detected in 40% and 60% of the sub-clade I and II, respectively.

3.3.3 Number of eggs

In the first trial, an average of 28.56 eggs was laid per single female whitefly at 3 d by the sub-clade I and II haplotypes (Table 6). Significantly ($P = 0.008$) more eggs were laid by sub-clade I (33.52) than sub-clade II (23.59) haplotypes. Slightly fewer eggs (27.29) were laid per female in the repeat trial. Similar to the first trial, sub-clade I (30.94) laid significantly ($P = 0.009$) more eggs than sub-clade II (23.63) whitefly haplotypes (Table 6). There were no significant differences ($P>0.05$) in the number of eggs laid by sub-clade I and II haplotypes on healthy, ACMV-[TZ: 01] and EACMV-KE-infected cassava plants in both the first and repeat trials (Table 7 & 8).

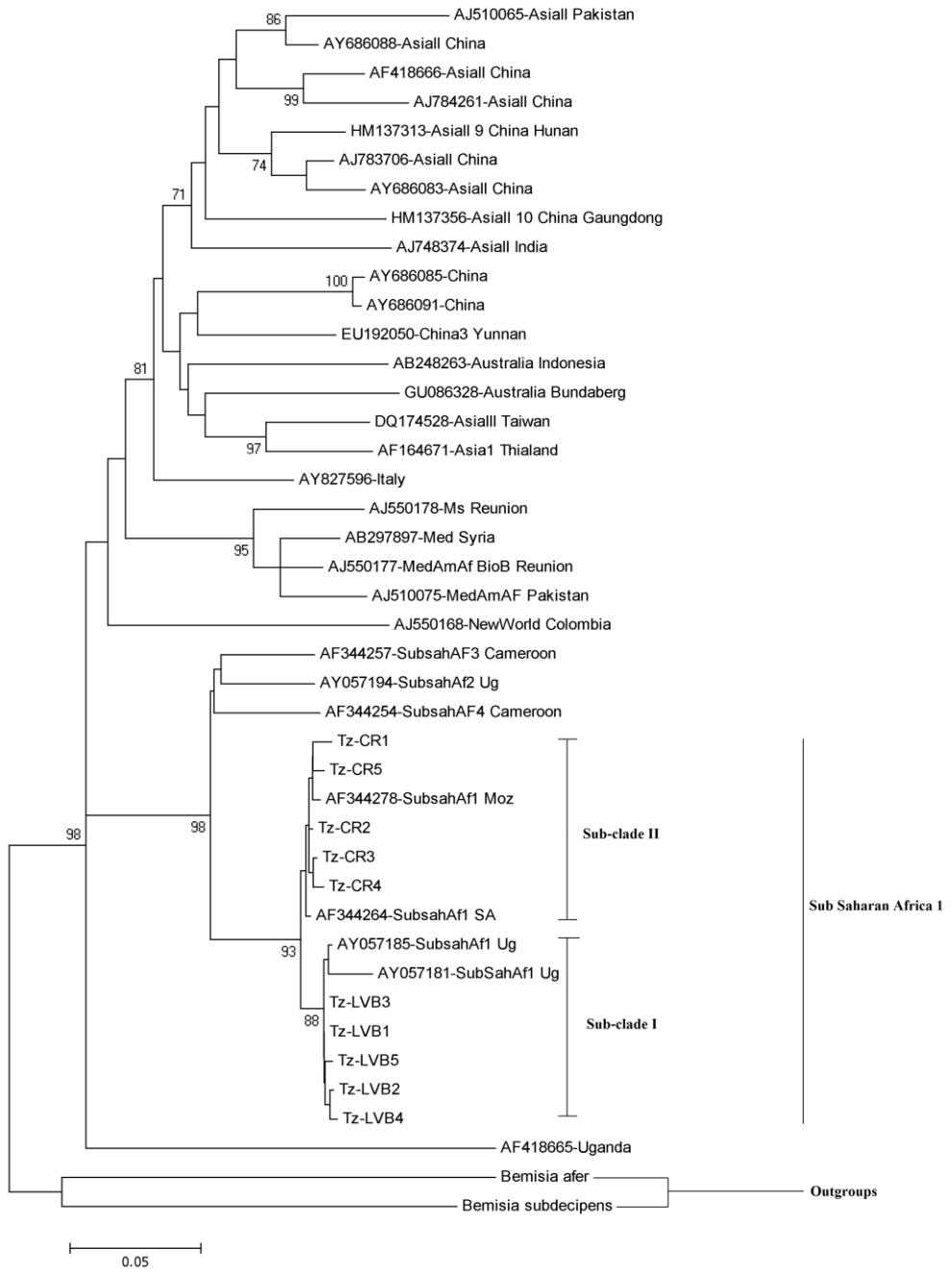


Figure 8 Phylogenetic tree of *Bemisia tabaci* SSA1 whitefly used in the fecundity study on cassava plants with healthy and CMB-infected based on the mitochondrial cytochrome oxidase I sequence generated using maximum likelihood available in MEGA5 software program. *B. subdecipens* and *B. afer* are included as outgroups. Whiteflies used in the study are labelled (Tz-LVB1, Tz-LVB2, Tz-LVB3, Tz-LVB4 and Tz-LVB5) from Lake Victoria Basin and (Tz-CR1, Tz-CR2, Tz-CR3, Tz-CR4 and Tz-CR5) from Coast region

Table 6 Number of eggs, nymphs and adults produced by a single female *B. tabaci* SSA1 whitefly from Lake Victoria Basin (sub-clade I) and Coast region (sub-clade II) of Tanzania over 3 days on healthy, ACMV-[TZ: 01] and EACMV-KE infected plants in the first trial, between April and May 2012

SSA1 Whitefly haplotypes	First trial					Repeat trial				
	Eggs	1st instar	2nd & 3rd instars	4th instar	Hatched adults	Eggs	1st instar	2nd & 3rd instars	4th instar	Hatched adults
Sub-clade I	33.52	27.02	24.44	23.83	22.29	30.94	22.4	19.91	19.11	18.05
Sub-clade II	23.59	16.74	14.66	13.62	13.13	23.63	17.32	15.21	14.12	13.55
Mean	**28.56**	**21.88**	**19.55**	**18.73**	**17.71**	**27.29**	**17.20**	**17.56**	**16.62**	**15.80**
P-value (5%)	**0.008**	**0.005**	**0.005**	**0.002**	**0.005**	**0.009**	**0.048**	**0.048**	**0.033**	**0.048**
LSD	**7.787**	**6.796**	**6.374**	**6.180**	**5.793**	**5.498**	**4.864**	**4.600**	**4.467**	**4.209**

3.3.4 Developmental instars

Developmental instars were assessed at 11 d, 17 d and 20 d for 1st, 2nd & 3rd and 4th instar, respectively for both sub-clades I and II *B. tabaci* haplotypes (Table 6). Generally, significant differences were observed in the number of 1st (P = 0.005 & 0.048), 2nd & 3rd (P = 0.005 & 0.048) and 4th (P = 0.002 & 0.033) instars between sub-clade I and II haplotypes in the first and repeat trials, respectively. On average 21.88 & 17.20 (1st), 19.55 & 17.56 (2nd & 3rd) and 18.73 & 16.62 (4th) instars were produced in the first and repeat trials (Table 6).

Comparison of the development of the immature instars on healthy, ACMV-[TZ: 01] and EACMV-KE-infected plants was significant for only the sub-clade II haplotypes in the first trial (Table 7). There was a drastic decline in the number of developmental instars (1st, 2nd & 3rd and 4th) on ACMV-[TZ: 01]-infected plants compared with the healthy and EACMV-KE-infected plants (Table 7). No significant differences were observed in the number of immature instars that developed on all the treatments for both sub-clades I and II haplotypes in the repeat trial (Table 7 & 8).

3.3.5 Hatched adults

Analysis of the number of hatched adult whiteflies showed significant differences (P = 0.005 & 0.048) between sub-clade I and II haplotypes in the first and repeat trials (Table 6). Significantly more hatched adults were produced by sub-clade I (22.29 & 18.05) than sub-clade II (13.13 & 13.55) haplotypes in the first and repeat trials. The number of hatched adults was significantly (P = 0.041) higher on healthy (17.42) and EACMV-KE (16.75) than ACMV-[TZ:01] (3.83) infected plants for sub-clade II haplotypes only in the first trial (Table

Table 7 Whitefly number of eggs, nymphs and adults produced by a single female *B. tabaci* SSA1 species from Lake Victoria Basin (sub-clade I) and Coast region (sub-clade II) of Tanzania over 3 days on healthy, ACMV-[TZ: 01] and EACMV-KE-infected plants, in the first and repeat trials conducted between April and May 2012

Cassava mosaic begomovirus status	Sub Saharan Africa 1 whitefly species									
	Sub-clade I (Lake Victoria Basin)					Sub-clade II (Coast Region)				
	Eggs	1st instar	2nd & 3rd instars	4th instar	Hatched adults	Eggs	1st instar	2nd & 3rd instars	4th instar	Hatched adults
Healthy	33.70	28.00	26.20	25.50	24.60	24.17	21.20	19.08	17.90	17.42
ACMV-[TZ: 01]	31.00	27.30	23.20	22.90	21.50	17.50	7.00	4.50	3.83	3.83
EACMV-KE	35.95	25.27	23.18	22.36	19.73	29.25	20.50	19.00	17.75	16.75
Mean	**33.52**	**27.02**	**24.44**	**23.83**	**22.29**	**23.59**	**16.74**	**14.66**	**13.62**	**13.13**
P-value (5%)	**0.588**	**0.842**	**0.758**	**0.765**	**0.519**	**0.532**	**0.091**	**0.055**	**0.051**	**0.046**
LSD	**9.76**	**9.91**	**9.38**	**9.24**	**8.68**	**17.21**	**13.18**	**12.23**	**11.62**	**10.87**

Table 8 Number of eggs, nymphs and adults produced by a single female *B. tabaci* SSA1 whitefly from Lake Victoria Basin (sub-clade I) and Coast Region (sub-clade II) of Tanzania over 3 days on healthy, ACMV-[TZ: 01] and EACMV-KE-infected plants in the repeat trial conducted between May and June 2012

Cassava mosaic begomovirus status	Sub Saharan Africa 1 whitefly species									
	Sub-clade I (Lake Victoria Basin)					Sub-clade II (Coast Region)				
	Eggs	1st instar	2nd & 3rd instars	4th instar	Hatched adults	Eggs	1st instar	2nd & 3rd instars	4th instar	Hatched adults
Healthy	34.50	24.00	22.15	21.10	20.35	23.29	19.62	16.58	15.67	15.17
ACMV-[TZ: 01]	26.50	20.35	17.25	16.90	16.05	22.17	11.42	9.67	9.08	8.83
EACMV-KE	30.46	22.26	19.48	18.55	16.85	25.62	20.13	19.00	17.13	16.12
Mean	**30.94**	**22.40**	**19.91**	**19.11**	**18.05**	**23.41**	**17.32**	**15.21**	**14.12**	**13.55**
P-value (5%)	**0.149**	**0.612**	**0.357**	**0.452**	**0.344**	**0.875**	**0.161**	**0.185**	**0.218**	**0.227**
LSD	**7.87**	**7.10**	**6.56**	**6.46**	**6.03**	**10.53**	**9.01**	**8.76**	**8.36**	**7.96**

7). However, the CMB treatments had no significant effect on the number of hatched adults for both sub-clade I and II haplotypes in the repeat trial (Table 8).

3.3.6 Mortality and mortality rates

Whitefly mortality (K_T = 16.22) was highest for sub-clade I immature instars on EACMV-KE- infected plants. In contrast the highest mortality (K_T = 13.67) occurred on the ACMV-[TZ:01] infected plants for sub-clade II immature instars.

Most deaths of sub-clade I haplotypes occurred between egg deposition and 1st instars on both the healthy (K_1 = 5.7) and EACMV-KE-infected (K_1 = 10.68) plants. On the other hand, death was highest between the 1st and 2nd & 3rd instars on the ACMV-[TZ: 01]-infected (K_2 = 4.1) plants (Fig. 8A). However, sub-clade II haplotypes experienced highest mortality between egg deposition and 1st instars for both ACMV-[TZ: 01] (K_1 = 10.5) and EACMV-KE-infected (K_1 = 8.75) plants (Fig. 8B). Interestingly, mortality rate was highest for immature instars developing on the healthy plants for both sub-clade I (R^2 = 0.74) and II (R^2 = 0.99) haplotypes. The mortality rates were nearly similar on ACMV-[TZ: 01] and EACMV-KE-infected plants (Fig. 8A & B).

Similar to the first trial, mortality was highest for sub-clade I and II immature instars on EACMV-KE (K_T = 13.62) and ACMV-[TZ: 01] (K_T = 13.33) infected plants, respectively (Fig. 8C & D). The highest number of deaths for both sub-clade I and II haplotypes occurred between egg deposition and 1st instars on EACMV-KE (K_1 = 8.20 & 5.50) and ACMV-[TZ: 01] (K_1 = 6.15 & 10.75) infected plants (Fig. 8C & D). Mortality rate was highest on ACMV-[TZ: 01] (R^2 = 0.84) and healthy (R^2 = 0.93) plants, respectively (Fig. 8C & D).

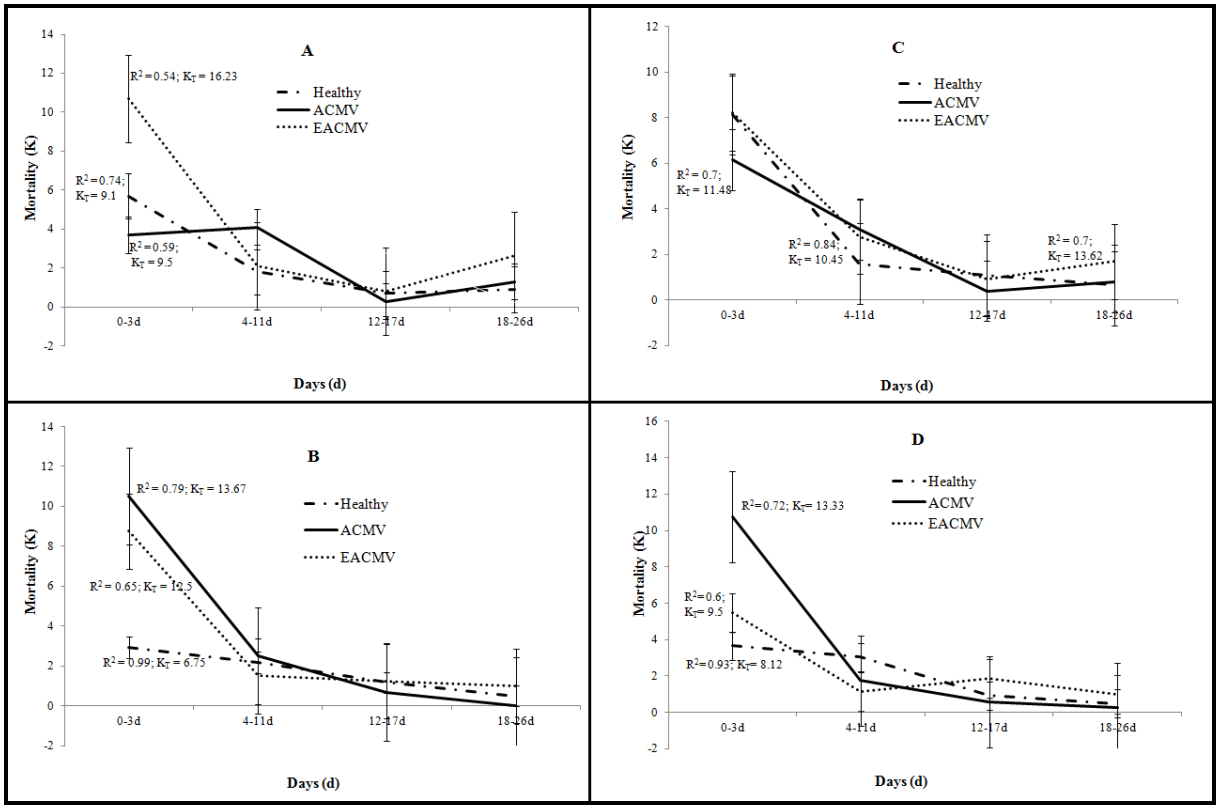


Figure 9 Mortality (K) and mortality rates of *B. tabaci* from Lake Victoria Basin: (A) first trial and (C) repeat trial; Coast Region: (B) first trial and (D) repeat trial of Tanzania on Healthy, ACMV-[TZ: 01] and EACMV-KE-infected plants conducted between April and May, and May and June, respectively, 2012.

3.3.7 Survivorship

Significant differences were observed in the survivorship of the 4th instar nymphs (P = 0.015) and hatched adults (P = 0.044) for sub-clade I and all the developmental stages for sub-clade II haplotypes in the first trial (Fig. 9A & B). In the repeat trial sub-clade I haplotypes had nearly similar survivorship levels on the healthy, ACMV-[TZ: 01] and EACMV-KE-infected plants (Fig. 9C). However, survivorship was significantly (P<0.01) higher on healthy and EACMV-KE than ACMV-[TZ: 01]-infected plants across all the developmental stages for sub-clade II haplotypes (Fig. 9D).

3.4 Discussion

We report here two *B. tabaci* haplotypes of SSA1 genetic group obtained on cassava plants in the Lake Victoria Basin and Coast Region in Tanzania that exhibited distinct biological differences in fecundity and development. The LVB and CR whitefly were confirmed to be SSA1 sub-clade I and II haplotypes, respectively as reported by Mugerwa *et al*. (2012).

A first report of biological differences between two cassava-associated *B. tabaci* haplotypes was made by Legg *et al*. (2002) at the peak of the 'severe CMD epidemic' that devastated cassava in the 1990s in Uganda. One of the haplotypes was a 'local/indigenous' whitefly that occurred on cassava in areas ahead of the CMD epidemic, and was associated with very low populations. The other haplotype was considered an 'invading' cassava whitefly that had superabundant populations at the 'severe CMD epidemic front' in the late 1990s in Uganda.

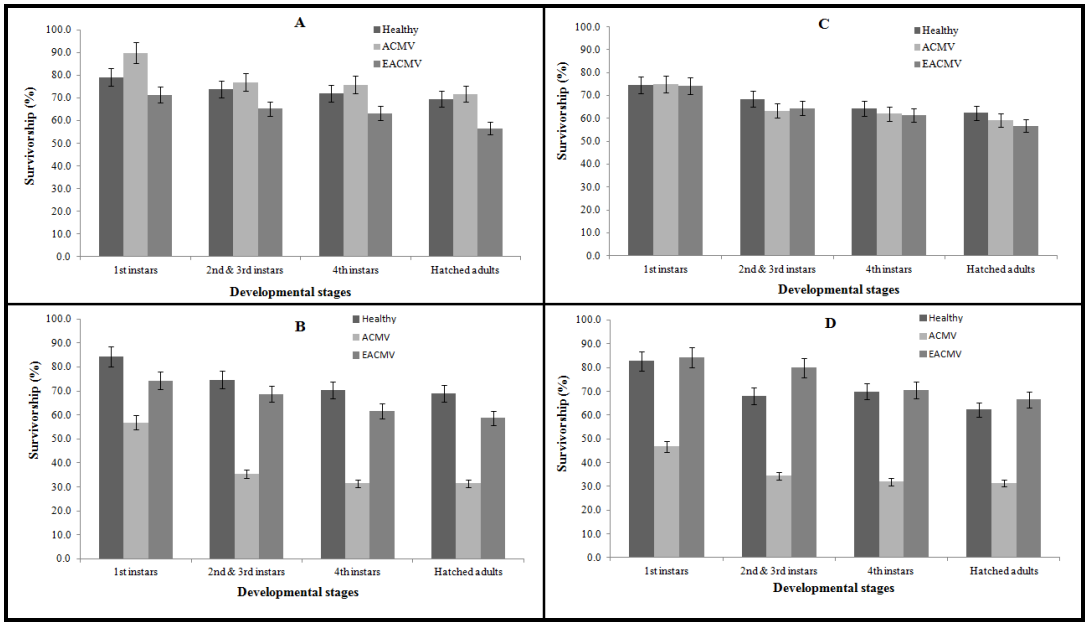


Figure 10 Percentage survivorship of different stages of *B. tabaci* from Lake Victoria Basin: (A) first trial and (C) repeat trial; Coast Region: (B) first trial and (D) repeat trial of Tanzania on Healthy, ACMV-[TZ:01] and EACMV-KE-infected plants conducted between April and May, and May and June, respectively, 2012.

In order to further investigate possible causes of the high whitefly populations on cassava, we studied the fecundity and development of the sub-clade I and II haplotypes from LVB and CR. Observations at the inception of the study clearly indicated general differences in the behaviour of the two populations. Sub-clade I haplotypes established rapidly compared with sub-clade II haplotypes, despite the latter being the resident whitefly in the Coast region. These observations were confirmed with sub-clade I producing significantly more eggs, immature instars and hatched adults than sub-clade II haplotypes in the study. Further, the CMB-infected plants had no influence on the number of eggs laid, developmental instars and hatched adults of the whiteflies. Rather, the healthy plants supported significantly higher whitefly population numbers than the virus infected plants. These results concur with findings by Thompson (2002) and Li et al. (2011), who reported a lack of effect by EACMV and *Tomato yellow leaf curl virus* (TYLCV) on the population of cassava and the Q (Mediterranean) & ZHJ2 (Asia II 1) whitefly that fed on the virus-infected cassava and, tomato and cotton plants, respectively. Additionally, Liu *et al*. (2009) reported no effect on the biology of the B biotype/Middle East-Asia Mirror 1 by TYLCV and *Tomato yellow leaf curl China virus* (TYLCCNV). Reduction in the survival and fecundity of the ZHJ1 biotype/Asia II 2 on the TYLCV and TYLCCNV-infected plants was also observed in the study.

In contrast, studies by McKenzie (2002), Colvin *et al*. (2006), Jiu *et al*. (2007) and Guo *et al*. (2010) obtained divergent results that suggested 'indirect' effects by plant viruses on whitefly biology. For example, Thomas *et al*. (1995) reported a reduction of up to 97% in whitefly reproduction on transgenic tobacco, which resulted from conversion of tryptophan to tryptamine and other derivatives during tryptophan decarboxylase (TDC) activity. Other studies reported the invasive B biotype/Asia II 2 to increase its fecundity and longevity on

Tobacco curl shoot virus (TbCSV) and TYLCCNV-infected tobacco plants (Jiu *et al*. 2007; Guo *et al*. 2010). However, no explicit conclusions can be made linking virus-infection and whitefly abundance, since there appears to be many contradictory findings on the effect of plant viruses on the population of insect vectors generally. Clearly, these and the earlier reports show that plant-virus-vector interactions are complex systems that require careful and standardized procedures to accurately compare and measure their effects on vector behaviour and biology.

In both the first and repeat trials, mortality was lowest on the healthy plants. The highest mortality was recorded between eggs to 1^{st} instars and 4^{th} instars to adult emergence. This is consistent with findings by Byrne & Bellows (1991), who reported that the principal mortalities affecting whitefly populations occurred between egg deposition and adult emergence. In addition, whitefly mortality occurred most on plants infected with CMBs they are least associated with in the field. That is, sub-clade I the dominant haplotypes in LVB had highest mortality on EACMV-KE; the coastal virus, while the sub-clade II haplotypes, which predominate the CR had most deaths on ACMV-[TZ: 01] the dominant virus in the LVB. This could have implications in the epidemiology of cassava viruses.

In conclusion, contradictory studies in the literature do not currently support a role for host-virus interactions in whitefly fecundity or development, although virus-vector interaction may (McKenzie, 2002; Jiu *et al*., 2007). Certainly, feeding preferences and adaptation for different whitefly haplotypes on different host plants has been clearly demonstrated (Byrne & Bellows, 1991), and this is likely linked to the chemical composition and/or other morphological features of the host plant (Byrne & Bellows, 1991). While the reasons for differences in population numbers and development between the two SSA1 haplotypes is

unknown, this study rather supports a genetic role in the biology (fecundity and development) of *B. tabaci* haplotypes. Identification of genes or transcriptomes, through deep sequencing, involved in biological processes will be necessary to shed more light on this subject (http://departments.agri.huji.ac.il/horticulture/staff-eng/czosnek-files/czosnek-whitefly.html; Wang *et al*., 2012). There have been some studies linking vector-virus mutualism to population increase of an invasive whitefly (Jiu *et al*., 2007) through increase of both the host plant suitability and differentiation of oocytes during oogenesis. The high number of oocytes in the ovaries consequently led to the high number of eggs laid. Because, the whitefly remains the most important vector for cassava viruses, our understanding of the factors controlling population dynamics is vital.

CHAPTER FOUR

General discussion, recommendations and conclusion

Data presented in chapter two identified the presence of two *B. tabaci* species belonging to two distinct genetic groups of whiteflies, namely sub-Saharan Africa clade 1 (SSA-1) and South West Indian Ocean Islands (SWIO), on cassava crops in the post epidemic area of Kenya, Tanzania and Uganda. The SSA-1 species had two closely related sub-clades (I and II), which were earlier reported on cassava as Uganda 1 (Ug1) with a nt sequence similarity of 97.8-99.7% and southern Africa (SA) genotypes with sequence similarity of 97.2-99.5% in Uganda (Legg *et al*., 2002; Sseruwagi, 2005) and southern Africa (Berry *et al*., 2004; Esterhuizen *et al*., 2012), respectively. The occurrence of a Reunion whitefly species that clustered among the SWIO genetic group with nt sequence similarity of 97.2-97.7% on cassava in Kenya, it's ability to colonise the crop is doubtable although they were reported to colonise a number of non-cassava plants species in Uganda (Sseruwagi *et al*., 2005). More definitive studies to ascertain the colonisation of cassava by these whiteflies needs to be carried out.

A clear geographical distribution of the cassava *B. tabaci* species in the East African region was noted. Previous studies reported SSA1 sub-clade I genotypes/indigenous population occurring widely in areas ahead of the severe CMD pandemic 'front', and associated with very low numbers (Legg *et al*., 2002; Maruthi *et al*., 2004), however, this genotype was not only wide spread in Kenya, Tanzania and Uganda, but also occurred in high population. Previously, the super abundant whitefly population associated with the severe CMD pandemic (Legg, 1999; Otim Nape *et al*., 2000; Colvin *et al*., 2004) commonly referred to as

the ‘invader/Ug2’ (Legg *et al*., 2002) or SSA2 was not detected on cassava in this study. The diminishing occurrence of the SSA2 whiteflies was reported by Sseruwagi (2005) in the severe CMD-affected areas in Uganda. The complete absence of SSA2 and the resurgence of the SSA1 sub-clade 1 whitefly species in high populations in EA could be the result of backcrosses between the indigenous SSA1 sub-clade I whitefly population and the SSA2 invasive population, that resulted in a hybrid population with SSA1 sub-clade I mtCOI and the invasive traits of the SSA2 species (J.K. Brown, personal communication). Further studies are required to affirm the hybrid hypothesis.

The complete absence of the SSA1 sub-clade I genotypes in Coast Kenya and Tanzania, and central and eastern Uganda could possibly be due to displacement by the SSA1 sub-clade II haplotypes as previously reported (Bedford *et al.*, 1994; Brown, 2001; Chu *et al*., 2010; Crowder *et al*., 2010; Esterhuizen *et al*., 2012; Wang *et al*., 2011). This study also reports for the first time the occurrence of SSA1 sub-clade II whitefly in East Africa where it predominated in the Coast province of Kenya, Southern and the coast region of Tanzania, and were low in the Lake Victoria Basin of Tanzania, and widespread in all four regions in Uganda. The SSA1 sub-clade II could be the indigenous whitefly species in the Coast province of Kenya, Southern and the Coast region of Tanzania. This whitefly is generally believed to be the indigenous whitefly in southern Africa (Berry *et al*., 2004; Esterhuizen *et al*., 2012); countries that share common boundaries and climate with Tanzania. However, the occurrence of the SSA1 sub-clade II whiteflies in the Lake Victoria Basin of Tanzania and in Uganda was unexpected and requires further investigation.

The two SSA1 sub-clades I & II whiteflies which group together as a single genetic group due to their less than 3.5% divergence in the mtCOI (Dinsdale *et al*., 2010), were speculated

to differ in important aspects of their biology, such as fecundity, virus transmission, and mating ability. Evidence obtained in this study (chapter three) showed that SSA1 sub-clade I haplotypes exhibited distinct biological differences in fecundity and development from SSA1 sub-clade II haplotypes.

Fecundity and development of the two key whitefly haplotypes SSA1 sub-clade I and II from LVB and CR was studied to understand further the possible cause of the high whitefly populations on cassava. Generally, the SSA1 sub-clade I performed better than the SSA1 sub-clade II haplotypes. This was clearly evident in the behaviour of the two colonies made in the screenhouse observations at the inception of the study. It took many attempts to establish the SSA1 sub-clade II colony in spite of these being the resident whitefly in the Coast region, where MARI is situated. The SSA1 sub-clade I produced significantly more eggs, immature instars and hatched adults than the SSA1 sub-clade II haplotypes. Compared to the healthy plants, the diseases plants had a negative effect on whitefly development. These results concur with findings by Thompson (2002) and Li et al. (2011), who reported a lack of effect by EACMV and *Tomato yellow leaf curl virus* (TYLCV) on the population of cassava and the Q (Mediterranean) & ZHJ2 (Asia II 1) whitefly that fed on the virus-infected cassava and, tomato and cotton plants, respectively. In contrast, studies by McKenzie (2002), Colvin *et al*. (2006), Jiu et al. (2007) and Guo et al. (2010) obtained divergent results that suggested 'indirect' effects by plant viruses on whitefly biology.

However, no explicit conclusions can be made linking virus-infection and whitefly abundance, since there appears to be many contradictory findings in literature on the effect of plant viruses on the population of insect vectors generally. Clearly, these and the earlier reports show that plant-virus-vector interactions are complex systems that require careful and

standardized procedures to accurately compare and measure their effects on vector behaviour and biology.

The highest mortality was recorded between eggs to 1st instars and 4th instars to adult emergence as observed by Byrne & Bellows (1991). In addition, sub-clade I the dominant haplotypes in LVB had highest mortality on EACMV-KE-infected plants; the coastal virus, while the sub-clade II haplotypes, which predominate the CR had most deaths on ACMV-[TZ: 01]-infected plants which dominant in the LVB. This could have implications in the epidemiology of cassava viruses.

Amid different hypotheses which suggest different causes of high population numbers, this study rather supports a genetic role in the biology (fecundity and development) of *B. tabaci* haplotypes. Identification of genes or transcriptomes, through deep sequencing, involved in fecundity and development will be necessary to shed more light on this subject. Because, the whitefly remains the most important vector for cassava viruses, our understanding of the factors controlling population dynamics is vital.

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