MOLECULAR VARIABILITY OF CASSAVA *Bemisia tabaci* AND ITS EFFECT ON THE EPIDEMIOLOGY OF CASSAVA MOSAIC GEMINIVIRUSES IN UGANDA

By

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Declaration

This thesis is my work and has not been presented for a degree in any other University

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Dedication

To my dear wife, Alexandria N. Sseruwagi and lovely children Darlene E.R. Nagitta and Dillon D. Lugolobi, and in memory of a true friend and colleague, the late Mr. W.S. Sserubombwe for the dedicated contribution to cassava research in Africa

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Table of contents

Content **Page**

List of tables

List of figures

List of plates

Abstract

Bemisia tabaci (Genn.) is the vector of cassava mosaic geminiviruses (CMGs), which are the main production constraint to cassava, both in Uganda and elsewhere in Africa. A severe form of cassava mosaic disease (CMD) was responsible for the devastation of cassava in Uganda beginning in the late 1980s. In subsequent years the severe CMD epidemic spread throughout Uganda, and to neighbouring countries, causing devastating effects to cassava production, and its geographical range continues to expand with the pandemic. To further understand the virus-vector dynamics involved in the spread of CMD in the post epidemic zone in Uganda, we investigated the current distribution of *B. tabaci* genotypes in selected cassava-growing regions. Additionally, the relationship between the vector genotypes and distribution of CMGs in the post-epidemic zone was examined also. CMD-affected cassava leaves were collected from 3 to 5 month-old cassava plants, and *B. tabaci* adults and fourth instar nymphs were collected from cassava and twenty-two other plant species occurring adjacent to the sampled cassava fields. The mitochondrial cytochrome oxidase I (mtCOI) sequence was used to establish the genotype of *B. tabaci* adults and nymphs associated with the sampled plant species. *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus*-Uganda 2 (EACMV-UG2) were confirmed to be present in the post-epidemic zone in Uganda, as reported previously. As expected, EACMV-UG2 predominated. However, unlike previous observations in which EACMV-UG2 was consistently associated with the severe disease phenotype, in this study EACMV-UG2 occurred almost equally in the severely and mildly diseased plants. Phylogenetic analyses of Ugandan *B. tabaci* genotypes (mtCOI) revealed that their closest relatives were other Old World genotypes, as might be expected. Two previously reported *B. tabaci* genotype clusters, Uganda 1 (Ug1) and Uganda 2 (Ug2), at ~8% nt divergence, were confirmed to occur on cassava in the post-epidemic zone. However, Ug1

occurred more frequently (83%) than Ug2 (17%), and no definite association was established of a particular vector genotype with cassava plants exhibiting the severe disease phenotype, in contrast to the *B. tabaci* genotype distribution and association with the CMGs reported there at the height of the spread of the severe CMD epidemic. Based on the presence of *B. tabaci* fourth instar nymphs, the Ug1 genotypes colonized five additional non-cassava plant species: *Manihot glaziovii*, *Jatropha gossypifolia, Euphorbia heterophylla, Aspilia africana* and *Abelmoschus esculentus,* suggesting that in Uganda the Ug1 genotypes are not restricted to cassava. However, no Ug2 genotypes were detected on the non-cassava plant species sampled. This study revealed also the presence in Uganda of five distinct previously unrecorded *B. tabaci* genotype clusters, Uganda 3 (Ug3), Uganda 4 (Ug4), Uganda 5 (Ug5), Uganda 6 (Ug6) and Uganda 7 (Ug7), and a sweetpotato colonizing genotype cluster, designated Uganda 8 (Ug8), among the collective Ugandan *B. tabaci* populations. Ug3 was the only exemplar representing one cluster, which was unlike any previously described genotype in Uganda or elsewhere, and diverged at 8%, 10% and 17% from Ug1, Ug2 and Ug8, respectively. The Ug3 genotypes colonized a single species, *Ocimum gratissimum*. Ug4, Ug5, Ug6 and Ug7 formed four closely related sub-clusters (93-97% nt identity), and diverged from one another by 1-7%, and by 15-18% from Ug1, Ug2, Ug3 and Ug8, respectively. The Ug4 genotypes had as their closest relatives (at 97-99% nt identity) previously reported *B. tabaci* from okra in the Ivory Coast, whereas, the Ug5 and Ug6 genotypes shared 95-99% and 99% nt identity, respectively, with their closest relatives from the Mediterranean-North Africa- Middle East (MED-NAFR-ME) region, which also includes the well studied B and Q biotypes. The Ug7 genotypes were closely related (at 98-99% nt identity) to *B. tabaci* from Reunion Island in the Indian Ocean. The Ug4, Ug5, Ug6 and Ug7 genotypes were identified on 54%, 8%, 8%, and 31% of the sampled plants species, respectively. Ug4 were most polyphagous, followed by Ug7 and Ug6. However, none of the new five genotypes (Ug3-Ug7) was found associated with, or colonizing,

cassava or sweetpotato plants in this study. Squash plants colonized by the Ug6 and Ug7 genotypes, both members of the B biotype/B-like cluster, developed the silvering phenotype, while those colonized by the Ug4 genotypes (most closely related to a non-B like genotype from okra in the Ivory Coast) did not. In addition to colonizing sweetpotato, the Ug8 genotypes also colonized *Lycopersicon esculentum* and *L nepetifolia*.

Chapter One

General Introduction

1.1 Cassava

Cassava, *Manihot esculenta* (Crantz) is a member of the family *Euphorbiaceae* (Purseglove, 1988). The crop is believed to be indigenous to tropical and sub-tropical areas of America, where it has been cultivated for about four thousand years (Charrier and Lefevre, 1987; Purseglove, 1988). Shrubby species of *Manihot* originated in the New World, with a geographic range extending from the southern United States (Arizona) to northern Argentina (Sauer, 1951). From tropical America, cassava spread to tropical and sub-tropical regions in many other parts of the world and it is now widely grown in Africa, India, Indonesia, Madagascar, Malaysia, the Philippines and Thailand (Guthrie, 1987). Cassava was introduced to Uganda by Arab traders between 1862 and 1875 (Langlands, 1972). The crop is grown in at least 40 African countries with an estimated annual production of 97 million tonnes in 2002 (FAO, 2003).

Cassava retains its importance as a major staple crop in Africa for over 200 million people (Horton, 1988; IITA, 1990; Dahniya, 1994). The starchy, thickened, tuberous roots are a valuable source of cheap calories, especially in developing countries, where calorie deficiency and malnutrition are widespread (Purseglove, 1988; IITA, 1990). Throughout Africa the cultivation of cassava has been encouraged as a famine reserve and against the ravages of the migratory African locust (*Locusta migratoria migratorioides*) to which it is almost immune (Purseglove, 1988). Propagation is normally done using 10-30 cm long stem cuttings

(Purseglove, 1988). Depending on variety, the tuberous roots store for long in soil and piecemeal harvesting can be done over a long period (Byabakama, 1996). The crop can withstand prolonged periods of drought, except at planting, making it adaptable to regions with low and uncertain rainfall.

1.2 The whitefly (*Bemisia tabaci*)

1.2.1 General background

Over 1200 whitefly species are known worldwide, although only a limited number have been closely studied on key herbaceous hosts (Mound and Halsey, 1978; Byrne *et al*., 1990a). Species of the genus *Bemisia* are among the most important on cultivated species and is believed to have originated in SouthEast Asia/Indian sub-continent (Gill, 1990; Mound and Halsey, 1978) or possibly Africa (Gill, 1990; Campbell *et al*., 1996). *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is the most widely distributed and economically important *Bemisia* species (Brown *et al*., 1995a). This whitefly has gained increased importance during the past thirty years as a pest and vector of plant viruses, particularly whitefly-transmitted geminiviruses in the genus: *Begomovirus* (family: *Geminiviridae*) in tropical and subtropical regions (Muniyappa, 1980; Duffus, 1987; Brown and Bird, 1992; Bedford *et al*., 1994; Fishpool and Burban, 1994; Poulston and Anderson, 1997).

Bemisia tabaci also causes damage to plants through direct feeding, such as chlorosis of leaves and irregular ripening of tomato fruits (Maynard and Cantliff, 1989; Byrne *et al*., 1990a; Perring *et al*., 1991), stem blanching and reduction in plant vigour (Byrne *et al*., 1990a; Costa *et al.,* 1993a; Legg *et al*., 2004), heavy phloem feeding (Byrne *et al*., 1990b: Byrne and Miller,

1990) leading to stunting of plants and yield loss, and indirectly, as through the production of sticky sugary exudates or 'honey dew', which encourage development and growth of saprophytic fungi (e.g. *Capnodium* spp.) on the affected plants (Byrne *et al*., 1990b) and induction of phytotoxic disorders resulting in silvering in some plants (Costa and Brown, 1991), which is restricted to a few genotypes (genetically distinct individuals for which formal taxonomic assignment has not been determined) (Brown and Bird, 1992; Brown *et al*., 1992, 1995a; Cohen *et al*., 1992; Bedford *et al*., 1994; Byrne *et al*., 1995). These effects are especially severe when large populations of *B. tabaci* colonize plants.

1.2.2 Biology

Adult whitefly normally feed on the under surfaces of young apical leaves, where they lay eggs and the immature stages develop. The eggs are oval and elongate and are attached to the leaf surface by a narrow stalk or 'pedicel' (Avidov, 1956; Paulson and Beardsley, 1985), which in addition is a guide for spermatozoa (Quaintance and Baker, 1913), and a passage for water into the egg (Wiggelsworth, 1965; Hinton, 1981; Byrne *et al*., 1990b). *B. tabaci* undergoes incomplete metamorphosis and therefore its development is divided into four nymphal instars (Lopez-Avila, 1986; Byrne and Bellows, 1991). The nymphal instars are found on the lower older leaves, with the youngest stages on the uppermost leaves (Robertson, 1987). The first instar, also referred to as a 'crawler', can move short distances from the eggshell to locate suitable feeding sites (Pollard, 1955). The second and third instars are sessile, closely resemble each other, but differ in size (Gill, 1990; Fishpool and Burban, 1994). The fourth instar (Plate I), also inappropriately referred to as the 'pupa' (Mound and Halsey, 1978; Lopez-Avilla, 1986; Byrne and Bellows, 1991) is shield-shaped, broadly elliptical (Gill, 1990) and has two small red eye spots at the anterior end, which are visible beneath the translucent

integument during the late fourth instar nymphal stage (Von Arx *et al*., 1983, 1984). The adults (Plate I) differ in size, and the females are larger (~1 mm) than the males (~0.8 mm) (Azab *et al.*, 1970; Gill, 1990). The males are also thinner and have more tapered abdomens with a pair of claspers at the tip (Fishpool and Burban, 1994).

The duration of the developmental stages and generation times of *B. tabaci* have been reviewed (Gerling *et al*., 1986; Lopez-Avila, 1986). On average, 12 generations are attained annually under field conditions (Husain and Trehan, 1933; Butler *et al*., 1983; Fishpool and Burban, 1994). From the egg to the adult, developmental times were 18.6 days on sweetpotato, 29.8 days on carrot in the laboratory (Coudriet *et al*., 1985), 14.5 days on aubergine in Israel (Avidov, 1956), 107 days on cotton in India (Husain and Trehan, 1933), and averaged 21 days (dry season) and 28 days (rainy season) on cassava in Ivory Coast (Fishpool *et al*., 1995) and in Uganda (Legg, 1995). Temperature is the key-determining factor and higher temperatures (30 - 33°C) result in faster developmental times (Gerling *et al*., 1986). Great variability has been reported on fecundity, which is affected by environmental conditions and host-plant species (Byrne and Bellows, 1991). Fecundity estimates range from 28 to 394 eggs per female (Byrne and Bellows, 1991; Fishpool and Burban, 1994; Palaniswami *et al*., 1996).

Several studies have been conducted on the mating behaviour of whiteflies (Byrne and Bellows, 1991; Costa *et al*., 1993b; Ronda *et al*., 1999; Maruthi *et al*., 2001, 2004a). *Bemisia tabaci* reproduce arrhenotokously/parthenogenetically, with the unmated females (virgins) producing males, which are haploid, and the mated females producing both males and diploid females (Byrne and Bellows, 1991). The males express either one or the other of the maternal allelic traits, while the females express both the maternal and paternal traits. The males are usually fewer than females, with a highly variable ratio of male to female (1:2 or 1:3) (Sharaf

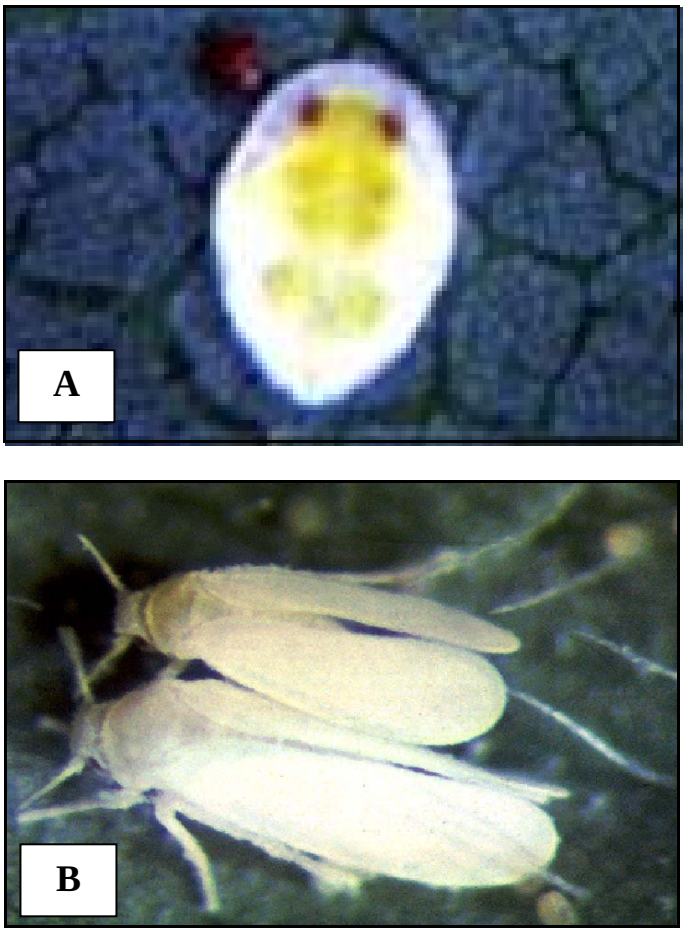

Plate I. Whitefly (*B. tabaci*) fourth instar nymph/pupa, with small red eye spots at the anterior end (A) and male (smaller) and female (bigger) adults (B).

and Batta, 1985), which depends on the time of the year, temperature and host species (Pruthi and Samuel, 1942; Sharaf and Batta, 1985) and also results from differences in the longevity of the adults, with the females on average having a longer life span (35 days) than the males (20 days) (Azab *et al*., 1972; Butler *et al*., 1983). There is great variability in the number of days before adults copulate. Upon emergence, both males and females are sexually immature and courtship takes place within 1 to 8 hours during summer (Avidov, 1956), 20 to 24 hours after emergence (Li *et al*., 1989) and after 3 days during fall and spring (Avidov, 1956).

Adults of different whitefly species are difficult to distinguish on the basis of morphological characters (Mound and Halsey, 1978; Lopez-Avilla, 1986; Rosell *et al*., 1996, 1997). Attempts were made to synonymize about 19 whitefly species into a single taxon, *B. tabaci*, in 1957 (Russell, 1957), but the lack of morphologically distinguishable features of the adult whiteflies presented taxonomic difficulties. Identification relied mainly on the morphology of the fourth instar nymph, also referred to as the 'pupae' (Mound and Halsey, 1978; Gerling *et al*., 1986; Martin, 1987; Gill, 1990). However, the influence of both genetic and environmental factors limited the use of the fourth instar nymphs in distinguishing between the different whitefly species (Mohanty and Basu, 1986; Rosell *et al*., 1997).

Whiteflies have been known to exhibit distinct biological traits as in host-plant range and adaptability (Costa and Russell, 1975; Gill, 1992; Burban *et al*., 1992; Legg, 1995), inducement of plant physiological disorders (Costa and Brown, 1991; Perring *et al*., 1991; Cohen *et al*., 1992) and plant virus transmission (Bird and Maramorosch, 1978; Brown *et al*., 1992, Bedford *et al*., 1994; McGrath and Harrison, 1995; Markham *et al*., 1996; Maruthi *et al*., 2002). Bird (1957) and Mound (1963) referred to morphologically indistinguishable *B. tabaci* populations, exhibiting distinct biological traits as biotypes or host-races, and the terms have

been used subsequently, to describe agriculturally important insects (Saxena and Barrion, 1987; Burban *et al*., 1992; Gill, 1992; Brown *et al*., 1995a). For example, in the southern U.S. a *B. tabaci* population capable of inducing silverleaf symptoms in squash (*Cucurbita* spp.), hence the name 'silverleaf whitefly' (Costa *et al*., 1993a; Brown *et al*., 1995a), was named the 'B biotype', due to the production of a distinct electromorph profile, using general esterases, from that of the indigenous American *B. tabaci* population, named the 'A biotype' (Costa and Brown, 1990; Perring *et al*., 1992; Costa *et al*., 1993a). Several other biotypes or host races, including the 'E' (on *Asystasia* spp., in Benin) (Bedford *et al.*, 1994), 'J' (polyphagous, Nigeria) (Bedford *et al*., 1994), 'N' (*Jatropha gossypifolia*) (Bird, 1957), 'cassava' and 'okra' (Burban *et al*., 1992), 'Sida' (Bird and Maramorosch, 1975, 1978) and a polyphagous non-cassava biotype in Brazil (Bird and Maramorosch, 1975, 1978; Brown and Bird, 1992; Costa and Russell, 1975) have been reported.

A key biological trait that has aided the understanding of the level of relatedness among morphologically indistinguishable *B. tabaci* populations is the ability of the different populations to interbreed. For example, Costa *et al*. (1993b), Perring *et al*. (1993) and Bedford *et al*. (1994) reported only very low reproductive compatibility between the B and Q biotypes. However, these studies could not demonstrate interbreeding between the A and B biotypes. On the basis of the failure to interbreed, the B biotype was referred to as a separate species: *Bemisia argentifolii* Bellows & Perring (Perring *et al*., 1993; Bellows *et al*., 1994). In contrast, successful mating compatibility was demonstrated between the Sudanese non-B males and Israeli B female biotypes (Byrne *et al*., 1995), the indigenous Q biotype and the B biotype in Spain, using RAPD PCR markers (Ronda *et al*., 1999), the Australian indigenous *B. tabaci* and the B biotype (Gunning *et al*., 1997), and more recently, between the A and B biotypes and the Jatropha biotypes (Brown *et al*., 2001), between the cassava-associated *B. tabaci* from Ghana,

Tanzania and Uganda, and between two cassava-associated Indian *B. tabaci* populations (Maruthi *et al*., 2004a). Uncertainty, however, still remains over the extent to which different *B. tabaci* populations interbreed and many other studies have revealed mating incompatibilities between different host-associated populations. For example, in Ivory Coast, the cassava and okra *B. tabaci* populations did not produce hybrids when they occurred on a common host-plant, eggplant (Burban *et al*., 1992). Similar results were obtained for the cassava, cotton and sweetpotato populations in Uganda, from the analysis of esterase profiles (Legg, 1995), and the cassava-colonizing *B. tabaci* populations from Africa and India, cassava and sweetpotato *B. tabaci* from Uganda, and cassava and *B. tabaci* populations from *Euphorbia geniculata* in India (Maruthi *et al*., 2004a). The variations in the biological, biochemical and molecular traits exhibited by the different *B. tabaci* populations, has led to the suggestion that *B. tabaci* may be a species complex (Brown *et al*., 1995a; 2001; Frohlich *et al*., 1999). However, De Barro *et al*. (2000, 2005) did not consider the current state of knowledge sufficient to justify new species assignments. This view is held also by Maruthi *et al*. (2004a), who, however, strongly support the species complex hypothesis, based on the recent establishment of several biologically discrete *B. tabaci* populations amongst different hosts and geographical locations.

1.2.3 Host range, colonization and population dynamics

Bemisia tabaci is a polyphagous species (Greathead, 1986), and some biotypes and genotypes are extremely polyphagous (Brown *et al*., 1995a). It colonizes mainly annual, herbaceous plants including over 500 species from 74 families (Mound and Halsey, 1978; Greathead, 1986; Fishpool and Burban, 1994). However, nearly monophagous *B. tabaci* populations (Jatropha race) (Bird, 1957; Brown and Bird, 1992; Brown *et al*., 1994) have been recognized

on *Jatropha gossypifolia* and *Croton lobatus* and the cassava *B. tabaci*, colonizes only cassava in Africa (Storey and Nichols, 1938).

Adult whiteflies occur on cassava throughout the crop's growing period (Fishpool and Burban, 1994), although their populations differ with the stage of plant growth (Fishpool *et al*., 1995; Otim-Nape *et al*., 1996). The adults will slowly and steadily invade and establish within the crop as soon as the plants are sufficiently grown (Fishpool and Burban, 1994). Small numbers of adults may occur on the plants 3 weeks after the initial colonization, which is followed by a rapid population build-up at 3 to 4 months after planting (Fishpool and Burban, 1994; Otim-Nape *et al*., 1996). This is the period when foliage is most able to support rapid whitefly multiplication. A steady population growth follows for a short period, followed by a rapid decline to a low residual level maintained throughout the rest of the crop's growth period (Silvestre and Arraudeau, 1983; Robertson, 1987; Fishpool and Burban, 1994; Fishpool *et al*., 1995).

The population dynamics and activity of *B. tabaci* are believed to depend on changes in the nutritional quality of the host-plant, natural enemy populations and climatic factors (temperature, rain, wind, relative humidity) (Fishpool *et al*., 1987; Fishpool and Burban, 1994; Legg, 1995). Adult whiteflies feed by inserting their stylets between host-plant cells and penetrating the phloem of the host-plant (Pollard, 1955; Janssen *et al*., 1989; Cicero *et al*., 1994; Rosell and Brown, 1994). The food quality of cassava phloem varies with time and maturity of the plant. In the early growth period (1 to 3 months) the food resource is devoted to aerial growth, which declines after 4 to 5 months when the process of root tuberisation begins. There is usually a greater whitefly population during the first 3 months than later when the plants are more mature (Silvestre and Arraudeau, 1983). According to Fargette *et al*.

(1992), Otim-Nape (1993) and Fishpool and Burban (1994), increase in whitefly population is favoured by high temperatures and radiation and low rainfall and relative humidity. However, according to Robertson (1987) and Legg *et al*. (1994), population growth was greatest when rapid leaf growth occurred, which was associated to both the high temperature and rainfall. The adults disperse mainly by the aid of wind and can move short or long distances (Cohen and Ben-Joseph, 1986; Blackmer and Byrne, 1993), but also by humans who transport the immature and adult stages on plant material (Joyce, 1981; Mound, 1983; Byrne and Bellows, 1991). Further, cropping practices such as planting date (Robertson, 1987; Fargette *et al*., 1990), crop disposition (Thresh *et al*., 1994a) and intercropping (Fargette and Fauquet, 1988) influence whitefly population dynamics and hence the spread of whitefly-transmitted geminiviruses.

1.2.4 Whitefly systematics

Outbreaks of *B. tabaci*, particularly in areas where it was previously unimportant, are linked to the appearance of new biotypes, strains or possibly species of the vector (Simone *et al*., 1990; Cohen *et al*., 1992; Brown *et al*., 1995a; Legg *et al*., 2002). The evolution of agriculture leading to irrigated monocultures, the requirement for intensive agriculture enabling two cropping seasons per year and the use of fertilisers and pesticides are key factors in biotype emergence (Brown *et al*., 1995a). *B. tabaci* gained increased importance as a pest and vector of diseases, due to its ability to cause damage to host-plants through direct feeding and as the main vector of whitefly-transmitted geminiviruses. The growing economic importance of *B. tabaci* generated renewed interest in the development of techniques for systematics and evolutionary studies (Nei, 1987; Doolittle, 1990; Hillis and Moritz, 1990; Li and Graur, 1991).

Whitefly (*B. tabaci*) systematics has been reviewed (Campbell *et al*., 1996). Among the most commonly studied molecular techniques are: protein polymorphism involving isozyme variation in esterases (Wool *et al*., 1989; Brown *et al*., 1995b) and DNA-based molecular techniques, like random amplified polymorphic DNA (RAPD) PCR fingerprinting (Gawel and Bartlett, 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, cytochrome oxidase I (mtCOI) (Simon *et al*., 1994; Frohlich *et al*., 1999), the ribosomal RNAs, 16S rDNA (prokaryotes) (Clark *et al*., 1992; Frohlich *et al*., 1999) and 18S rDNA (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).

Isozyme variation in esterases have been studied extensively in insects and sufficient variability to allow the typing of *B. tabaci* populations using esterase patterns was demonstrated (Costa and Brown 1990, 1991; Brown *et al*., 1992, 1995b; Wool *et al*., 1989, 1993). Based on distinct esterase patterns, three whitefly species (Prabhaker *et al*., 1987) and cotton, sweetpotato and poinsettia host-associated populations (Costa and Brown 1991; Costa *et al*., 1993a), and 20 distinct general esterase patterns (designated A to S) (Wool *et al*., 1989; 1993) were distinguished, which revealed that *B. tabaci* was more polymorphic than previously expected (Bedford *et al*., 1994; Brown *et al*., 1995b; Rosell *et al*., 1997). In Ivory Coast, Burban *et al*. (1992) were able to distinguish cassava and okra biotypes, while Legg *et al*. (1994) distinguished cassava, cotton and sweet potato *B. tabaci* populations in Uganda using esterase profiles, confirming the existence of host-specific biotypes among African *B. tabaci*. However, according to Brown *et al*. (1995a), the allozymic esterase markers are limited in their capacity to distinguish variability in *B. tabaci* from a broad range of hosts and geographical locations, but are able to differentiate Old and New World populations.

The first attempt to distinguish *B. tabaci* biotypes using a mitochondrial DNA gene, 16S rDNA found in eubacterial endosymbionts of whiteflies, aphids and mealybugs was of limited use, as the endosymbionts were indistinguishable in the different insect hosts because the 16S rDNA is highly conserved (Simon *et al*., 1991; Clark *et al*., 1992). However, using the insect host 18S rDNA, which has relatively fast base-substitution rates, Campbell *et al*. (1993, 1994) detected only two base differences between the A and B biotypes, and concluded that the two biotypes were not separate species. Evidence enabling a clear distinction between the A and B biotypes of *B. tabaci* was obtained when DNA-based RAPD-PCR was used to study the genetic similarity between the two biotypes (Gawel and Bartlett, 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). In Uganda, Maruthi *et al*. (2001) used RAPD-PCR to establish whether a genetically distinct *B. tabaci* population was associated with the CMD epidemic. The results obtained showed clear differences, but there was no pattern associated with either the epidemic or non-epidemic zones.

The mitochondrial cytochrome oxidase I (mtCOI) marker (Frohlich *et al*., 1999) and ITSI region sequences (De Barro *et al*., 2000, 2005; Abdullahi *et al*., 2003) have been used also to study the genetic variability and evolutionary relationships among *B. tabaci* from different geographical locations and host-plant species. In Uganda, using the mtCOI marker, Legg *et al*. (2002) identified two distinct cassava-associated *B. tabaci* genotype clusters, designated, Uganda 1 (Ug1) and Uganda 2 (Ug2), which at the time of the study in 1997/8 occurred in areas 'ahead' of and 'behind' the CMD epidemic 'front', respectively. It was suggested that Ug1 could be 'indigenous' or 'local' genotypes, while the Ug2 genotypes were suggested to be an 'invasive' population with closest relatives in Cameroon in West Africa (Legg *et al*., 2002).

The Ug1-like and Ug2-like genotypes were shown to be sexually compatible (Maruthi *et al*., 2004a), although few females were obtained from the crosses. However, the identification of the hybrids still awaits the development of a nuclear marker, as the *mtCOI* is a maternal gene, enabling the identification of female *B. tabaci* only. The mtCOI marker has been used also to detect a distinct New World group of *B. tabaci* and the Old World B biotype in Argentina (Viscarret *et al*., 2003), and more recently five distinct geographic populations of *B. tabaci* in sub-Saharan Africa (Berry *et al*., 2004).

1.3 Cassava mosaic disease

1.3.1 General background

Cassava mosaic disease (CMD) was first reported in what is now Tanzania under the name 'Krauselkrankheit' by Warburg in 1894 (Storey, 1936), but was not known to cause serious losses until the 1920s. The disease was first reported in Uganda as 'curly leaf' (Hall, 1928) and as 'mosaic' (Martin, 1928). Subsequently, CMD was reported throughout mainland Africa and the surrounding islands (Deighton, 1926; Golding, 1936; Storey and Nichols, 1938) and also in India (Abraham, 1956) and Sri Lanka (Austin, 1986).

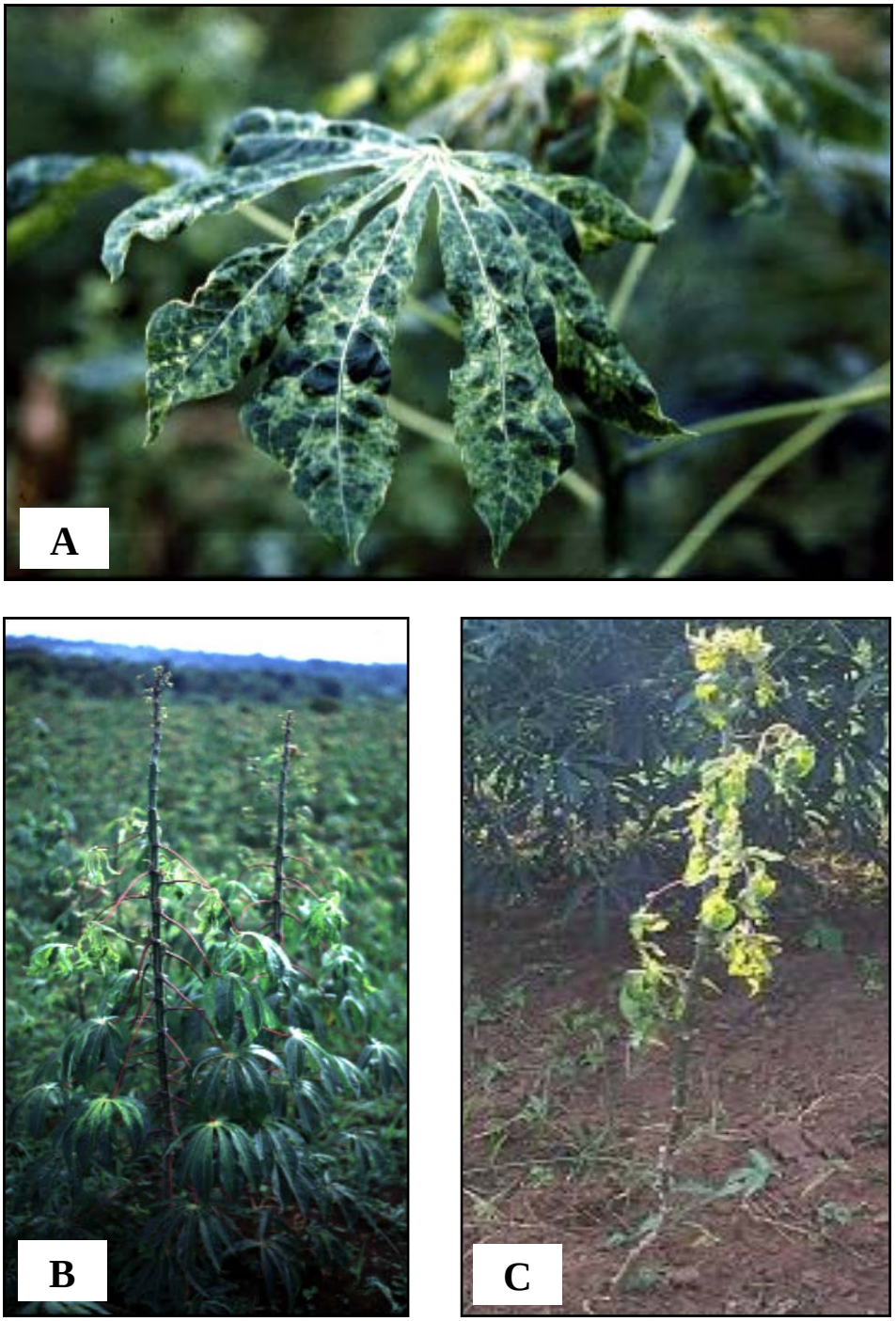

Plate II. Cassava leaf showing symptoms of cassava mosaic disease (CMD) (A), whitefly infection (B) and cutting infection (C).

A ‘mosaic’ pattern of light green, yellow or white areas intermingled with the green of uninfected leaf portions (Agrios, 1988; IITA, 1990) characterises CMD-affected plants (Plate II). The symptoms may be mild, where the affected plants exhibit patchy leaf chlorosis and little or no mottling, while severely affected plants have reduced leaf size and chlorosis, which result in reduced photosynthetic efficiency and hence retarded growth, leading to stunting and reduced tuberous root production (Agrios, 1988). The disease is spread by the whitefly vector (*B. tabaci*) and disseminated in CMD-affected cassava cuttings (Harrison, 1987).

Cassava mosaic disease is the most economically important vector-borne pathogen of cassava (Fargette *et al*., 1988; Geddes, 1990; Thresh *et al*., 1997). The disease was estimated to cause yield losses of between 19-27 million tonnes in Africa, compared to actual production in 2002 (FAO, 2003). About US$ 74 million was lost annually due to the disease in the pandemic-affected areas of Uganda and Kenya, compared to US$ 19 million in the unaffected areas of Kenya and Tanzania (Sseruwagi *et al*., 2004a).

1.3.2 Cassava mosaic geminiviruses

1.3.2.1 Aetiology, variability and geographical distribution

The presumed etiologic agent of cassava mosaic disease was first isolated in Kenya in 1975 (Bock, 1975; Bock *et al*., 1978), and was shown to be transmissible to *Nicotiana clevelandii* Grey through mechanical inoculation using sap from some CMD-affected cassava plants. Hence the initial reference to Cassava latent virus. The virus isolate, currently referred to as *African cassava mosaic virus* [Kenya] (ACMV-[KE]) was shown to contain DNA (Harrison *et al*., 1977). Its bipartite genome was demonstrated, the nucleotide sequence determined

(Stanley and Gay, 1983) and the virus shown to cause mosaic in cassava when back transmitted from herbaceous hosts to cassava (Bock *et al*., 1981; Bock and Woods, 1983).

In 1984 the first attempt was made to detect ACMV using nucleic acid hybridisation (Robinson *et al*., 1984). Using polyclonal antibodies (Sequeira and Harrison, 1982) and murine monoclonal antibodies (mAbs) (Thomas *et al*., 1986), raised against ACMV particles, geminivirus infection was detected in cassava mosaic-affected plants. Subsequently, three CMG isolates (A, B and C) produced distinct characteristic epitope profiles when a panel of 17 mAbs to ACMV were used in tests on extracts from 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 mainly along the east coast of Africa, while group C isolates occurred only in India and Sri Lanka (Hong *et al*., 1993; Swanson and Harrison, 1994). The A, B and C isolates were ascribed: ACMV, EACMV and *Indian cassava mosaic virus* (ICMV) later ascribed under the genus *Begomovirus*, family *Geminiviridae*.

Five other CMGs have been described of which four occur in Africa (Fauquet and Stanley, 2003). ACMV is the most widely distributed species and occurs in most areas in the cassava-growing belt of Africa. In contrast, EACMV is mainly found in coastal East Africa, western Kenya, western Tanzania, although reports of its presence were also made in some West African countries under the name *East African cassava mosaic Cameroon virus* (EACMCV) (Ogbe *et al*., 1998, 2003; Fondong *et al*., 2000; Pita *et al*., 2001a, b) and in Malawi as *East African cassava mosaic Malawi virus* (EACMMV-[MH]/[K]) and *East African cassava mosaic virus*-[Malawi] (EACMV-[MW]) (Ogbe *et al*., 1998; Pita *et al*., 2001b) and in South Africa

(Berry and Rey, 2001). ICMV only occurs in India and Sri Lanka (Swanson and Harrison, 1994). Additionally, new virus species including the *South African cassava mosaic virus* (SACMV) mainly occurring in South Africa and Swaziland (Rey and Thompson, 1998; Berrie *et al*., 2001), *East African cassava mosaic Zanzibar virus* (EACMZV) in Zanzibar Island (Maruthi *et al*., 2004b) and *Sri Lankan cassava mosaic virus* (SLCMV) in Sri Lanka (Saunders *et al*., 2002) have also been reported.

1.3.2.2 Genome organization, gene function and DNA replication

Cassava mosaic geminiviruses (CMGs) have geminate particles (Bock *et al*., 1978; Bock and Woods, 1983; Robinson *et al*., 1984), which measure 30 x 20 nm (Fig. 1a) and consist of a protein coat of *c*. 30 Kda, with a single molecule of circular single-stranded DNA (ssDNA) of Mr 0.8 x 106 (Harrison *et al*., 1977; Sequeira and Harrison, 1982). The bipartite genomic DNA of CMGs is comprised of two such ssDNA components (A and B) (Fig. 1b) of similar sizes with 2500 to 2900 nucleotides (Stanley and Gay, 1993). The two components share an 'intergenic region' (IR), which is a 200 bp noncoding region, identical in each DNA and which lies between the initiation codons of AV1, the virus coat protein (CP), and AC1 in DNA-A, and BV1 and BC1 in DNA-B. The IR contains promoter and sequence elements responsible for DNA replication and transcription (Chatterji *et al*., 2000), which are similar in both DNA-A and DNA-B (Harrison and Robinson, 1999) and as a result is referred to as the 'common region' (CR).

The open reading frames (ORFs) of DNA-A and DNA-B are comprised of six protein-coding sequences (four in DNA-A and two in DNA-B) in the plus/virus and minus/complementary-sense

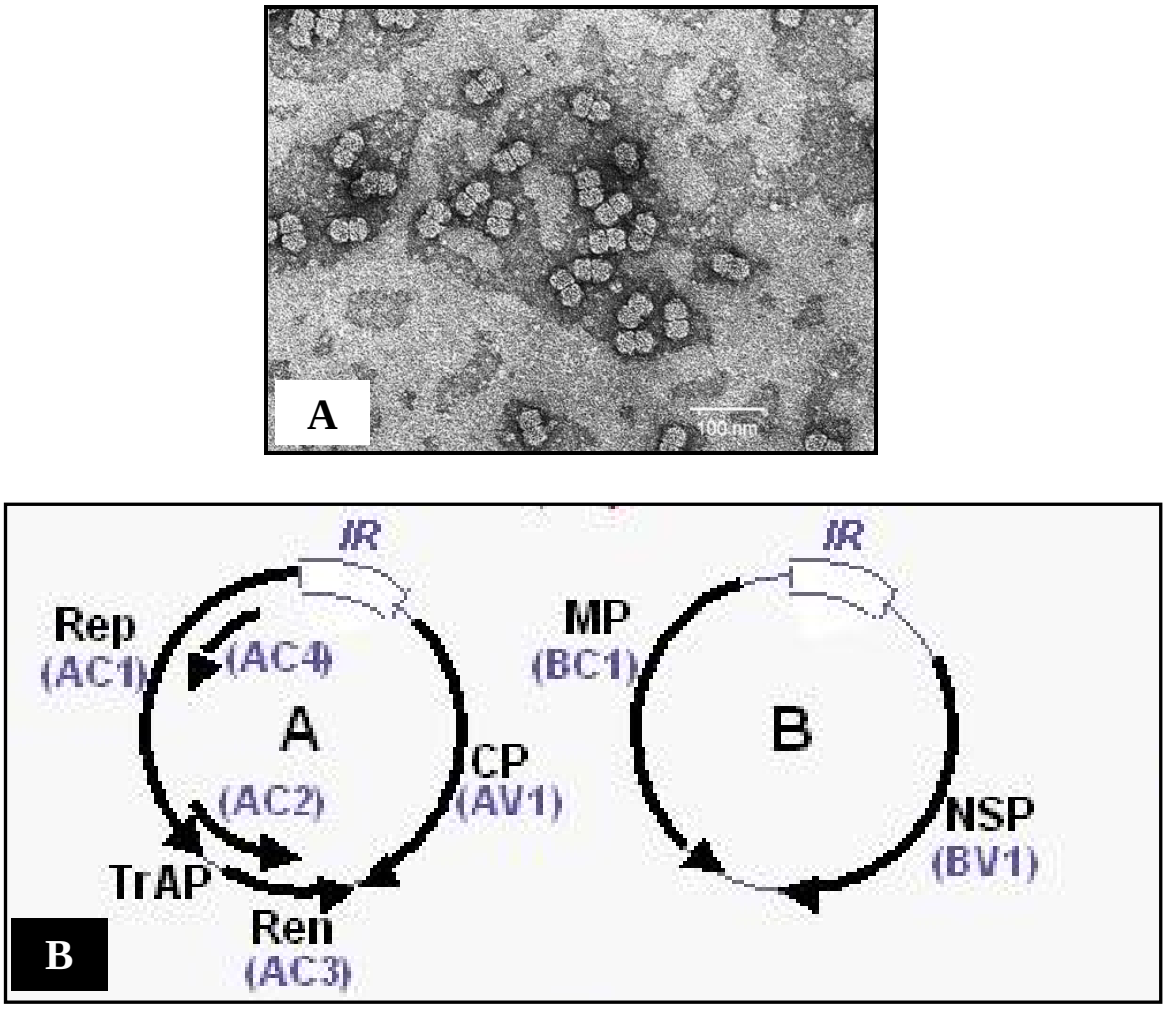

Figure 1. Electron micrograph showing germinate particles (A) and genomic organisation (B) of cassava mosaic geminiviruses

strands, which occur in a conserved arrangement when compared with those of other begomoviruses including *Tomato golden mosaic virus* (TGMV) (Hamilton *et al*., 1984) and *Bean golden mosaic virus* (BGMV) (Howarth *et al*., 1985). DNA-A encodes viral coat proteins required for replication and encapsidation of both DNA-A and B (Rogers *et al*., 1986). The AV1 has properties that play a crucial role in insect vector specificity (Roberts *et al*., 1984; Stanley *et al*., 1986; Briddon *et al*., 1990; Harrison and Robinson, 1999), although it is not required for infectivity (Etessami *et al*., 1988). DNA-A can replicate independently, while DNA-B does not (Townsend *et al*., 1986). The AC1 ORF encodes a viral DNA replication gene and is required for DNA synthesis (Elmer *et al*., 1988). However, the accumulation of ssDNA is encoded by the AC2 ORF, a transcription activator (TrAP) gene for virus sense genes in both DNA-A and B (Sunter and Bisaro, 1992), while the maximal replication of both the ssDNA and dsDNA is enhanced by the *AC3/AL3* gene (Sunter *et al*., 1990; Hanley-Bowdoin *et al*., 1999). The DNA-B component encodes transport proteins, BC1 and BV1, which are responsible for movement and symptom expression of the virus in the plant (Ingham *et al*., 1995; Sanderfoot and Lazarowitz, 1996; Sanderfoot *et al*., 1996).

The origin of replication (ori) '<u>A</u>' in CMGs is initiated in a conserved nonanucleotide (TAATATT<u>A</u>C) sequence (Heyraud-Nitschke *et al*., 1995) in a stable loop of a stem-like loop structure found in all geminiviruses (Stanley, 1995) and a binding site (Chatterji *et al*., 2000, 2001) for host enzymes required for priming complementary strand synthesis (Davies *et al*., 1987). Both DNA-A and DNA-B are required for infectivity (Stanley, 1983) and the two ORFs of DNA-B in CMGs are essential for infectivity (Etessami *et al*., 1988). The DNA-A nucleotide sequences are less diverse and hence have more conserved elements than those of DNA-B. The greatest variation, however, is obtained in the CR (Rybicki, 1994; Harrison and Robinson, 1999).

1.3.3 Vector-virus specificity and virus transmission

The most important role of *B. tabaci,* however, arises from its ability to vector plant viruses (Storey, 1938; Muniyappa, 1980; Duffus, 1987; Brown, 1990). A disease agent assumed to be a virus was first demonstrated to be transmissible in the 1930s in Africa (Kufferath and Ghesquiére, 1932; Storey, 1938) and in the 1950s in tropical America (Costa, 1955).

It was believed that CPs of specific geminiviruses from the same area were more adapted for transmission by locally adapted *B. tabaci* than those from elsewhere (Harrison *et al*., 1991; McGrath and Harrison, 1995; Harrison and Robinson, 1999). However, Bedford *et al*. (1994) and Markham *et al*. (1996) demonstrated the transmission of more than 15 geminiviruses from different countries by each of about 20 *B. tabaci* populations from three different continents. The specificity of transmission of plant viruses by different insect vectors was also demonstrated (Briddon *et al*., 1990; Höfer *et al*., 1997; Noris *et al*., 1998). For example, A CP of the leaf hopper-transmitted *Beet curly top virus* (BCTV) was substituted for the ACMV CP in the ACMV genome, enabling the transmissibility of the modified ACMV by leafhoppers (Briddon *et al*., 1990). In related studies, the substitution of a CP of a non-*B. tabaci* transmissible *Abutilon mosaic virus* (AbMV) by a CP of a *B. tabaci*-transmitted *Sida golden mosaic virus* (SGMV) enabled the transmission of AbMV by *B. tabaci* (Höfer *et al*., 1997), while the transmissibility of *Tomato yellow leaf curl virus* (TYLCV) by *B. tabaci* was affected by the substitution of a single amino acid in the CP (Noris *et al*., 1998). However, there were no significant differences between the transmissions of *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and *East African cassava mosaic virus*-Uganda2 (EACMV-UG2) by cassava-associated *B. tabaci* originating from widely separated geographical locations in Africa (Tanzania, Uganda and Ghana) (Maruthi *et al*., 2002).

1.3.4 The epidemic of severe cassava mosaic disease in Uganda

In 1988 serious crop failure was reported in northern Luwero district in Uganda (Otim-Nape, 1993; Otim-Nape *et al*., 1994), which was later attributed to an unusually severe form of cassava mosaic disease (Gibson *et al*., 1996). Early serological tests in the 1990s (Swanson and Harrison, 1994) on isolates from epidemic and non-epidemic areas showed ACMV to be the cause of the epidemic, because the mAbs could not distinguish between ACMV and the *Uganda variant* (UgV).

Using oligonucleotide primers designed to bind and amplify specific virus DNA sequences during polymerase chain reaction (PCR), a more severe virus, the Uganda variant or EACMV-UG2 a strain of EACMV with more than 90% homology in the DNA-A sequence with EACMV (Deng *et al*., 1997; Pita *et al*., 2001a; Fauquet and Stanley, 2003) and also a hybrid/recombinant of ACMV and EACMV in which *c.* 400 nucleotides of the CP of ACMV replace the similar region of EACMV (Deng *et al*., 1997; Zhou *et al*., 1997) was reported in Uganda. EACMV-UG2 was reported to be responsible for the severe epidemic of CMD (Harrison *et al*., 1997; Otim-Nape *et al*., 1997a; Legg, 1999), which was estimated to spread southwards across the country at an annual rate of 20 to 30 Km (Otim-Nape *et al*., 1997a; Legg and Ogwal, 1998).

A key feature of the epidemic was the formation of a 'front' characterized by unusually severe disease symptoms (Gibson *et al*., 1996; Harrison *et al*., 1997), rapid disease spread and high whitefly populations (Otim-Nape *et al*., 1997a; Legg and Ogwal, 1998; Colvin *et al*., 1999). Further investigation of the cause of the epidemic in Uganda revealed even more severe symptoms resulting from dual infections by ACMV and EACMV-UG2, believed to be due to a

synergistic interaction between the two viruses (Harrison *et al*., 1997; Pita *et al*., 2001a). Additionally, a pseudo-recombinant virus, resulting from intraspecific recombination between DNA-A of EACMV-UG2 and DNA-B of *East African cassava mosaic virus*-Uganda 3 (EACMV-UG3) was also reported to be responsible for the severe CMD epidemic in Uganda (Pita *et al*., 2001a).

Major efforts were launched in the 1990s to control the epidemic, and significant success was achieved through the use CMD-resistant varieties (Thresh *et al*., 1994a; Otim-Nape *et al*., 1997b, 2000, 2001; Legg *et al*., 1999). Consequently, the use of resistant varieties, reduction in area of cassava cultivated as a result of the epidemic and the emergence of tolerant local varieties (Otim-Nape *et al*., 2000, 2001), and less virulent viruses resulted in a general reduction in CMD inoculum pressure in Uganda. Coupled with the spread of the epidemic from Uganda into neighbouring countries, these changes led to the use of the term 'post-epidemic' to describe the current status of CMD in Uganda (Otim-Nape *et al*., 1998, 2000, 2001; Sseruwagi *et al*., 1998; Legg, 1999). Meanwhile, CMD continues to cause devastating effects to the production of cassava in many parts of sub-Saharan Africa and most especially in the pandemic-affected zone in East and Central Africa (Thresh *et al*., 1994b; Legg, 1999). The epidemic of severe CMD has been reported in southern Sudan (Harrison *et al*., 1997), western Kenya, northern and north-eastern Tanzania (Legg, 1999), Rwanda (Legg *et al*., 2001; Sseruwagi *et al*., 2005a), south western Democratic Republic of Congo (Neuenschwander *et al*., 2002), Gabon (Legg *et al*., 2003) and Burundi (Bigirimana *et al*., 2004) and its geographical range continues to expand with the pandemic.

1.4 Problem statement and justification

The recent discovery of new CMGs and genotypes of *B. tabaci* in Africa and elsewhere has led to new insights into the epidemiology of CMD. The significance of recombination and how it influences whitefly adaptation in the changing virus situations needs to be investigated. In trying to account for the boosted whitefly populations in the pandemic affected zone, a synergistic/mutually beneficial interaction between severely diseased plants infected with EACMV-UG2 and *B. tabaci* has been suggested (Colvin *et al*., 1999, 2004; Omongo, 2003). A change in the composition of the amino acids: asparagine, tryptophane, glutamine and tyrosine (P. Stevenson, unpublished data) in the severe phenotype plants was suggested as the possible cause of the observed increased fecundity and development rate of *B. tabaci*, which led to elevated vector population levels. However, more studies are still required to verify this assertion.

An alternative and perhaps not mutually exclusive hypothesis proposed the appearance of a more fecund biotype/genotype as the possible cause of the elevated *B. tabaci* populations in the pandemic zone (Gibson *et al*., 1996; Otim-Nape *et al*., 1996). More recently, using the mtCOI marker, it has been confirmed that a distinct *B. tabaci* genotype cluster, Ug2, also referred to, as the 'invader' was later associated with the epidemic of severe CMD in Uganda (Legg *et al*., 2002). Although the epidemiology of CMD has been widely investigated and documented (Otim-Nape *et al*., 1996), the role of virus mixtures and how these might impact on whitefly virus acquisition remains largely unknown. Elsewhere, evidence has accumulated that *B. tabaci* populations have different biological characteristics (Byrne and Miller, 1990), and that some recent devastating outbreaks of the vector, particularly in areas where it was previously unimportant, are linked to the appearance of new biotypes, strains or possibly species of *B.*

tabaci (Simone *et al*., 1990; Bedford *et al*., 1994; Brown, 1994, Brown *et al*., 1995a). For example, unprecedented outbreaks of the B biotype led to high populations that 'displaced' the A biotype in the United States of America, leading to the spread of devastating crop viruses (Brown, 1994; Brown *et al*., 1995a). However, the B biotype, which is also invasive in southern Spain, has failed to displace the Q biotype, the indigenous population (Moya *et al*., 2001). Most recently, outbreaks of the B biotype have occurred in Australia, China, and elsewhere in SouthEast Asia (De Barro *et al*., 2000; Chowda *et al*., 2003; Coombs *et al*., 2003), spreading with it several devastating crop viruses. Over the past decade, severe outbreaks of *B. tabaci* in many parts of East Africa have become more frequent, leading to increased spread of CMD and yield losses due to the disease.

In order to ensure the sustainable control of CMGs and the future development and expansion of cassava production in Africa and in Uganda in particular, it is crucial that the molecular variability of cassava *B. tabaci* genotypes and its effects on the epidemiology of cassava mosaic geminiviruses (CMGs) in Uganda is established. The interaction between synergism and the different genotypes also requires further investigation. High populations of *B. tabaci* continue to be reported in areas affected by the CMD pandemic (J.P. Legg, unpublished data) and a definitive answer is needed on the possibility that there is an epidemic-associated genotype with higher fecundity, virus acquisition or transmission efficiencies and whether this is linked to severity of symptoms should be investigated. If this is demonstrated, a practical objective of further studies on *B. tabaci* will be the development of a molecular marker, usable within laboratories in East and Central Africa, which would allow the specific detection of the epidemic-associated genotypes. This would then facilitate genotype 'tracking' and would aid in forecasting patterns of spread of the CMD pandemic. The findings of studies of this type will greatly support on-going efforts to control the CMD pandemic in East and Central Africa.

In contrast, if it is shown that the primary reason for population outbreaks of *B. tabaci* is the synergistic/mutually beneficial interaction with CMD-diseased cassava, a greater understanding of interactions between *B. tabaci* populations and CMGs/CMG mixtures will be vital for the prediction of future patterns of spread of the CMD pandemic.

1.5 Objectives of the study

This study sought to establish the molecular variability of cassava *B. tabaci* and its effect on the epidemiology of cassava mosaic geminiviruses in Uganda, following the 1990s epidemic of severe CMD. Possible associations between different *B. tabaci* genotypes and cassava mosaic geminivirus species, and whether a particular genotype is associated with the CMD pandemic were also investigated. The specific objectives of the study were:

(1) To establish the molecular variability of the principal *B. tabaci* genotypes on cassava through comparison of sequences of portions of the mitochondrial DNA cytochrome oxidase I (*mtCOI*) gene

(2) To establish the identity of the cassava mosaic geminiviruses occurring in Uganda, following the 1990s epidemic of severe cassava mosaic disease

(3) To establish the association between the geographical distributions of the cassava *B. tabaci* genotypes and that of the cassava mosaic geminiviruses occurring in the epidemic-affected areas of Uganda

(4) To establish the colonization and/or host range of cassava-associated *B. tabaci* genotypes in nature in cassava, and cultivated and uncultivated plant species occurring adjacent to cassava fields in the epidemic-affected areas of Uganda

Chapter Two

The cassava mosaic geminiviruses occurring in Uganda following the 1990s epidemic of severe cassava mosaic disease

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Key words: Cassava, cassava mosaic geminiviruses, virulence, distribution, Uganda

Abstract

The cassava mosaic geminiviruses (CMGs) isolated from cassava plants expressing mild and severe symptoms of cassava mosaic disease (CMD) in 2002 in Uganda were investigated using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) molecular techniques and DNA sequencing. Two previously described cassava mosaic geminiviruses: *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* – Uganda variant (EACMV-UG2) were detected in Uganda. The RFLP technique distinguished two polymorphic variants of ACMV (ACMV-UG1 and ACMV-UG2) and three of EACMV-UG2 (EACMV-UG2[1], EACMV-UG2[2] and EACMV-UG2[3]). ACMV-UG1 produced the fragments predicted for the published sequences of ACMV-[KE]/UGMld/UGSvr, while ACMV-UG2, which produced RFLP fragments predicted for the West African ACMV isolates ACMV-[NG], ACMV-[CM], ACMV-[CM/DO2] and ACMV-[CI] was shown to be ACMV-UGMld/UGSvr after DNA sequencing. EACMV-UG2[1] produced the RFLP fragments predicted for the published sequences of EACMV-UG2/UG2Mld/UG2Svr. However, both EACMV-UG2[2] and EACMV-UG2[3], which produced *East African cassava*

mosaic virus-[Tanzania]-like polymorphic fragments with RFLP analysis, were confirmed to be isolates of EACMV-UG2 after DNA sequencing. Thus, this study emphasises the importance of DNA sequence analysis for the identification of CMG isolates. EACMV-UG2 was the predominant virus and occurred in all the surveyed regions. It was detected in 73% of the severely and 53% of the mildly diseased plants, while ACMV was less widespread and occurred most frequently in the mildly infected plants (in 27% of these plants). Mixed infections of ACMV and EACMV-UG2 were detected in only 18% of the field samples. Unlike previously reported results the mixed infection occurred almost equally in both plants exhibiting the mild or severe disease symptoms (21% and 16%, respectively). The increasing frequency of mild forms of EACMV-UG2 together with the continued occurrence of severe forms in the field warrants further studies of virus-virus and virus-host interactions.

2.1 Introduction

An unusually severe form of cassava mosaic virus disease (CMD) was first reported in Uganda in 1988 in northern Luwero district, where it led to serious crop failure (Otim-Nape, 1993; Otim-Nape *et al*., 1994, 1997a; Gibson *et al*., 1996). CMD is caused by cassava mosaic geminiviruses (CMGs) (family *Geminiviridae*; genus *Begomovirus*) and is spread by the whitefly vector *Bemisia tabaci* Gennadius and disseminated through the use of CMD-affected cuttings used as planting material (Harrison, 1987). CMGs have geminate particles (Robinson *et al*., 1984) comprised of two ssDNA components (A and B). The DNA-A component encodes viral coat proteins required for replication and encapsidation of both DNA-A and B (Rogers *et al*., 1986), while the DNA-B component encodes transport proteins responsible for movement and symptom expression of the virus in the plant (Ingham *et al*., 1995). However, both DNA-A and B are required for infectivity (Stanley and Gay, 1983).

A virus referred to as African cassava mosaic virus (ACMV) was first reported as the causative virus of CMD in Africa (Bock and Harrison, 1985), but further investigation using monoclonal antibodies (Mabs) in enzyme-linked immunosorbent assay (ELISA) distinguished what were later regarded as two distinct viruses, now known as *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) (Thomas *et al*., 1986; Hong *et al*., 1993; Swanson and Harrison, 1994). Six other CMGs have been described of which four occur in Africa (Fauquet and Stanley, 2003). In Uganda the severe form of CMD was caused by a recombinant of ACMV and EACMV in which *c*. 400 nucleotides of the CP of ACMV replace the similar region of EACMV (Deng *et al*., 1997; Zhou *et al*., 1997). The recombinant was designated EACMV-UG2 on the basis of its more than 90% homology in the DNA-A sequence with EACMV (Deng *et al*., 1997; Pita *et al*., 2001a, Fauquet and Stanley, 2003). EACMV-UG2 was reported to be responsible for the severe epidemic of CMD (Harrison *et al*., 1997; Otim-Nape *et al*., 1997a, 1997b; Legg, 1999; Legg and Okao-Okuja, 1999), which was estimated to spread southwards across the country at an annual rate of 20 to 30 km (Otim-Nape *et al*., 1997a; Legg and Ogwal, 1998). A key feature of the epidemic was the formation of a 'front' characterized by unusually severe disease symptoms (Gibson *et al*., 1996; Harrison *et al*., 1997), rapid disease spread and high whitefly populations (Otim-Nape *et al*., 1997a; Legg and Ogwal, 1998; Colvin *et al*., 1999). Further investigation of the cause of the epidemic in Uganda revealed even more severe symptoms resulting from dual infections by ACMV and EACMV-UG2, believed to be due to a synergistic interaction between the two viruses (Harrison *et al*., 1997; Pita *et al*., 2001a). Major efforts were launched in the 1990s to control the epidemic, and significant success was achieved through the multiplication and distribution of CMD-resistant varieties (Thresh *et al*., 1994a; Otim-Nape *et al.,* 1997b, 2000, 2001; Legg *et al*., 1999). As the epidemic spread from Uganda into neighbouring countries, it was considered that there was a general reduction in CMD inoculum pressure in Uganda arising

from the reduction in area of cassava cultivated as a result of the epidemic, the increasing prevalence of officially released improved resistant varieties and the emergence of somewhat tolerant local varieties (Otim-Nape *et al*., 2000, 2001) and less virulent viruses. These changes led to the use of the term 'post-epidemic' to describe the current status of CMD in Uganda (Otim-Nape *et al*., 1998, 2000, 2001; Sseruwagi *et al*., 1998; Legg, 1999). The current study aimed at establishing the identity of the CMGs and their distribution in the post-epidemic affected areas in Uganda. The analysis was conducted using the DNA-A component. DNA sequencing, polymerase chain reaction (PCR) and restriction fragment polymorphism (RFLP) procedures were adopted for the virus analyses.

2.2 Materials and Methods

2.2.1 Virus sources, PCR amplification and RFLP analysis

Fresh leaf samples were collected from symptomatic young shoots of CMD-affected plants from a total of 100 cassava fields sampled in six important cassava-producing regions of Uganda comprising: central (*Kayunga*, *Kiboga*, Luwero, Masaka, Mpigi, Mubende, Mukono and *Wakiso* districts), eastern (*Bugiri*, *Busia*, Iganga, Jinja, Kamuli, Kumi, Mbale, Pallisa and Tororo districts), northern (Masindi–*Buruli*, Masindi–*Kibanda*, Nakasongola and Soroti districts), southern (Bushenyi, Mbarara and Rakai districts), western (Hoima, Kabarole, Kasese, Kibaale, *Kyenjojo*, Masindi–*Budongo* and Masindi–*Buliisa* districts) and Kalangala (Island) district, in September 2002 (Fig. 2). The locations indicated in italics are recently created districts. Cassava fields 3 to 5 months after planting were sampled at regular intervals along main roads traversing each of the sampled districts. In order to avoid cross-contamination, separate 'microcentrifuge tubes' were used to collect each leaf sample. One

sample was taken from a plant with mild CMD symptoms and another from a severely diseased plant of the same variety at each site. The samples were kept on ice in a cool box until laboratory analysis. DNA was extracted using the method of Dellaporta *et al*. (1983).

The universal primers Uni/F (5' KSGGGTCGACGTCATCAAGACGTTRTAC 3') and Uni/R (5' AARGAATTCATKGGGGCCCARARRGACTGGC 3') (Briddon and Markham, 1994), where K = G+T, R = A + G, S = G + C were used to amplify near full- length (*c*. 2760-2780 bp) DNA-A fragments. PCR with Taq DNA polymerase (Sigma Genosys Ltd, UK) was performed with a first cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1.5 min, 72°C for 2 min and a final cycle of 94°C for 1 min, 55°C for 1.5 min and 72°C for 10 min using a thermocycler (Hybaid-Omni-E, UK).

The virus-specific primers: ACMV-AL1/F (5' GCGGAATCCCTAACATTATC 3') and ACMV-ARO/R (5' GCTCGTATGTATCCTCTAAGGCCTG 3') and UV-AL1/F (5' TGTCTTCTGGGACTTGTGTG 3') and ACMV-CP/R3 (5' GCCTCCTGATGATTATATGTC 3') (Zhou *et al*., 1997) were used to identify the presence of ACMV and EACMV-UG2, respectively. ACMV and EACMV-UG2 positive controls were obtained from previously characterised CMG DNA, while the negative control comprised of the PCR reaction mixture less the DNA template. The PCR products were detected by electrophoresis.

In order to investigate the variability of the CMGs, RFLPs were analysed using *Eco*RV and *Mlu*I (R W Briddon, unpublished). The PCR-amplified products were digested independently with *Eco*RV and *Mlu*I for 1.5 hrs at 37ºC and separated by electrophoresis in a 1.8% agarose gel in TAE buffer. The results obtained were compared with those for the reference full-length CMG DNA-A sequences obtained from GenBank (National Center for Biotechnology

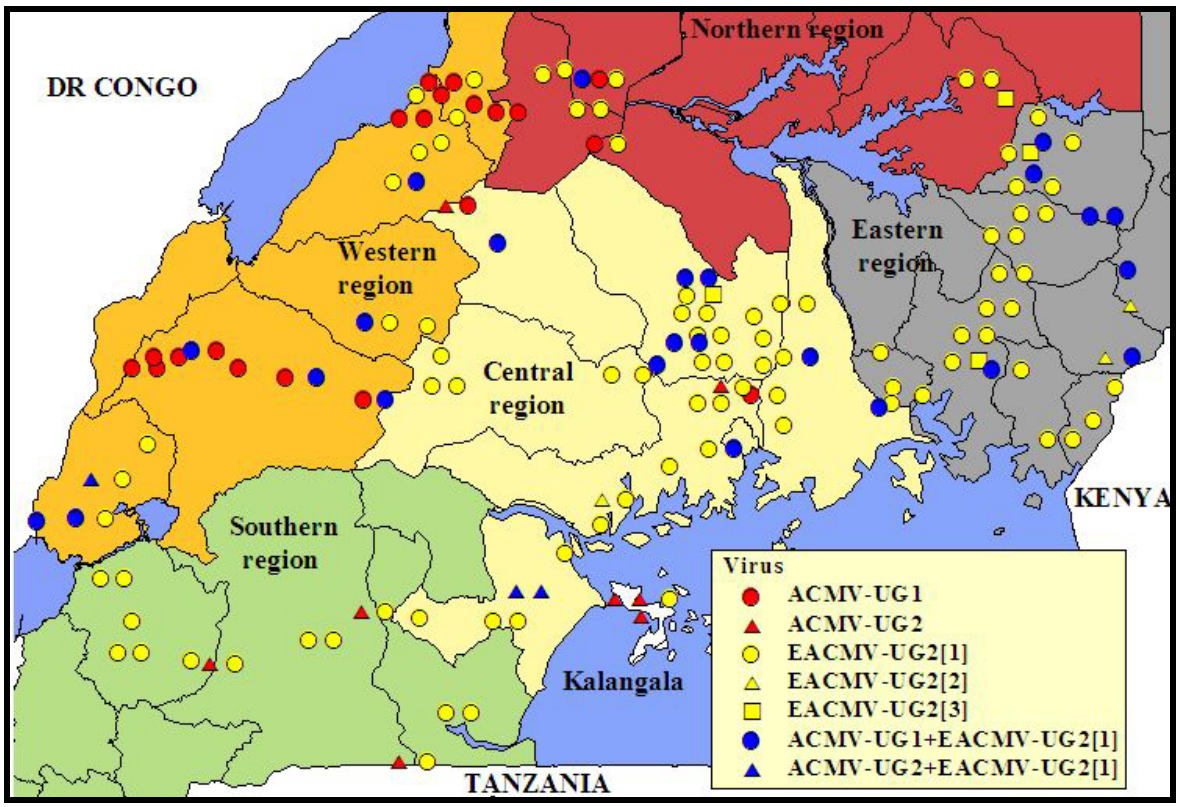

Figure 2. Map showing areas surveyed and the distribution of cassava mosaic geminiviruses in Uganda, September 2002

Information [NCBI]) using the DNA Strider 1.2 computer-based programme. The CMGs used in the analysis and their respective GenBank accession numbers are presented in Table 1. Nomenclature is as described in Fauquet and Stanley (2003).

2.2.2 Cloning, sequencing and phylogenetic analysis

To further analyse the data obtained with the PCR-RFLP-based technique, PCR products were subjected to DNA sequencing. Specific primers were designed to amplify a product of *c.* 1375 bp based on conserved CMG sequences beginning at 5′ position 1 (using ACMV as the reference virus) and flanking the common region (CR) and *CP* genes for the DNA-A components. The primers were: ACMV-A-CPV (ACCGGTTGGCCCCGCCCCCCTTT) and ACMV-A-CPC (GGCACCAACAACGACCATTCCTG) for ACMV and EACMV-A-CPV (GGCCGCGCCCGAAAAAGCAGGTGGA) and EACMV-A-CPC (AACCACGACATCATCACACTCCAGA) for EACMV-TZ and EACMV-UG2. The PCR products were ligated into pGEMT-Easy vector (Promega, Madison, Wisconsin, USA) and then used to transform *Escherichia coli* strain DH5α. The presence of inserts was confirmed by digestion of miniprep DNA with *Eco*RI.

Two representative clones were selected for each polymorphic variant as detected by the PCR-RFLP analysis and were sequenced bi-directionally using the virus-specific primers in an automated sequencer at the Laboratory of Molecular Systematics and Evolution facility, University of Arizona, Tucson, AZ 85621, USA. Consensus sequences were obtained using the computer programs Align and EditSeq (DNASTAR Lasergene, Madison, Wisconsin, USA). The CP open reading frames (ORFs) were identified using EditSeq. The sequences were compared with the published CMG sequences using the Basic Local Alignment Search

Table 1. Begomovirus sequences used for phylogenetic analysis

Begomovirus	Acronym [1]	GenBank Accession No. DNA-A
Abutilon mosaic virus	AbMV	X15983
African cassava mosaic virus – [Cameroon]	ACMV-[CM]	AF112352
African cassava mosaic virus – [Cameroon-DO2]	ACMV-[CM/DO2]	AF366902
African cassava mosaic virus – [Ivory Coast]	ACMV-[CI]	AF259894
African cassava mosaic virus – [Kenya]	ACMV-[KE]	J02057
African cassava mosaic virus – [Nigeria]	ACMV-[NG]	X17095
African cassava mosaic virus – [Nigeria-Ogo]	ACMV-[NG-Ogo]	AJ427910
African cassava mosaic virus – Uganda Mild	ACMV-UGMld	AF126800
African cassava mosaic virus – Uganda Severe	ACMV-UGSvr	AF126802
Beet curly top virus	BCTV	M24597
East African cassava mosaic Cameroon virus	EACMCV	AF112354
East African cassava mosaic Cameroon virus – [Ivory Coast]	EACMCV-[CI]	AF259896
East African cassava mosaic virus – [Kenya-K2B]	EACMV-[KE-K2B]	AJ006458
East African cassava mosaic Malawi virus – [MH]	EACMMV-[MH]	AJ006459
East African cassava mosaic Malawi virus – [K]	EACMMV-[K]	AJ006460
East African cassava mosaic virus – [Malawi]	EACMV-[MW]	AJ006461
East African cassava mosaic virus –[Tanzania]	EACMV-[TZ]	Z83256
East African cassava mosaic virus – Uganda2 (Uganda variant)	EACMV-UG2	Z83257
East African cassava mosaic virus – Uganda2 Mild	EACMV-UG2Mld	AF126804
East African cassava mosaic virus – Uganda2 Severe	EACMV-UG2Svr	AF126806
East African cassava mosaic Zanzibar virus	EACMZV	AF422174
East African cassava mosaic Zanzibar virus – [Kenya-Kilifi]	EACMZV-[KE-Kil]	AJ516003
South African cassava mosaic virus	SACMV	AF155806
South African cassava mosaic virus – [M12]	SACMV-[M12]	AJ422132
Tomato yellow leaf curl virus	TYLCV	X15656

[1] The abbreviations of the begomovirus acronyms are as listed by Fauquet and Stanley (2003)

Tools (BLASTX) (Altschul *et al*., 1997) at the National Center for Biotechnology Information (NCBI) and deposited in the GenBank under accession numbers AY562421-AY562430.

Sequence alignment was obtained with the ClustalW option (Thompson *et al*., 1994) in the multiple alignment programme, version 1.4 (Mac Vector 7.2 Package, Pharmacia Inc., San Diego, California, USA). The ClustalW alignments were used to generate a phylogenetic tree using parsimony and the maximum likelihood methods in the PAUP version 3.1.1 computer software (Swofford, 1993), with *Beet curly top virus* (BCTV) as the out-group. Bootstrap analysis (Felsenstein, 1985) was performed using 1000 replications. Pairwise nucleotide distance estimates for the partial DNA-A sequences were obtained with ClustalW.

2.2.3 Mapping

Geo-coordinates (latitude and longitude) were recorded using a geographical positioning system (GPS) for each sampled site and were used to map the geographical distribution of the CMGs in Uganda (Fig. 2) using ArcView software (Environmental Systems Research Institute, Inc., Redlands, CA).

2.3 Results

2.3.1 CMD symptom expression by infected cassava plants

CMD symptoms varied widely among the infected plants in the field, but deliberate steps were taken during this study to collect only samples from plants of a single variety that showed extreme mild and severe symptoms in each field. The symptoms generally consisted of severe mosaic; leaf distortion, abscission and necrosis, and reduced leaf size in the severely diseased

plants (Fig. 3a), whereas the mildly diseased plants exhibited a patchy green or yellow mosaic but generally lacked leaf distortion and abscission (Fig. 3b).

2.3.2 Polymerase chain reaction analysis of CMGs

Eighty-seven percent (152/174) of the virus samples produced a near full-length (*c.* 2760 - 2780 bp) DNA-A fragment after PCR amplification of CMG genomic DNA using the universal primers Uni/F and Uni/R (Fig. 4a). Two main CMGs, ACMV and EACMV-UG2, were detected in Uganda in 2002. Based on the overall occurrence of the CMGs in the single and mixed infections, ACMV was confirmed in 36% (55/152) of the positive samples, while EACMV-UG2 was the predominant virus and occurred in 82% (124/152) of the positive samples.

The mixed infections occurred between ACMV and EACMV-UG2 and were detected in only 18% (27/152) of the samples. EACMV-UG2 predominated in both the mildly (53%) and severely (73%) diseased plants, while ACMV was detected in 27% of the mildly and only 12% of severely diseased plants. The mixed infections comprised 21% of the total for the mildly diseased plants and only 16% for those with severe symptoms. Representative samples of agarose gels showing the PCR amplification of ACMV (*c.* 1024 bp) and EACMV-UG2(*c.* 1700 bp) using the virus-specific primers are presented in Fig. 4b and c, respectively.

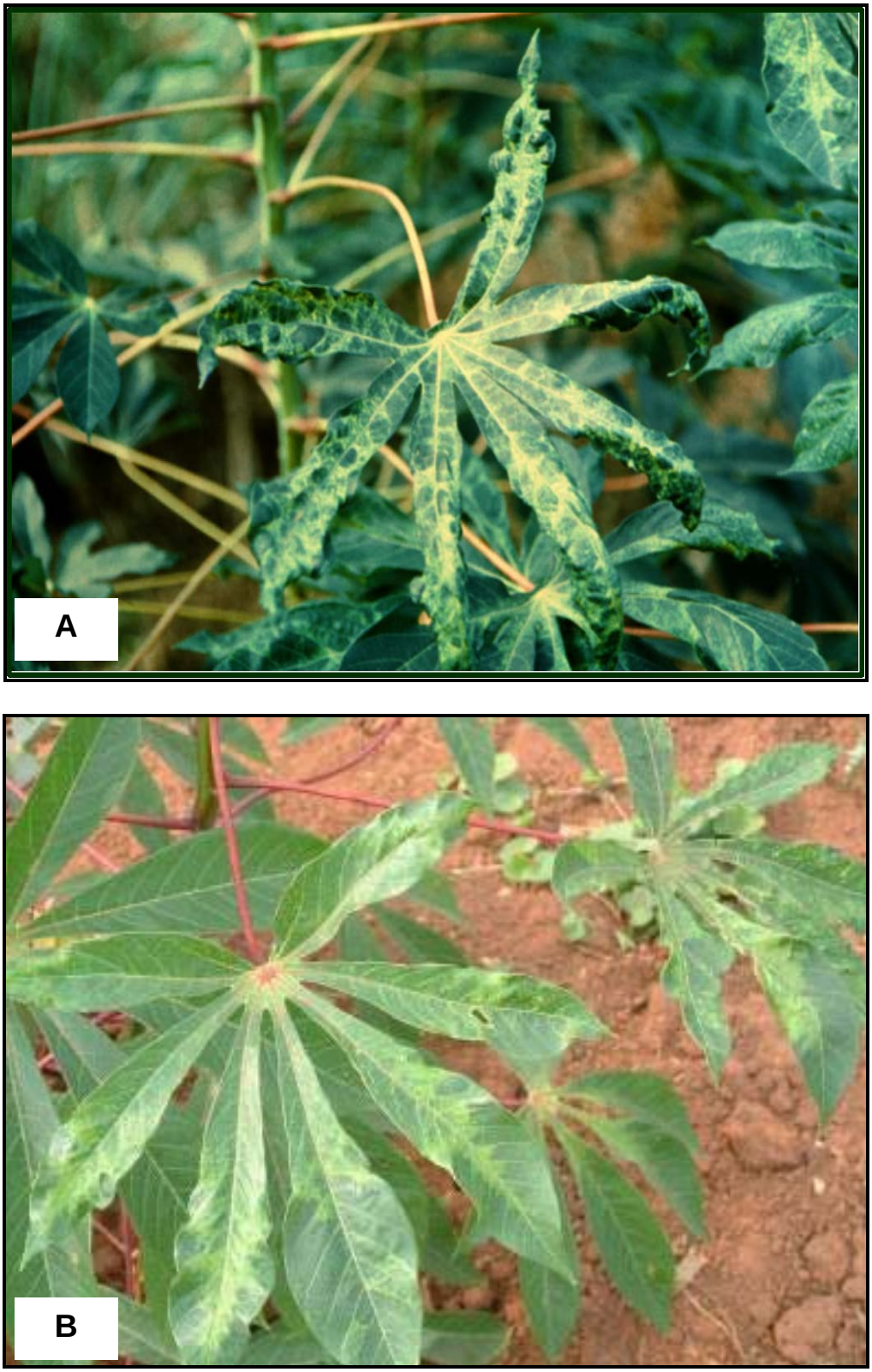

Figure 3. Symptom expression of (A) severe and (B) mild cassava mosaic on infected cassava plants of the CMD susceptible local cultivar Ebwanatereka

2.3.3 RFLP analysis of CMG variability in Uganda

Restriction fragment polymorphisms were used to investigate CMG variability in the PCR-amplified products, using the endonucleases *Eco*RV (Fig. 4d) and *Mlu*I (Fig. 4e). The band pattern and fragments produced following digestion were compared with those predicted for CMGs in the GenBank (Table 2).

As expected, two polymorphic fragments (1.48 and 1.28 kbp) were produced for the ACMV-infected plants (lanes 1, 2 and 9) and similarly, diagnostic fragments (2.19 and 0.59 kbp) were produced for EACMV-UG2 infections (lanes 3-5, and 10) following *Eco*RV digestion (Fig. 4d). Dual infections of ACMV and EACMV-UG2 occurred (lane 6 and 7) and were characterised by four polymorphic fragments (2.19, 1.48, 1.28 and 0.59 kbp) with the *Eco*RV digest (Fig. 4d). Digestion with *Mlu*I (Fig. 4e) produced two distinct polymorphic variants for ACMV: ACMV-UG1 (lane 1) and ACMV-UG2 (lane 2). In addition, three distinct polymorphic variants were established for EACMV-UG2 by the *Mlu*I digest (Fig. 4e). These were designated EACMV-UG2[1] (lane 4), EACMV-UG2[2] (lane 5) and EACMV-UG2[3] (lane 3). ACMV-UG1 was characterised by two polymorphic fragments (1.55 and 1.21 kbp), while ACMV-UG2 had one fragment (2.76 kbp).

The ACMV-UG1 fragments were similar to those predicted of ACMV-[KE]/UGMld/UGSvr, whereas ACMV-UG2 produced ACMV-[CI]/[CM]/[CM/DO2]/[NG]-like fragments (Fig. 4e, Table 2). EACMV-UG2[1] was characterised by four distinct polymorphic fragments (1.21, 0.67, 0.52 and 0.39 kbp) and was similar to EACMV-UG2/UG2Mld/UG2Svr (Table 2), while EACMV-UG2[3] produced three polymorphic fragments (1.21, 1.06 and 0.52 kbp), which were similar to those predicted of EACMV-[KE-K2B]/[MW]/[TZ]/EACMCV/[CI] (Table 2).

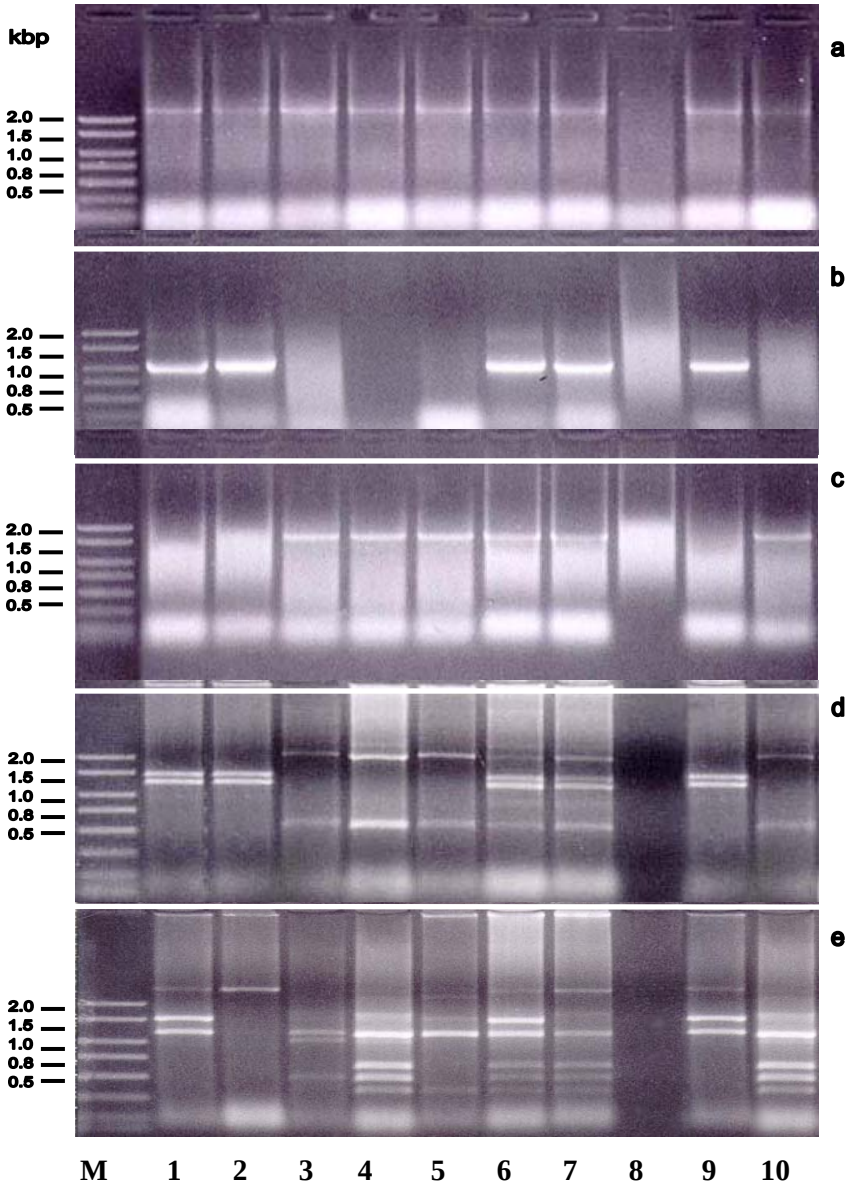

Figure 4. Gel electrophoresis of PCR-amplified DNA fragments using (a) universal oligonucleotide primers - Uni/F & Uni/R for full-length DNA-A, (b) ACMV-specific primers - ACMV-AL1/F & ACMV-ARO/R and (c) EACMV-UG2-specific primers - UV-AL1/F & ACMV-CP/R3 and restriction digestion of the PCR-amplified products using (d) *Eco*RV and (e) *Mlu*I. 1 - ACMV-UG1, 2 - ACMV-UG2, 3 - EACMV-UG2[3], 4 - EACMV-UG2[1], 5 - EACMV-UG2[2], 6 - ACMV-UG1 + EACMV-UG2[1], 7 - ACMV-UG2 + EACMV-UG2[1], 8 - Negative control (reaction mixture without DNA template), 9 and 10 are ACMV and EACMV-UG2 positive controls, M - Molecular marker (2 kbp).

Table 2. Predicted RFLPs for published cassava mosaic geminivirus sequences following computer-based digestion of near full-length DNA-A with the restriction enzymes *Eco*RV and *Mlu*I

Enzyme	Fragment	ACMV-[NG]/ [CI]/[CM]/ [CM/DO2]	ACMV-UGMld/ UGSvr/[KE]	EACMV-UG2/ UG2Svr	EACMV-UG2Mld	EACMV-[KE-K2B]/[MW]/ [TZ]/EACMCV /[CI]
*Eco*RV	1[1]	1.48 [2]	1.48	2.19	2.19	2.19
	2	1.28	1.28	0.59	0.59	0.59
*Mlu*I	1	2.76	1.55	1.21	1.21	1.21
	2	-	1.21	0.67	0.67	1.06
	3	-	-	0.52	0.46	0.52
	4	-	-	0.39	0.39	-
	5	-	-	-	0.06	-

[1] Number of fragments

[2] approximate size (kbp) of each fragment

However, EACMV-UG2[2] produced three distinct polymorphic fragments (1.21, 1.19 and 0.39 kbp), which did not correlate with any of the published CMGs. Products from plants co-infected with ACMV-UG1 and EACMV-UG2[1] or ACMV-UG2 plus EACMV-UG2[1] produced five polymorphic fragments (1.48, 1.21, 0.67, 0.52 and 0.39 kbp) or (2.76, 1.21, 0.67, 0.52 and 0.39 kbp), respectively following *Mlu*I digestion (Fig. 4e). None of the samples was co-infected with either ACMV or EACMV-UG2.

2.3.4 Cloning and analysis of CMG sequences

In order to further elucidate the results obtained with the PCR-RFLP technique, two representative samples were selected for each polymorphic variant for DNA sequencing. The specific primers, ACMV-A-CPV and ACMV-A-CPC for ACMV and EACMV-A-CPV and EACMV-A-CPC for EACMV-UG2, were used to amplify PCR products of *c*.1375 bp from the regions flanking the CR and CP. The PCR products were cloned and the partial nucleotide sequences obtained for clones *aug115* and *aug124* identified as ACMV-UG1. Clones *aug226* and *aug2110* corresponded to ACMV-UG2, *eaug112* and *eaug137* to EACMV- UG2[1], *eaug228* and *eaug237* to EACMV-UG2[2], and *eaug313* and *eaug322* to EACMV-UG2[3]. Phylogenetic analysis was conducted for the field isolates together with sequences of already published begomoviruses (Fig. 5) using parsimony and maximum likelihood methods. Results are reported only for the parsimony method because the two methods did not differ.

Based on the DNA sequencing results, isolates *aug115, aug124, aug226* and *aug2110* clustered with ACMV, while isolates *eaug112, eaug137, eaug228, eaug237, eaug313* and *eaug322* clustered with EACMV-UG2 (Fig. 5). Interestingly, none of the ACMV isolates clustered with the West African isolates ACMV-[CI]/[CM]/[CM/DO2]/[NG]/[NG-Ogo] and

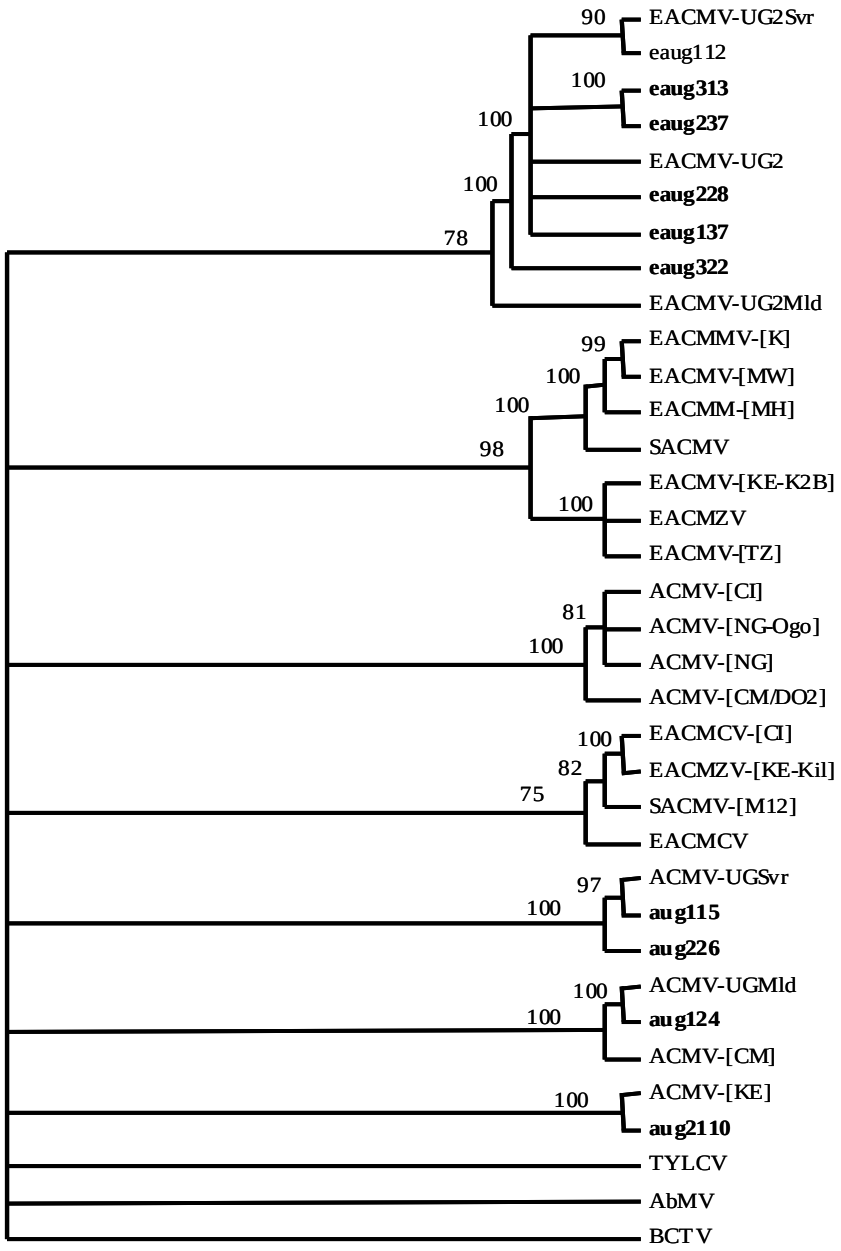

Figure 5. Dendrogram showing the phylogenetic relationship of cassava mosaic geminiviruses based on a partial sequence flanking the CR and CP. The abbreviated reference GenBank sequences are as described in Table 1 and the field isolates are presented in a lower case. The numbers at nodes indicate bootstrap scores using 1000 replicates and 70% majority-rule consensus tree. The horizontal distance equivalent was 0.05 substitutions per site

none of the EACMV-UG2 isolates clustered with EACMV-[KE-K2B]/[MW]/[TZ]/EACMCV/[CI], despite the distinct polymorphisms obtained for ACMV-UG2, EACMV-UG2[2] and EACMV-UG2[3]. Distance analysis revealed that the ACMV isolates shared a 95-100% sequence homology with their closest relatives, ACMV-[CI]/[CM]/[CM/DO2]/[NG]/[NG-Ogo]/[KE]/UGMld/UGSvr, while the EACMV-UG2 isolates shared a 98-100% sequence homology with their closest relatives: EACMV-UG2/UG2Mld/UG2Svr (Table 3). There was a higher percentage sequence identity within (≥ 95%) than between the virus species (69-88%).

2.3.5 Geographical distribution of CMGs in Uganda

EACMV-UG2 was the most widespread virus and occurred throughout the surveyed regions (Fig. 2). It predominated in the central, eastern, northern and southern regions, where it was detected in 89%, 100%, 84% and 81% of the samples, respectively (Table 4). In contrast, ACMV was less widespread and predominated in the western region (69%) and Kalangala Island (83%). Mixed infections occurred in the eastern (4.6%), western (5.3%), northern (3.9%), central (2.6%) and Kalangala (1.3%) regions, but were not identified in the southern region (Table 4). Relative proportions of plants exhibiting mild or severe symptoms were: 44%:56%, 38%:62%, 44%:56% and 48%:52% in the eastern, central, southern and northern regions, respectively. In the western and Kalangala regions each comprised 50% of the populations sampled (Table 4).

Table 3. Comparison of pairwise nucleotide identity (ClustalW) of sequences flanking the CR and CP of isolated cassava mosaic begomoviruses and some selected well-studied cassava mosaic begomoviruses

	CMG isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1.	ACMV-UGSvr	-	**98**[1]	**97**	**97**	**100**	**98**	**97**	**96**	80	80	80	80	80	79	80	80	80	72	73	70	74
2.	ACMV-UGMld		-	**97**	**96**	**98**	**96**	**97**	**97**	79	79	79	78	79	78	79	79	79	71	71	69	73
3.	ACMV-KE			-	**96**	**97**	**96**	**97**	**96**	79	79	79	79	79	78	79	79	79	71	71	69	72
4.	ACMV-NG				-	**97**	**96**	**96**	**95**	80	79	79	79	80	79	79	80	79	72	73	70	74
5.	aug115					-	**98**	**98**	**96**	80	80	80	80	80	79	80	80	80	72	73	70	74
6.	aug226						-	**96**	**95**	79	79	79	79	79	78	79	79	79	72	72	70	73
7.	aug124							-	**97**	80	80	80	79	80	79	80	80	80	72	72	70	73
8.	aug2110								-	78	78	78	78	78	77	78	78	78	70	70	69	72
9.	EACMV-UG2									-	**99**	**100**	**99**	**99**	**99**	**99**	**99**	**99**	89	89	82	80
10.	EACMV-UG2Mld										-	**99**	**99**	**99**	**98**	**99**	**99**	**99**	89	90	82	81
11.	EACMV-UG2Svr											-	**99**	**99**	**98**	**99**	**99**	**100**	89	90	81	81
12.	Eaug313												-	**99**	**98**	**98**	**98**	**98**	89	89	81	80
13.	Eaug137													-	**98**	**99**	**99**	**99**	89	89	81	80
14.	Eaug228														-	**98**	**98**	**98**	88	88	81	80
15.	Eaug237															-	**99**	**99**	89	89	81	80
16.	Eaug322																-	**99**	89	89	81	81
17.	Eaug112																	-	89	89	81	81
18.	EACMV-TZ																		-	96	88	81
19.	EACMV-KE-K2B																			-	88	82
20.	EACMV-CM																				-	75
21.	SACMV																					-

[1]Isolates (values over 90% sequence identity) are indicated in bold

Table 4. Occurrence and distribution of cassava mosaic geminiviruses in Uganda, September 2002

Region[1]		CMGs isolated							
		ACMV-UG1	ACMV-UG2	EACMV-UG2[1]	EACMV-UG2[2]	EACMV-UG2[3]	EACMV-UG2[1] + ACMV-UG1	EACMV-UG2[1] + ACMV-UG2	No. of samples (n)
Eastern	M[2]	0	0	11	1	0	4	0	16
	S	0	0	14	1	2	3	0	20
		0 (7)[3]	0 (0)	25(32)	2 (2)	2 (2)	7	0	36
Western	M	7	0	4	0	0	4	1	16
	S	7	0	6	0	0	3	0	16
		14 (21)	0 (1)	10 (18)	0 (0)	0 (0)	7	1	32
Central	M	1	2	10	1	0	0	0	14
	S	1	0	17	0	1	4	0	23
		2 (6)	2 (2)	27 (31)	1 (1)	1 (1)	4	0	37
Southern	M	0	3	4	0	0	0	0	7
	S	0	0	9	0	0	0	0	9
		0 (0)	3 (3)	13 (13)	0 (0)	0 (0)	0	0	16
Northern	M	3	0	5	0	0	4	0	12
	S	1	0	9	0	1	2	0	13
		4 (10)	0 (0)	14 (20)	0 (0)	1 (1)	6	0	25
Kalangala	M	0	2	0	0	0	0	1	3
	S	0	1	1	0	0	0	1	3
		0 (0)	3 (5)	1 (3)	0 (0)	0 (0)	0	2	6
	M	11	7	34	2	0	12	2	68
	S	9	1	56	1	4	12	1	84
	Total	20 (44)	8 (11)	90 (117)	3 (3)	4 (4)	24	3	152

[1]Regions are the relative geographical locations of the surveyed areas and do not represent the administrative boundaries of the regions of Uganda. [2]Symptoms phenotype, M = Mild, S = Severe. [3]Figures in parenthesis are occurrences (single and double infections) of the CMGs in samples collected from each region.

2.4 Discussion

CMGs occurring in Uganda in 2002 were investigated using DNA-based PCR-RFLP molecular techniques and the results extended by DNA sequencing. The PCR-RFLP technique was reproducible and enabled the preliminary identification of the CMGs affecting cassava, although some (13%) symptomatic plants produced no products with PCR. This could be the result of either the degradation of the samples or viral DNA during handling and storage before laboratory analysis or low virus titre in these samples. The PCR-RFLP technique distinguished between ACMV and EACMV-UG2, but further variability within the two species was not evident following sequence analysis, despite the potential usefulness of the technique in showing variations. This emphasises the need for DNA sequencing when seeking to fully characterise field isolates.

As in previous studies (Zhou *et al*., 1997; Harrison *et al*., 1997; Pita *et al*., 2001a, b) both ACMV and EACMV-UG2 occurred in Uganda in 2002. EACMV-UG2 predominated and was comprised of three isolates: EACMV-UG2 (Deng *et al*., 1997; Zhou *et al*., 1997), EACMV-UG2Mld and EACMV-UG2Svr (Pita *et al*., 2001a, b). In contrast ACMV was less prevalent and was represented by isolates ACMV-[KE] (Stanley, 1983), ACMV-UGMld and ACMV-UGSvr (Pita *et al*., 2001a, b). The unique polymorphisms observed for ACMV-UG2 did not correlate with the DNA sequencing results as both isolates *aug226* and *aug2110* clustered with ACMV-UGMld/UGSvr (Pita *et al*., 2001a, b) and not with the West African isolates ACMV-[NG] (Morris *et al*., 1990), ACMV-[CM]/[CM/DO2] and ACMV-[CI] (Fondong *et al*., 2000). Additionally, both the EACMV-UG2[2] isolates *eaug228* and *eaug237*, which generated unique RFLPs unlike any of the published CMGs and the EACMV-UG2[3], and isolates *eaug313* and *eaug322*, which generated a RFLP pattern

similar to that predicted of EACMV-[KE-K2B]/[MW] (Zhou *et al*., 1998), EACMV-[TZ] (Zhou *et al*., 1997) or EACMCV/[CI] (Pita *et al*., 2001b) clustered with EACMV-UG2/EACMV-UG2Mld/EACMV-UG2Svr. These data provide no evidence for the occurrence of EACMV-[KE-K2B]/ [MW]/[TZ] or EACMCV/[CI] in Uganda. Rather the unique polymorphisms could be the result of single base substitutions in the sequences.

EACMV-UG2 was found to be more widespread in post-epidemic Uganda than previously reported (Harrison *et al*., 1997; Pita *et al*., 2001a). Unlike previous observations, in which the virus was consistently associated with severe CMD symptoms (Harrison *et al*., 1997; Zhou *et al*., 1997; Pita *et al*., 2001a), in the current study EACMV-UG2 occurred in both the mildly (74%) and severely (89%) diseased plants. This is probably due to the increased occurrence of mild forms of EACMV-UG2 compared with 5 yr previously, when mild EACMV-UG2 was seemingly confined to isolated localities (Pita *et al*., 2001a). Furthermore, as a result of change in attitude farmers are increasingly selecting the mildly diseased plants, which provide more planting material and ensure higher yields than the severely diseased plants (Owor, 2003). As a consequence, many local varieties are now retained in farmers' fields despite the high incidence of infection (Otim-Nape *et al*., 2001). Moreover, the widespread occurrence of both the mildly and severely diseased plants in the field, irrespective of location, inherent plant resistance and/or virus type is further evidence that Uganda was in a 'post-epidemic' situation by the time of the current study. As expected, ACMV was mainly associated with mild (48%) than severe (28%) CMD. The virus (ACMV) has been reported in most of the cassava producing areas of Africa (Swanson and Harrison, 1994) and has not been associated with severe CMD. The higher incidence of ACMV than EACMV-UG2 in Kalangala Island suggests that the latter may have only recently spread to this area. By contrast, the predominance of ACMV in the western region, an area affected by EACMV-UG2 during the

1990s (Harrison *et al*., 1997), is more likely to be the result of recent whitefly-borne spread of ACMV in the region.

Mixed infections occurred in the eastern (4.6%), western (5.3%), northern (3.9%), central (2.6%) and Kalangala (1.3%) regions. Mixed infections have been reported previously in Uganda (Harrison *et al*., 1997; Pita *et al*., 2001a, b) and were shown to elicit extremely severe symptoms in the affected plants. Surprisingly, data from this study did not provide evidence of the previously reported synergism in the mixed infections that leads to severe symptoms. However, both mild and severe forms of EACMV-UG2 and ACMV have been reported (Pita *et al*., 2001*a*), and confirmed in our study, and it may be that there are mixtures in which synergism does not occur, possibly involving mild strain combinations. Furthermore, the fact that no plants were identified that showed co-infection with different isolates of either ACMV or EACMV-UG2 is significant and could suggest that infection of a plant by one strain prevents superinfection by another strain of the same virus. It has been reported that initially healthy plants become more severely diseased than plants initially infected by mild virus strains under high CMD pressure conditions in Uganda (Owor, 2003; Owor *et al*., 2004a).

The study established the current situation of the CMGs in the post-epidemic affected areas of Uganda. The widespread occurrence of EACMV-UG2 throughout the surveyed areas is evidence that the disease remains a major limiting factor to cassava production in the country. However, the increasing occurrence of mildly diseased plants in farmers' fields warrants further investigation. This information will be integral to an understanding of the molecular mechanisms and biological significance of the virus-virus and virus-host interactions.

Chapter Three

Mitochondrial DNA variation and geographical distribution of cassava *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) genotypes and associated cassava begomoviruses in the post-CMD epidemic regions of Uganda

Submitted: *Bulletin of Entomological Research*

Key words: cassava mosaic disease, EACMV-UG2, *mtCOI*, whitefly

Abstract

Unprecedented upsurges in populations of the cassava whitefly *Bemisia tabaci* (Genn.) have occurred in several regions of Uganda that have been affected by an epidemic of a severe form of cassava mosaic disease (CMD), which began there in the late 1980's. More recently, the increased whitefly populations and the severe form of CMD have spread to previously unaffected areas in Uganda and beyond. The objective of this study was to investigate the identity and geographical distribution of cassava-associated *B. tabaci* genotypes in the post-CMD epidemic affected areas of Uganda, using the mitochondrial cytochrome oxidase I (mtCOI) sequence as a molecular marker. The presence in the post-epidemic zone of the two previously described genotype clusters, Ug1 and Ug2, that diverged at ~8% (nt. identity), as was also reported previously, was confirmed. A comparison of the mtCOI nucleotide sequences (n = 66) for *B. tabaci* within the Ug1 and Ug2 genotype clusters revealed intra-group variances of 0.1 to 2.8% and 0.1 to 0.6%, respectively. However, our data showed that

the Ug1 genotypes occurred more frequently (83%) than the Ug2 types (17%), in contrast to the genotypes distribution reported for vector populations that occurred on cassava in 1996-97, at the height of the spread of the severe epidemic. In addition, unlike previously reported, in the current study no definitive association was established of a particular vector genotype cluster with cassava plants exhibiting the severe disease phenotype. However, generally, the Ug1 genotypes predominated with EACMV-UG2 in the central, southern, northern and eastern regions, while the Ug2 types occurred most frequently in the western and Kalangala Island regions where ACMV infection was likewise dominant.

3.1 Introduction

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) transmit begomoviruses (genus, *Begomovirus*; family, *Geminiviridae*) (Fauquet and Stanley, 2003) that cause mosaic disease (CMD) of cassava in Africa (Bock and Woods, 1983; Fishpool and Burban, 1994; Legg, 1995; Otim-Nape *et al*., 1996). During the past fifteen years, upsurgence of *B. tabaci* in certain parts of sub-Saharan Africa has become more frequent and increased yield losses have occurred in cassava crops heavily colonized by *B. tabaci*, with disease incidences ranging from 20% to 100% in the most affected cultivars (Thresh *et al*., 1994b). Using a conservative estimate of 100 United States (U.S.) dollars per tonne, annual losses in cassava due to CMD are estimated at $60 million in Uganda (Otim-Nape and Thresh, 1998), and more than $1,200 million in Africa (Thresh *et al*., 1997).

Evidence has been provided that *B. tabaci* populations exhibit distinct biological traits as in host-plant range and adaptability (Bird, 1957; Costa and Russell, 1975; Gill, 1992; Burban *et al*., 1992; Legg, 1995), induction of plant physiological disorders (Costa and Brown, 1991;

Cohen *et al*., 1992) and plant virus transmission (Brown and Bird, 1992, Bedford *et al*., 1994; Markham *et al*., 1996; Maruthi *et al*., 2002). Using different molecular tools, including the random amplified polymorphic DNA (RAPD) PCR fingerprinting (Gawel and Bartlett, 1993; De Barro and Driver, 1997; Guirao *et al*., 1997; Maruthi *et al*., 2001; Moya *et al*., 2001), the internal transcribed spacer I (ITSI) region (De Barro *et al*., 2000, 2005; Abdullahi *et al*., 2003), and the mitochondrial DNA cytochrome oxidase I (*mtCOI*) gene (Frohlich *et al*., 1999; Legg *et al*., 2002; Viscarret *et al*., 2003; Berry *et al*., 2004; Maruthi *et al*., 2004a), evidence has been provided also for the occurrence of genetically distinct *B. tabaci* populations worldwide.

Increased populations of *B. tabaci,* particularly in areas where the whitefly was previously unimportant, have been linked to the appearance of new biotypes or strains of the vector (Brown, 1990, 1994, 2001; Brown and Bird, 1992; Cohen *et al*., 1992; Bedford *et al*., 1994). In the south-western U.S. for example, the B biotype of *B. tabaci* was introduced to the region by way of ornamental plants (Costa and Brown, 1991; Costa *et al*., 1993a; Brown *et al*., 1995a,b; Frohlich *et al*., 1999; Brown, 2000). The B biotype subsequently increased in distribution and abundance, ultimately displacing the 'local' A biotype (Costa *et al*., 1993a). However, the B biotype, which has also invaded southern Spain, has failed to displace the Q biotype, the indigenous population (Moya *et al*., 2001). The propensity of certain *B. tabaci* to colonize a wide range of plant hosts (Cock, 1993) has led to outbreaks of previously undescribed begomovirus diseases in the Americas (Brown, 1990, 1994, 2000, 2001; Brown and Bird, 1992; Brown *et al*., 1995b).

In Uganda a key feature of the epidemic of severe form of CMD that devastated cassava was the occurrence of a disease 'front' characterized by an unusually severe disease symptom, rapid CMD spread (Legg and Ogwal, 1998), and high whitefly populations (Otim-Nape *et al*.,

1997a; Legg and Ogwal, 1998; Colvin *et al*., 1999). In trying to establish the cause of the increased whitefly populations at the CMD epidemic 'front', research has focussed mainly on cassava-colonizing *B. tabaci* populations, in search of a pandemic-associated biotype (Maruthi *et al*., 2001; Legg *et al*., 2002), and the interaction between the cassava mosaic geminiviruses (CMGs), the whitefly vector and their cassava host (Colvin *et al*., 1999, 2004; Maruthi *et al*., 2002; Omongo, 2003).

Preliminary evidence has been advanced for a synergistic/mutually beneficial interaction between severely diseased plants infected with *East African cassava mosaic virus*–Uganda 2 (EACMV-UG2) and *B. tabaci* (Colvin *et al*., 1999, 2004; Omongo, 2003). A change in the composition of the amino acids: asparagine, tryptophane, glutamine and tyrosine (P. Sevenson, unpublished data) in the severe phenotype plants was suggested as the possible cause of the observed increased fecundity and development rate of *B. tabaci*, which led to increased vector population densities. However, these results remain to be verified with further studies. An alternative and not mutually exclusive hypothesis, proposed the appearance of a more fecund biotype/genotype of *B. tabaci* as the likely cause of the increased whitefly populations in the epidemic-affected areas (Gibson *et al*., 1996; Otim-Nape *et al*., 1996). *B. tabaci* were collected on cassava in the pandemic and non-pandemic areas in Uganda in 1996-97 (Maruthi *et al.*, 2001; Legg *et al.*, 2002), and used to test the hypothesis.

Maruthi *et al*. (2001) found no discernible differences in the fecundity, nymphal development, or survival to adult eclosion in the cassava-associated *B. tabaci* populations from pandemic and non-pandemic areas. Further, no clear association was established between plants exhibiting the severe disease phenotype, and 'pandemic' or 'non-pandemic'-associated whiteflies. Moreover, in a related study, Maruthi *et al*. (2002) established no significant differences in

virus transmission efficacy between whiteflies from the 'pandemic' and 'non-pandemic' areas. This led to the conclusion that the severe pandemic was probably not associated with a novel or reproductively-isolated *B. tabaci* biotype with improved fitness attributes. However, both the 'pandemic' and 'non-pandemic' populations grouped in the same phylogenetic clade, based on RAPD-PCR (Maruthi *et al*., 2001). This led to the suggestion that the two populations were genetically similar, and hence, they would not have been expected to exhibit biological differences, which was consistent with experimental results.

Using the mtCOI molecular marker, Legg *et al*. (2002) confirmed that a distinct *B. tabaci* genotype cluster, designated Uganda 2 (Ug2), was associated with the epidemic of severe CMD in 1997/8 in Uganda. More recently, the occurrence of the Ug2-like genotypes in the pandemic zone in Uganda was confirmed also by Maruthi *et al*. (2004) from the mtCOI analysis of a whitefly population collected at Namulonge, which was within the pandemic-affected zone in 1996-97. In the Legg *et al*. (2002) study, another genotype cluster, referred to as Uganda 1 (Ug1) was identified, which diverged at 8% from Ug2, and was most closely related to the other genotypes from surrounding countries that were unaffected by the CMD epidemic together with *B. tabaci* from southern Africa (Berry *et al*., 2004), and in localities of Uganda 'ahead' of the epidemic 'front'. Since 1997, the EACMV-UG2-associated epidemic has continued to spread, affecting the whole of Uganda (Sseruwagi *et al*., 2004b) and the high *B. tabaci* populations persist in many parts of the country including the newly affected areas. In this context, it is essential to understand the current status of previously identified *B. tabaci* genotypes, in order both to clarify their relationship with the CMD epidemic and to determine the biological significance of the genetic differences identified and their epidemiological implications.

In this study, we sought to establish the geographical distribution of the Ug1 and Ug2 *B. tabaci* genotypes in the post-epidemic zone of Uganda. Additionally, the relationship between the vector genotypes and distribution of cassava-infecting begomoviruses in the post-epidemic zone was examined also. We used the terms genotype and genotype cluster throughout to refer to genetically distinct sequences and for a group of closely related sequences, respectively.

3.2 Materials and Methods

3.2.1 Whitefly and virus collections

Whitefly (*B. tabaci*) adults and mosaic-affected cassava leaves were collected from 3 to 5 month-old cassava plants during September 2002 at twenty-two locations in six major cassava-producing regions of Uganda (Table 5). At each location, at least 20 adult whiteflies were collected using an aspirator. Whiteflies were transferred to vials containing 80% ethanol and stored at -20ºC until analysis. Two leaf samples were collected from symptomatic cassava plants at each location, and stored in a cool box. Samples were frozen at -20ºC until virus identification was carried out.

3.2.2 Extraction of whitefly DNA

Three adult female whiteflies were selected randomly from each field collection/sample. Each individual insect was ground in 40µl of lysis buffer (5mM Tris-HCl, pH8.0, 0.5 mM EDTA, 0.5% Nonidet P-40, 1 mg/ml proteinase K) using the tips of 0.5 ml microfuge tubes. The lysis product was incubated for 15 min at 65°C and for a further 10 min at 95°C. The lysis was

briefly centrifuged (~ 60 sec) and immediately placed on ice before PCR amplification. Lysis was done as described by Frohlich *et al*. (1999).

3.2.3 PCR and cloning of mtCOI DNA

Amplification of a fragment (~ 850 bp) of the *mtCOI* gene was achieved by the primer pair: MT10/C1-J-2195 (5´-TTGATTTTTTGGTCATCCAGAAGT-3´) and MT12/L2-N-3014 (5´-TCCAATGCACTAATCTGCCATATTA-3´) obtained from the UBC Insect Mitochondrial DNA Primer Oligonucleotide Set, complied by B. J. Crespi and C. Simon (Simon *et al*., 1994, per Frohlich *et al*., 1999). A DNA template of 5µl was used in a reaction mixture of 25µl, containing 2.5µl *Taq* 10x buffer with Mg^{2+}, 3.0µl 5x *Taq* Master enhancer, 1.5µl deoxynucleotide triphosphates (dNTPs), 1.0µl of 25 mM Mg^{2+}, 0.75µl each of primers MT10 and MT12 and 0.15 µl of *Taq* DNA polymerase.

Template DNA was denatured at 95°C for 1 min, followed by primer annealing at 52°C for 1 min and DNA amplification for 1 min at 72°C using 30 cycles. A final extension of 20 min was run at 72°C and the reaction held at 4°C in a Perkin Elmer DNA thermal cycler. Electrophoresis of the PCR-amplified products was conducted using 1% agarose gels in 1x TAE buffer in a submarine gel unit (Hoefer Scientific Instruments, San Francisco, U.S.A) and the bands viewed with UV transillumination following ethidium bromide staining. The gels were photographed using an Electrophoresis Documentation and Analysis System 120 digital camera (Kodak Digital Science, Japan).

Table 5. Geographical distribution and percentage of the total population examined for cassava-associated *B. tabaci* genotypes, and of two cassava mosaic begomoviruses, associated with cassava plants in six regions of Uganda during September 2002

Geographical location		Source plant	Number of *B. tabaci* samples	*B. tabaci* genotype distribution (%)		Cassava mosaic begomovirus distribution (%)	
Region[1]	District/s			Ug1	Ug2	ACMV	EACMV-UG2
Central	Wakiso, Masaka, Kibaale, Mukono	Cassava	12	83	17	20	80
Southern	Rakai, Mbarara, Bushenyi	Cassava	12	100	0	19	81
Western	Kabarole, Kyenjojo, Masindi, Hoima	Cassava	12	58	42	55	45
Northern	Nakasongola, Masindi, Soroti	Cassava	12	92	8	32	68
Eastern	Iganga, Kumi, Busia, Bugiri	Cassava	12	83	17	16	84
Kalangala Island	Kalangala	Cassava	6	75	25	62	38

[1]Regions are relative geographical locations of the surveyed areas and do not follow the administrative boundaries of the regions of Uganda

A PCR product of the expected size (~ 850 bp) was obtained. Bands were excised from the agarose gel and purified for DNA sequencing using a QIAquick PCR Purification Kit (Qiagen Inc, USA) and the protocol supplied by the manufacturer. The sequence for each PCR product was determined bi-directionally to achieve at least a 200 base overlap, using the PCR primers MT10 and MT12 as sequencing primers. Sequences were determined using an automated sequencer (ABI 3700) located at the Laboratory of Molecular Systematics and Evolution (LMSE) Facility, University of Arizona, Tucson, Arizona, USA.

3.2.4 Phylogenetic analysis of mtCOI sequences

Whitefly mtCOI sequences were edited manually to produce a consensus sequence (800 bp) for each individual using Faktory, which is based on the on-line program, FAKtory 1.41 (Ewing *et al*., 1998), available through the Biotechnology Computing Facility of the University of Arizona (http:bcf.arl.Arizona.edu/biodesk) and the EditSeq programme available in the DNASTAR software package (Lasergene, Madison, Wisconsin, USA). Sequences were aligned using the Clustal W (weighted) (Thompson *et al.*, 1994) algorithm option in the Megalign computer programme (DNASTAR). Mt COI sequences were subjected to a heuristic search and subtree-pruning-regrafting branch swapping using the maximum likelihood (ML) and parsimony methods available in Phylogenetic Analysis Using Parsimony* (PAUP*4.0b4) (Swofford, 1998). The ML tree was reconstructed using the maximum likelihood 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 PAUP using the heuristic option for 1000 replications at a 70% confidence limit. Sequences were subjected to phylogenetic

analysis using reference mtCOI sequences available in the EMBL/DDBJ/NCBI GenBank databases (Table 6).

3.2.5 DNA extraction, PCR and analysis of cassava begomoviruses

Viral DNA was extracted using the method of Dellaporta *et al*. (1983). PCR amplification of begomoviral DNA was conducted with the universal primers: Uni/F (5' KSGGGTCGACGTCATCAAGACGTTRTAC 3') and Uni/R (5' AARGAATTCATKGGGGCCCARARRGACTGGC 3') (Briddon and Markham, 1994), where K = G+T, R = A + G, S = G + C to obtain near full-length (*c*. 2760-2780 bp) DNA-A fragments. The virus-specific primers: ACMV-AL1/F (5' GCGGAATCCCTAACATTATC 3') and ACMV-ARO/R (5' GCTCGTATGTATCCTCTAAGGCCTG 3') and UV-AL1/F (5' TGTCTTCTGGGACTTGTGTG 3') and ACMV-CP/R3 (5' GCCTCCTGATGATTATATGTC 3') (Zhou *et al*., 1997) were used to identify the presence of ACMV (*c*. 1024 bp) and EACMV-UG (*c*. 1700 bp), respectively. Viral DNA from previously characterised samples was used as positive controls for ACMV and EACMV-UG and the PCR reaction mixture as the negative control. The PCR products were detected by electrophoresis. The conditions and reagents used for the PCR analyses are as described in Sseruwagi *et al*. (2004b).

3.2.6 Genotype distribution mapping

Latitude and longitude were recorded for each sample site using a Geographical Positioning System (GPS) for each sampled location and were used to map the geographical distribution of the *B. tabaci* genotypes in Uganda, using ArcView software (Environmental Systems Research Institute Inc., Redlands, California, USA).

Table 6. Whitefly genotypes and location of reference whiteflies used in phylogenetic analysis of mitochondrial cytochrome oxidase I sequences and their respective GenBank accession numbers

Whitefly/Genotype	Location	GenBank Accession No.
AB A Benin	Benin	AF110693
ARG2 Santiago	Argentina	AF340213
AZ A Arizona	Arizona, USA	AY057122
AZ B Arizona	Arizona, USA	AY057123
Bemisia afer[1]	-	AY057218
CAL A Brawlee CA	California, USA	AY057124
CUL Mexico	Mexico	AY057125
HC China	China	AF342777
IS B Israel	Israel	AF110705
Ivory Coast, cassava	Ivory Coast	AY057135
Ivory Coast, okra	Ivory Coast	AY057136
IW India	India	AF110702
JAT Puerto Rico	Puert Rico	AF110705
Morocco 1	Morocco	AF342773
Moz-Kal 1	Mozambique	AF344278
PC91 Pakistan 1	Pakistan	AF342778
SA Lucia 2	South Africa	AF344260
SC Sudan 1	Sudan	AF110706
SP92 Spain Q	Spain	AF342775
SwazMap1	Swaziland	AF344269
Trialeurodes vaporariorum[2]	-	AF342774
TC Turkey	Turkey	AF342776
Thailand cotton	Thailand	AF164670
Uganda sweetpotato	Uganda	AY057174
Zam 2	Zambia	AF344281
30MNten (Ug1)	Uganda	AY057171
70Namu (Ug1)	Uganda	AY057209
59Bmatu (Ug1)	Uganda	AY057199
131Igan (Ug2)	Uganda	AY057154
17Ikul (Ug2)	Uganda	AY057158

[1,2]The species and genus outgroups used in the analysis were *Bemisia afer* (Priesner & Hosny) and *Trialeurodes vaporariorum* (Westwood)

3.3 Results

3.3.1 PCR amplification of mtCOI DNA

At least six *B. tabaci* adults were obtained from each of six major cassava-producing regions of Uganda (Table 5), to provide a total of 66 samples to study the genetic variability and distribution of cassava-associated *B. tabaci* genotypes. A PCR fragment of the *mtCOI* gene (~850 bp) was obtained for each adult whitefly examined using the primer pair: MT10/C1-J-2195 and MT12/L2-N-3014 (Fig. 6).

3.3.2 Phylogenetic analysis of whitefly mtCOI sequences

A consensus sequence was obtained for each mtCOI nucleotide sequence. The sequences have been deposited in the GenBank database as accession numbers AY563637 to AY563704. Phylogenetic trees that were predicted using parsimony and the maximum likelihood methods did not differ with respect to clade affiliations for whitefly genotypes, thus, only the parsimony tree is shown here (Fig. 7).

Based on the phylogenetic analyses of the mtCOI sequences, members of the *B. tabaci* complex grouped into four main clusters in either the New or Old World (Fig. 7). All four major clusters were supported by high bootstrap (bs) values (>90), with several other sub-groups having bs scores of ≥80. The first main cluster contained genotypes from the New World (Argentina, Puerto Rico, Mexico and USA). The second, third and fourth main clusters contained only Old World *B. tabaci*. Members of *B. tabaci* from the Mediterranean-North Africa-Middle East (MED-NAFR-ME) region, which also includes the well-studied B and Q

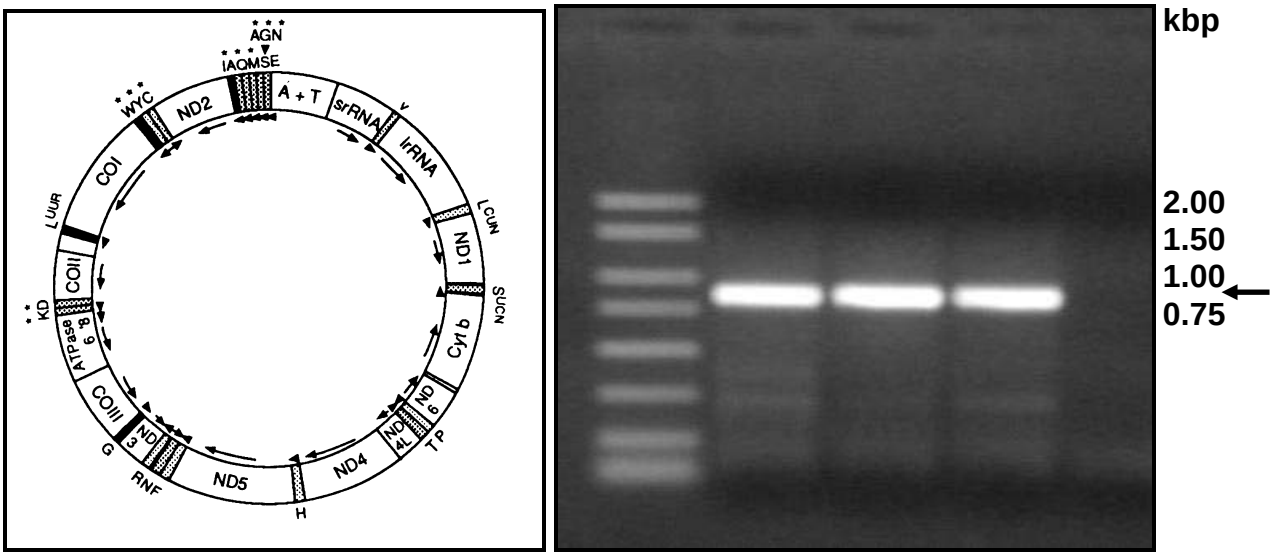

Figure 6. (A) Map of mitochondrial DNA showing position of cytochrome oxidase I (*COI*) gene, adapted from Crozier and Crozier (1993) in Marjorie (1994). (B) Agarose gel of PCR-amplified fragments (850 bp) of *Bemisia tabaci* mtCOI DNA (lanes 2-4). Lane 1: DNA ladder of 2 kbp

biotypes grouped in the second main cluster, while those from SouthEast Asia and the Far East, including India, China, Pakistan, Thailand and Australia grouped in the third main cluster. The cassava-associated *B. tabaci* genotypes from sub-Saharan Africa grouped into three sub-clusters within the fourth main cluster, together with a non-cassava genotype on *Asystasia* spp. in Benin. However, the Ugandan sweetpotato and Ivory Coast cassava genotypes clustered separately from the other African genotypes.

Whiteflies (*B. tabaci*) collected on cassava in the post-CMD epidemic affected areas of Uganda (this study), grouped definitively in two sub-clusters (bs >90) within the fourth main cluster, together with other cassava-associated *B. tabaci* populations from sub-Saharan Africa. *B. tabaci* (n = 55) in the first sub-cluster shared close sequence homology (97-99%) with the previously reported Ug1 genotypes in Uganda, together with *B. tabaci* on cassava from Mozambique, South Africa, Swaziland and Zambia in southern Africa, and collectively, from locales outside the CMD epidemic zone (Fig. 7). The intra-group variance was 0.1 to 2.8% (Table 7). *B. tabaci* (n = 11) in the second sub-cluster closely resembled (>99% nt identity) the Ug2 genotypes, also reported previously in Uganda (Fig. 7), with an intra-group variance of 0.1 to 0.6%. A pairwise comparison of the nucleotide identity of the Ug1- and Ug2-like genotypes revealed an inter-group variance of ~ 8% (Table 7).

3.3.3 Geographical distribution and association of *B. tabaci* genotypes and CMGs in Uganda

Interestingly, the Ug1 genotypes comprised the majority (83%) of the *B. tabaci* sampled on cassava and occurred more frequently than the Ug2 types (17%) in the post-CMD epidemic-affected areas in Uganda in 2002 (Table 5, Fig. 8). The Ug1 types predominated throughout the sampled areas, comprising the entire population in southern region, 92% of the total

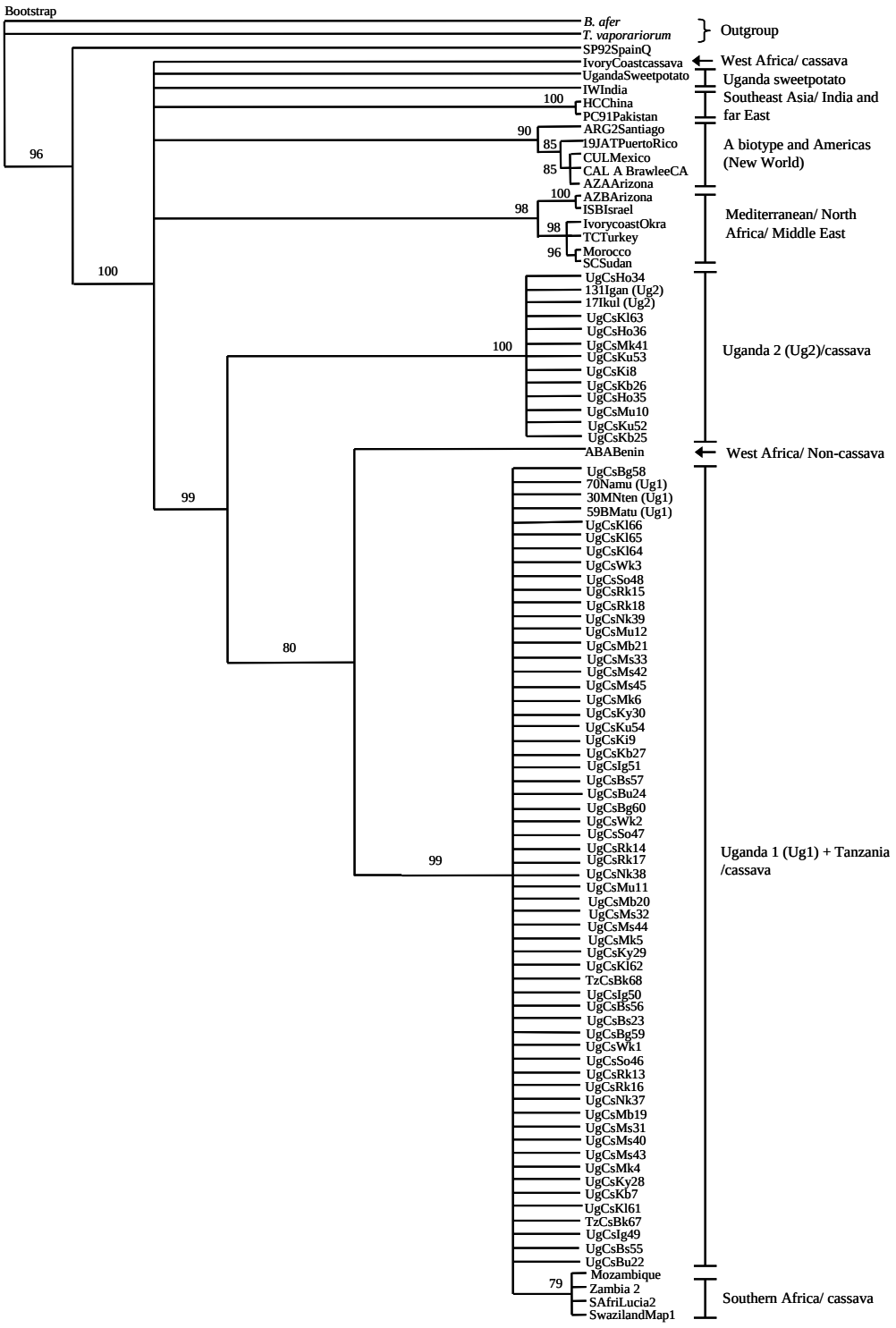

Figure 7. Predicted genotype phylogeny based on the mitochondrial cytochrome oxidase I sequence for *B. tabaci* collected in six cassava-growing regions of Uganda during September 2002, and well-studied *B. tabaci* reference populations, using the maximum parsimony algorithm available in PAUP* (Swofford, 1998). *T. vaporariorum* and *B. afer* are included as the outgroup sequences. Samples are indicated by country, source-plant species and location from where they were collected. Country: Ug = Uganda, source-plant species: Cs = cassava and location (district): Bg = Bugiri, Bs = Busia, Bu = Bushenyi, Ho = Hoima, Ig = Iganga, Kb = Kabarole, Ki = Kibaale, Kl = Kalangala, Ku = Kumi, Ky = Kyenjojo, Mb = Mbarara, Mk = Masaka, Mu = Mukono, Ms = Masindi, Nk = Nakasongola, Rk = Rakai, So = Soroti, Wk = Wakiso

population in the northern region, 83% in both central and eastern regions, 75% in Kalangala Island and 58% in the western region (Table 5). In contrast, the Ug2 types occurred most frequently in western region (42%) and Kalangala Island (25%), and least frequently in northern region (8%). In several eastern, western and central region locations, the Ug1- and Ug2-like genotypes occurred together (Fig. 8).

No definitive relationship was observed with respect to presence or geographical distribution of the two vector genotype clusters, Ug1 and Ug2, and the two cassava-infecting begomoviruses, ACMV and EACMV-UG2, detected in samples examined here. However, generally, the Ug1-like genotypes predominated the central, southern, northern and eastern regions, where EACMV-UG2 infection was also most frequent (Table 5, Fig. 8). In contrast the Ug2 types occurred most frequently in the western and Kalangala Island regions where ACMV infection was likewise dominant (Table 5, Fig. 8).

3.4 Discussion

This study sought to establish the current status of the two cassava-associated *B. tabaci* genotype clusters (Ug1 and Ug2), previously identified in Uganda (Legg *et al*., 2002), in order both to clarify their relationship with the CMD epidemic and to determine the biological significance of the genetic differences identified and their epidemiological implications.

As expected, the mtCOI analysis confirmed the presence of the Ug1 and Ug2 genotypes in the post-epidemic zone in Uganda in 2002. The mtCOI sequences of the Ug1- and Ug2-like genotypes were 8% divergent, as shown also by Legg *et al*. (2002) and Maruthi *et al*. (2004a),

Table 7. A pairwise comparison of the mitochondrial cytochrome oxidase I (mtCOI) nucleotide sequence[1], expressed as percent nucleotide identity between whitefly populations, as calculated by the *Clustal W* algorithm (Thompson *et al.*, 1994)

	Percentage Identity																
Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. UgCsWk3	-	99.9	99.7	92.6	92.6	92.1	97.2	93.2	86.7	85.3	85.2	82.7	82.1	80.9	78.9	48.5	34.9
2. UgCsBg58		-	99.7	92.6	92.6	92.1	97.2	93.2	86.7	85.3	85.2	82.7	82.1	80.9	78.9	48.5	34.9
3. 30MNten (Ug1)			-	92.6	92.6	92.1	97.2	93.2	87.0	85.5	85.3	82.9	82.3	81.0	79.0	48.5	35.0
4. UgCsKb25				-	99.9	99.5	91.0	90.7	87.3	85.2	85.0	82.9	82.3	81.6	79.5	49.4	34.7
5. UgCsHo34					-	99.5	91.0	90.7	87.3	85.2	85.0	82.9	82.3	81.6	79.5	49.4	34.7
6. 17Ikul (Ug2)						-	90.6	90.3	86.9	84.7	84.6	82.4	81.8	81.2	79.3	49.4	34.4
7. SAfriLucia2							-	91.4	85.6	84.2	84.0	81.6	82.1	79.9	77.9	48.0	36.1
8. ABA Benin								-	87.3	84.7	84.6	81.5	81.2	80.9	78.9	48.6	35.0
9. Ivory Coast cassava									-	85.8	85.6	81.0	80.1	79.5	79.3	48.0	34.1
10. IS B Israel										-	99.8	83.0	84.9	81.5	79.8	48.3	34.1
11. AZ B Arizona											-	82.9	84.7	81.6	79.6	48.5	34.1
12. Uganda sweetpotato												-	81.9	82.9	77.3	46.5	33.8
13. IW India													-	80.7	78.5	46.9	35.8
14. JAT Puerto Rico														-	89.7	48.1	34.6
15. AZ A Arizona															-	47.1	33.8
16. *T. vaporariorum*																-	34.6
17. *B. afer*																	-

[1]Selected, representative sequences are shown for cassava-associated *B. tabaci* genotypes identified in Uganda in 2002

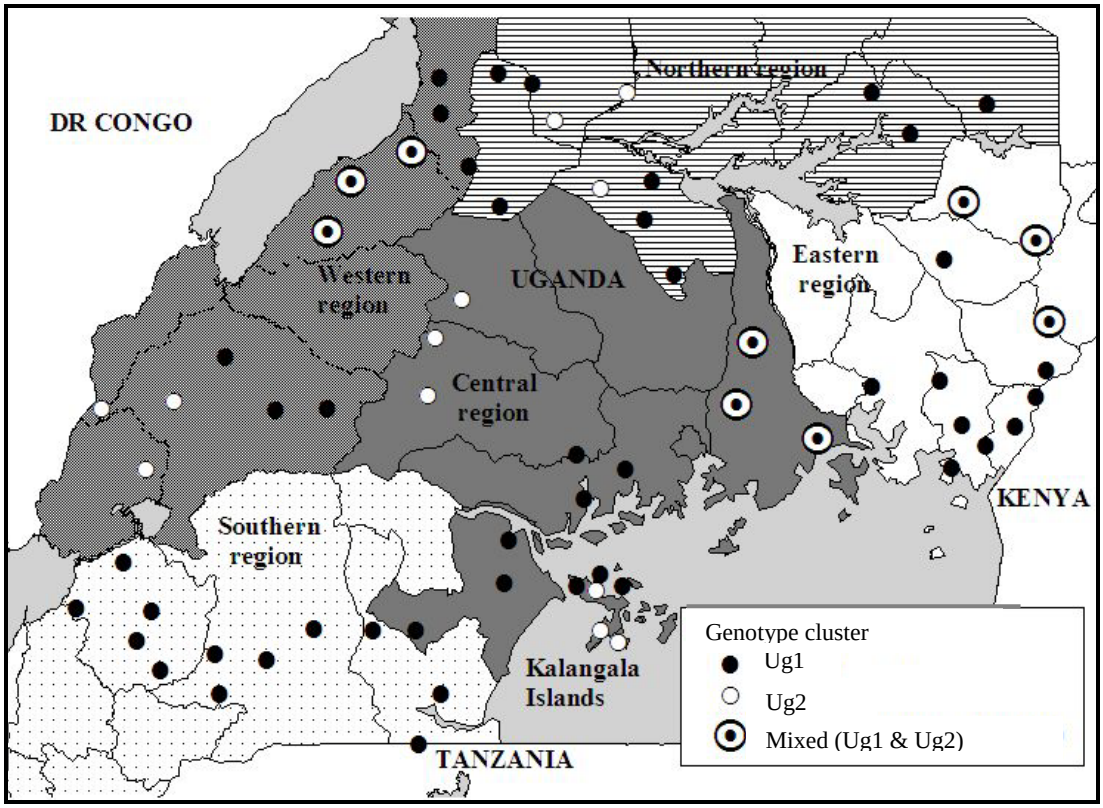

Figure 8. Map of Uganda illustrating the geographical distribution of cassava-associated *B. tabaci* genotypes in six cassava-producing regions sampled: northern (hatched area), eastern (solid white), western (dark spotted area), central (solid black), southern (light spotted area) and Kalangala (in L. Victoria), based on field collections during September 2002

thus confirming that the genotypes detected here using mtCOI as a molecular marker are the same as those previously documented. Both the Ug1 and Ug2 genotypes clustered together with other cassava-associated *B. tabaci* genotypes from sub-Saharan Africa, confirming the suggestion that African cassava *B. tabaci* populations form a distinct group (Maruthi *et al*., 2001, 2004a; Abdullahi *et al*., 2003; Berry *et al*., 2004).

In 1997, the Ug2 genotypes predominated (with 100% occurrence) in the epidemic-affected areas, and occurred together with the Ug1 genotypes 'at' the epidemic 'front', while the Ug1 types predominated 'ahead' of the 'front' (Legg *et al*., 2002). The occurrence of the Ug2 genotypes in the CMD epidemic-affected areas in Uganda in 1996/7 was confirmed also by Maruthi *et al*. (2004a), whose Ugandan *B. tabaci* population, UgCas-Nam (AF418669), which was collected in Namulonge, an area within the CMD epidemic-affected zone in 1996-97, shared 100% nt identity with the Ug2 genotype cluster of Legg *et al*. (2002). More recently, it was further established that a Ug2-like genotype occurred also at Ssanji in Rakai district of south-west Uganda, an area well ahead of the epidemic front in 1996-97, from the analysis of *B. tabaci* samples (stored in 80% ethanol) collected on cassava at the time (J. Colvin, unpublished data). Collectively, these data suggest the wide occurrence of the Ug2 genotypes in Uganda in 1996-97. In contrast, however, the data presented here indicate a larger proportion and wider occurrence of the Ug1 (83%) than Ug2 (17%) types among the collective *B. tabaci* population on cassava in the post-CMD epidemic zone in Uganda in 2002. The apparently diminishing frequency of the Ug2 genotypes in the epidemic-affected areas was reported also in 1999 (Legg *et al*., 2002), but as in the current study it was unclear why or how it was disappearing.

Although both the originally occurring ACMV and the recent epidemic-associated EACMV-UG2 continue to occur in the post-epidemic affected regions of Uganda, currently the newly occurring EACMV-UG2 predominates in most of the country (this study & Sseruwagi *et al*., 2004b). However, the fact that the Ug1 whitefly genotypes predominated with EACMV-UG2, unlike previously where the Ug2 genotypes were more closely associated with the severe disease phenotype (due to EACMV-UG2) in Uganda (Legg *et al*., 2002), suggests that the Ug1 genotypes, which are closely related to *B. tabaci* populations in the CMD epidemic-threatened areas and locales 'ahead' of the epidemic 'front', may be the key vector of the spreading severe epidemic in these areas. Moreover, whiteflies that are presently associated with the epidemic 'front' in western Kenya exhibit high fecundity and the dispersal behaviour (J.P. Legg, pers. observ.), characteristic of the whitefly population that occurred in Uganda at the peak of the CMD epidemic in the late 1990s (Otim-Nape *et al*., 1997a; Legg and Ogwal, 1998; Colvin *et al*., 1999), and the recombinant virus is associated with the epidemic front where it continues to spread in East and Central Africa (Legg, 1999; Legg *et al*., 2001; Bigirimana *et al*., 2004). In the light of these observations, the circumstances leading to the dramatically increased frequency of occurrence of the Ug1 genotypes amongst the collective cassava-associated *B. tabaci* population, and its now wide distribution in the post-epidemic zone in Uganda, compared to that in 1996-97, merit further investigation.

Severe CMD has reached pandemic proportions and has been reported in numerous countries neighbouring Uganda, including western Kenya and the Lake Victoria region of Tanzania and to the east and south (Legg, 1999), Rwanda (Legg *et al*., 2001: Sseruwagi *et al*., 2005a), DR Congo (Neuenschwander *et al*., 2002) to the southwest, and most recently, to Burundi (Bigirimana *et al*., 2004). The need to discern the genetic identity, biology, field population dynamics and relationship of the whitefly vector and begomoviruses associated with the

spreading pandemic is now more compelling than ever. Elucidating the biological significance and epidemiological implications of the genetic differences identified of the *B. tabaci* populations in the pandemic-threatened areas in relation to the virus population structure will enable present and future forecasting of the pandemic front, and ensure that sustainable control practices are adopted to combat this plant disease that threatens the livelihood of more than 200 million people in the affected regions.

Chapter Four

Colonization of non-cassava plant species by cassava whiteflies (*Bemisia tabaci*) in Uganda

Key words: Whitefly, genetic diversity, mitochondrial cytochrome oxidase I DNA, host races

In press: *Entomologia Experimentalis et Applicata*

Running title: Cassava *B. tabaci* ecology

Abstract

The restriction of cassava *Bemisia tabaci* (Genn.) genotypes to cassava and the colonization of alternative host species in selected cassava-growing areas of Uganda were studied in 2003/4. *B. tabaci* adults and fourth instar nymphs were collected from cassava and 22 other cultivated and uncultivated species occurring adjacent to the sampled cassava fields. The mtCOI sequence was used to establish the genotype of the *B. tabaci* collected. Phylogenetic analysis of mtCOI sequences revealed that only a single genotype cluster, Ug1, occurred on both cassava and non-cassava plant species sampled. The Ug1 genotypes (n = 23) shared 97-99% nt identity with the previously reported cassava-associated *B. tabaci* populations in southern Africa, and were ~8% divergent from the Ug2 genotypes. The Ug1 types were identified on 52% of the plant species sampled. Based on the presence of *B. tabaci* fourth instar nymphs, the Ug1 genotypes colonized five non-cassava plant species: *Manihot glaziovii*, *Jatropha gossypifolia, Euphorbia heterophylla, Aspilia africana* and *Abelmoschus esculentus,*

suggesting that cassava *B. tabaci* (Ug1 genotypes) are not restricted to cassava in Uganda. No Ug2-like genotypes were detected on any of the non-cassava plant species sampled in this study. The identification of additional hosts for at least one genotype cluster, Ug1, known also to colonize cassava, and which was hitherto thought to be 'cassava-restricted' may have important epidemiological significance for the spread of CMGs in Uganda.

4.1 Introduction

The whitefly *Bemisia tabaci* (Genn.) (Brown *et al*., 1995a) is typically a polyphagous insect, and some biotypes and genotypes are extremely polyphagous (Greathead, 1986; Brown *et al*., 1995a). Populations of *B. tabaci* colonize mainly annual, herbaceous plants, including over 500 species (Mound and Halsey, 1978; Greathead, 1986). However, near monophagous *B. tabaci* populations, such as the Jatropha race, which colonizes *Jatropha gossypifolia* L. and *Croton lobatus* L. (Euphorbiaceae) in Puerto Rico, the *Asystasia* spp.-restricted *B. tabaci* from Benin (Brown and Bird, 1992; Brown *et al*., 1995a) and cassava- (*Manihot esculenta* Crantz) (Euphorbiaceae) colonizing *B. tabaci* in Africa (Storey and Nichols, 1938; Burban *et al*., 1992) are also recognized.

B. tabaci is the vector of cassava mosaic geminiviruses (CMGs), the causal agents of cassava mosaic disease (CMD) in Africa (Bock and Woods, 1983; Otim-Nape *et al*., 1996) During the past decade, outbreaks of *B. tabaci* in many parts of sub-Saharan Africa have become more frequent and crops colonized by *B. tabaci* have suffered major yield reductions due to CMD, with losses ranging from 20% to 100% in the most affected cultivars (Thresh *et al*., 1994b, 1997). In Africa cassava is colonized by cassava whiteflies (*B. tabaci*) (Burban *et al*., 1992; Legg, 1996; Abdullahi *et al*., 2003), although the occurrence of non-cassava genotypes on

cassava has also been reported in the Ivory Coast and Zimbabwe (Berry *et al*., 2004). Using esterase profiles, Legg *et al*. (1994) detected polymorphisms amongst the cassava whitefly populations from different locations in Uganda. The high degree of genetic variability in whiteflies, however, precluded the identification of distinct biotypes. Brown *et al*. (1995b) highlighted the limitations of esterase markers for distinguishing variability in *B. tabaci* from a broad range of hosts and geographical locations, although the approach has been helpful for differentiating certain Old and New World populations (Costa and Brown, 1991; Costa *et al*., 1993b; Brown *et al*., 1995b; Brown, 2000). The characterisation of the Ugandan cassava-associated *B. tabaci* populations using the mitochondrial cytochrome oxidase I (*mtCOI*) gene has revealed two genotype clusters, Uganda 1 (Ug1) and Uganda 2 (Ug2), which diverge at approximately 8% (Legg *et al*., 2002). When Ugandan populations were sampled in 1997, the Ug2 genotypes were associated with the severe CMD epidemic, while the Ug1 genotypes occurred primarily 'at' and 'ahead' of the epidemic 'front' (Legg *et al*., 2002). Subsequent observations (P. Sseruwagi *et al*., unpublished data) in 2002, which were made after the expansion of the CMD epidemic to all of the country's cassava-growing areas, have shown that both genotype clusters occur throughout the country. The Ug1-like genotypes were more frequently detected in 2002, occurring in 83% of the fields, compared to 17% for the Ug2 types.

Storey and Nichols (1938) were the first to suggest that cassava-colonizing *B. tabaci* were host-specialized. This assertion has generally been supported by subsequent studies, including those of Burban *et al*. (1992) in the Ivory Coast, Legg (1996) in Uganda, and Abdullahi *et al*., 2003 and Berry *et al*. (2004) on cassava-associated whitefly populations in sub-Saharan Africa, although in all the studies, except that of Abdullahi *et al*., 2003, cassava-associated *B. tabaci* were found associated with and/or colonizing certain other plant hosts.

Certain specific virus and vector-related factors are driving the epidemic of severe CMD in Uganda and the East and Central African region (Pita *et al*., 2001a; Legg *et al*., 2002; Colvin *et al*., 2004). For example, it is known that the epidemic is primarily associated with the spread of a recombinant CMG, EACMV-UG2, whose DNA A contains sequences from both ACMV and EACMV (Harrison *et al*., 1997; Zhou *et al*., 1997). EACMV-UG2 now occurs throughout Uganda (Sseruwagi *et al*., 2004b) and has also been reported from other parts of East and Central Africa affected by the so-called 'pandemic' (Legg and Fauquet, 2004). In addition, it is clear that a previously unknown and possibly 'invasive' variant of *B. tabaci*, together with a putative indigenous *B. tabaci* variant, are important constituents of the collective vector population that colonizes cassava and transmits CMGs in eastern Africa (Legg *et al*., 2002; Maruthi *et al*., 2002, 2004a).

However, important details surrounding the ecology of *B. tabaci* variants associated with cassava in general in Africa, and their role(s) as CMG vectors remain poorly understood. One important, as yet poorly understood ecological aspect involves the host range of cassava-colonizing *B. tabaci*, including their potential to colonize uncultivated or cultivated plant species other than cassava, some of which may also serve as hosts. This aspect is further important to understand because a wider host range would allow cassava-colonizing *B. tabaci* to reproduce on additional plant species thereby facilitating their survival in areas where the cassava crop is devastated.

In this study, the distribution of cassava-associated *B. tabaci* genotypes was investigated in cassava and non-cassava crop and weed species adjacent to the sampled cassava plantings in the post-epidemic affected areas of Uganda. The terms genotype and genotype cluster are used

throughout to refer to genetically distinct sequences, and for a group of closely related sequences, respectively.

4.2 Materials and Methods

4.2.1 Whitefly collection

At least ten *Bemisia tabaci* adults and fourth instar nymphs were collected in 2003/4 from cassava and eleven other cultivated and uncultivated plant species occurring adjacent to cassava fields in the selected cassava-producing areas of Uganda (Table 8), and were used to establish the colonization and/or host range of cassava *B. tabaci* genotypes in nature. Three female adult whiteflies and two fourth instar nymphs were randomly selected from each sample for analysis.

4.2.2 DNA extraction, PCR, cloning and sequencing of mtCOI DNA

Single adult whitefly and fourth instar nymphs were lysed to release total DNA (De Barro and Driver, 1997). Polymerase chain reaction (PCR) (Mullis and Fallona, 1987) was carried out to amplify a fragment (~ 850 bp) of the mtCOI gene using primers MT10/C1-J-2195 (5´-TTGATTTTTTGGTCATCCAGAAGT-3´) and MT12/L2-N-3014 (5´-TCCAATGCACTAATCTGCCATATTA-3´) as described in Frohlich *et al*. (1999). PCR amplification, cloning and sequencing of *mtCOI* gene was conducted as described in Maruthi *et al*. (2004a). Sequencing was carried out by the John Innes Biotechnology Centre, Norwich, UK.

Table 8. Source-plant species and geographical location of *Bemisia tabaci* (adults and fourth instar nymphs) collected on cultivated and uncultivated species occurring adjacent to cassava fields in Uganda, 2003/4

Source-plant species		Family	Geographical location	Accession number
Common name	Botanical name			
Cassava	*Manihot esculenta* Crantz	Euphorbiaceae	Namulonge	AY903461-68
Jatropha	*Jatropha gossypifolia* L.	Euphorbiaceae	Namulonge	AY903469-77
Mexican Fireplant	*Euphorbia heterophylla* L.	Euphorbiaceae	Busukuma	AY903483-89
Tree cassava	*Manihot glaziovii* Muell. Arg.	Euphorbiaceae	Namulonge	AY903517-18
Lablab	*Lablab purpureus* L.	Leguminosae	Mukono	AY903478-82
'Makayi'	*Aspilia africana* Pers. Adams	Asteraceae	Namulonge	AY903490-92
Sunflower	*Helianthus annus* L.	Asteraceae	Namulonge	AY903498-01
Okra	*Abelmoschus esculentus* L.	Malvaceae	Namulonge	AY903493-97
Sweetpotato	*Ipomoea batatas* L.	Convolvulaceae	Namulonge	AY903502-05
Tobacco	*Nicotiana tabacum* L.	Solanaceae	Namulonge	AY903506-07
Tomato	*Lycopersicon esculentum* Mill.	Solanaceae	Namulonge	AY903508-16
Wild sunflower	*Tithonia diversifolia* L.	Compositae	Namulonge	AY903519-20

4.2.3 Phylogenetic analysis

Whitefly mtCOI sequences were edited manually using the EditSeq programme available in the DNASTAR software package (Lasergene, Madison, Wisconsin, USA) to produce a consensus sequence (~780-800 bp) for each individual insect. Sequences were aligned using the Clustal W (weighted) (Thompson *et al.,* 1994) algorithm option in MegAlign available in DNASTAR and compared with *B. tabaci* reference mtCOI sequences available in the EMBL/DDBJ/GenBank databases.

The DNA sequences were subjected to a heuristic search and subtree-pruning-regrafting branch swapping using the maximum likelihood (ML) and parsimony methods available in Phylogenetic Analysis Using Parsimony* (PAUP*4.0b10) (Swofford, 2002). The ML tree was reconstructed using the maximum likelihood 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 PAUP using the heuristic option for 1000 replications at a 70% confidence limit. The GenBank accession numbers for *B. tabaci* reference mtCOI sequences were as follows presented in Table 9.

4.3 Results

4.3.1 Phylogenetic analyses of adult *B. tabaci* mtCOI sequences

The mtCOI sequence (~ 800 bp) was obtained for the field-collected female adult *B. tabaci* from: cassava (*Manihot esculenta* Crantz), 'Makayi' (*Aspilia Africana* Pers. Adams), okra

Table 9. Whitefly genotypes and location of reference whiteflies used in phylogenetic analysis of mitochondrial cytochrome oxidase I sequences and their respective GenBank accession numbers

Whitefly/Genotype	Location	GenBank Accession No.
AB A Benin	Benin	AF110693
ARG2 Santiago	Argentina	AF340213
AZ A Arizona	Arizona, USA	AY057122
AZ B Arizona	Arizona, USA	AY057123
Bemisia afer[1]	-	AY057218
CAL A Brawlee CA	California, USA	AY057124
CUL Mexico	Mexico	AY057125
HC China	China	AF342777
IS B Israel	Israel	AF110705
Ivory Coast cassava	Ivory Coast	AY057135
Ivory Coast okra	Ivory Coast	AY057136
IW India	India	AF110702
JAT Puerto Rico	Puert Rico	AF110705
Morocco 1	Morocco	AF342773
Moz-Kal 1	Mozambique	AF344278
PC91 Pakistan 1	Pakistan	AF342778
Reunion 1	Reunion Island	AJ550172
Reunion 2	Reunion Island	AJ550178
SA Lucia 2	South Africa	AF344260
SC Sudan 1	Sudan	AF110706
SP92 Spain Q	Spain	AF342775
SwazMap1	Swaziland	AF344269
TC Turkey	Turkey	AF342776
Thailand cotton	Thailand	AF164670
Uganda sweetpotato	Uganda	AY057174
Zam 2	Zambia	AF344281
30MNten (Ug1)	Uganda	AY057171
17Ikul (Ug2)	Uganda	AY057158

[1]*Bemisia afer* (Priesner & Hosny) was used as the outgroup species

(Abelmoschus esculentus L.), Mexican Fireplant (Euphorbia heterophylla L.), Sunflower (Helianthus annus L.), sweetpotato (Ipomoea batatas L.), jatropha (Jatropha gossypifolia L.), tomato (Lycopersicon esculentum Mill.), wild sunflower (Tithonia diversifolia L.), lablab (Lablab purpureus L.), tree cassava (Manihot glaziovii Muell. Arg.), and tobacco (Nicotiana tabacum L.), plants occurring adjacent to cassava fields in selected cassava-producing areas of Uganda in 2003/04. The sequences are deposited in the EMBL/DDBJ/GenBank under the accession numbers indicated in Table 8.

Alignment of the partial mtCOI sequences established 67 constant, 213 variable and 419 parsimony informative characters, respectively. A search for the most-parsimonious tree was performed with parsimony and maximum likelihood options. The two options predicted the same phylogenetic relationships for the *B. tabaci* populations examined in the current study, hence only the parsimony tree is presented here (Fig. 9).

Based on the phylogenetic analyses, members of *B. tabaci* grouped within four major clusters, supported by high bootstrap (bs) values (>80), with either the New or Old World (Fig. 9). A cluster was formed comprising *B. tabaci* from SouthEast Asia, India and the Far East. *B. tabaci* from the New World formed the second major cluster, whereas, genotypes from the Mediterranean-North Africa-Middle East (MED-NAFR-ME) region, which also includes the B and Q biotypes comprised the third major cluster. The fourth major cluster comprised *B. tabaci* genotypes from cassava in sub-Saharan Africa, which also includes a non-cassava genotype sub cluster on *Asystasia* spp. in Benin in West Africa.

The *B. tabaci* genotypes (n = 49) examined in this study grouped within a single cluster, at 97-99% nt identity together with the previously described Ug1 genotype cluster in Uganda and *B.*

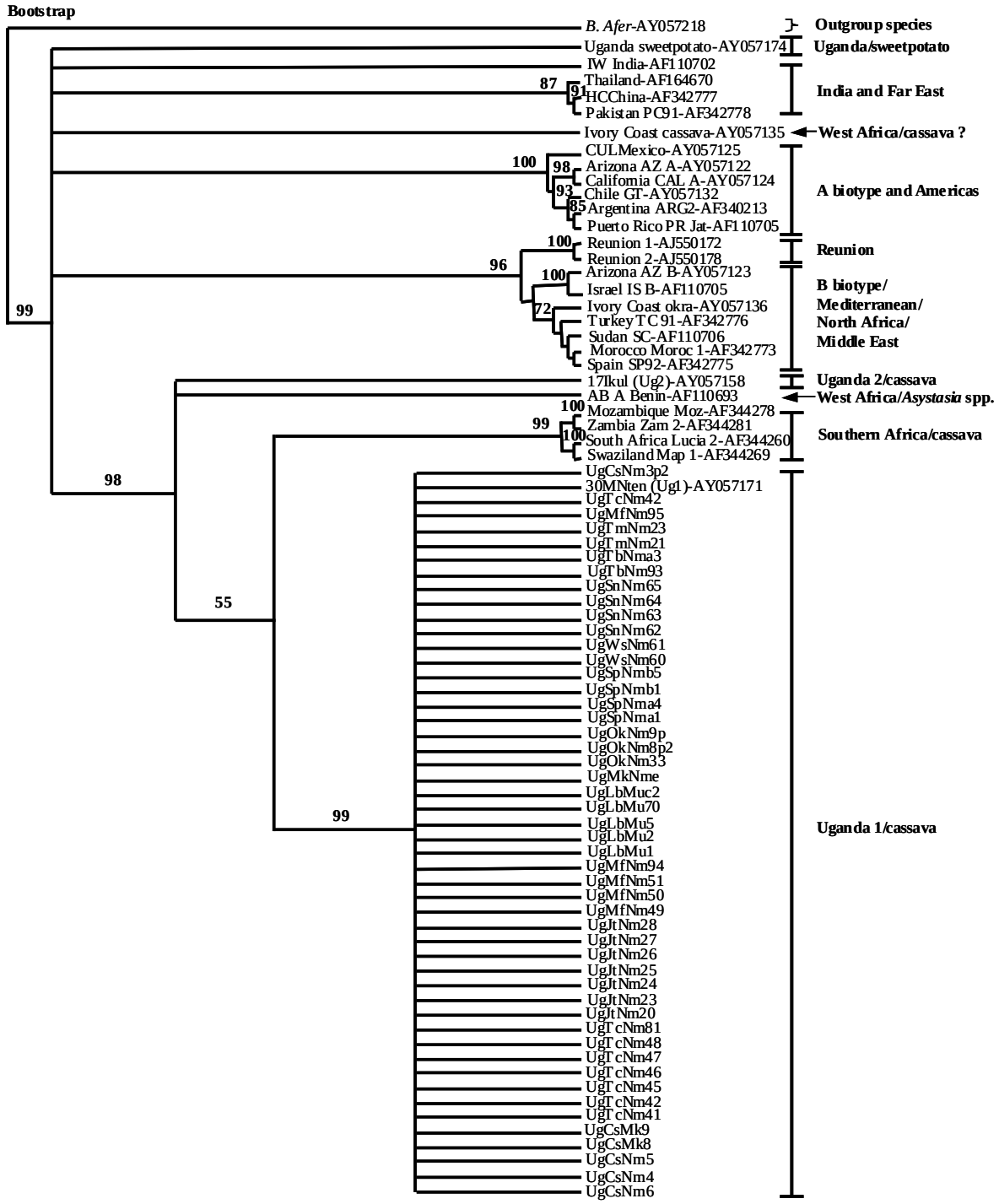

Figure 9. Dendrogram of the mitochondrial cytochrome oxidase I sequence for adult *Bemisia tabaci* collected on cassava, and cultivated and uncultivated species occurring adjacent to cassava fields in Uganda during 2003/4, and well-studied *B. tabaci* reference populations, using the maximum parsimony algorithm available in PAUP* (Swofford, 2002). *B. afer* is included as the outgroup sequence. Samples are indicated by country, source-plant species and location from where they were collected. Country: Ug = Uganda. Source-plant species: Tm = tomato, Tb = tobacco, Sn = sunflower, Ws = 'wild sunflower', Mf = Mexican Fireplant, Mk = 'makayi', Ok = okra, Cs = cassava, Tc = tree cassava, Jt = jatropha, Sp = sweetpotato and Lb = lablab. Sample location: Nm = Namulonge, Bk = Busukuma, Mk = Masaka, Mu = Mukono

tabaci populations on cassava in Mozambique, Zambia, Swaziland and South Africa (Fig. 9). However, the Ug1-like genotypes shared only 87% nt identity with the previously described 'Ivory Coast cassava' genotype cluster in Ivory Coast. No Ug2-like genotypes were detected on any of the twelve source-plant species sampled, including cassava, in this study.

The field occurrence and source-plant distribution of adult female Ug1 *B. tabaci* genotypes in Uganda in 2003/4 was determined as follows: *A. Africana* (3/3), *A. esculentus* (2/4), *E. heterophylla* (3/3), *H. annus* (2/2), *I. batatas* (3/5), *J. gossypifolia* (4/4), *L. esculentum* (2/3), *T. diversifolia* (3/3), *L. purpureus* (4/4), *M. esculenta* (3/3), *M. glaziovii* (3/3) and *N. tabacum* (2/3). The rest of the *B. tabaci* identified on *A. esculentus* (2/4), *I. batatas* (2/5), *L. esculentum* (1/3) and *N. tabacum* (1/3) were non-cassava genotypes (data not presented).

4.3.2 Phylogenetic analyses of *B. tabaci* fourth instar nymphs mtCOI sequences

The mtCOI DNA sequence (~ 800 bp) was obtained for the field-collected *B. tabaci* fourth instar nymphs on: *A. Africana, A. esculentus, E. heterophylla, H. annus, I. batatas, J. gossypifolia, L. esculentum, T. diversifolia, L. purpureus, M. esculenta, M. glaziovii* and *N. tabacum*, and the sequences were deposited in the GenBank under the assigned accession numbers shown in Table 8. Phylogenetic analysis of the mtCOI sequences for fourth instar nymphs produced one most parsimonious tree, with a topology similar to that obtained for the analogous adult whitefly sequences (Fig. 10). Similar to the results of the adult whiteflies, the thirteen *B. tabaci* fourth instar nymphs genotypes examined here grouped within a single cluster, at 97-99% nt identity, together with the Ug1-like genotypes in Uganda, and *B. tabaci* on cassava from southern Africa (Fig. 10). None of the nymph mtCOI sequences examined here

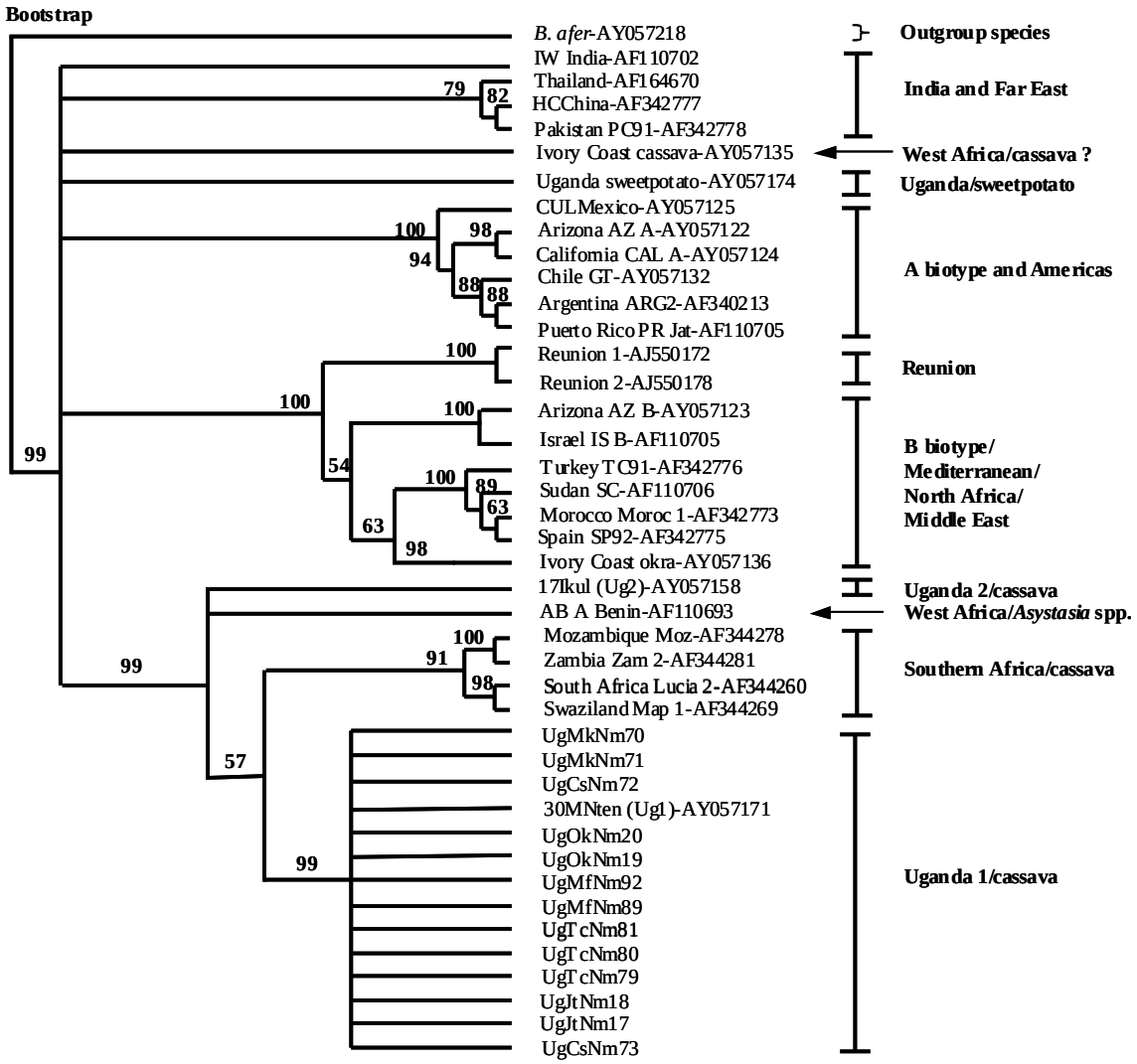

Figure 10. Dendrogram of the mitochondrial cytochrome oxidase I sequence for *Bemisia tabaci* fourth instar nymphs collected on cassava, and cultivated and uncultivated species occurring adjacent to cassava fields in Uganda during 2003/4, and well-studied *B. tabaci* reference populations, using the maximum parsimony algorithm available in PAUP* (Swofford, 2002). *B. afer* is included as the outgroup sequence. Samples are indicated by country, source-plant species and location from where they were collected. Country: Ug = Uganda. Source-plant species: Mf = Mexican Fireplant, Mk = 'makayi', Ok = okra, Cs = cassava, Tc = tree cassava and Jt = jatropha. Sample location: Nm = Namulonge, Bk = Busukuma, Mk = Masaka, Mu = Mukono

Table 10. Uncorrected pairwise nucleotide distances, as calculated by the *Clustal W* algorithm (Thompson *et al*., 1994), for reference mtCOI sequences of adult *B. tabaci* and fourth instar nymphs collected on different host-plant species in Uganda

B. tabaci nymphs' sequences[1]	Ugandan[2] cassava cluster (n = 2)[3]	Southern Africa cassava cluster (n = 4)	North Africa and Mediterranean cluster (n = 4)	Reunion non-B cluster (n = 1)	B biotype cluster (n =2)	India and Far East cluster (n = 6)	A biotype and New World cluster (n = 6)	Ugandan sweetpotato cluster (n = 1)
Ug*cs*Nm73	0.1 - 7.6	1.7 - 3.1	15.3 - 16.3	17.2	15.3 - 15.5	16.9 - 61.9	19.0 - 20.3	17.2
Ug*mk*Nm70	0.2 - 7.7	1.8 - 3.2	15.3 - 16.4	17.3	15.3 - 15.7	16.9 - 61.9	19.0 - 20.3	17.2
Ug*ok*Nm20	0.2 - 7.6	1.7 - 3.3	15.3 - 16.3	17.1	15.3 - 15.5	16.9 - 61.9	19.0 - 20.3	17.2
Ug*mf*Nm92	0.1 - 7.6	1.7 - 3.1	15.3 - 16.3	17.2	15.3 - 15.5	16.9 - 61.9	19.0 - 20.3	17.2
Ug*tc*Nm81	0.1 - 7.6	1.7 - 3.1	15.3 - 16.3	17.2	15.3 - 15.5	16.9 - 61.9	19.0 - 20.3	17.2
Ug*jt*Nm18	0.2 - 7.7	1.7 - 3.2	15.3 - 16.4	17.3	15.3 - 15.7	16.9 - 61.9	19.0 - 20.3	17.2

[1]Representative sequences are shown for *B. tabaci* genotypes identified on cassava and non-cassava host-plant species in Uganda, 2003/4. [2]The within-group estimates of the minimum and maximum nucleotide divergence are presented for the major *B. tabaci* phylogenetic clusters in Fig. 9. [3]Number of clusters of closely related genotypes

clustered with the reference Ug2 genotype (17 Ikul Ug2) from Uganda. A pairwise comparison (Clustal W) to estimate nucleotide distances for the fourth instar nymphs sequences and reference *B. tabaci*, produced the similar results as had been obtained when adult sequences were analysed. The Ug1 genotypes had a within-group variance of 0.1 to 1.7%, and diverged with the Ug2 genotypes at ~8%, as expected (Table 10).

Based on the presence of the *B. tabaci* fourth instar nymphs on plant species sampled, the Ug1 genotypes were shown to colonize cassava and five other non-cassava plant species including: *M. glaziovii, J. gossypifolia, E. heterophylla, A. africana* and *A. esculentus* (Figure 10). However, the Ug1 genotypes did not colonize: *H. annus, T. diversifolia, L. purpureus, L. esculentum, N. tabacum* or *I. batatas*.

4.4 Discussion

The study investigated the field occurrence and host-plant distribution of cassava-colonizing *B. tabaci* genotypes on cultivated and uncultivated species occurring adjacent to cassava fields in 2003/4 in Uganda. The genetic variability of morphologically indistinguishable *B. tabaci* populations on cassava and other plant species in Uganda (Legg, 1996; Chapter three) and elsewhere in Africa (Burban *et al*., 1992; Abdullahi *et al*., 2003, 2004; Berrie *et al*., 2004) was investigated previously using field-collected adult whitefly. However, field-collected adult whitefly are of limited use in establishing host-associated *B. tabaci* genotypes, since the occurrence of the adults on plants in the field does not necessarily indicate colonization. Therefore, in addition to using adult whitefly in this study, we analysed also the field-collected fourth instar nymphs to establish both the host range (colonized hosts) and associated *B. tabaci* genotypes in nature.

Analysis of adult whitefly and fourth instar nymphs mtCOI sequences generated similar results, indicating that the fourth instar nymphs could be used for identification of host-associated genotypes, especially where colonization studies have not been conducted. Furthermore, under field conditions, other approaches, other than sequencing followed by phylogenetic analysis, such as polymerase chain reaction, followed by restriction fragment length polymorphisms (PCR-RFLP) are available (Abdullahi *et al*., 2004) or can be developed for the identification of host-associated genotypes.

The results of the study reported herein indicate that in Uganda, cassava is only colonized by cassava types of *B. tabaci*, which is consistent with previous findings in Africa (Burban *et al*., 1992; Legg, 1996; Abdullahi *et al*., 2003, 2004; Omondi *et al.*, 2005; Sseruwagi *et al*., 2005b). The occurrence of non-cassava *B. tabaci* genotypes on cassava in the Ivory Coast and Zimbabwe (Berry *et al*., 2004) has been reported, based on the analysis of adult whitefly collected on the crop in the field. However, it is possible that these whiteflies could have been visiting cassava at the time of collection, in which case it would be erroneous to call them 'cassava whitefly'. Whiteflies visit several plant species to feed and to look for suitable sites for mating and oviposition (van Lenteren and Noldus, 1990). However, subsequent colonization and adaptation to a particular plant host depends on the feeding preferences of the whiteflies and the plant quality (Byrne and Bellows, 1991), which probably explains why fewer plant hosts were colonized than were found to have the Ug1 adult cassava whiteflies in our study.

In addition to cassava, the Ug1 genotypes colonized five other non-cassava plant species including: *A. africana, A. esculentus, M. glaziovii, J. gossypifolia* and *E. heterophylla* in this study, establishing that in Uganda cassava *B. tabaci* (Ug1) is not restricted to cassava. *A.*

esculentus is cultivated as a vegetable crop in central and northern Uganda, and *A. africana* is a wild species that is ubiquitous in Uganda. *E. heterophylla* occurs in thicket edges and cultivated areas under fallow in east and central Uganda (Anonymous, 1957; Mabberley, 1987). *M. glaziovii* and *J. gossypifolia* are wild species, and the latter is used to support vanilla (*Vanilla planifolia* Andrews., Orchidaceae) plants during growth in central and western Uganda.

Previous studies in Uganda (Legg, 1996) have shown that certain *B. tabaci* that colonize cassava are able to survive on alternative hosts including cotton and sweetpotato, but that *B. tabaci* found naturally colonizing cotton and sweetpotato do not survive when transferred to cassava under laboratory conditions. Cassava-associated *B. tabaci*, which were reported to be monophagous in Africa (Storey and Nichols, 1938; Abdullahi *et al*., 2003, 2004), were detected also on: *Centrosema molle* Mart. ex Benth., Fabaceae (Burban *et al*., 1992; Legg, 1996); *Solanum nigram* L., *Solanum aethiopicum* L. and *Solanum melongena* L., Solanaceae (Burban *et al*., 1992), *Lantana camara* L., Verbenaceae (Legg, 1996), and *E. heterophylla* (Thompson, 2003). However, the Ug1 genotypes were neither associated nor found colonizing *S. melongena* or *L. camara* in this study. Further, it was not possible to determine whether the species recorded in this study as new hosts of cassava whiteflies simply went undetected by earlier workers (Burban *et al*., 1992; Legg, 1996), or if this observation is linked to a 'shift' in the host range for the Ug1 genotypes, which are now widely distributed in the CMD-epidemic zone in Uganda (P Sseruwagi, JP Legg, MEC Rey, J Colvin, D Rogan and JK Brown, unpublished data). Moreover, the Burban *et al.* (1992) and Legg (1996) studies were not host range studies like one described in this study; hence it is difficult to make firm conclusions on any possible change in host range. The ability of the Ug1 genotypes to colonize other plant species besides cassava could effectively facilitate its survival in areas where the crop has been

devastated by the CMD epidemic. This also raises the possibility that cassava *B. tabaci* (Ug1) could acquire other whitefly-transmitted viruses (WTVs) from non-cassava hosts, which are not known to infect cassava, but which could feasibly become of epidemiological importance if they become adapted to cassava. In view of this possible threat, it would clearly be prudent to make an assessment of the field populations of the Ug1 *B. tabaci* genotypes, and the occurrence of WTVs, particularly begomoviruses, in non-cassava hosts occurring adjacent to cassava fields both in Uganda and the wider East African region.

Chapter Five

Genetic diversity of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) populations and presence of the B biotype and a non-B biotype that can induce silverleaf symptoms in squash, in Uganda

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Running title: Genetic diversity of *B. tabaci* populations in Uganda

Abstract

The extent of genetic variability and host-plant distribution of *Bemisia tabaci* (Gennadius) genotypes colonizing 13 cultivated and uncultivated species occurring adjacent to cassava fields in selected cassava-producing areas of Uganda were investigated. Phylogenetic analysis of the mitochondrial cytochrome oxidase I (*mtCOI*) gene revealed eight distinct genotype clusters, Ug1-Ug8, which are supported by high bootstrap (bs) values (≥80%), at 3-18% nt divergence, among the collective Ugandan *B. tabaci* populations. Ug1 and Ug2 (both cassava-associated) and Ug8 (sweetpotato-associated) have been reported previously in Uganda. Ug3 (n = 3) was the only exemplar representing one cluster, which was unlike any previously described genotypes in Uganda or elsewhere, and diverged at 8%, 10% and 17% from Ug1, Ug2 and Ug8, respectively. The Ug3 genotypes colonized a single species, *Ocimum gratissimum*. Ug4, Ug5, Ug6 and Ug7 formed four closely related sub-clusters (93-97% nt identity), and diverged from one another by 1-7%, and by 15-18% from Ug1, Ug2, Ug3 and

Ug8, respectively. The Ug4 genotypes (n =11) had as their closest relatives (at 97-99% nt identity) previously reported *B. tabaci* from okra in the Ivory Coast, whereas, the Ug5 (n = 1) and Ug6 (n = 3) genotypes shared 95-99% and 99% nt identity, respectively, with their closest relatives from the Mediterranean-North Africa- Middle East (MED-NAFR-ME) region, which also includes the well-studied B and Q biotypes. The Ug7 genotypes (n = 8) were closely related (at 98-99% nt identity) to *B. tabaci* from the Reunion Island in the Indian Ocean. The Ug4, Ug5, Ug6 and Ug7 genotypes were identified on 54%, 8%, 8%, and 31% of the sampled plants species, respectively. Ug4 were most polyphagous and, colonized: *Cucurbita pepo*, *Cucumis sativus, Leonotis nepetifolia* and *Pavonia urens*, followed by Ug7, which colonized: *Commelina benghalensis, Gossypium hirsutum* and *Phaseolus vulgaris,* and Ug6, which colonized: *Abelmoschus esculentus* and *C. benghalensis* only. None of the Ug4-Ug7 genotypes was found associated with, or colonizing, cassava or sweetpotato plants. Squash plants colonized by the Ug6 and Ug7 genotypes, both members of the B biotype/B-like cluster, caused silvering of squash, while those colonized by the Ug4 genotypes (most closely related to a non-B like genotype from Okra in the Ivory Coast) did not. In addition to colonizing sweetpotato, the Ug8 genotypes colonized *Lycopersicon esculentum* and *L nepetifolia* also.

5.1 Introduction

Over 1200 whitefly species are known worldwide, although only a limited number have been closely studied on key herbaceous hosts (Mound and Halsey, 1978; Byrne *et al*., 1990). Species of the genus *Bemisia* are among the most important on cultivated species and are believed to have originated in the SouthEast Asia/Indian subcontinent (Gill, 1990; Mound and Halsey, 1978) or possibly Africa (Gill, 1990; Campbell *et al*., 1996).

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is the most widely distributed and economically important *Bemisia* species (Brown *et al*., 1995a). It has increased in importance during the past thirty years as a direct pest and also as a vector of plant viruses, particularly whitefly-transmitted geminiviruses (family *Geminiviridae*; genus *Begomovirus*) (Muniyappa, 1980; Duffus, 1987; Brown, 1990; Brown and Bird, 1992; Brown, 1994; Bedford *et al*., 1994; Fishpool and Burban, 1994; Otim-Nape *et al*., 1996; Poulston and Anderson, 1997). *B. tabaci* can damage plants through direct feeding and by the induction of phytotoxic symptoms (Costa and Brown, 1991; Cohen *et al*., 1992), the latter of which is a feature of only some biotypes (Brown and Bird, 1992; Bedford *et al*., 1994).

Beginning in 1986-87 until the mid-1990s, unprecedented outbreaks of *B. tabaci*, which are now known to be caused by infestations of the B biotype (Costa and Brown, 1991), occurred throughout the Americas, and also in Europe (Brown and Bird, 1992; Bedford *et al*., 1994; Costa *et al*., 1993; Viscarret *et al*., 2003). Based on differences in general esterase patterns, fecundity on the cotton, squash, and poinsettia and differences in ability to induce (phytotoxic) silvering symptoms in squash plants, two distinct populations of *B. tabaci* were distinguished, and subsequently referred to as the AZ-A and AZ-B biotype (Costa and Brown, 1991). The B biotype increased in distribution in the southwestern US and ultimately was shown to have displaced the local A biotype (Costa *et al*., 1993). Most recently, outbreaks of the 'B' biotype have occurred in Australia, China, and elsewhere in SouthEast Asia (De Barro *et al*., 2000; Coombs *et al*., 2003; Chowda *et al*., 2003).

The extent of genetic polymorphism has been examined for many representative *B. tabaci* populations from throughout the world using general esterases as genetic markers (Wool *et al*., 1989, 1993). The analysis yielded 20 distinct general esterase patterns (designated A to S), and

revealed that *B. tabaci* was more polymorphic than previously assumed (Bedford *et al*., 1994; Brown *et al*., 1995b; Rosell *et al*., 1997). The subsequent analysis of *B. tabaci* populations collected from different parts of the world using esterase analysis and random amplified polymorphic DNA (RAPD) PCR fingerprinting revealed that the B biotype was an exotic *B. tabaci* (Costa *et al*., 1993; Gawel and Bartlett, 1993). Further, the DNA sequencing of the mitochondria 16S rRNA and cytochrome oxidase I (COI) genes for the same or similarly representative *B. tabaci* populations, indicated that the B biotype originated from Africa or the Middle East (Brown *et al*., 1995a,b; Frohlich *et al*., 1999). Using molecular genetics approaches, several distinct genotypes and/or biotypes of *B. tabaci* have been distinguished (Costa and Brown, 1991; Costa *et al*., 1993; Brown *et al*., 1995a,b; DeBarro and Driver, 1997; Frohlich *et al*., 1999; Brown, 2000; De Barro *et al*., 2000, 2005; Delatte *et al*., 2005), even though populations are morphologically indistinguishable (Rosell *et al*., 1997; Gill, 1990). This has led one group of researchers to suggest that *B. tabaci* may best be described as a species complex (Brown *et al*., 1995a; Frohlich *et al*., 1999) and studies indicating that the A and B biotypes were reproductively isolated, led to the proposal that the A and B biotypes were distinct species. On the basis of the failure to interbreed, the B biotype was referred to as: *Bemisia argentifolia* Bellows and Perring (Perring *et al*., 1993; Bellows *et al*., 1994).

Although *B. tabaci* generally is considered polyphagous (Greathead, 1986; Brown *et al*., 1995a), evidence for host adaptation among certain *B. tabaci* populations, such as *Jatropha gossypifolia* L., and *Croton lobatus* L. (Euphorbiaceae) in Puerto Rico (Bird, 1957; Bird and Maramorosch, 1978; Brown and Bird, 1992); the *Asystasia* spp.-restricted *B. tabaci* from Benin (Brown and Bird, 1992; Brown *et al*., 1995a); biotype T on *Euphorbia characias* L., from Italy (Simon *et al*., 2003) and cassava- (*Manihot esculenta* Crantz) (Euphorbiaceae) colonizing *B. tabaci* in Africa (Storey and Nichols, 1938; Burban *et al*., 1992; Legg *et al*.,

1994; Legg, 1996; Abdullahi *et al*., 2003, 2004; Omondi *et al*., 2005; Sseruwagi *et al*., 2005c) have been reported. In Brazil, Costa and Russell (1975) showed that *B. tabaci* does not colonize cassava.

In Uganda, a key-driving factor in the spread of the 1990s severe CMD epidemic (Gibson *et al*., 1996; Harrison *et al*., 1997) caused by *East African cassava mosaic virus*-Uganda variant (EACMV-UG2) (Deng *et al*., 1997; Zhou *et al*., 1997; Pita *et al*., 2001; Fauquet and Stanley, 2003) was the high population density of whiteflies (Otim-Nape *et al*., 1997; Legg and Ogwal, 1998; Colvin *et al*., 1999) at the epidemic 'front'. In trying to establish the cause of the increased whitefly populations at the CMD epidemic 'front', much of the research on *B. tabaci* in Uganda has focused on: (i) cassava-colonizing populations mainly in search of a pandemic-associated biotype (Maruthi *et al*., 2001; Legg *et al*., 2002), (ii) the interaction between the CMGs and the whitefly vector (Colvin *et al*., 1999, 2004; Maruthi *et al*., 2002; Omongo, 2003), and (iii) the host range of cassava-colonizing *B. tabaci* (Sseruwagi *et al*., 2005c).

The development of the mtCOI marker (Frohlich *et al*., 1999; Brown, 2000) has enhanced the ability to differentiate biotypes and distinct genotypes of *B. tabaci*, hence the marker has been used increasingly to assess the genetic variability of *B. tabaci* populations in Africa (Legg *et al*., 2002; Berry *et al*., 2004; Maruthi *et al*., 2004; Sseruwagi *et al*., 2005c), and elsewhere (Frohlich *et al*., 1999; Brown, 2000; Viscarret *et al*., 2003). Using this approach Legg *et al*. (2002) distinguished two divergent (8%) genotype clusters associated with CMD-affected cassava, and referred to them as Uganda 1 (Ug1) and Uganda 2 (Ug2). The CMD pandemic has now spread throughout Uganda (Sseruwagi *et al*., 2004b), and has been reported also in some areas of East and Central Africa (Legg, 1999; Legg and Fauquet, 2004; Sseruwagi *et al*., 2005a), where it continues to have devastating effects on cassava.

This study sought to determine the genetic diversity and host-plant distribution of *B. tabaci* genotypes on cultivated and uncultivated plant species occurring adjacent to cassava planted in the post-epidemic affected areas of Uganda. The term 'genotype' is used throughout to refer to genetically distinct sequences, while 'genotype-cluster' refers to a group of closely related sequences.

5.2 Materials and Methods

5.2.1 Insect material, DNA extraction and PCR analysis

At least ten whitefly (*B. tabaci*) adults and fourth instar nymphs were collected from thirteen cultivated and uncultivated species occurring adjacent to cassava fields in selected cassava-producing areas (n = 10) of Uganda (Table 11), and were used to establish the genetic diversity and host-plant distribution of *B. tabaci* genotypes in 2003/4. Three female adult whiteflies and two fourth instar nymphs, respectively, were selected randomly from each sample for DNA analysis.

Total nucleic acids were extracted from individual nymphs and adult whiteflies according to De Barro and Driver (1997). Polymerase chain reaction (PCR) (Mullis and Fallona, 1987) was conducted to amplify a fragment (~ 850 bp) of the mtCOI gene using primers MT10/C1-J-2195 (5´-TTGATTTTTTGGTCATCCAGAAGT-3´) and MT12/L2-N-3014 (5´-TCCAATGCACTAATCTGCCATATTA-3´) according to Frohlich *et al*. (1999). For each sample a 25µl reaction mixture was made up containing 2.0µl DNA template, 2.5µl 10x reaction buffer IV (ABgene), 2.0µl 25 mM $MgCl_2$, 1.5µl dinucleotide triphosphates (dNTPs), 0.5µl each of primers MT10 and MT12 and 0.1 µl 'Red-hot' Taq polymerase (ABGene,

Table 11. Source plant species, geographical location, genotype and induction of squash silverleaf (SSL) symptoms by *Bemisia tabaci* populations in Uganda, 2003/4

Source plant species			Geographical location	Accession number	Genotype	SSL
Common name	Botanical name	Family				
Beans	*Phaseolus vulgaris* L.	Leguminosae	Masindi	AY903521-22	Ug7	+
			Namulonge	AY903523-26	Ug7	+
Cucumber	*Cucumis sativus* L.	Cucurbitaceae	Namulonge	AY903531-32	Ug4	-
			Wakiso	AY903533-35	Ug4	-
Pumpkin	*Cucurbita pepo* L.	Cucurbitaceae	Luwero	AY903571-72	Ug4	-
			Namulonge	AY903573-74	Ug4	-
Dew flower	*Commelina benghalensis* L.	Commelinaceae	Busukuma	AY903536-39	Ug7	+
			Namulonge	AY903540-44	Ug6/7	+
Eggplant	*Solanum melongena* L.	Solanaceae	Busukuma	AY903545-46	Ug4	-
			Masaka	AY903547-48	Ug7	+
			Namulonge	AY903549-50	Ug4	-
Tobacco	*Nicotiana tabacum* L.	Solanaceae	Masindi	AY903578	Ug1/5	NA
Tomato	*Lycopersicon esculentum* Mill.	Solanaceae	Namulonge	AY903579-81	Ug1/8	-
Lion's ear	*Leonotis nepetifolia* L.	Labiatae	Kabarole	AY903551-52	Ug4	-
			Namulonge	AY903553-55	Ug4/8	-
Mint/wild basil	*Ocimum gratissimum* L.	Labiatae	Mukono	AY903556-58	Ug3	-
			Namulonge	AY903559-63	Ug3	-
Cotton	*Gossypium hirsutum* L.	Malvaceae	Namulonge	AY903527-29	Ug7	+
			Pallisa	AY903530	Ug7	+
'Muwogola omusajja'	*Pavonia urens* Cav.	Malvaceae	Namulonge	AY903564-66	Ug4/6	+
Okra (Ladies finger)	*Abelmoschus esculentus* L.	Malvaceae	Namulonge	AY903567-70	Ug1/6	+
Sweetpotato	*Ipomoea batatas* L.	Convolvulaceae	Namulonge	AY903575-77	Ug1/8	-

Epsom, Surrey, UK). The PCR conditions were: 94°C for 1 min, followed by primer annealing at 52°C for 1 min and extension for 1.20 min at 72°C using 35 cycles. A final extension of 10 min at 72°C was included and the reaction was held at 4°C in a Gene Amp PCR System 9700 thermal cycler (Applied Bio-Systems). The PCR products were electrophoresed through a 1% agarose gel in 0.5x TBE buffer and amplified DNA was viewed under UV trans-illumination after staining in ethidium bromide.

5.2.2 Cloning, sequencing and phylogenetic analysis

The amplified DNA of the expected size was eluted from the agarose gel using a QIAquick PCR Purification Kit (Qiagen Inc, USA), cloned in the pGEMT-Easy vector (Promega, Madison, Wisconsin, USA) and transformed into *Escherichia coli* strain 109, following the manufacturers' instructions. The presence of an insert of the expected size was confirmed by PCR using the universal primers T7 (TAATACGACTCACTATAGGG) and SP6 (TATTTAGGTGACACTATAG) (Promega, Madison, Wisconsin, USA) in the polylinker of the pGEMT-Easy vector. DNA was bi-directionally sequenced at the John Innes Biotechnology Centre, Norwich, UK.

Whitefly mtCOI sequences were edited manually using the EditSeq programme available in the DNASTAR software package (Lasergene, Madison, Wisconsin, USA) to produce a consensus sequence (~780-800 bp) for each individual adult. Sequences were aligned using the Clustal W (weighted) (Thompson *et al.*, 1994) algorithm option in MegAlign available in DNASTAR and compared with *B. tabaci* reference mtCOI sequences available in the EMBL/DDBJ/GenBank databases.

The DNA sequences were subjected to a heuristic search and subtree-pruning-regrafting branch swapping using the maximum likelihood (ML) and parsimony methods available in Phylogenetic Analysis Using Parsimony* (PAUP*4.0b10) (Swofford, 2002). The ML tree was reconstructed using the maximum likelihood 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 PAUP using the heuristic option for 1000 replications at a 70% confidence limit. The GenBank accession numbers for reference mtCOI sequences are presented in Table 12.

5.2.3 Analysis of molecular variance

Analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was performed in ARLEQUIN, version 2.000 (Schneider *et al*., 2000): software for population genetics data analysis to establish the population structure of the *B. tabaci* populations examined in the current study using the pairwise difference and Tajima and Nei distance methods (Excoffier *et al*., 1992). Variance components and percentage variation were calculated for among groups, among populations within groups and within populations. The significance tests (i.e., *F*-statistics and *P*-value) for the variance components were tested at the 5% significance level using 1023 permutations.

5.2.4 Squash silverleaf bioassay

In order to determine whether the B-like whiteflies identified in Uganda could induce squash silverleaf (SSL) symptoms in squash (*Cucurbita pepo* L.) (Tozer Seeds Ltd., UK), several B

Table 12. Whitefly genotypes and location of reference whiteflies used in phylogenetic analysis of mitochondrial cytochrome oxidase I sequences and their respective GenBank accession numbers

Whitefly/genotype	Location	GenBank Accession No.
AB A Benin	Benin	AF110693
ARG2 Santiago	Argentina	AF340213
AZ A Arizona	Arizona, USA	AY057122
AZ B Arizona	Arizona, USA	AY057123
Bemisia afer[1]	-	AY057218
CAL A Brawlee CA	California, USA	AY057124
CUL Mexico	Mexico	AY057125
HC China	China	AF342777
IS B Israel	Israel	AF110705
Ivory Coast cassava	Ivory Coast	AY057135
Ivory Coast okra	Ivory Coast	AY057136
IW India	India	AF110702
JAT Puerto Rico	Puert Rico	AF110705
Morocco 1	Morocco	AF342773
Moz-Kal 1	Mozambique	AF344278
PC91 Pakistan 1	Pakistan	AF342778
Reunion 1	Reunion Island	AJ550172
Reunion 2	Reunion Island	AJ550178
SA Lucia 2	South Africa	AF344260
SC Sudan 1	Sudan	AF110706
SP92 Spain Q	Spain	AF342775
SwazMap1	Swaziland	AF344269
TC Turkey	Turkey	AF342776
Thailand cotton	Thailand	AF164670
Uganda sweetpotato	Uganda	AY057174
Zam 2	Zambia	AF344281
30MNten (Ug1)	Uganda	AY057171
17Ikul (Ug2)	Uganda	AY057158

[1]The outgroup species was *Bemisia afer* (Priesner & Hosny)

biotype variants (Costa *et al*., 1993a), colonies of Ug4, Ug6 and Ug7 were subsequently established at Namulonge, Uganda. About 40 female whitefly adults were collected of each of the three genotype clusters and introduced separately in clip cages on whitefly-free squash plants as described by Costa *et al*. (1993a). The plants were maintained in a screenhouse at 12L:12D and 22-26ºC at the International Institute of Tropical Agriculture (IITA), Namulonge, Uganda. The whiteflies were allowed to feed and oviposit for 10 days and the plants observed daily for the SSL symptoms development. Silvering was determined as positive when the SSL symptoms were observed and negative where no symptoms developed.

5.3 Results

5.3.1 Phylogenetic analyses of adult *B. tabaci* mtCOI sequences

The mitochondrial DNA sequences (~ 800 bp) obtained for the field-collected female adult *B. tabaci* from: beans (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita pepo* L.), Dew flower (*Commelina benghalensis* L.), eggplant (*Solanum melongena* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon ensculentum* Mill.), Lion's ear (*Leonotis nepetifolia* L.), mint/wild basil (*Ocimum gratissimum* L.), cotton (*Gossypium hirsutum* L.), 'Muwogola omusajja' (*Pavonia urens* Cav.), okra (*Abelmoschus esculentus* L.) and sweetpotato (*Ipomoea batatas* L.) are deposited in the GenBank and the assigned accession number for each shown in Table 11. Comparative sequence analysis was used to assess the genetic variability of *B. tabaci* populations.

Based on phylogenetic analysis with PAUP, alignment of the mtCOI sequences established 74 constant, 209 variable and 417 parsimony-informative characters. Both parsimony and

maximum likelihood options predicted the same phylogenetic relationships for the *B. tabaci* populations examined in this study: hence only the parsimony results are presented (Fig. 11).

The mtCOI sequence grouped the members of *B. tabaci* into either the Old or New World clusters. The Ugandan *B. tabaci* populations grouped into eight distinct sub-clusters within the three major clusters comprising the Old World *B. tabaci* collections from sub-Saharan Africa, the Mediterranean-North Africa-Middle East (MED-NAFR-ME) region and Ugandan sweetpotato, as might be expected (Fig. 11). All eight sub-clusters had high bootstrap (bs) values (≥80). The cassava-associated genotypes, included here as reference sequences sorted into two distinct sub-clusters, Uganda (Ug1) and Uganda (Ug2), as reported previously.

A third sub-cluster was formed comprising a distinct genotype cluster, herein designated Uganda 3 (Ug3), whose members (n = 3) were unlike any *B. tabaci* genotypes reported previously in Uganda or elsewhere.

In addition, four closely related (at 93-97% nt identity) sub-clusters, designated as: Uganda 4 (Ug4), Uganda 5 (Ug5), Uganda 6 (Ug6) and Uganda 7 (Ug7) were evident (Fig. 11). The Ug4 genotypes (n = 11) had as their closest relatives (97-99% nt identity) the *B. tabaci* genotype described by Burban *et al.* (1992) from okra in the Ivory Coast, West Africa, whereas, the group containing the Ug5 (n = 1) and Ug6 (n = 3) types shared 95-99% and 99% nt identity, respectively, with their closest relatives from Morocco, Sudan, Spain and Turkey (Mediterranean-North Africa) and Israel (Middle East) (MED-NAFR-ME), which also include the well studied B and Q biotypes (Fig. 11). Despite a relatively high-shared sequence identity(>93%) between the Ug4, MED-NAFR-ME (Ug5 and Ug6), and Ug7 types (n = 8), the

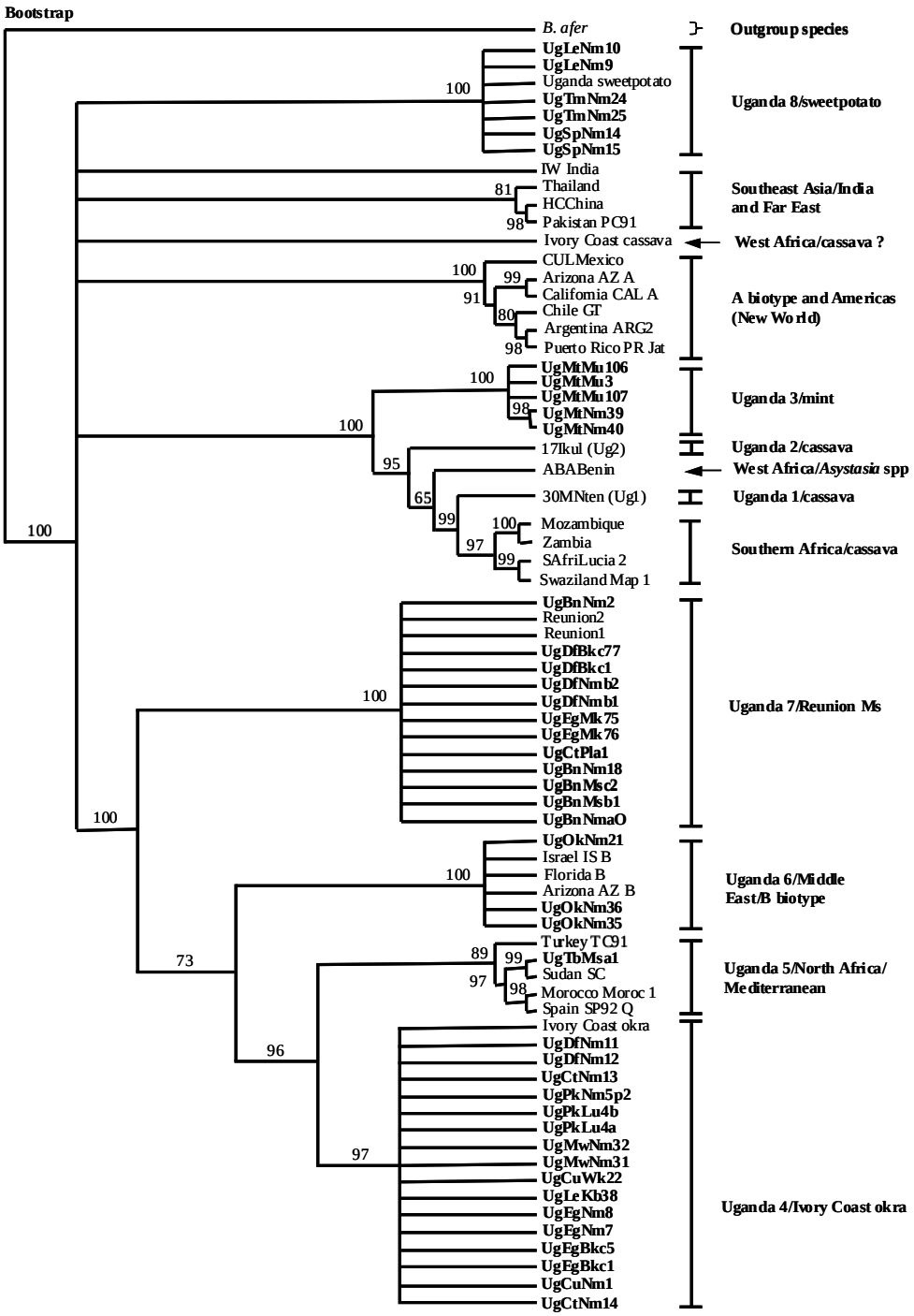

Figure 11. Dendrogram of the mitochondrial cytochrome oxidase I sequence for adult *B. tabaci* collected on the cultivated and uncultivated species occurring adjacent to cassava fields in Uganda (in bold) during 2003/4, and well-studied *B. tabaci* reference populations, using the maximum parsimony algorithm available in PAUP* (Swofford, 2002). *B. afer* is included as the outgroup sequence. Samples are indicated by country, source-plant species and location from where they were collected. Country: Ug = Uganda, source-plant species: Tm = tomato, Tb = tobacco, Eg = eggplant, Ct = cotton, Bn = beans, Df = dew/day flower, Ok = okra, Cu = cucumber, Pk = pumpkin, Sp = sweetpotato, Le = Lion's ears, Mt =mint/wild basil and Mw = 'muwogola omusajja, and sample location (district): Nm = Namulonge, Ms = Masindi, Pl = Paliisa, Km = Kamuli, Mu= Mukono, Bk = Busukuma, Wk = Wakiso, Lu = Luwero, Mk = Masaka, Kb = Kabarole

closest Ug7-like genotype cluster relatives (at 98-99% nt identity) were *B. tabaci* genotypes from the Reunion Islands in the Indian Ocean.

The eighth distinct sub-cluster, referred to herein as Uganda 8 (Ug8), contained three members that grouped (at 98-99% nt identity) with the previously described sweetpotato genotype in Uganda.

A pairwise comparison of the nucleotide identity of the Ug3, Ug4, Ug6, Ug7 and Ug8 genotypes revealed a within-group variance of 0.1 to 0.4%, 0.1 to 1.2%, 0.5 to 2%, 0.1 to 1% and 0.1 to 0.6%, respectively (data not presented). Comparison of the nucleotide identity of the Ug3 genotypes with sequences of well-studied *B. tabaci* available from the GenBank database revealed that Ug3 genotypes shared 90-92% nt identity with the Ug1 and Ug2 genotypes, and *B. tabaci* from southern Africa (Table 13). The Ug3 genotypes diverged from Ug1 by ~ 8%, which is similar to the divergence between Ug1 and Ug2, but were ~ 10% divergent from Ug2 (Table 13), suggesting that Ug3 could be another distinct genotype cluster. In addition Ug3 diverged by 18% from Ug8. In contrast, Ug4 and the two MED-NAFR-ME (Ug5 and Ug6) genotype clusters diverged from one another by 3-7% and further, were 16-17% and 15-18% divergent from Ug3 and Ug1, Ug2 and Ug8, respectively (Table 13).

The field occurrence and source-plant distribution of adult female *B. tabaci* genotypes in Uganda in 2003/4 was determined as follows. Ug1 occurred on *N. tabacum*, *L. esculentum*, *A. esculentus* and *I. batatas*, representing 30.8% of the source-plants (data not presented). The Ug3 genotypes occurred on *O. gratissimum* (7.7%) only, while the Ug4 types were identified on 54% of the source-plant species including: *C. benghalensis, C. pepo, C. sativus, G.*

Table 13. A pairwise comparison of the mitochondrial cytochrome oxidase I (mtCOI) nucleotide sequence[1], expressed as percent nucleotide divergence between adult *B. tabaci* populations on different plant species in Uganda, as calculated by the *Clustal W* algorithm (Thompson *et al.* 1994)

Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. UgDfNm11	-	0.1	3.0	5.1	4.6	6.7	6.7	6.7	17.0	17.0	17.0	16.6	16.1	16.6	3.4	5.1	7.0	0.7	17.3	17.0	28.1
2. UgPkLu4a		-	3.1	5.0	4.4	7.0	7.0	7.0	17.0	17.0	17.0	16.3	16.3	16.7	3.6	5.0	7.1	0.9	17.4	17.0	28.3
3. UgTbNma1			-	5.3	4.3	6.7	6.7	6.7	17.4	17.4	16.6	15.9	15.3	16.3	0.7	5.3	6.9	2.6	17.1	17.4	27.9
4. UgOkNm35				-	2.1	6.9	6.9	6.9	17.1	17.1	16.3	15.6	15.4	15.6	5.7	0.1	7.0	5.6	16.6	17.1	26.9
5. UgOkNm36					-	6.7	6.7	6.7	16.9	16.9	16.2	15.4	15.3	15.3	5.6	0.2	6.9	5.5	16.6	16.9	27.1
6. UgBnNm18						-	0.1	0.1	18.3	18.3	16.4	16.0	17.0	16.3	7.1	6.9	0.1	7.0	18.0	18.3	29.1
7. UgCtPaa1							-	0.1	18.3	18.3	16.6	16.1	17.0	16.3	7.1	6.9	0.1	7.0	18.0	18.3	29.1
8. UgDfNmb1								-	18.3	18.3	16.6	16.1	17.0	16.3	7.1	6.9	0.1	7.0	18.0	18.3	29.1
9. UgTmNm25									-	0.1	17.6	16.9	17.0	17.3	17.9	17.1	18.1	18.9	17.1	0.1	28.1
10. UgSpNm14										-	17.4	16.7	17.0	17.3	17.9	17.1	18.1	18.9	17.3	0.1	28.1
11. UgMtNm40											-	0.4	8.0	9.6	17.0	16.5	16.6	16.9	9.0	17.6	27.2
12. UgMtMu106												-	7.6	9.5	16.3	15.6	16.4	16.1	8.9	16.9	26.4
13. 30Mnten (Ug1)													-	7.6	16.3	15.3	17.1	16.4	2.1	17.1	26.1
14. 17Ikul (Ug2)														-	16.7	15.6	16.4	16.9	8.0	17.3	26.3
15. Sudan															-	5.7	7.3	3.0	17.6	17.9	27.6
16. Israel B																-	7.0	5.6	16.6	17.1	26.9
17. Reunion																	-	7.1	18.1	18.1	29.3
18. Ivory Coast okra																		-	17.6	17.3	28.0
19. SAfricaLucia																			-	21.3	26.7
20. Ugsweetpotato																				-	28.1
21. *B. afer*																					-

[1]Representative sequences are shown for *B. tabaci* genotypes identified on the different host-plant species in Uganda, 2003/4

hirsutum, L. nepetifolia, P. urens and *S. melongena* (Table 11). The Ug5 and Ug6 genotypes occurred on *N. tabacum* (7.7%) and *A. esculentus* (7.7%), respectively, while the Ug7 types were identified on *C. benghalensis, G. hirsutum, P. vulgaris* and *S. melongena,* which comprised 31% of the source-plant species sampled. The Ug8 genotypes were identified on 23% of the source-plant species including: *I. batatas*, *L. esculentum* and *L. nepetifolia* (Table 11). Ug2 was not identified on any of the source-plants sampled in the current study.

5.3.2 Phylogenetic analyses of *B. tabaci* fourth instar nymphs mtCOI sequences

The mtCOI DNA sequences (~ 800 bp) were obtained for the field-collected *B. tabaci* fourth instar nymphs and the sequences deposited in the GenBank under the assigned accession numbers shown in Table 11. Phylogenetic analysis produced one most parsimonious tree, with a topology similar to that obtained for the analogous adult whitefly sequences (Fig. 12). Other than Ug1 and Ug2, which are not reported here, and Ug5, which was not identified in the nymphs sampled, the *B. tabaci* examined in this study grouped into five distinct sub-clusters within the Old World cluster, as expected. Similar to the results of the adult whiteflies, the Ug3 genotypes (n = 2) formed a distinct sub-cluster unlike any previously reported genotypes. The Ug4 (n = 4), Ug6 (n = 2), Ug7 (n = 3) and Ug8 (n =2) genotypes clustered with the Ivory Coast okra, Israeli, Reunion and the Ugandan sweetpotato genotypes, respectively.

A pairwise comparison of the nucleotide distances of the fourth instar nymphs' sequences and reference mtCOI sequences of adult *B. tabaci* in the GenBank produced similar results to those obtained with the adult whitefly sequences (Table 14). Ug3 diverged at 8-10% from Ug1 and Ug2, respectively, while Ug4, Ug6 and Ug7 diverged at 3-7% from one another and at 15-18% from Ug1, Ug2, Ug3 and Ug8, respectively.

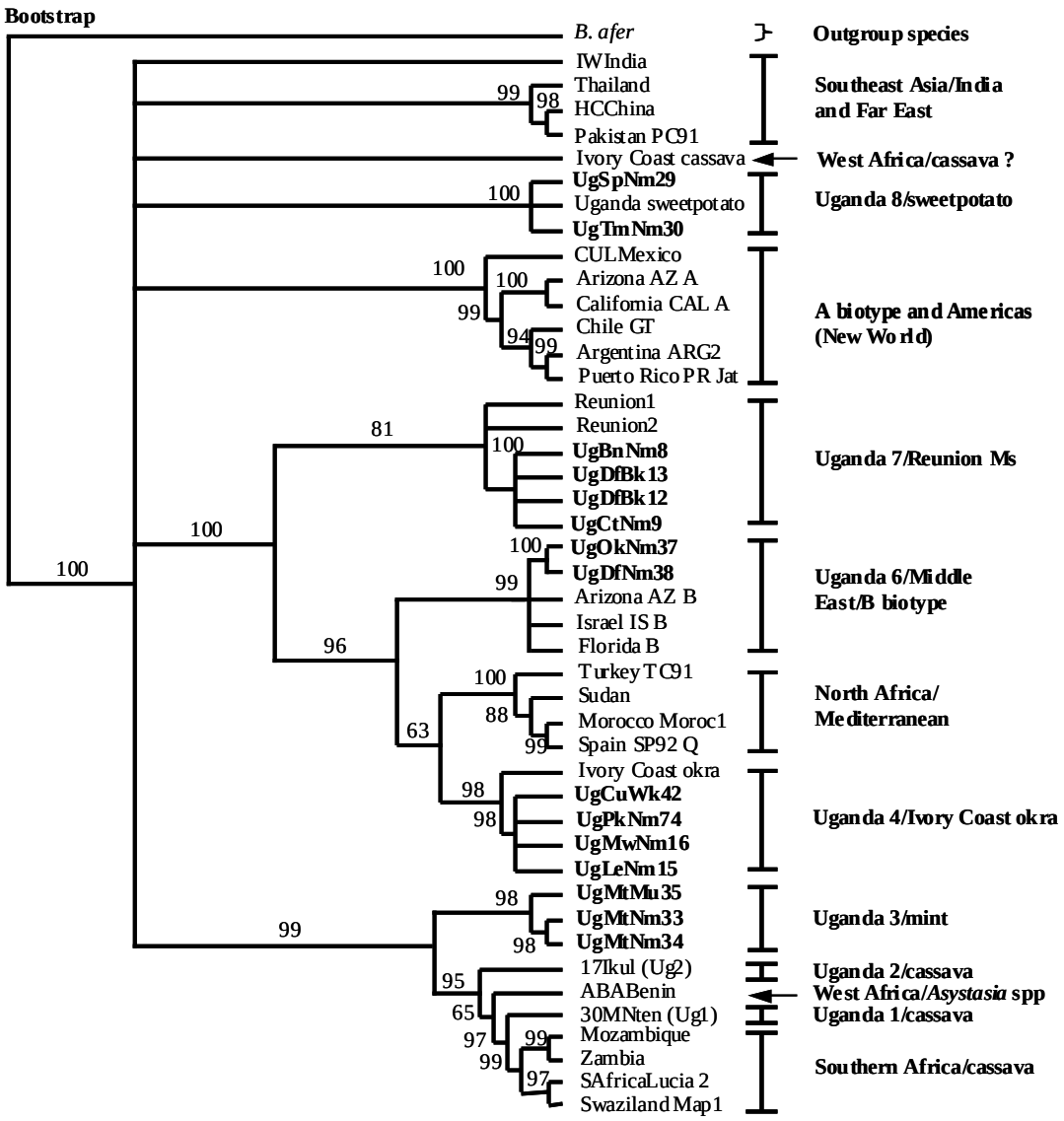

Figure 12. Dendrogram of the mitochondrial cytochrome oxidase I sequence for *B. tabaci* fourth instar nymphs collected on cultivated and uncultivated species occurring adjacent to cassava fields in Uganda (in bold) during 2003/4, and well-studied *B. tabaci* reference populations, using the maximum parsimony algorithm available in PAUP* (Swofford, 2002). *B. afer* is included as the outgroup sequence. Samples are indicated by country, source-plant species and location from where they were collected. Country: Ug = Uganda. Source-plant species: Tm = tomato, Ct = cotton, Bn = beans, Df = dew/day flower, Ok = okra, Cu = cucumber, Pk = pumpkin, Sp = sweetpotato, Le = Lion's ears, Mt =mint/wild basil and Mw = 'muwogola omusajja'. Sample location: Nm = Namulonge, Mu= Mukono, Bk = Busukuma, Wk = Wakiso

Based on the presence of *B. tabaci* fourth instar nymphs on the host-plant species sampled, the Ug3 types colonized *O. gratissimum* only, whereas the Ug4 genotypes colonized: *C. pepo, C. sativus, L. nepetifolia* and *P. urens.* The Ug6 types colonized *A. esculentus* and *C. benghalensis* in the field, and *Bidens pilosa* - (L.) (Asteraceae), *G. hirsutum, L. esculentum, P. vulgaris* and two unidentified weeds in cages at Namulonge, Uganda (data not presented). The Ug7 genotypes colonized *C. benghalensis, G. hirsutum* and *P. vulgaris,* while in addition to colonizing sweetpotato (*I. batatas*); the Ug8 types colonized *L. esculentum* also. None of the Ug3-Ug8 genotypes described in this study colonized cassava plants in the field or in the screen houses at IITA, Namulonge, Uganda.

5.3.3 Analysis of molecular variance

A hierarchical analysis of molecular variance (AMOVA) conducted to assess the genetic differentiation of the Ugandan *B. tabaci* populations (Table 15) grouped according to the genotype clusters, Ug3, Ug8 and Ug4-MED-NAFR-ME-Ug7 (B biotype-like), revealed significant differences among groups ($P<0.0001$, $F_{ST} = 0.4347$), among populations within groups ($P<0.0001$, $F_{ST} = 0.4019$) and within populations ($P<0.0001$, $F_{ST} = 0.6619$). The highest contribution to the total variance was due to differences among groups (43.47%). A similar result was obtained with the Tajima and Nei distance method (data not shown).

Table 14. Uncorrected pairwise nucleotide distances, as calculated by the *Clustal W* algorithm (Thompson *et al*., 1994), for reference mtCOI sequences of adult *B. tabaci* and fourth instar nymphs collected on different host-plant species in Uganda

B. tabaci nymphs' sequences[1]	Ivory Coast[2] okra cluster (Ug4) (n = 1)	North African and Mediterranean cluster (Ug5) (n = 4)	B biotype cluster (Ug6) (n =3)	Reunion non-B cluster (Ug7) (n = 1)	India and Far East cluster (n = 6)	Ugandan cassava cluster (Ug1 & Ug2) (n = 2)	Southern Africa cassava cluster (n = 4)	A biotype and New World cluster (n = 6)	Ugandan sweetpotato cluster (Ug8) (n = 1)
Ug*pk*Nm74	0.9	2.6 - 5.0	5.0 - 5.2	7.2	14.2 - 63.5	16.3 - 16.7	16.5 - 17.7	18.1 - 18.8	17.0
Ug*bn*Nm8	7.2	6.8 - 7.3	7.0 - 7.3	0.3	13.9 - 63.9	16.6 - 17.2	17.0 - 18.5	19.3 - 19.7	18.3
Ug*sp*Nm29	17.5	17.3 - 18.0	17.3 - 17.5	18.6	18.9 - 63.1	17.3 - 17.5	17.2 - 18.7	20.5 - 22.9	0.1
Ug*tm*Nm30	17.3	17.2 - 17.9	17.2 - 17.3	18.5	18.7 - 63.1	17.3 - 17.5	17.0 - 18.6	20.3 - 22.7	0.1
Ug*df*Nm38	5.0	4.1 - 5.0	2.4 - 2.7	6.7	14.4 - 63.1	15.4 - 15.5	15.4 - 17.0	17.4 - 18.4	16.9
Ug*ok*Nm37	4.7	3.9 - 4.7	2.1 - 2.4	6.4	14.4 - 63.0	15.3 - 15.4	15.4 - 17.0	17.1 - 18.1	16.9
Ug*mt*Mu35	16.6	16.0 - 16.7	16.0 - 16.2	16.3	16.2 - 61.5	7.5 - 9.8	8.2 - 9.6	18.9 - 20.4	17.3

[1]Representative sequences are shown for *B. tabaci* genotypes identified on different host-plant species in Uganda, 2003/4. [2]The within-group estimates of the minimum and maximum nucleotide divergence are presented for the major *B. tabaci* phylogenetic clusters in Fig. 11

Table 15. Hierarchical analysis of molecular variance and *F*-statistics of genetic differentiation for Ugandan *Bemisia tabaci* populations grouped according to genotype clusters (groups), among populations within groups and within populations, respectively. The population structure was obtained using a pairwise difference distance method (Excoffier *et al*., 1992) in ARLEQUIN Version 2.000 (Schneider *et al*., 2000)

Source of variation	d.f.	Sum of squares	Variance components	% of variation	*F*-Statistic	*P*-Value[1]
Among groups	2	1226.260	33.4675 Va	43.47	0.4347	< 0.0001
Among populations within groups	3	567.121	17.4937 Vb	22.72	0.4019	< 0.0001
Within populations	54	1405.686	26.0312 Vc	33.81	0.6619	< 0.0001
Total	59	3199.067	79.9923			

[1]P < 0.05

Va = variation due to differences among groups (Ug3-like, Ug8-like and B biotype-like)

Vb = variation due to population differences within groups

Vc = variation due to population differences

5.3.4 Squash silver leaf bioassay

Colonies of the Ug4, Ug6 and Ug7 genotypes were evaluated for their ability to induce SSL symptoms in *C. pepo.* The Ug6 and Ug7 genotypes induced SSL in squash, respectively, whereas the Ug4 did not (Plate III, Table 11). The SSL symptoms developed 18 and 25 days after squash plants were colonized by the Ug6 and Ug7 genotypes, respectively.

5.4 Discussion

The genetic variability of *B. tabaci* populations on thirteen cultivated and uncultivated species occurring adjacent to cassava fields in selected cassava-producing areas of Uganda was investigated using the *mtCOI* gene (Frohlich *et al*., 1999) as the molecular marker. Because adult whitefly that occur on a plant in the field may not necessarily indicate host preference/colonization, the fourth instar nymphs were collected also and used to establish both the genetic identity and host range of the populations studied.

In addition to the two previously described cassava-associated *B. tabaci* genotype clusters, Ug1 and Ug2 (Legg *et al*., 2002), and the sweetpotato-colonizing genotype (Maruthi *et al*., 2004a), designated here as Ug8, the mtCOI sequence enabled the detection of five distinct additional and previously undocumented *B. tabaci* genotype clusters, Ug3, Ug4, Ug5, Ug6 and Ug7, among the collective Ugandan whitefly populations. These data show a wider diversity (3-18% nt divergence) among the Ugandan *B. tabaci* populations than previously expected. This was confirmed also by the highly significant results obtained with AMOVA analysis, which showed the highest contribution to the total variance to be due to differences among groups, as expected.

Plate III. *Cucurbita pepo* L. plants showing squashsilver leaf (SSL) symptoms by Ug6 (B biotype-like) and Ug7 (Reunion type-like) *B. tabaci* genotypes (white bucket) and negative control – with no whiteflies introduced (blue bucket)

Previously, Legg *et al*. (1994) distinguished cassava, sweetpotato and cotton whitefly populations from different locations in Uganda using esterase profiles, although the high degree of genetic variability in whiteflies precluded the identification of distinct biotypes, owing to the limitations of esterase markers distinguishing between *B. tabaci* from a broad range of hosts and geographical locations (Brown *et al*., 1995b).

Here, we report for the first time the occurrence of a previously unreported *B. tabaci* genotype cluster, Ug3, which differs from both Ug1 (92% nt identity) and Ug2 (90% nt identity). The Ug3 genotypes occurred exclusively on wild mint (*O. gratissimum*), and were not found to colonize any other plant species including cassava sampled in the same localities during this study. *O. gratissimum* occurs mainly as a roadside shrub in grasslands and waste lands in east, west and central Uganda (Anonymous, 1957; Mabberley, 1987) and is also grown as a medicinal herb around homesteads, together with other food crops.

In addition, four closely related genotype clusters, Ug4-Ug7, were identified in Uganda that shared close (>93%) sequence homology. The Ug4 genotypes have as their closest relatives the polyphagous okra-associated *B. tabaci* genotype that colonized: *Chromolaena odorata* - (L.) RM King and H. Robinson (Asteraceae); *Euphorbia heterophylla* - (L.) (Euphorbiaceae); *Centrosema molle* Mart. ex Benth, *Crotalaria* sp, and *Pueraria phaseloides* - (L.) (Fabaceae); A. *esculentus* and *Sida* sp. - (L.) (Malvaceae) and *L. esculentum* in the Ivory Coast (Burban *et al*., 1992), and was similarly as polyphagous in this study. The Ug4 genotypes were identified on a wide range of plant species in the field and were shown to colonize members of the *Cucurbitaceae*, including *C. pepo* and *C. sativus* and the uncultivated species *L. nepetifolia* and *P. urens*. The presence of the Ug5 and Ug6 genotypes in Uganda, which were closely related to the B and Q biotypes: members of the MED-NAFR-ME clade (Guirao *et al*., 1997; Frohlich

et al., 1999; Moya *et al*., 2001), respectively is a significant finding. Currently, the 'hypothesized' center of origin for the B and Q biotypes is the Mediterranean-North Africa-Middle East (MED-NAFR-ME) region (Brown *et al*., 1995a,b; Frohlich *et al*., 1999). New evidence, presented here, for the occurrence of both the B and B-like biotypes in Uganda, situated in East Africa, suggests that this hypothesis may have to be reassessed.

The B and Q biotypes were reported to be extremely polyphagous (Brown *et al*., 1995a; Moya *et al*., 2001). However, in this study, Ug5 (the Q-like genotype) and Ug6 (the B-like genotype) were not found to be as polyphagous. The Ug5 genotypes occurred only on *N. tabacum*, whereas the Ug6 types colonized only *A. esculentus* and *C. benghalensis* in the field. However, that the Ug6 genotypes colonized several other plant species including: *B. pilosa, G. hirsutum, L. esculentum, P. vulgaris* and two unidentified (as yet) weeds in cages at Namulonge, Uganda is significant, and evidence that the B biotype could indeed be just as highly polyphagous in the field. More extensive sampling and sequencing work may be required, however, to confirm the apparent restricted polyphagy of the B and Q biotypes in the field.

Despite having very close sequence homology with the Ug4-Ug6 genotypes, the Ug7 genotypes were related most closely to *B. tabaci* genotypes from the Reunion Island in the Indian Ocean (Delatte *et al*., 2003, 2005). The Ug7 genotypes occurred on *C. benghalensis, G. hirsutum, P. vulgaris* and *S. melongena,* which is evidence that they are highly polyphagous. In addition to colonizing sweetpotato, the Ug8 genotypes colonized *L. esculentum* also. Legg (1996) reported that the sweetpotato genotypes occur on several other plant species in the field, but with less success for colonization, as observed also in the current study. However, the

absence of the Ug3-Ug8 genotypes on cassava in the field is further evidence that in Uganda, cassava is colonized only by cassava *B. tabaci*.

A hallmark phenotype of the B biotype and several closely related variants is the ability to induce SSL symptoms in *C. pepo* (Costa and Brown, 1991; Brown *et al*., 1995a; Bedford *et al*., 1994). Of the four closely related *B. tabaci* genotype clusters (Ug4-Ug7), the Ug6 and Ug7 types induced SSL in *C. pepo*. As in this study, silver-inducing non-B populations were reported elsewhere in the world (Bedford *et al.*, 1994; Brown *et al*., 1995a; Delatte *et al*., 2003, 2005). The Ug7 genotypes clustered with Ug6 (the B biotype-like) at >98% nt identity, suggesting that this is another variant of the B biotype. However, it was not possible to establish whether the genotypes (Ug4-Ug7) described in this study are recent introductions or indigenous populations in Uganda. It should be highlighted, however, that there is little trade in ornamental plants in Uganda, reducing the likelihood of foreign introductions. This fact, coupled with the low abundance of all of the Ugandan *B. tabaci* genotypes discussed here (data not shown), suggests strongly that these populations are indigenous.

This study presents the most comprehensive assessment of the genetic variability of *B. tabaci* populations carried out to date in Uganda or elsewhere in the East and Central African region. The discovery of five previously identified *B. tabaci* genotype clusters, Ug3-Ug7, in Uganda, among which are some of the world's most economically important biotypes, namely B and Q is particularly significant. *B. tabaci* is a prolific and widespread vector of many plant viruses, which include some of the most devastating biotic constraints to crop production in Africa. Additional studies are required to establish the distribution, field populations and host range for the Ug3-Ug7-like genotypes, and whether they mate freely and produce viable offspring. The discovery of economically 'insignificant' populations of the B and B-like genotypes in Uganda

in this study raises important questions, such as how these genotypes do not become more abundant and cause more damage. An answer to this question may be of substantial value in enhancing the management of B biotype populations in countries and regions where they are a major pest.

Chapter Six

General discussion, conclusions and recommendations

Data reported in Chapter two (this study) confirmed the presence, in the post-epidemic zone, of two different CMGs, ACMV and EACMV-UG2, which were also previously associated with the CMD epidemic in Uganda. As expected, EACMV-UG2 predominated; confirming that much of Uganda is in a post-epidemic situation. However, unlike previous observations in which EACMV-UG2 was consistently associated with the severe disease phenotype symptom (Harrison *et al*., 1997; Zhou *et al*., 1997; Pita *et al*., 2001a), the virus occurred with almost equal frequency in the severely and mildly diseased plants in the current study. Both mild and severe strains of EACMV-UG2 were found to occur in Uganda in collections made there in 1998 (Pita *et al*., 2001a), but the mild strains then occurred with much less frequency, and were seemingly confined to isolated localities. The detection of increased proportions of mildly diseased plants in farmers' fields in the current study is significant. The mild strains have less devastating effects on plant growth (Harrison *et al*., 1997) and are known to cause less yield loss than the severe strains (Fauquet *et al*., 1998; Owor *et al*., 2004a, b). In the 1990s, farmers virtually abandoned growing cassava in much of the epidemic-affected areas in Uganda, e.g., eastern districts, where the severe disease led to widespread food shortages and famine (Thresh and Otim-Nape, 1994). During this period, key phytosanitary approaches for managing CMD, like roguing (the removal of CMD-diseased plants from a crop stand) and selection (the use of symptom-free cassava planting material) were unpopular with the farmers and seldom practised. Although we could not establish, in the current study, why and how the mildly diseased plants were increasing in frequency in the farmers' fields, one possibility could be that farmers have changed their attitudes and have taken seriously the use of selection in the

management of the CMD epidemic. By selecting the mildly diseased plants, which provide more planting material, and ensure higher yields than the severely diseased plants (Thresh and Otim-Nape, 1994; Thresh *et al*., 1998; Owor *et al*., 2004a, b), the farmers have been able to retain many CMD-susceptible local varieties (with desirable attributes), despite the high incidence of infection.

The occurrence of mixed infections in Uganda was reported previously (Harrison *et al*., 1997; Pita *et al*., 2001a, b), and they were shown to cause extremely severe symptoms in the affected plants. However, the data described here (Chapter two) did not provide evidence of the previously reported synergism associated with mixed infections that leads to severe symptoms. It seems that there are mixed infections in which synergism does not occur, possibly involving mild strain combinations. Mild strain protection, in which plants initially infected with mild virus strains become less severely diseased than the initially healthy plants, was suggested (Owor *et al*., 2004a). However, further studies are required to establish the molecular mechanisms and biological significance of the virus-virus and virus-host interactions. Information from such studies would be integral to developing an IPM package involving mild strain protection.

This study sought also to establish and/or clarify the relationship between the *B. tabaci* genotypes and the different CMGs occurring singly or together on cassava in post-epidemic areas. The data presented in Chapter three confirmed the presence of two previously reported cassava-associated mtCOI *B. tabaci* genotype clusters, Ug1 and Ug2, in the post-epidemic zone in Uganda. The two genotype clusters diverged at ~8% as previously reported also (Legg *et al*., 2002). Two key findings were evident from this study. The first was the higher frequency of occurrence of the Ug1 (83%) than the Ug2 (17%) genotypes in the post-epidemic

zone (P Sseruwagi, JP Legg, MEC Rey, J Colvin, D Rogan and JK Brown, unpublished data), in contrast to the genotypes distribution reported for the vector populations that occurred on cassava in 1997-98, at the height of the spread of the severe CMD epidemic. Ug2 were then the predominant genotypes at all sites sampled in the post-epidemic zone, while the Ug1 types occurred mainly 'at' and 'ahead' of the epidemic 'front' (Legg *et al*., 2002). Coupled with the evidence that the Ug2 genotypes were less prevalent in the epidemic-affected zone in 1999 (Legg *et al*., 2002; chapter 3 and 4, this study), collectively these data seem to suggest that Ug1 may be increasing, while Ug2 are diminishing from the collective *B. tabaci* population on cassava in Uganda. Although it was not possible in this study, as in the previous study, to establish why and how the Ug1 types were recovering and the Ug2 types disappearing from the overall *B. tabaci* populations on cassava in the post-epidemic zone, a number of suggestions are advanced as follows: (i) the possibility that the Ug1 genotypes, the hypothesized 'local/indigenous' genotypes (Legg *et al*., 2002) could be re-occupying their original habitat and hence displacing the Ug2 genotypes, the 'invader', in these areas, (ii) that due to the mating compatibility between the Ug1- and Ug2-like genotypes (Maruthi *et al*., 2004a), members in the two genotype clusters may have hybridized leading to the increased occurrence of hybrid offspring with the Ug1 genotype in the epidemic-affected areas, and (iii) that the Ug2 genotypes could have failed to establish permanently in the 'invaded' areas, owing to habitat-related or other incompatibilities. Further studies are required to verify these suggestions.

Secondly, there was no clear association of a particular vector genotype cluster with plants exhibiting the severe disease phenotype symptom in the current study. The Ug2 genotypes were at least in 1997/8 closely associated with the severe epidemic in Uganda (Legg *et al*., 2002). Generally, however, the Ug1 genotypes predominated with EACMV-UG2, while Ug2 occurred with ACMV in the current study. The epidemiological significance of this apparent

'shift' in association between the vector and the virus in the continued spread of the CMD epidemic in Uganda and elsewhere is yet to be determined. Significantly, however, the EACMV-UG2-associated severe CMD epidemic has reached pandemic proportions and has been reported also in western Kenya and the eastern and southern parts of the Lake Victoria region in Tanzania (Legg, 1999), north eastern Rwanda (Legg *et al*., 2001; Sseruwagi *et al*., 2005a), south western DR Congo (Neuenschwander *et al*., 2002) and Burundi (Bigirimana *et al*., 2004), which calls for more studies to be conducted on the genetic identity, biology, field populations, dynamics and relationship of the whitefly vector and CMGs associated with the spreading pandemic.

Previous studies investigating the genetic variability of morphologically indistinguishable *B. tabaci* populations in Uganda (Legg *et al*., 1994; Chapter three) and elsewhere, in Africa (Burban *et al*., 1992; Abdullahi *et al*., 2003; Berry *et al*., 2004) have used field-collected adult whiteflies. However, these are of limited use in establishing host-associated populations, since the whiteflies could be just visiting, feeding or resting on the plants from which they were collected at the time of sampling. Hence, the use of adults alone could easily result in the erroneous assignment of *B. tabaci* genotypes to host-plant species they would otherwise not normally colonize. To overcome this problem, the studies described in Chapters four and five used both field-collected adult *B. tabaci* and fourth instar nymphs to establish both the genetic identity and associated host-plant species for the populations studied. The results obtained with the fourth instar nymphs corroborated those obtained from the analysis of the adult *B. tabaci*, thereby providing a unique tool to examine the molecular systematics of host-associated whitefly populations.

The role of alternative host-plant species in the ecology of cassava *B. tabaci* genotypes and their involvement in the epidemiology of CMD remain largely poorly understood. Although it was previously suggested that cassava-colonizing *B. tabaci* were host-specialized (Storey and Nichols, 1938; Abdullahi *et al*., 2003), evidence obtained from this study (Chapter four) suggests that the Ug1 genotypes, the only genotype cluster detected on both cassava and the non-cassava plant species sampled during the alternative host colonization studies, is oligophagous. The Ug1 genotypes colonized five additional non-cassava plant species: *Manihot glaziovii, Jatropha gossypifolia, Euphorbia heterophylla, Aspilia africana* and *Abelmoschus esculentus*. Burban *et al*. (1992) and Legg (1996) both reported the occurrence of cassava *B. tabaci* adults on non-cassava species. However, their approach differed from that described in Chapters four and five, which used field-collected adults and fourth instar nymphs to establish the distribution and colonization of host-plant species by *B. tabaci* genotypes. Therefore, there was no basis for determining, in my study, whether the alternative hosts identified are actually new host species defining a widening host range for the cassava-colonizing *B. tabaci* in Uganda or merely additional hosts that were simply undetected in the previous studies. Significantly, however, the additional host-plant species identified in the post-epidemic areas in this study, could facilitate the survival of the Ug1 genotypes in areas where cassava is devastated, hence enabling the acquisition of other whitefly-transmitted geminiviruses (WTVs), which could potentially become of epidemiological importance if adapted to cassava. However, further studies to assess the field populations of the Ug1 genotypes and the occurrence of WTVs in the additional host-plant species should be conducted countrywide.

A major finding of the study described in Chapter five was the discovery of five distinct previously unrecorded *B. tabaci* genotype clusters, Ug3-Ug7, among the collective Ugandan *B.*

tabaci populations. Legg *et al*. (1994) provided the first evidence of the occurrence of discrete cassava, sweetpotato and cotton-associated *B. tabaci* populations in Uganda, based on the analysis of general esterases. The data described here (Chapter five), using the mtCOI as the molecular marker provide further evidence of the occurrence of additional discrete populations of *B. tabaci* in Uganda. Further, these data show a wider diversity (3-18% nt divergence) among the Ugandan *B. tabaci* populations than was previously expected. The Ug3 genotypes have not been reported before in Uganda or elsewhere, and occurred exclusively on wild mint (*O. gratissimum*) in this study. Four closely-related (at 1-7% nt divergence) genotype clusters, Ug4, Ug5, Ug6 and Ug7, which also include the well-studied B and Q biotypes, and *B. tabaci* from the Ivory Coast, the MED-NAFR-ME region and the Reunion, respectively, are reported also for the first time in Uganda. The Ug4 and Ug7 genotypes were most polyphagous, while the Ug6 genotypes colonized only a few hosts. These data did not show the high polyphagy associated with the Ug5 (Q biotype-like) and Ug6 (B biotype-like) genotypes in other parts of the world, where members of the two genotype clusters are of significant economic importance (Brown *et al*., 1995a; Moya *et al*., 2001).

Collectively, however, none of the newly reported genotype clusters (Ug3-Ug7) was found colonizing cassava or sweetpotato plants in this study, providing further evidence that in Uganda, cassava is only colonized by cassava *B. tabaci*, which is consistent with previous findings on cassava-colonizing whitefly biotypes in Africa (Burban *et al*., 1992; Legg, 1996; Abdullahi *et al*., 2003). Further studies should, however, be conducted to establish the countrywide occurrence, field population dynamics and host range of the Ug3-Ug7 genotypes, and whether there is gene exchange between them. The geminiviruses transmitted by members of the Ug3-Ug7 genotype clusters should be established also, and their economic status in the affected crops.

Finally the findings of this study provide more understanding of the *B. tabaci* and CMGs situation in Uganda. CMD remains the main constraint to cassava production in Uganda. However, the biological property of virulence is seemingly more important than the biochemical property of identity for CMGs. Severe and mild disease phenotypes could be associated with either ACMV or EACMV-UG2, and interestingly also with mixed infections. Moreover, the apparent 'shift' in distribution and association of cassava-associated *B. tabaci* genotypes, and CMGs in the post-epidemic-affected areas, are new developments for which the significance to the epidemiology of the CMD pandemic has yet to be determined.

B. tabaci is taking on increasingly significant importance in Africa. Recent studies (Legg *et al*., 2004) and field observations (C. Omongo, pers. observ) indicate upsurges in the populations of cassava *B. tabaci* in newly released CMD-resistant varieties, which have caused physical damage and significant yield loss. Coupled with the discovery of previously identified *B. tabaci* genotypes, among which are some of the world's economically important biotypes (B and Q), it is clear that more work still needs to be done to elucidate the biological significance and epidemiological (virus transmission) implications of the genetic differences identified of the *B. tabaci* populations in Uganda.

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