DIVERSITY AND ROLE OF BACTERIAL ENDOSYMBIONTS INFECTING THE CASSAVA WHITEFLY, *Bemisia tabaci* (GENNADIUS) IN SUB-SAHARAN AFRICA

SAPTARSHI GHOSH

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DECLARATION

"I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of (Doctor of Philosophy) being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others".

Saptarshi Ghosh _____ Date_____

(Student)

Dr Maruthi M. N. Gowda_____ Date_____

(First supervisor)

Dr Simon Richardson_____ Date_____

(Second supervisor)

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ABSTRACT

The main aims of this study were to investigate the diversity of endosymbiotic bacteria infecting cassava whiteflies, understand their putative role on insect population dynamics and cassava virus disease epidemics in sub-Saharan Africa (SSA). The genetic diversity of field-collected cassava whiteflies, *Bemisia tabaci*, and their endosymbionts from Tanzania, Uganda, Malawi and Nigeria were described. Cassava in these countries is infested by five whitefly populations, which were infected with diverse endosymbiotic bacteria with varied prevalence. *Wolbachia* was the most predominant symbiont with infection levels varying from 21 to 97%. *Arsenophonus* varied from 17 to 64% and that of *Rickettsia* was 0 to 53%. Sequence analysis of the bacteria revealed high genetic diversity. Several strains of *Rickettsia*, *Arsenophonus* and *Wolbachia* were found infecting cassava whiteflies. *Hamiltonella* and *Fritschea* were absent in all the samples tested. Fluorescent in situ hybridisation was used to localise *Arsenophonus*, *Rickettsia* and *Wolbachia*. These were confined within specialised cells called bacteriocytes in both nymphs and adults. Isofemale lines of different whitefly populations with varied symbiont infections were developed for comparing the effect of bacterial infection on the whitefly fitness and biology.

Isofemale colonies of sub-Saharan Africa 1-subgroup 3 (SSA1-SG3), infected with *Arsenophonus* and *Rickettsia* (AR+) and those free of AR (AR-) were compared for fecundity, nymph development, adult emergence and development time on healthy and *East African cassava mosaic virus*-Uganda (EACMV-UG)-infected cassava plants var. Ebwanateraka. The fecundity (number of eggs laid) and nymph development was not affected by the presence of endosymbionts or the infection of cassava by the virus. AR- whiteflies, however, produced significantly higher number of adults than AR+ on both healthy and virus-infected plants, indicating that bacterial infections negatively affect cassava whitefly development. The AR+ whiteflies took longer time to emerge on healthy and virus-infected plants than AR-. AR+ and AR- populations were further compared for EACMV-UG acquisition and retention after 48 hours each of acquisition access and inoculation access on diseased and healthy cassava var. Ebwanateraka, respectively. Higher proportion of AR- acquired and retained EACMV-UG than AR+. Similarly, AR- also retained higher (~9 folds) titres of virus than AR+. This indicated that bacteria-free SSA1-SG3 AR- whiteflies had higher adult emergence, quicker life cycle and better vectoring abilities than those infected with bacteria. However, innate immune response genes were upregulated in AR+ than AR- populations, possibly indicating a protective role of bacterial infections in AR+ whiteflies against pathogenesis.

Endosymbiotic bacteria infecting cassava whiteflies during the Uganda cassava mosaic disease (CMD) pandemic in 1997 were characterized. No statistically significant association was found between symbiont infections of whiteflies and the CMD pandemic. However, SSA1-SG2 and SSA2, which were predominant in the 1990s and have become rare recently, had higher prevalence and titres of *Wolbachia*. Simulation experiments to investigate the effect of bacterial infections on different whiteflies showed that *Wolbachia*-infected SSA1-SG2 was quickly outcompeted and almost eliminated by the *Wolbachia*-free SSA1-SG3. These results indicated fitness costs of *Wolbachia* infections on cassava whitefly biology, which may have led to the decline of SSA1-SG2 and SSA2 in cassava fields. SSA2 whiteflies with different symbiont infections were also compared for EACMV-UG retention and expression of immune genes. SSA2 with *Arsenophonus* retained higher titres of virus than those infected with *Wolbachia* and free of symbionts. SSA2 with *Wolbachia*, however, had higher expression of antimicrobial peptides, which may have led to reduced virus retention. Put together, these results indicate the various effects of bacterial symbionts on whitefly and viruses they transmit. These should be studied further to better understand and control superabundant whitefly populations and cassava disease pandemics in eastern Africa.

LIST OF ABBREVIATIONS

The following table describes the abbreviations and acronyms used in the thesis.

Abbreviation	Explanation
µg	Micro gram
µl	Micro litre
µM	Micro moles
˚C	Degree Centigrade
AAP	Acquisition access period
Atg-9	Autophagy related protein 9
atg-9	Autophagy related protein 9 gene
B. tabaci	*Bemisia tabaci*
BLAST	Basic local search alignment tool
CBSV	*Cassava brown streak virus*
CBSVs	Cassava brown streak viruses
CBSD	Cassava brown streak disease
CI	Cytoplasmic incompatibility
CMBs	Cassava mosaic begomoviruses
CMD	Cassava mosaic disease
coxA	Cytochrome oxidase A
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EACMV-UG	*East African cassava mosaic virus*-Uganda variant
EDTA	Ethylene di-amine tetra acetic acid
et al	And others
FAO	Food and Agriculture Organisation
fbpA	Fructose bi-phosphate aldolase
FISH	Fluorescent in situ hybridisation
ftsZ	Filamenting temperature sensitive mutant Z
gatB	Aspartyl/glutamyl-tRNA amidotransferase B
HCl	Hydrochloric acid
hcpA	Hypothetical conserved protein A
IAP	Inoculation access period
K1	Knottin 1
K2	Knottin 2
K3	Knottin 3
L12:D12	Light period-12 hour: Dark period- 12 hour
MAP	Months after planting
MEAM1	Middle east Asia minor 1
MED	Mediterranean
MLST	Multi locus sequence typing
mtCO1	Mitochondrial cytochrome oxidase I
mg	Milligram

ml	Millilitre
mm	Millimetre
mM	Millimoles
NaCl	Sodium Chloride
NCBI	National centre for biotechnology information
PCR	Polymerase chain reaction
p-endosymbionts	Primary endosymbionts
pmoles	Pico moles
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
rDNA	Ribosomal deoxyribonucleic acid
SSA	Sub-Saharan Africa
SSA1-SG1	Sub-Saharan Africa 1 subgroup 1
SSA1-SG2	Sub-Saharan Africa 1 subgroup 2
SSA1-SG3	Sub-Saharan Africa 1 subgroup 3
SSA1-SG5	Sub-Saharan Africa 1 subgroup 5
SSA2	Sub-Saharan Africa 2
SSA3	Sub-Saharan Africa 3
S-endosymbionts	Secondary endosymbionts
Tris	Tris (hydroxymethyl) amino methane
UCBSV	*Uganda cassava brown streak virus*
UV	ultraviolet
v/v	Volume by volume
w/v	Weight by volume
wsp	*Wolbachia* surface protein

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1 GENERAL INTRODUCTION AND OBJECTIVES OF STUDY

Cassava (*Manihot esculenta* Crantz) together with rice and maize constitutes the most important source of energy in the tropics (Ceballos *et al*., 2012). It is the main source of carbohydrate for an estimated 800 million people in the tropical and sub-tropical world (Howeler *et al*., 2013). Cassava is the most produced food commodity in Africa both in quantity and value. Africa contributes 56% of the world's total cassava production in quantities and provided a per capita daily energy of 160 Kcal (FAOSTAT, 2014). In addition to the economic value of the products and by-products of the crop, it has several advantages including easy propagation through stem cuttings, drought tolerance, capacity to produce in poor soils, flexibility in planting and harvesting time. Most importantly cassava can be stored in the field for harvest when other foods are scarce, which provides food security during adverse times (Legg and Hillocks, 2003). Cassava has recently been identified as a crop with great potential to adapt to climate change (Jarvis *et al*., 2012). This high promise of cassava to meet the increased food demand with the growing population in Africa is however, seriously limited by two important viral diseases; cassava mosaic disease (CMD) and cassava brown streak disease (CBSD). The causal agents of both diseases are transmitted by an insect vector, the whitefly, *Bemisia tabaci* (Gennadius) (Dubern, 1994; Maruthi *et al*., 2005).

Bemisia tabaci is a species complex containing at least thirty six morphologically indistinguishable species (Firdaus *et al*., 2013) and has a proposed origin in sub-Saharan Africa (SSA) (De Barro, *et al*., 2011). This hemipteran transmits more than 200 plant viruses (Jones, 2003; Polston *et al*., 2014) that cause devastating plant disease epidemics, undermining the food security and livelihoods of millions around the world. The efficiency of virus transmission varies significantly across the different whitefly species (Polston *et al*., 2014). *B. tabaci* is a major vector of viruses causing CMD and CBSD which account for almost 50% losses to total cassava production in East and Central Africa (Legg *et al*., 2013). CMD in Africa is caused by nine different virus species belonging to the genus *Begomovirus* (Thresh *et al*., 1997; Legg *et al*., 2013). Symptoms of CMD include chlorotic mottle with distortion of leaflets and stunting of plants with reduced root development (Thresh and Cooter, 2005). CBSD is caused by two viruses, *Cassava brown streak virus* (CBSV) and *Uganda cassava brown streak virus* (UCBSV) (Mbanzibwa *et al*., 2009; Winter *et al*., 2010),

which lead to chlorotic mottling of leaves but most importantly corky necrosis and rotting of starchy roots (Hillocks and Jennings, 2003).

Insects commonly possess obligate mutualistic symbionts which provide essential nutrients or help with the degradation of food materials (Kikuchi, 2009). There is high complementarity between the symbionts and the whitefly host with the existence of shared biosynthetic pathways for some amino acid synthesis which highlights the role of symbionts in the host adaptation to their low protein diets (Xie *et al*., 2012). *B. tabaci* has one of the highest number of endosymbiotic elements, with eight different vertically transmitted bacteria reported so far (Gueguen *et al*., 2010; Bing *et al*., 2013b; Marubayashi *et al*., 2014). One obligate primary symbiont, *Portiera* and seven facultative secondary endosymbionts, *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, *Hamiltonella defensa*, *Hemipteriphilus* and *Fritschea bemisae* have been detected in the *B. tabaci* (Zchori-Fein and Brown, 2002; Gueguen *et al*., 2010; Ahmed *et al*., 2013; Marubayashi *et al*., 2014). The prevalence of the symbionts varies among whitefly populations, geographical regions and host plants with high diversity within the bacterial communities. Several of these endosymbionts have been found to affect the biology and behaviour of *B. tabaci*. *Rickettsia* provided fitness benefits by increasing the fecundity and greater rate of survival (Himler *et al*., 2011), from heat stress (Brumin *et al*., 2011), while it can also increase susceptibility to chemical insecticides (Kontsedalov *et al*., 2008). *Fritschea* had negative impact with reduced fecundity and reduced the host range (Everett *et al*., 2005). *Hamiltonella* and *Arsenophonus* facilitated virus transmission by protecting the transit of virions in the haemolymph of insects through chaperonins (GroEL) and protein complexes that aid in protein folding and repair mechanisms (Gottlieb *et al*., 2010; Rana *et al*., 2012). Presence of endosymbionts facilitated acquisition, retention and transmission of *Tomato yellow leaf curl virus* (Su *et al*., 2013b).

Bacterial infections can also affect insect biology in many other ways. Cytoplasmic incompatibility (CI) caused by *Wolbachia* is the most common type of reproductive manipulation of hosts in all major insect orders as well as mites and woodlice (Werren *et al*., 2008). CI can be unidirectional, where only crosses between infected males and uninfected females are incompatible and also bidirectional when crosses between males and females infected with different strains of *Wolbachia* are incompatible (Duron *et al*., 2006). The ability of *Wolbachia* that can cause CI to spread in arthropod populations has generated much interest for generating desirable phenotypes (Brelsfoard and Dobson, 2009). CI can be used as an alternative strategy to spread *Wolbachia*, and in the process shorten the life

cycle of harmful insects such as *Aedes* mosquitoes (Cook *et al*., 2008; McMeniman and O'Neill, 2010; Iturbe-Ormaetxe *et al*., 2011). The presence of *Wolbachia* can also induce robust antiviral activity against insect viruses infecting *Drosophila* and mosquito-borne human pathogens such as dengue virus, chikungunya virus and *Plasmodium* (Moreira *et al*., 2009; Walker *et al*., 2011).

In the last 20 years, there has been an upsurge in the number of whiteflies feeding on cassava in SSA resulting in the development of super abundant populations (>1000 insects per plant) (Legg and Raya, 1998; Colvin *et al*., 2004; Legg *et al*., 2006, 2014). This has led to the rapid spread of CMD and CBSD in East and Central Africa. The superabundance and the spread of CMD pandemic were associated with a genetically distinct invasive group of whiteflies, which were previously described as Uganda 2 (Ug2) (Legg *et al*., 2002) but now described as sub-Saharan Africa 2 (SSA2) (Dinsdale *et al*., 2010). However, there has been a temporal and spatial shift in whitefly populations on cassava with a sharp decline in SSA2 in the last 10 years (Legg *et al*., 2013). SSA2, was predominant (63.9%) in East and Central Africa from 1997 to 1999. It was subsequently reduced to mere 1.5% by 2010 and displaced by another population described as sub-Saharan Africa subgroup 1 (SSA1-SG1) (Legg *et al*., 2014). Many theories including the emergence of invader whitefly species (Legg *et al*., 2002), symbiotic interaction between whiteflies and viruses (Colvin *et al*., 2004) and endosymbiotic bacteria have been mooted to explain the superabundance. The underlying reasons for this natural shift in whitefly populations, however, remain unknown. Many studies have shown the role of symbiotic bacteria living within the body cavity of whiteflies to drive population explosion by providing fitness benefits (Himler *et al*., 2011). The main objective of this study was therefore to understand the prevalence and genetic diversity of endosymbionts, and their effect on whitefly biology affecting cassava in eastern African countries.

Understanding the prevalence and genetic diversity of symbionts harboured in cassava whiteflies is crucial to understand the phenomenon of whitefly superabundance and population dynamics (Tajebe *et al*., 2015b). Tajebe *et al*. (2015b) have previously described the diversity of endosymbionts infecting cassava whiteflies in SSA. However, the sample numbers in the previous study were few for SSA1-SG2 (N=6) and SSA1-SG3 (N=8) populations and prevalence of *Wolbachia* was also an underestimate. The genetic diversity of symbionts by Tajebe *et al*. (2015b) produced preliminary results and thus there was need for further characterisation of symbionts infecting cassava whiteflies to understand the

specific association of strains with whiteflies. High numbers (38%) of SSA1-SG1, the current highly abundant population was free of secondary symbionts (Tajebe *et al*. 2015b) but its significance was still unknown. The role of endosymbionts on the biology of the whiteflies specialised on cassava and their interaction with the two CMBs and CBSVs was therefore essential to understand cassava whitefly-symbiont relationship. This study was therefore initiated with the following objectives to clearly understand the diversity and effect of secondary symbionts infecting cassava whiteflies.

Objective 1: To determine the prevalence and genetic diversity of endosymbionts in cassava whiteflies by PCR detection, sequencing and phylogenetic analysis of conserved nucleotide sequences.

Objective 2: To study the effect of symbionts on whitefly biology parameters such as fecundity, nymph development and adult emergence on healthy and virus-infected cassava plants.

Objective 3: To study the effect of symbionts on whitefly immune response and vector abilities such as EACMV-UG acquisition, retention and transmission.

Objective 4: To determine the prevalence of endosymbionts and *Wolbachia* titres in historical whitefly population during the Uganda CMD pandemic areas in 1999 and its association with the changing whitefly population diversity.

2 LITERATURE REVIEW

2.1 Bemisia tabaci- *The global pest*

Bemisia tabaci (Hemiptera: Aleyrodidae) is a sap-feeding pest belonging to the group of insects commonly known as whiteflies. *B. tabaci* was first described in 1889 as a pest of tobacco in Greece and described as *Aleyrodes tabaci*, the tobacco whitefly (Gennadius, 1889). The whitefly thrives worldwide in tropical and subtropical habitats (Basu, 1995), though the proliferation of protected agriculture and mass movement of plants and produce has extended its geographical range to include temperate climate areas which makes it a global pest found in all continents except Antarctica (Oliveira *et al*., 2001; Stansly and Naranjo, 2010). *B. tabaci* rose to international prominence in the mid- to late 1970s in Sudan and again in the 1980s in the south-western United States and since then has risen in status globally as one of the most damaging pests of open field and protected cropping systems (De Barro *et al*., 2011). *B. tabaci* is a cryptic species complex infesting eudicot plants but are highly specific and diverse in host preference. For example, the species that feeds on mint is restricted to a single host *Ocimum gratissimum* (Sseruwagi *et al*., 2005), whereas the highly polyphagous Middle East Asia Minor 1 (MEAM1) species colonises more than 150 different species from 43 different plant families and even a monocotyledonous plant (Bayhan *et al*., 2006; Quintela *et al*., 2016) but fails to infest cassava (Sseruwagi *et al*., 2005).

This tiny insect impedes crop production in many ways, firstly through direct damage by piercing of leaf surface with its mouth and removing nutrients from phloem tubes which result in chlorotic spots and withering of leaves which can cause more than 50% yield reduction (Lloyd, 1922). It also caused damage by excretion of a sticky substance called honeydew on leaves and fruits, inducing the growth of sooty moulds, which reduce photosynthesis and affect growth (Pollard, 1955). The sooty mould on vegetables require thorough washing after harvesting, which raises processing costs for the grower (http://cisr.ucr.edu/silverleaf_whitefly.html) and deposition of sticky honeydew on cotton lint causes serious problems in ginning and spinning of cotton (Horowitz *et al*., 1984). Certain populations of *B. tabaci* can cause specific damage to certain host plants, such as "silverleafing" (whitening of leaf) on cucurbits (Segarra-Carmona *et al*., 1990; Yokomi *et al*., 1990), irregular ripening of tomatoes (Schuster *et al*., 1990), white stalk of broccoli,

cauliflower, white stem on poinsettia, and light root in carrots (Costa *et al*., 1993a; Bedford *et al*., 1994). The most important problem caused by the whitefly, however, is through the transmission of over 200 plants virus species (Jones, 2003; Polston *et al*., 2014) that causes devastating crop disease epidemics affecting food security and undermining the livelihood of people around the world.

2.1.1 Origin and taxonomy

The genus *Bemisia* contains at least 37 species and estimated to have separated from other members 87 million years ago (Mound and Halsey, 1978; Boykin *et al*., 2013). Evolutionary affiliations of the *Bemisia* taxa within the family *Aleyrodidae* suggest that *B. tabaci* may have originated in tropical Africa 70 million years ago and was possibly introduced into the Neotropics and southern North America (Campbell *et al*., 1996; Boykin *et al*., 2013). Previously, it was suggested to be native to India or Pakistan, where the greatest diversity of the species' parasitoids has been found, a criterion that has been considered a good indication of a genus epicentre previously (Brown *et al*., 1995). sub-Saharan Africa (SSA) has been proposed as its centre of origin due to the high variability in mitochondrial cytochrome oxidase I nucleotide sequence amongst other major geographical clades (Brown, 2010; De Barro *et al*., 2011). Molecular dating based on mitochondrial cytochrome oxidase I gene estimated that all other species of whiteflies diverged from SSA whiteflies infesting cassava 48 million years ago (Boykin *et al*., 2013).

After the first description, as *Aleurodes tabaci* (Gennadius, 1889), *B. tabaci* has been subsequently re-described under 22 additional names (Mound and Halsey, 1978). The taxonomy of the family *Aleyrodidae* has traditionally been based on differences in the morphology of the fourth instar nymph/pupa but these pupal characteristics in *B. tabaci* are extremely plastic and determined by the host rather than the insect genome (Mound, 1963). These fourth instar pupal characteristics were more likely associated with avoidance of natural enemies, desiccation, exposure to UV, and host plant characteristics (De Barro *et al*., 2011). This high level of morphological plasticity combined with the geographical isolation and lack of communication between the taxonomists contributed to the serial re-descriptions by different taxonomists leading to the assignment of multiple names to a globally distributed insect (De Barro *et al*., 2011).

Lack of distinguishable morphological characters within the *B. tabaci* complex forced dependency on other techniques based on allozymes; random amplified polymorphic DNA (RAPD), phylogenetic analysis using mitochondrial 16S, mitochondrial cytochrome oxidase1 (mtCO1), nuclear ribosomal intergenic transcribed spacer 1 (ITS1) and on biological characters such as capacity to transmit viruses, host range, mating compatibility and capacity to induce silverleafing in squash but none formed a clear set of criteria to define a biotype or a species (De Barro *et al*., 2011). This combined usage of biological characters and molecular tools led to the proliferation of up to 36 biotypes (De Barro *et al*., 2011). Boykin *et al*. (2007) resolved the *B. tabaci* species complex into 12 genetic groups based on phylogenetic analysis of mtCo1 gene from samples collected worldwide.

Dinsdale *et al*. (2010) however felt scope for more accurate and consistent resolution of the genetic groups by re-interpretation of results with a more refined data-set using phylogenetic group clustering to identify the species-level boundary for *B. tabaci*. They demonstrated that the frequency distribution of the pairwise divergences between mtCo1 sequences of *B. tabaci* across the world reflected two distinct gaps, one at 11% and the other at 3.5%. These breaks identified 11 distinct genetic groups at 11% divergence limits and 24 distinct genetic groups at 3.5% divergence limits.

Mating experiments between the different genetic groups indicated that the groups at 3.5% divergence level were completely or partially reproductively isolated whereas the groups at 11% divergence had complete reproductive isolation within the groups (De Barro and Hart, 2000; Liu *et al*., 2012; Xu *et al*., 2010). Also the groups at 3.5% divergence level were not uniformly dispersed across genetic space and thus suggested the lack of gene flow between the groups (De Barro *et al*., 2011). 3.5% mtCO1 sequence divergence was therefore suggested as the mark for species delimitation. Although this level of classification is yet to be fully acceptable by the whitefly research community, *B. tabaci* is presently considered as a cryptic species complex of at least 36 morphologically indistinguishable species (Firdaus *et al*., 2013; Boykin and De Barro, 2014) (Figure 2-1).

2.1.2 Biology

B. tabaci encompasses six developmental stages throughout its life cycle: eggs, three nymphal stages, pupae and adult (Basu, 1995; Byrne and Bellows, 1991). Adult females lay whitish subelliptical 0.2 mm long eggs in circular groups which gradually turn brown on the

abaxial surface of leaves and are anchored by a pedicel spike at the base inserted in the leaf surface with glue like secretion (Basu, 1995). The fecundity and time duration of egg hatching vary with whitefly species, host plant, virus infection of host plant and temperature (Basu, 1995; Guo *et al*., 2010, 2013). Fecundity range from 5.5 to 400 eggs per female and egg incubation period from 3 to 39 days at an optimum temperature of 27 °C (Verma *et al*., 1990; Byrne and Bellows, 1991; Basu, 1995; Guo *et al*., 2013). However, the fecundity is an overestimate as most studies calculated average eggs laid by multiple females. All nymphal stages are sessile except the first instar larva known as 'crawler', a mobile, translucent white, oval-scale like in shape, about 0.27 mm long, which moves to a suitable feeding location on the lower surface of the leaf and becomes sessile as it loses its legs in the ensuing moult (Basu, 1995).

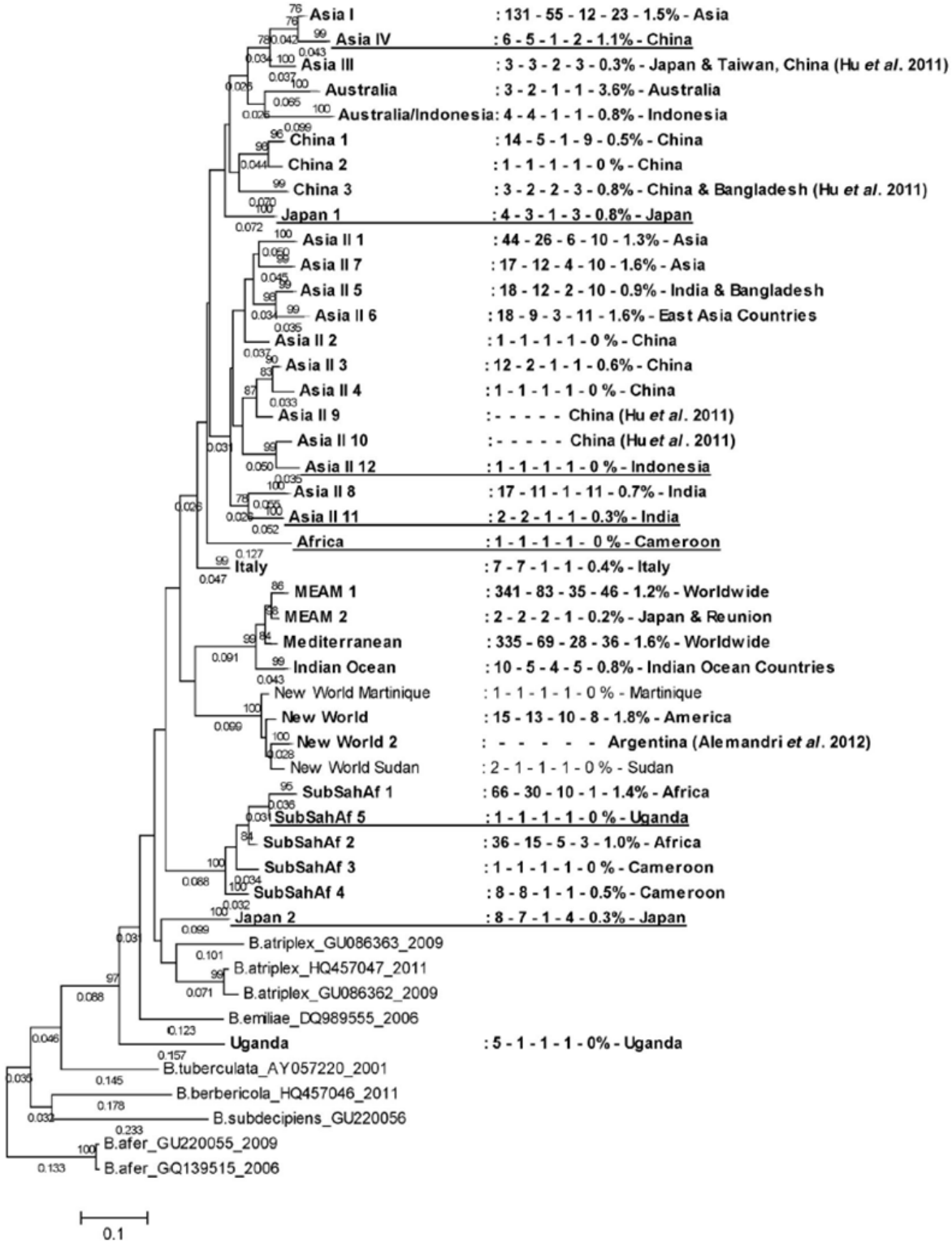

Figure 2-1: Evolutionary relationships of global *B. tabaci* populations. The groups are followed by number of accessions, haplotypes, countries, host plants, mean of genetic divergence percentage and distribution. Reproduced from Firdaus *et al*. (2013).

The first three nymphal stages last 2-10 days each (variable with temperature and host plant) and the fourth nymphal stage, called the 'puparium' is about 0.7 mm long and during this stage the metamorphosis to adult takes place in 2-11 days (Gerling *et al*., 1986; Mohanty and Basu, 1987). The fourth instar/puparium is used to distinguish between *B. tabaci* and *Trialeurodes vaporariorum,* another species of whitefly, which has predominantly been a glasshouse pest. In comparison with the glasshouse whitefly, *B. tabaci* pupae appears flatter, and do not have a marginal 'fringe' of wax filaments or long waxy rods on the dorsum.

The adult *B. tabaci* is about 1-2 mm long, the male slightly smaller than the female and the body and both pairs of wings are covered with a powdery, waxy secretion, white to slightly yellowish (Basu, 1995). The adult emerges through a "T"-shaped rupture in the skin of the puparium and maximum emergence occurs between 6:00 to 9:30 AM (Byrne and Bellows, 1991). Copulation begins within 10 hours after emergence and takes place several times throughout the life of the adult (Li *et al*., 1989). *B. tabaci* is arrhenotokous, wherein unfertilised eggs develop into males. Unmated females produce male offspring (XO) while mated females produce both male and female offspring (XO and XX) (Basu, 1995) and sex-ratio is also under influence of temperature (Sharaf and Batta, 1985). Adult longevity is dependent upon sex, whitefly species, host plants, temperature and ranges from 4-34 days for males and 8-61.5 days for females (Basu, 1995). The total developmental period from egg to adult vary with whitefly species, host plants and temperatures and range from 11-107 days (Basu, 1995). The number of generations of *B. tabaci* per year range between 10-15 depending upon the environmental conditions, host plants and species (Basu, 1995).

2.1.3 Economic impact

B. tabaci species complex have caused excessive annual crop losses over the last three decades. Reliable estimates of the economic impact of the *B. tabaci* species complex on worldwide agriculture have been difficult to obtain because of the extensive areas affected, the numbers of crops and ornamentals involved, and different monetary systems (Oliveira *et al*., 2001). The global spread of the polyphagous MEAM1 (formerly known as B biotype) as a ‘hitch-hiker’ on traded plant material, increased monoculture of crops with overlapping periods of growth and increase in resistance to insecticides are major factors in the world-wide increase in whitefly transmitted virus diseases (De Barro, 1995; Jones, 2003). *B. tabaci*

is a vector of more than 200 plant viruses recognized as species in the genera *Begomovirus* (*Geminiviridae*), *Crinivirus* (*Closteroviridae*), *Carlavirus* (*Betaflexiviridae*), *Ipomovirus* (*Potyviridae*) or *Torradovirus* (*Secoviridae*) and in addition vector of many other named viruses that are either in the same genera or in unidentified genera (Jones, 2003; Polston *et al*., 2014). Geminiviruses infecting tomatoes, beans and cassava have been the most widespread and important (Oliveira *et al*., 2001).

Extensive economic losses caused by diseases of whitefly-transmitted viruses have threatened food security and poverty alleviation efforts globally with losses ranging from 20 to 100% depending on the host plant, season and other factors (Basu, 1995; Brown and Bird, 1992). Some important examples of epidemics caused by *B. tabaci* transmitted are provided in (table 2-1).

Table 2-1: Major disease epidemics caused by *B. tabaci*-transmitted viruses since the 1970s

Crop	Year	Estimated losses	Location	Reference
Cotton	1970s	Heavy	Sudan	Horowitz, 1986
Cotton	1990s	4.98 billion US $	Pakistan	Mansoor *et al*., 1999
Cotton	1990-98	75% reduced production	Indian Punjab	Singh *et al*., 1999
Tomato	1983-84	90% losses	South India	Saikia and Muniyappa, 1989
Tomato	1970s	100% loss	Middle east	Czosnek and Laterrot, 1997
Tomato	1996-00	11,000 jobs lost in tomato industry	Brazil	Oliveira *et al*., 2001
Beans	1990s	> 1 million ha of crop land abandoned	Latin America	Morales and Anderson, 2001
Legumes	1990	300 million US $	India	Varma *et al*., 1992
Lettuce	1981	70% yield reduction	USA	Duffus *et al*., 1986
Sugar beet	1981	30% yield reduction	USA	Duffus *et al*., 1986
Cassava	1987	50% loss~ 2 billion US$	Africa	Fauquet and Fargette, 1990
Cassava-CMD	1990s	1.2-2.4 billion US$	SSA	Thresh *et al*., 1997
Cassava-CBSD	2014	726 million US$	East and Southern Africa	Maruthi *et al*. unpublished

2.2 Cassava as a host of B. tabaci

Cassava is a shrubby perennial plant grown mainly for its carbohydrate rich tuberous roots. It belongs to the family *Euphorbiaceae* and is one of ninety other species of the genus *Manihot*, but it is the only widely cultivated member. Cassava originated in South America and was introduced into Africa in the 16th century, and later into Asia in the late 17th century by Portuguese traders. Today, cassava is cultivated in more than 80 countries mainly between 30 °C south and 30 °C north of the equator (Fauquet and Fargette, 1990). Nigeria, Brazil, Thailand, Congo, Ghana, India, Tanzania, Mozambique are the leading producers of cassava. Africa has the highest land area (13.8 million ha) cultivated for cassava which is 67% of the total land area (20.6 Million ha) in the world and produces 147.6 million tonnes, 56% of the world production in 2011 (FAOSTAT, 2014). Cassava is called Africa's food insurance because it provides stable yields even in the face of drought, low soil fertility and low intensity management (Dixon *et al*., 2003).

2.2.1 Diversity of whitefly populations colonising cassava in SSA

Cassava crops in sub-Saharan Africa are colonised by five genetically distinct groups of *B. tabaci*, sub-Saharan Africa 1 to 5 (SSA-1 to SSA-5). SSA1 occurs throughout SSA, SSA2 in East and West Africa, SSA3 in Cameroon and Togo, SSA4 only in Cameroon and SSA5 only recently reported from South Africa (Legg *et al*., 2013).

The SSA1 group was further categorised into four subgroups 1 to 4 (SSA1-SG1 to SG-4) on the basis of phylogenetic analysis in six countries in East and Central Africa in a time course survey study from 1997 to 2010 (Legg *et al*., 2013). This study revealed a shift in *B. tabaci* genotype in East and Central Africa, with the relative frequency of SSA1-SG1 increasing from 24.6 to 89.2%, while the frequencies of SSA2 and SSA1-SG2 declined significantly from 63.9 to 1.4%, and 11.5 to 1.4%, respectively (Figure 2-2).

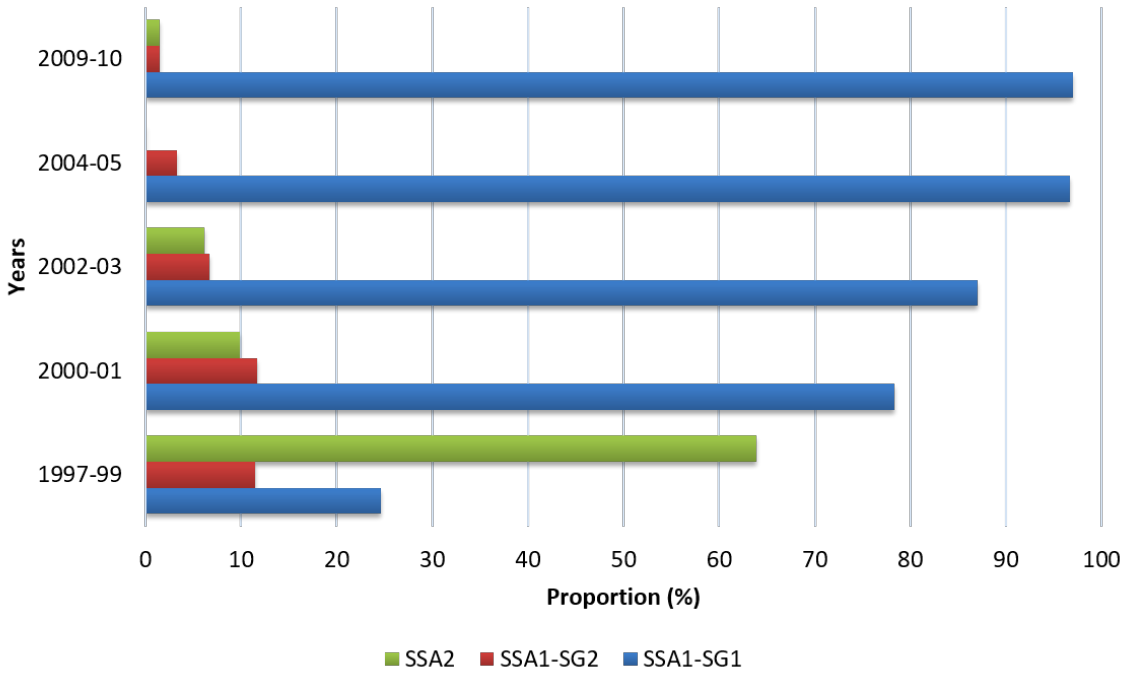

Figure 2-2: Proportion of cassava whitefly populations in East and Central Africa between 1997 and 2010. Data adapted from Legg *et al*. (2013).

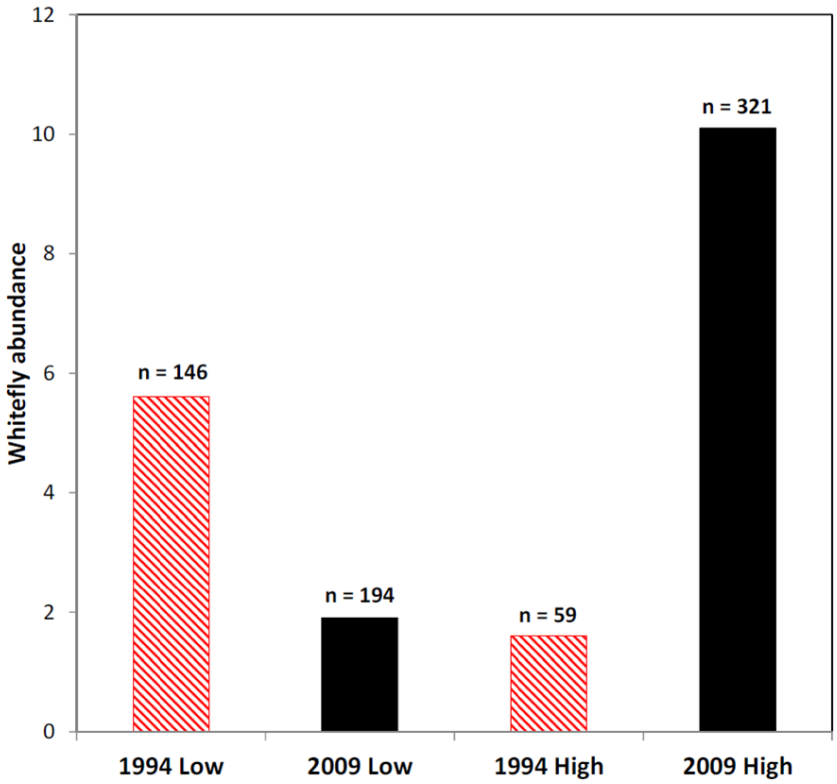

Figure 2-3: Mean abundance of *B. tabaci* adults on the top five leaves of cassava plants in low altitude (coastal) and high altitude (lake) zone districts of Tanzania between 1994 and 2009 (Jeremiah *et al*., 2015).

2.2.2 High whitefly populations on cassava in CMD-pandemic affected zones

Records show that the native African populations of *B. tabaci* readily colonised the crop soon after its introduction. Significantly, *B. tabaci* has been one of only a small number of insects that have been able to effectively colonise cassava in Africa, a complete contrast to the situation in Latin America where the crop supports a diverse arthropod fauna (Bellotti and van Schoonhoven, 1978). In spite of the successful colonisation of cassava by *B. tabaci*, there were no reports about their level of abundance that could cause physical damage to the crop until 1993. Recently super abundance of *B. tabaci*, commonly numbering more than 1000 adults per top five leaves have been widely reported from CMD pandemic areas (Legg and Raya, 1998; Colvin *et al*., 2004; Legg *et al*., 2006). Increase of vector populations have resulted in rapid spread of the CMD and CBSD in areas of East and Central Africa. Whitefly abundance was higher in the coastal zone (Newala, Mtwara, Masasi, and Tanga) and central eastern areas of Tanzania (Morogoro-Segera and Dar-es-Salaam) and lower in the lake zone (Mwanza, Nzega and Tabora) during 1994 (Legg and Raya, 1998). A recent survey in 2009, however identified the change in whitefly population dynamics with higher whitefly abundance in the lake zone than in the coastal zone (Jeremiah *et al*., 2015) (Figure 2-3).

Two hypothesis have been proposed to explain super abundance, one attributing a synergistic interaction between virus infected cassava plants and *B. tabaci* (Colvin *et al*., 2006) and the other considers genetic changes in the *B. tabaci* population itself (Legg *et al*., 2002) with the evidence of association of a genetically distinct *B. tabaci* group (SSA2/Ug2) with the CMD epidemic.

2.3 Endosymbionts

Symbiotic bacteria are omnipresent in nature, having a significant impact on eukaryotic evolution and diversity. They have diverse relationships with the host from being harmful/lethal (parasitism/pathogenicity) to beneficial (mutualism or symbiosis) (Kikuchi, 2009). The cohesive form of symbiotic association, where the symbiotic bacteria live inside the host body (thus called endosymbiotic) and allow spatial intimate interactions between partners is named as endosymbiosis (Kikuchi, 2009).

The class *Insecta,* composed of over 1.2 million species, is regarded as the most diverse animal group. Of these, almost half of all insects are expected to harbour endosymbionts (Buchner, 1965). Insects dependent upon nutritionally restricted diets like plant saps,

vertebrate blood and woody material, commonly possess obligate mutualistic symbionts involved in the provision of essential nutrients or degradation of food materials (Kikuchi, 2009). The insects like aphids, psyllids, whiteflies and mealy-bugs which utilise plant sap as food, require 10 essential amino acids that are absent or less abundant in their diets. Endosymbionts are thought to upgrade the diet by synthesising these essential amino acids and providing it to the host (Baumann *et al*., 2006).

The endosymbionts are often located in vesicles within specialised cells called bacteriocytes and their aggregation within the body cavity is called the bacteriome (Baumann *et al*., 2006). They are heritable through the acquisition of mechanisms ensuring vertical, maternal transmission to progeny which is referred as 'transovarial transmission'.

2.3.1 Types of endosymbionts

The endosymbionts which are present in all the species of an insect group and which appears to be of essential for host survival and reproduction are referred to as primary endosymbionts (P-endosymbionts). P-endosymbionts are morphologically similar to each other and are restricted to the bacteriocytes (Baumann *et al*., 2006). The phylogenetic trees based on the P-endosymbionts and the host genes are congruent in most insects, indicating the relationships appear to be the result of single ancient infection of a direct ancestor, co-speciation with the insect host and strict vertical transmission (Ahmed *et al*., 2013; Moran and Baumann, 2000; Thao and Baumann, 2004). Most P-endosymbionts have been characteristically shown to have reduced and static genomes (Moran, 1996; Moran and Mira, 2001) suggesting long association with the hosts and thus inability to survive outside the host (Kikuchi, 2009).

Endosymbionts which are not systematically associated with hosts and whose contribution is not essential to the survival and reproduction of its host are referred to as secondary endosymbiont (S-symbiont). S-symbionts are morphologically different and are not always restricted to bacteriocytes but can be in almost all types of insect host cells (Baumann *et al*., 2006). Although most S-symbionts are transmitted vertically, the phylogenies of S-Symbionts and the hosts show no congruence, thus indicating that these bacteria have been acquired recently and come from multiple origins through horizontal transmission (O'Neill *et al*., 1993; Thao and Baumann, 2004; Ahmed *et al*., 2013). These associations are less fixed than those of host and P-symbionts but may serve as portals for the import of new

genes that affect host ecology and subject the host to greater evolutionary forces (Rosell *et al*., 2010). S-symbionts provide advantages to hosts such as resistance to heat, and plant defence and protection from natural enemies (Clark *et al*., 2010). However the imperfect vertical transmission, costs associated with infections and conditional advantage they provide is a possible explanation of the diversity of symbionts within a population (Zchori-Fein and Bourtzis, 2011). *Wolbachia* the most common S-symbiont is estimated to infect an estimated 40% of terrestrial arthropod species (Zug and Hammerstein, 2012), *Cardinium* 6% (Zchori-Fein and Perlman, 2004), *Arsenophonus* 4.4% (Duron *et al*., 2008), *Rickettsia* 0.7% and *Spiroplasma ixodetis* 6.6% of arthropods (Duron *et al*., 2008).

2.3.2 Phenotypic reproductive manipulations by S-symbionts

Reproductive manipulation in arthropod hosts by inherited microorganisms is widespread in nature (Engelstädter and Hurst, 2009). Lack of vertical transmission through male hosts has led to the evolution of five commonly recognised reproductive manipulation phenotypes (Figure 2-4); four of which induce a shift in the sex ratio toward females by embroyonic (early) male killing, larval (late) male killing, feminisation and parthenogenesis induction (Engelstädter and Hurst, 2009). Cytoplasmic incompatibility (CI) is the fifth manipulation by sperm-egg incompatibility between infected male and uninfected female (Laven, 1956; Engelstädter and Hurst, 2009).

Many phylogenetically diverse microorganisms have been associated with reproductive manipulations of arthropods and *Wolbachia* is the most common of them (Engelstädter and Hurst, 2009). *Wolbachia* has been associated with all the manipulative phenotypes (Werren *et al*., 2008; Hurst *et al*., 1999); *Cardinium* with CI, feminisation and parthenogenesis (Hunter, *et al*., 2003; Ros and Breeuwer, 2009; Weeks *et al*., 2001; Zchori-Fein *et al*., 2001); *Rickettsia* with parthenogenesis and male killing (Perlman *et al*., 2006); *Arsenophonus* with male killing (Gherna *et al*., 1991) and *Spiroplasma* with male killing (Hurst *et al*., 2003). Cytoplasmically (maternally) inherited symbionts *Wolbachia*, *Cardinium*, *Arsenophonus* and *S. ixodetis* are found to infect more than 30% of the sampled arthropod species (Duron *et al*., 2008) and in many cases manipulate host reproduction by strong virulence in males, often killing them or rendering them sterile with minimal effect on the viability and fertility of infected females (Engelstädter and Hurst, 2009). Interestingly, the reproductive manipulation phenotype exhibited by the symbiont can also vary with host context and host

genetic background. For example, a strain of *Wolbachia* in the butterfly *Hypolimnus bolina* induces male killing in one host genetic background but causes CI in others (Hornett *et al*., 2008).

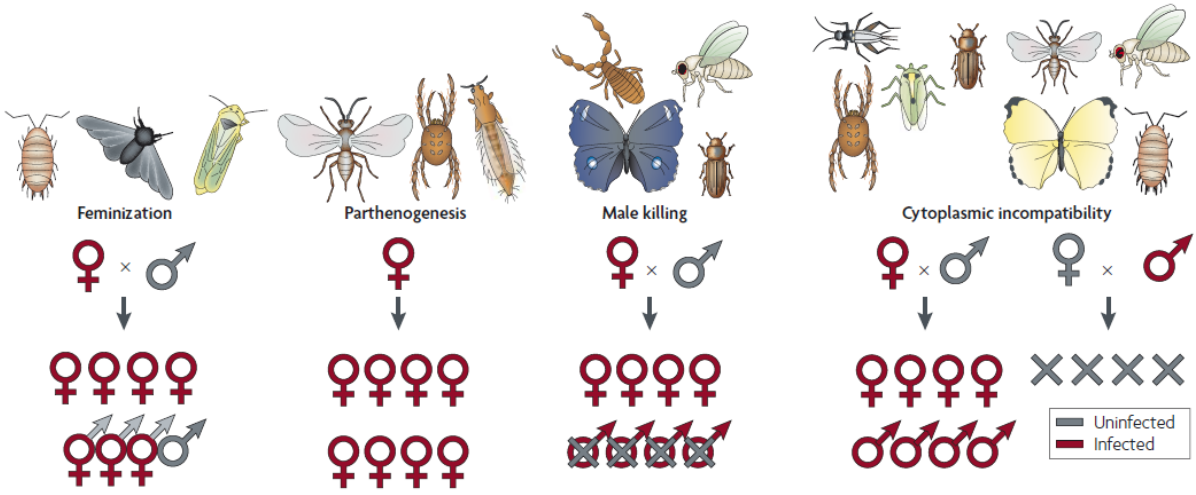

Figure 2-4: Reproductive manipulations induced by endosymbionts such as *Wolbachia* (Werren *et al*., 2008).

2.3.3 Endosymbionts and B. tabaci

Whitefly feed exclusively on phloem sap, which is rich in carbohydrate but deficient in amino acids. Symbiotic relationships of sap feeding insects with intracellular bacteria was proposed and confirmed to be based on the nutritional need of the hosts (Buchner, 1965; Douglas *et al*., 2006; Xie *et al*., 2012; Snyder and Rio, 2013; Rao *et al*., 2015). *B. tabaci* has one of the highest number of endosymbiotic elements with one obligate and seven different facultative bacteria reported so far which are vertically transmitted (Gueguen *et al*., 2010; Bing *et al*., 2013b; Marubayashi *et al*., 2014). Other sap sucking insects such as aphids, psyllids, mealy-bugs, leaf hoppers and sharpshooters have one (sometimes 2) P and variable number (1-5) of S-endosymbionts (Subandiyah *et al*., 2000; Von Dohlen *et al*., 2001; Tsuchida *et al*., 2002; Moran *et al*., 2003; Wu *et al*., 2006; Ishii *et al*., 2013; Wang *et al*., 2013). Most whiteflies contain orange-yellow, paired oval sac-like organs called bacteriomes composed of bacteriocytes, which are visible at low magnification (Buchner, 1965). Pyrosequencing analysis of total RNA and bacterial 16S rRNA from *B. tabaci* reflected low diversity of microbial community in comparison to *Drosophila* and were

dominated mostly by gram negative phylum of *Proteobacteria* (92%) (Xie *et al*., 2012; Jing *et al*., 2014).

Two morphologically distinct type of microorganism were observed during the ultrastuctural studies of the bacteriocytes and bacteriome of *B. tabaci* and *Trialeurodes vaporarium*. The predominant type was pleomorphic with a surrounding vacuole and lacked a distinct cell wall, whereas the other was coccoid in shape with inner and outer cell membranes (Figure 2-5, Costa *et al*., 1993). The pleomorphic bacteria that lacked the cell wall was later confirmed to be the P-endosymbiont and were designated as "*Candidatus* Portiera" gen.nov., with a single species, "*Candidatus Portiera aleyrodidarum*" sp.nov. (Thao and Baumann, 2004). *Portiera* have a reduced genome with a short chromosome (357,472 bp) (Santos-Garcia *et al*., 2012). The coccoid microorganisms were morphologically distinct in different *B. tabaci* populations (Costa *et al*., 1993) and were later confirmed to be S-endosymbionts.

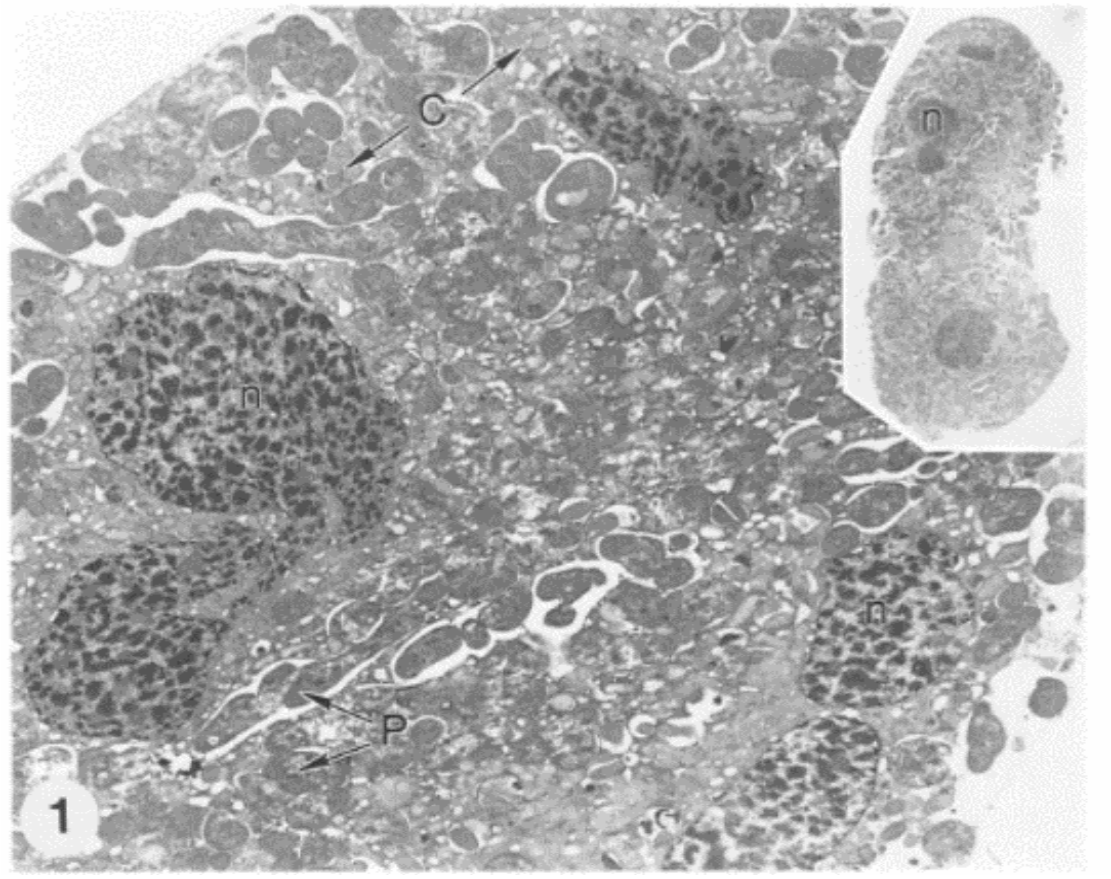

Figure 2-5: Bacteriome of an immature *B. tabaci* showing bacteriocytes with large large nuclei (n), pleomorphic (P) and coccoid (C) microorganisms (x3220). Inset: lower magnification of mycetome (x578) (Costa *et al*., 1993b).

Portiera aleyrodidarum, the P-symbiont supplies and complements the host metabolic activities for the synthesis of essential amino acids threonine and tryptophan along with non-essential amino acid serine but was unable to supply phenylalanine, valine, leucine and isoleucine, methionine pathways (Santos-Garcia *et al*., 2012). It is the only insect endosymbiont to supply carotenoids to its host, which are source of insect pigments, antioxidant action and can use light for reduction of NAD^+ (Santos-Garcia *et al*., 2012). *Portiera* has long co-evolutionary history with all members of the *Aleyrodinae* subfamily (Thao and Baumann, 2004). The phylogenetic tree of *Portiera* was not strictly congruent with the *B. tabaci* tree (Zchori-Fein and Brown, 2002), though high congruence was shown between *B. tabaci* mtCoI and 16Sr RNA phylogenies (Thao and Baumann, 2004; Ahmed *et al*., 2013).

Six facultative S-endosymbionts, *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus*, *Hamiltonella defensa* and *Fritschea bemisae* have been detected in the *B. tabaci* (Zchori-Fein and Brown, 2002; Thao *et al*., 2003; Gottlieb *et al*., 2006; Gueguen *et al*., 2010; Marubayashi *et al*., 2014). Recently, a new bacterium named *Candidatus hemipteriphilus asiaticus* was also found to infect *B. tabaci* from China (Bing *et al*., 2013b).

2.3.4 Role of secondary endosymbionts

As mentioned previously, S-endosymbionts *Wolbachia*, *Cardinium*, *Rickettsia* and *Arsenophonus* have the ability to manipulate host reproduction (Duron *et al*., 2008; Werren *et al*., 2008), while *Hamiltonella* confers parasitoid resistance in pea aphid (Oliver *et al*., 2003). Essential amino acids synthesised for the metabolism of the host are provided by the bacterial community inside *B. tabaci*, the non-essential amino acids are in return supplied by the insect host and some biosynthetic pathways such as glutamate and aspartate are intertwined indicating complementarity between the symbionts and host (Xie *et al*., 2012). They can also significantly affect host's ability to detoxify toxic compounds such as insecticides (Ghanim and Kontsedalov, 2009; Pan *et al*., 2013). While the presence of *Rickettsia* increases susceptibility of the host to insecticides (Kontsedalov *et al*., 2008).

Presence of symbionts can influence key biological parameters of the host. Whiteflies infected with *Rickettsia* exhibited high fitness benefits such as increased fecundity and greater rate of survival (Himler *et al*., 2011). *Wolbachia* decreased juvenile development time and increased adult longevity (Xue *et al*., 2012), whereas *Frischea* had deleterious

effect with reduced fecundity and narrowed host range (Everett *et al*., 2005). *Arsenophonus* also decreased fecundity, nymph survival, juvenile development time and adult life span (Raina *et al*., 2015). Some symbionts increase host defence against heat stress, pathogens and parasitoids (Brumin *et al*., 2011; Xue *et al*., 2012; Hendry *et al*., 2014).

Hamiltonella and *Rickettsia* facilitate plant virus transmission with increased acquisition and retention (Kliot *et al*., 2014; Su *et al*., 2013b). This is done by protection and safe transit of virions in haemolymph of insects through chaperonins (GroEL) and protein complexes (Figure 2-6) that aid in protein folding and repair mechanisms (Gottlieb *et al*., 2010; Rana *et al*., 2012).

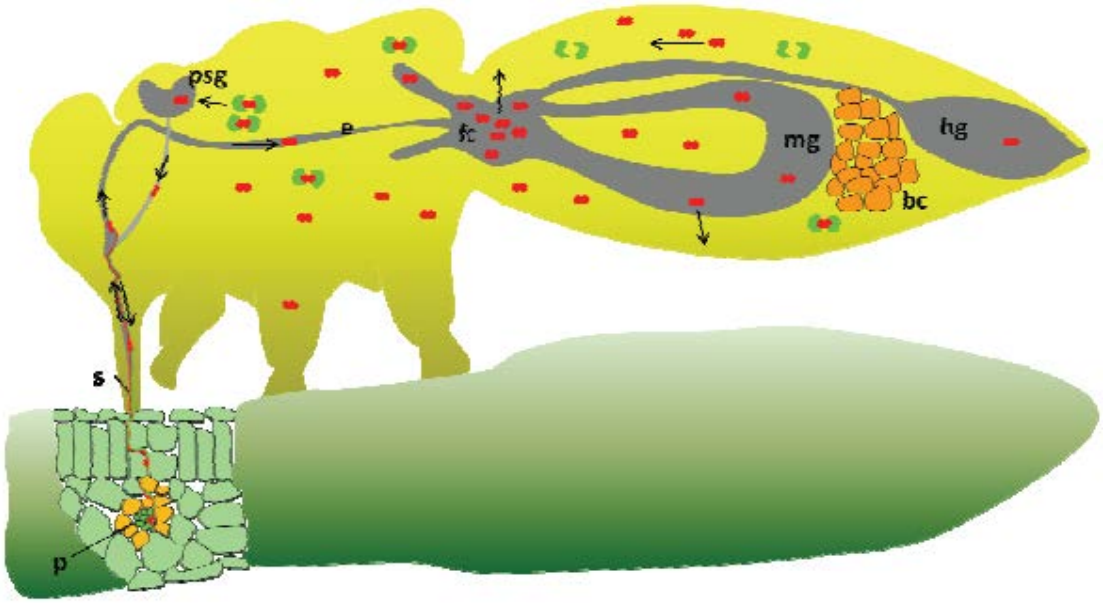

Figure 2-6: Role of GroEL in begomovirus transmission. Red particles: virion, green particle: GroEL, p: phloem, s: stylet, psg: primary salivary glands, fc: filter chamber, mg: mid gut, hg: hind gut, bc: bacteriocytes, black arrows: route of virions while translocating inside whitefly (Kliot and Ghanim, 2013).

2.3.5 Effect of antibiotic treatment and curing of symbionts

The success of antibiotics in eliminating S-endosymbionts depended upon the endosymbiont species, the *B. tabaci* biotype, and the relative efficiency of the antibiotics (Ahmed *et al*., 2010a). Rifampicin was most efficient for eliminating *Arsenophonus* across all *B. tabaci* biotypes, whereas tetracycline was better for curing whiteflies from *Wolbachia* (Ahmed *et al*., 2010a).

Treatment of *B. tabaci* (MEAM1) females with oxy-tetracycline hydrochloride adversely affected adult oviposition, growth and development of offspring, reduced the size of the bacteriome along with the microorganisms and most importantly reduced the ability of the offspring to induce silverleafing in Zucchini (Costa *et al*., 1997). Treatment of adults (MEAM1) with tetracycline or ampicillin trihydrate accelerated development with increased survival of offspring whereas treatment with rifampicin significantly retarded the rate of development of offspring with no effect on their survival (Ruan *et al*., 2006). Treatment of indigenous (Asia II 3) adults with tetracycline or ampicillin trihydrate accelerated the juvenile development without effecting its survival while treatment with rifampicin significantly retarded development with reduced survival of off springs (Ruan *et al*., 2006). Thus removal or reduction of endosymbiont populations had contrasting effects on the fitness of host insects.

Treatment of *B. tabaci* (Mediterranean) with rifampicin at a concentration of 1mg/ml for 48 hours was able to eliminate *Wolbachia* selectively (Xue *et al*., 2012). *Wolbachia* removal increased juvenile development time, decreased the percentage of nymphs which developed into adults, reduced adult life span, reduced percentage of female progeny, significantly decreased the body size of the 4th instar and decreased host defence against parasitisation by *Encarsia bimaculata* (Xue *et al*., 2012).

2.3.6 Prevalence and diversity of endosymbionts in B. tabaci

Endosymbiont composition within *B. tabaci* varied with different whitefly populations, sexes, host plants and geographical locations (Bing *et al*., 2013a; Gnankine *et al*., 2013; Gueguen *et al*., 2010; Marubayashi *et al*., 2014; Pan *et al*., 2013; Pan *et al*., 2012; Tajebe *et al*., 2015b). The relative amount of symbionts in *B. tabaci* also varied with host plants (Pan *et al*., 2012, 2013).

Over 95% of MEAM1 and Mediterranean (MED) adults were infected with one to four S-endosymbionts (Gueguen *et al.,* 2010). Highly diverse bacterial species with multiple strains are harboured in different whitefly species (Gueguen et al., 2010; Ahmed et al., 2013). The prevalence of symbionts varies significantly among the different whitefly species and also geographical locations. For example, *Hamiltonella* was nearly fixed in MEAM1 all over the world (Chiel *et al*., 2007; Gueguen *et al*., 2010; Bing *et al*., 2013a; Gnankine *et al*., 2013; Marubayashi *et al*., 2014) and MED in China and Tanzania (Bing *et al*., 2013a; Tajebe *et*

al., 2015b) but absent in MED in Israel (Chiel *et al*., 2007). Similarly, low frequency of *Rickettsia* is found in MED in China, Morocco, France, Uruguay and Burkina Faso (Gueguen *et al*., 2010; Bing *et al*., 2013a; Gnankine *et al*., 2013) but high percentage (>75%) in MED in Israel (Chiel *et al*., 2007). *Hamiltonella* infection rates in both MEAM1 and MED were higher in females (88.9%) than males (12.5%) (Pan *et al*., 2012). Most whitefly individuals harboured multiple symbionts (>65%) with double infections (>60%) being more common (Chiel *et al*., 2007; Gueguen *et al*., 2010). Interestingly, double infections with certain symbiont combinations such as *Rickettsia* + *Hamiltonella* in MEAM1 and *Hamiltonella* + *Cardinium* in MED were found more frequently (Gueguen *et al*., 2010; Skaljac *et al*., 2010). Similarly, certain combinations like *Arsenophonus* and *Hamiltonella* have never been found together in the same individual in any population tested (Gottlieb *et al*., 2008).

Hamiltonella was absent in indigenous whitefly populations across different geographical locations (Bing *et al*., 2013a; Gnankine *et al*., 2013; Marubayashi *et al*., 2014; Singh *et al*., 2012), while *Fritschea* has been only detected in New World populations of whiteflies (Everett *et al*., 2005; Marubayashi *et al*., 2014). *Wolbachia* among all the secondary symbionts is known to infect all the populations of *B. tabaci* (Ahmed *et al*., 2013; Ahmed *et al*., 2010a; Chiel *et al*., 2007; Singh *et al*., 2012; Tajebe *et al*., 2015b). *Wolbachia* infection rates as high as 80.5% (293/364) were reported from *B. tabaci* individuals from different host plants in China and interestingly the indigenous biotypes were infected with *Wolbachia* of the 'B subgroup' whereas the invasive biotypes (MEAM1 and MED) had infections mostly by the 'A subgroup' (Ahmed *et al*., 2010b; Li *et al*., 2007).

Cassava whiteflies from Tanzania (SSA1) were found to harbour *Arsenophonus*, *Hamiltonella*, *Cardinium* and *Wolbachia* (Tajebe *et al*., 2015b), although a definitive proof for the presence of *Hamiltonella* was not obtained. *Arsenophonus* was the most common S-endosymbiont in SSA1 whiteflies collected on cassava (Tajebe *et al*., 2015b). Interestingly, more than one third of the SSA1 populations tested (33%) were free from all S-symbionts. Majority of SSA1-SG1 whiteflies were free of S-symbionts whereas, SSA1-SG3 were predominantly doubly infected with *Arsenophonus* and *Rickettsia* (Tajebe *et al*., 2015b). SSA1 from West Africa were also mostly infected with *Arsenophonus* and few had infections of *Wolbachia* and *Rickettsia* (Gnankine *et al*., 2013).

2.3.7 Localisation of endosymbionts in B. tabaci

Understanding the location of the endosymbionts is important to understand their biological functions and interactions with the host and other symbionts. The technology of visualisation of target nucleic acids with a complementary probe labelled with a reporter molecule resulted in significant advances in resolution and speed, allowing simultaneous detection of multiple targets along with quantitative analysis and live-cell imaging (Levsky and Singer, 2003). Fluorescent *in situ* hybridisation (FISH) is based on DNA probes labelled with fluorescent reporter molecule which, when annealed to specific target sequences of a sample DNA, confirms the presence or absence of the target when viewed under a fluorescent microscope (Bishop, 2010). FISH has been widely used to analyse bacteria in environmental samples or inside insect samples which cannot be cultured (Koga, *et al*., 2009).

FISH has been used for localisation of *B. tabaci* endosymbionts (Gottlieb *et al*., 2006; Gottlieb *et al*., 2008; Skaljac *et al*., 2010; Brumin *et al*., 2012; Priya *et al*., 2012). *Portiera* was consistently found within the bacteriocyte and mainly confined to the circumference of the giant cells. *Hamiltonella* and *Arsenophonus* were also found inside the bacteriocyte in patches and their distribution was similarly towards the periphery of the giant cells. *Wolbachia* has been found in several insect hosts in different organs such as ovaries, salivary glands, guts, malpighian tubules, fat bodies and brain. *Wolbachia* in *B. tabaci* was found in bacteriocytes and mostly distributed along the circumference of the cells (Skaljac *et al*., 2010). Recently, it was also detected outside the bacteriocytes in the adult abdomen (Gottlieb *et al*., 2008; Bing *et al*., 2014; Marubayashi *et al*., 2014). *Cardinium* showed variable distribution inside its host body and was present in bacteriocytes, fat bodies with high concentrations in head and abdomen in nymphs and in the fat bodies and bacteriocytes of adult females (Gottlieb *et al*., 2008). *Rickettsia* display a confined phenotype wherein it is limited within the bacteriocytes, and also a scattered phenotype where it is distributed all across the body cavity (Gottlieb *et al*., 2006; 2008; Skaljac *et al*., 2010; Brumin *et al*., 2012).

2.3.8 Wolbachia

Intracellular bacteria were first reported within the reproductive tissues of the mosquito *Culex pipiens* by Hertig and Wolbach in 1924, and these *Rickettsiae* were subsequently named *Wolbachia pipientis*, to honour the pioneering work of Wolbach.

Wolbachia are intracellular bacteria that are transmitted within the egg cytoplasm and found in reproductive (ovary and testis) and other tissues of invertebrates (Werren, 1997a). *Wolbachia* are extremely common and widespread in insects (Werren and Windsor, 2000). More than 65% of insect species harbour *Wolbachia*, making it among the most abundant intracellular bacteria genus discovered so far, infecting at least 106 insect species (Werren *et al*., 2008).

2.3.9 **Wolbachia *taxonomy***

Wolbachia belong to the alpha subdivision of proteobacteria of *Rickettsiales* order and *Rickettsiae* family. Most bacteria belonging to *Rickettsiae* are obligate parasites and cannot be cultured *in vitro* which limits taxonomical studies (Werren, 1997b). *Wolbachia* taxonomy is currently based on diversity of nucleotide sequences of 16S rDNA, *Wolbachia* surface protein (*wsp*) or multiple locus sequence typing of five housekeeping genes (Baldo and Werren, 2007; Bing *et al*., 2014; Werren 1997). Based on 16S ribosomal sequences and other sequence information, *Wolbachia* spp. have so far been divided into 13 super-groups (A-N), with the exception of super-group G, which is considered as a recombinant between A and B subgroups (Baldo and Werren, 2007; Augustinos *et al*., 2011). Two subgroups C and D are commonly found in filarial nematodes, whereas the other six super groups are found primarily in arthropods in which A and B are most common (Werren *et al*., 2008). Two super groups A and B show around 2% 16S rDNA sequence divergence. *W. pipientis*, belongs to the B subgroup (Werren *et al*., 2008). Zhou *et al*. (1998) proposed to subdivide the A and B *Wolbachia* into smaller groups based on a minimum sequence divergence of 2.5% of the *wsp* gene sequences. The new group name was based on first three letters of the species from which the *Wolbachia* strain was identified.

There is no consensus on whether all bacteria within the *Wolbachia* clade should be given *W. pipientis* designation and presently is referred as *Wolbachia*, with strain designation that are based on host and super group identification (Werren *et al*., 2008).

2.3.10 Detection

Wolbachia cannot be cultured in chemically defined media thus its accurate detection can be difficult and time consuming (Jeyaprakash and Hoy, 2000). For this reason, detection of *Wolbachia* infection has been based largely on amplification of DNA using allele specific

polymerase chain reaction (PCR). Primers designed from 16S rDNA initially were used to amplify *Wolbachia* DNA from a diverse array of arthropods (O'Neill *et al*., 1992).

ftzZ is a bacterial cell-cycle gene involved in regulation of cell division in *Wolbachia*, containing conserved and highly divergent regions, making it suitable for finer scale phylogenetic analysis (Werren *et al*., 1995). Specific primers were designed for specific amplification of *ftsZ* and 16SrDNA regions of A and B groups of *Wolbachia* (Werren *et al*., 1995). The 16S rDNA primers (W-E and W-Spec) primers were found to be more sensitive in detecting *Wolbachia* infections (18.5%) than the general *ftsZ* primers (11.3%) and was attributed to degradation of DNA which affects the amplification of the *ftsZ* product more adversely (Werren and Windsor, 2000).

The *wsp* gene which encodes a major cell surface protein of *Wolbachia* was sequenced and used for its detection (Braig *et al*., 1998). The *wsp* gene evolves at a faster rate than any other gene previously reported and phylogenetic analysis based on *wsp* sequences results in an improved phylogenetic resolution of *W. pipientis* assemblage (Zhou *et al*., 1998).

Long PCR, which uses two enzymes (*Taq* and *Pwo*), consistently amplified *Wolbachia* DNA and was six manifold more sensitive than standard PCR (Jeyaprakash and Hoy, 2000). Use of long PCR increased *Wolbachia* detection limits across arthropod species producing lesser false negatives and thus *Wolbachia* infections were found to be more frequent than previously estimated by Werren and Windsor (2000) and Werren *et al*. (1995).

2.3.11 Multiple locus sequence typing (MLST)

Strain typing using the *wsp* gene was found unreliable due to extensive recombination and strong diversifying selection at this gene (Baldo *et al*., 2006). Recombination both within and between *Wolbachia* genes suggested that a single locus approach for strain characterisation would be misleading (Baldo *et al*., 2006).

MLST is a generic typing method, aimed at robust and portable method for characterising bacterial isolates at a molecular level, providing a common language for bacterial strain typing, which can also be used for evolutionary and population studies of wide range of bacteria (Maiden, 2006). Curated and freely accessible nucleotide sequence data are made available in the database, which has been used as a common dictionary for direct comparison of bacterial isolates.

A standard MLST system using five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) that are broadly distributed across the genome was developed for *Wolbachia* strains (Baldo *et al*., 2006). The *gatB* gene encodes aspartyl/glutamyl-tRNA amidotransferase subunit B that synthesizes charged tRNAs in organisms lacking certain tRNA synthetases. *coxA* encodes a catalytic subunit of cytochrome oxidase of the respiratory chain. *hcpA* encodes a functionally uncharacterized protein whose length and amino acid sequence are highly conserved in many bacteria. *fts*Z encodes a protein involved in bacterial cell division and *fbpA* encodes fructose-bisphosphate aldolase that in *Wolbachia* is likely to be involved in gluconeogenesis (Baldo *et al*., 2006). These five conserved genes were chosen for MLST as they were present throughout the *Rickttsiales* order, existed as a single copy with wide distribution in the wMel genome and were evidently stable within the genus *Wolbachia* (Baldo *et al*., 2006).

The *Wolbachia* pubMLST is a web-accessible central database and toolkit for storage, organization, and analysis of data. This provided accurate strain typing system and a genetic framework for tracing the movement of *Wolbachia* globally and within insect communities as well as for associating *Wolbachia* strains with geographic regions, host features (e.g., ecology and phylogeny), and phenotypic effects on hosts (Baldo *et al*., 2006). Identical nucleotide sequences at a given locus from different strains are assigned the same allele number, and each strain is then further characterised by the combination of the five MLST allelic numbers, representing its allelic profile. Each unique allelic profile is defined as a sequence type and characterises a unique strain (Baldo *et al*., 2006). For strain typing by MLST, the number of nucleotide differences between alleles is ignored and sequences are considered different alleles whether they differ at a single nucleotide site or at many sites but the level of divergence can be studied by phylogenetic analysis (http://pubmlst.org/wolbachia/info/general.shtml).

The MLST system provides an effective, universal and unambiguous tool for *Wolbachia* strain typing and detecting diversity among strains within a single host species, as well as for identification of closely related strains found in different arthropod hosts (Baldo *et al*., 2006). The similarities of *Wolbachia* strains in different host organisms may suggest recent horizontal transfer of *Wolbachia* across host species. The reproductive phenotype produced by a *Wolbachia* strain in its host can also be predicted and associated with the corresponding sequence type (Baldo *et al*., 2006).

2.3.12 **Wolbachia-*based strategies to control disease vectors and diseases***

Development of insecticidal resistance, negative impact on environment and resurgence of vector-borne diseases are the resulting effects of long-term usage of pesticides to control insect vectors. Thus, the need for novel environmentally friendly control strategies was suggested to complement current insect control measures (Brelsfoard and Dobson, 2009).

By inducing feminization of genetic males, parthenogenesis, male-killing, and CI, *Wolbachia* typically gives reproductive advantage to infected individuals and allow its rapid spread through a population (Brelsfoard and Dobson, 2009). CI manipulations by *Wolbachia* can be applied as strategies for insect control by using it as a form of sterility for a mass male release strategy, analogous to a sterile insect technique. CI can also be employed by using the reproductive advantage afforded as a population replacement strategy to drive infected phenotypes into natural populations (Brelsfoard and Dobson, 2009). *Wolbachia* has also been used for vector control by shortening the lifespan of the vector host and reducing the multiplication of the virus inside the vector (Iturbe-Ormaetxe *et al*., 2011; McMeniman and O'Neill, 2010).

A variant strain of *Wolbachia* designated as *W. pipientis* 'popcorn' (wMelPop) described from a laboratory stock of *Drosophila melanogaster*, widely proliferates in the brain, muscle and retina of *Drosophila*. This has often lead to tissue degeneration and early death of its adult host with the lifespan of infected flies being reduced by about 50% relative to uninfected controls and resulted in 100% mortality of infected flies by 14 days (Min and Benzer, 1997). The ability of *w*MelPop to induce strong CI without major fitness costs when introduced in a novel host can be used for vector control by its life shortening but stable invasion in uninfected populations (Cook *et al*., 2008). *Wolbachia* strain *w*MelPop transinfected into *Aedes aegyptii* mosquitoes, resulted in 50% reduction adult life span, compared with uninfected counterparts (McMeniman and O'Neill, 2010).

2.3.13 **Wolbachia *interference with viruses and parasites***

Wolbachia in *Drosophila* protects the host from virus infections, resulting in a selective advantage to flies with *Wolbachia* infections and this antiviral property can be exploited to reduce insect transmitted diseases (Hedges *et al*., 2008).

Drosophila infected naturally with *Wolbachia* delayed virus induced mortality caused from several pathogenic RNA viruses, including *Drosophila C virus* (DCV), *Cricket paralysis virus* (CrPV), *Flock house virus* (FHV), Nora virus and *West Nile virus* (WNV) by reducing virus load in infected flies (Hedges *et al*., 2008; Teixeira *et al*., 2008; Glaser and Meola, 2010). The high prevalence of *Wolbachia* in fruit fly populations and the benefits of increased resistance to viruses explained a positive role of *Wolbachia* (Teixeira *et al*., 2008). Stably transinfected mosquitoes, *A. aegypti* with wMelPop (mosquito cell adapted line) were more resistant when challenged with important human pathogens, dengue and chikungunya viruses and with avian malarial parasite *Plasmodium gallinaceum* (Moreira *et al*., 2009). Avirulent *w*Mel successfully transinfected into *A. aegypti*, under semi-field conditions showed increased invasion into mosquito population starting from an initial frequency of 0.65 to near fixation within a few generations and also blocked transmission of dengue serotype 2 (DENV-2) (Walker *et al*., 2011). Field released *Wolbachia* infected mosquitoes had reduced dengue virus replication and dissemination to the insect head compared to *Wolbachia* uninfected controls (Frentiu *et al*., 2014). This highlights the impact of the use of *Wolbachia* as a strategy for reducing dengue transmission in field.

Wolbachia-mediated antiviral protection and its mechanism of protection varied across its different strains, in some protection from was due to delayed virus accumulation and in some, infected flies were tolerant to high titres of virus (Osborne *et al*., 2009). This emphasises the importance of characterisation of *Wolbachia* in natural insect populations and identification of the different phenotypes by them.

2.4 Whitefly management

B. tabaci, being mostly a vector for semi-persistently and persistently transmitted viruses requires long hours of probe-feeding for successful acquisition and transmission of viruses and thus management through prophylactic insecticide usage was the main means of vector control. However, problems with its effective control on many crops are now being experienced worldwide due to insecticide resistance and the increased fecundity of the MEAM1, MED and other species. High prices and limited accessibility of chemical insecticides in developing countries further diminish its application, especially for the subsistence farming systems. No single control treatment can be used on a long-term basis

against this pest and there is need of integrating a number of different control agents needs implementing for an effective level of control (integrated pest management).

Cultural management practices targeted to restrict the ability of virus vectors to contact the host crop and manipulating the environment are effective elements in integrated pest management (Hilje *et al*., 2001). Cultural practices for management of whiteflies like crop-free periods, alteration in planting dates, weed removal, cultivation within insect proof screens, barrier crops, high density planting, intercropping, mulches, fertilisation and irrigation have been found effective in different horticultural cropping systems (Hilje *et al*., 2001). Whitefly vision is very important for its navigation and orientation due to lack of olfactory senses (Mound, 1962) and light nearing UV wavelength (280-380 nm) is necessary for dispersion and migratory behaviour of whiteflies, while near yellow wavelengths induces settling behaviour (Antignus *et al*., 1996, 1998). Cultural methods devised on formation of an optical barrier to manipulate sunlight spectrum have been successfully used for management of whiteflies and their transmitted diseases by usage of UV-absorbing films in glasshouses (Antignus *et al*., 1996, 1998) and yellow soil mulches and polythene sheets (Antignus *et al*., 2004, 2005). Row covers and other insect exclusion and reflective type materials for repelling *B. tabaci* have been adopted and are partially effective in some cropping systems (Hilje *et al*., 2001). Indoor tomato cultivation within tightly closed insect-proof screens effectively reduces *Bemisia* populations and slows down the spread of the vector borne virus (Ausher, 1997). Water management has been implemented in cotton, because higher *B. tabaci* populations occur in water-stressed cotton (Flint *et al*., 1996). Cultivation of cassava as intercrops with legumes and sweet potato and use of tall barrier crops like sorghum did not have significant benefits in whitefly management in cassava in sub-Saharan Africa (Legg *et al*., 2014).

The activity of different natural enemies (*Coccinelidae, Heteroptera, Neuroptera, Phytoseiidae*) against whiteflies especially *Amblyseius swirskii* (predatory mite) and *Nesidiocoris tenuis* (mirid bug) have been effectively used to manage *B. tabaci* populations in vegetable crops in greenhouses (Calvo, *et al*., 2009, 2011). Importation of parasitoids belonging to the genera *Encarsia* and/or *Eretmocerus* and of various predators has been successfully employed in greenhouses and open field (Gerling *et al*., 2001). Apparent parasitism of *Encarsia sophia* and *Eretmocerus mundus* on whitefly populations on cassava varieties ranged from 20-58% with a significant negative relationship between percentage of parasitism and whitefly numbers (Otim *et al*., 2008). The highest mean rate of marginal

mortality across all stages of cassava whiteflies was attributed to parasitism (Asiimwe *et al*., 2007b). Each larva of the coccinelid predator *Serangium* spp. consumed over 1000 nymphs of cassava whiteflies in its lifetime however, their population in cassava fields was most abundant late in the growing season (7-8 months after planting) which was after the peak in *B. tabaci* populations (4-6 MAP) (Asiimwe *et al*., 2007a). Entomopathogenic fungi like *Beauveria bassiana, Paecilomyces fumosorosea, Isaria fumosorosea* and *Laecanicillium muscarium* are virulent against whitefly nymphs especially the 2nd instar and have the potential to be bio-control agents (Wraight *et al*., 1998; Cuthbertson, 2013; Mascarin *et al*., 2013).

Breeding for resistance to whiteflies is based both on the morphological characteristics of the host plant and the presence of insect repellent/toxic plant chemicals (Basu, 1995). Leaf hair density, leaf thickness, leaf pH and nitrogen content are positively correlated with whitefly abundance (Butter and Vir, 1989; Jauset *et al*., 2000). Smaller size, glaborous leaves and open plant canopy reduce whitefly susceptibility of okra by 75% (De Ponti *et al*., 1990). Host plant resistance against whiteflies have been identified in *Lycopersicon hirsutum,* a relative species of tomato. The resistance has been attributed to exudation of 2-tridecanone, a natural insect toxic plant chemical by glandular leaf hairs (Williams *et al*., 1980) and sticky exudates from type VI C trichome glands on leaf surface (Channarayappa *et al*., 1992) but the introgression of resistance to the cultivated tomato has been slow. Identification of Mi-1 gene from tomato which imparts resistance to *Bemisia* and other sucking pests has been promising (Nombela *et al*., 2003). Cassava varieties with green petioles, soft leaves and erect leaf orientation support more whitefly colonisation and multiplication than varieties with red or red-green petioles, coarse leaves and horizontal or downward leaf orientation (Nair and Daniel, 1983). South American cassava genotypes with pubescent leaves were resistant while varieties with root cyanide were susceptible to whitefly species, *Aleurotrachelus socialis* (Parsa *et al*., 2015). Screening of Ugandan local cassava landraces for whitefly resistance identified four promising genotypes, MEcu7 (south American), Ofumba chai, Nabwire 1 and Mercury (Omongo *et al*., 2012) and resistance to whiteflies is considered to exist in local African cassava germplasm (Legg *et al*., 2014).

Chemical insecticides like nicotinoids and insect growth regulators have been effective for whitefly management. Efficient usage with rotation of chemicals could be used to combat development of insecticide resistance (Palumbo *et al*, 2001; McKenzie *et al*., 2014). However, subsistence cropping systems like cassava cultivation in Africa where there are

constraints with high expenses and non-availability of resources, makes growers unable and unwilling to use chemical pesticides (Legg *et al*., 2014). Thus there is need for identification of alternative, cost effective, easily available and most importantly sustainable management strategy for management of whiteflies.

2.5 Whitefly-borne virus diseases of cassava

B. tabaci threatens the food security in SSA by transmitting CMBs and CBSVs, the causative agents of CMD and CBSD, respectively. CMD and CBSD together account for almost 50% losses in total potential cassava production in the disease affected areas of East and Central Africa (Legg *et al*., 2013), causing rapidly spreading epidemics driven by superabundant populations of whiteflies.

2.5.1 Cassava mosaic disease

CMD was first reported from Tanzania in 1894 and prevalent in many countries in SSA particularly in Ghana, Nigeria, Cameroon, Madagascar and several other countries of West and Central Africa (Thresh and Cooter, 2005). A severe outbreak of CMD with unusually severe symptoms was reported in Uganda in early 1990s with rapid subsequent spread to other countries.

CMD in Africa is caused by nine CMBs; *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East Africa cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Kenya virus* (EACMKV) *East African cassava mosaic Zanzibar virus* (EACMZV), *Cassava mosaic Madagascar virus* (CMMGV), *African cassava mosaic Burkina Faso virus* (ACMBFV) and *South African cassava mosaic virus* (SACMV) (Legg *et al*., 2015). A recombinant Uganda variant of EACMV designated as EACMV-UG was associated with the severe epidemic in the 1990s but is considered to be a strain of EACMV (Legg and Fauquet, 2004).

B. tabaci transmit CMBs in a persistent circulative mode (Figure 2-6) throughout its life. The minimal (optimal) transmission times for successful persistent transmission process of *African cassava mosaic virus* were 3.5 hours (5 hours) for acquisition access, 3.5 hour (6 hours) latent period and 10 minutes (30 minutes) for inoculation, the vector retains the virus for at least 9 days (Dubern, 1994). The virus persists during moulting but is not transmitted

transovarially. Optimal transmission occurred with 10 insects per plant which resulted in 13% of the individuals to be infected (Dubern, 1994).

CMD is prevalent in all cassava growing regions of Africa including the adjacent islands of Cape Verde, Zanzibar, Seychelles, Mauritius and Madagascar (Calvert and Thresh, 2002). Three distinct CMD incidence situations of 'epidemic', 'endemic' and 'benign' have been recognised in SSA (Thresh *et al*., 1997). In epidemic areas CMD incidence is high, rapidly spread by superabundant whitefly populations with severe symptoms (Calvert and Thresh, 2002). Endemic areas too have high disease incidence but the symptoms are usually not severe with stable disease situation whereas in benign areas the incidence is low and rarely exceeds 25% (Calvert and Thresh, 2002). The epidemic region is predominantly infected by EACMV-UG commonly found both as single or mixed infection with ACMV. However, EACMV in West and Central Africa rarely occur as single infections and are mostly associated with ACMV (Legg and Fauquet, 2004).

Yield losses in range of 20-95% have been estimated in multiple locations under diverse conditions (Fauquet and Fargette, 1990). The yield losses and symptom severity vary with different viruses and virus associations, yield losses of 12%, 42%, 68% and 82% have been estimated on infection with mild strains of EACMV-UG, ACMV, severe strains of EACMV-UG and ACMV+EACMV-UG, respectively in Uganda (Legg and Fauquet, 2004). Various attempts have been made to assess the continent wide impact of CMD on African cassava production. Yield losses in the range of 30-40% in all the major producer countries accounting for a continental losses in the range of 19 to 27 million tons in 2003 which accounted to an annual economic loss of 1.9-2.7 billion USD (Legg and Fauquet, 2004).

2.5.2 Cassava brown streak virus disease (CBSD)

Two distinct RNA virus species, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), members of *Potyviridae* family and genus *Ipomovirus* are the causal agents of the CBSD (Mbanzibwa *et al*., 2009; Monger *et al*., 2001; Winter *et al*., 2010). CBSD is transmitted semi-persistently by *B. tabaci* (Maruthi *et al*., 2005). Semipersistent viruses are retained in the anterior foregut and cibarium of the whiteflies.

For most of its known history, CBSD seems to have been confined to coastal East Africa and the shores of Lake Malawi. The reason for this pattern though unknown was attributed

to distribution of vector and alternative hosts in the natural vegetation (Legg and Hillocks, 2003). From 2004, CBSD-like symptoms were seen in central-southern areas of Uganda (Alicai *et al*., 2007). However, this outbreak of CBSD caused by a different virus than the coastal one was reported from higher altitude areas (>1000m above the sea level) in Uganda and Lake Victoria basin (Alicai *et al*., 2007; Mbanzibwa *et al*., 2009). Highest incidences of CBSD was reported in southern Uganda, and Mara region of Tanzania and the disease has now spread to Burundi, Rwanda and eastern DRC in a recent survey of six countries in the Great Lakes region (Legg *et al*., 2011). Recently, very high incidence of CBSD in three districts of Lake Zone namely, Ukerewere, Ilemela and Musoma Rural was reported (Jeremiah *et al*., 2015). Spread of CBSD in field due to increased whitefly numbers have been reported previously in Tanzania (Maruthi *et al*., 2005) and Kenya (Mware *et al*., 2009). The recent spread of CBSD from coastal East Africa to the Great Lakes Region of East and Central Africa is believed to be driven by the sudden increase of whitefly numbers in these areas (Legg *et al*., 2011, 2013).

3 PREVALENCE AND GENETIC DIVERSITY OF ENDOSYMBIOTIC BACTERIA INFECTING CASSAVA WHITEFLIES IN AFRICA

This Chapter is published (Ghosh S, Bouvaine S, Maruthi MN, 2015, *BMC Microbiology*, 15:93). The paper is attached as an appendix T. This chapter is slightly modified from the paper version to fit into the thesis by removing sections described in other chapters (to avoid duplications), and added information on whitefly symbiont diversity in Introduction as suggested during my PhD transfer examination.

3.1 Introduction

Cassava, a key food security crop throughout sub-Saharan Africa (SSA), suffers devastating yield losses due to *B. tabaci*-borne cassava mosaic begomoviruses (CMBs) and cassava brown streak viruses (CBSVs). Superabundant *B. tabaci* populations, commonly numbering more than 1000 adults per top five leaves, of cassava plants, have been associated with the rapid spread of CMD pandemic in East and Central Africa since the late 1990s. SSA2, the then super abundant population which was also described as the 'invader or UG2', was associated with the spread of the CMD pandemic (Legg *et al*., 2002, 2006). In recent years, a shift in the *B. tabaci* population has occurred with the relative frequency of SSA1-SG1 increasing from 24.6% to 89.2%, while the frequencies of SSA2 and SSA1-SG2 decreasing significantly from 63.9% to 1.4%, and 11.5% to 1.4%, respectively, between 1997 and 2010 (Legg *et al*., 2013). The reasons for this observed shift in cassava whitefly population remain unknown. Other occurrence of shift in genetic diversity of populations have been reported for other *B. tabaci* species, mainly the population replacement by the invasive Middle East-Asia Minor 1 (MEAM1, previously B-biotype) and Mediterranean (MED, previously Q-biotype) populations (McKenzie *et al*., 2009; Pan *et al*., 2011; Simón *et al*., 2007).

In addition to the primary endosymbiont *Portiera aleyrodidarum*, the species *B. tabaci* has been reported to harbour six vertically transmitted secondary endosymbionts, *Arsenophonus*, *Hamiltonella*, *Cardinium*, *Fritschea*, *Rickettsia* and *Wolbachia* (Ahmed *et al*., 2010a; Gueguen *et al*., 2010; Zchori-Fein and Brown, 2002). Recently, a new bacterium named *Candidatus hemipteriphilus asiaticus* was also found to infect *B. tabaci* from China (Bing *et al*., 2013b). Several of these endosymbionts can affect the biology and behaviour of *B. tabaci*. Endosymbionts infecting *B. tabaci* are known to provide fitness benefits (Himler *et al*., 2011; Hendry *et al*., 2014; Brumin, *et al*., 2011) and can also alter their vector ability (Kliot *et al*., 2014; Su *et al*., 2013b).

Endosymbiont diversity within *B. tabaci* varies significantly within different whitefly populations, sexes, host plants and geographical locations (Bing *et al*., 2013a; Gnankine *et al*., 2013; Gueguen *et al*., 2010; Marubayashi *et al*., 2014; Pan *et al*., 2013; Pan *et al*., 2012; Tajebe *et al*., 2015b). Diversity of endosymbionts within a population also varies considerably, for example *Hamiltonella* infections in MED were common in Brazil, China and Africa but are absent in Israel (Chiel *et al*., 2007; Marubayashi *et al*., 2014; Pan *et al*., 2012; Skaljac *et al*., 2010; Tajebe *et al*., 2015b). Over 93% of the *B. tabaci* sampled were infected by at least one secondary symbiont (Bing *et al*., 2013a; Gueguen *et al*., 2010), but only *Wolbachia*, among all the secondary symbionts is known to infect all the populations of *B. tabaci* (Chiel *et al*., 2007; Ahmed *et al*., 2010a; 2013; Gueguen *et al*., 2010; Singh *et al*., 2012; Bing *et al*., 2013a). Cassava whiteflies from East and West Africa (SSA1) were found to harbour *Arsenophonus*, *Hamiltonella*, *Cardinium* and *Wolbachia* as secondary symbionts (Tajebe *et al*., 2015b; Gnankine *et al*., 2013).

The study of intracellular bacterial communities in these whiteflies and their impact on the host was essential for understanding the dynamics of insect populations and their vector abilities. In this study, we identified the endosymbionts infecting cassava whiteflies, determined their infection frequencies in different populations and characterised the diverse bacterial species by sequencing. We have also developed a cost effective and reliable restriction fragment length polymorphism (RFLP) diagnostic method for the molecular typing of the cassava whitefly populations.

3.2 Materials and Methods

3.2.1 Whitefly sampling and populations studied

Adult whiteflies were collected on cassava plants in four countries; Tanzania, Uganda, Malawi and Nigeria (Table 3-1) and preserved in alcohol. Two laboratory populations of cassava whiteflies were originally collected from Uganda and Tanzania (Maruthi *et al*., 2001) and subsequently maintained on cassava plants in insectary conditions (27±5 °C, 60% relative humidity and L12:D12). These were used for detecting endosymbionts and studying their genetic diversity.

3.2.2 Detection and molecular characterisation of endosymbionts

Total DNA was extracted from individual adult whiteflies using the Chelex method (Walsh *et al*., 1991) with slight modifications. Each whitefly was ground in 100 µl TE solution (10 mM Tris-Hcl and 1 mM EDTA, pH 8.0) containing 20% Chelex (BIO-RAD, UK) and 300 µg Proteinase K. Samples were incubated at 60 °C for 1.5 hours followed by protein denaturation at 96 °C for 10 minutes. Samples were then centrifuged at 13,000 rpm and the supernatant was collected and stored at -20 °C. Whitefly mtCOI genes and the endosymbiont 16S or 23S rDNA were amplified by polymerase chain reactions (PCR) using genus specific primers (Table 3-2). New primers were designed for *Cardinium* and *Wolbachia* to increase efficiency and specificity of detection. Multilocus sequence typing (MLST) based on the diversity of five conserved housekeeping genes; *coxA, fbpA, ftsZ, gatB* and *hcpA* have been used as a standard tool for strain typing and evolutionary studies of *Wolbachia*. This method was used to characterize the *Wolbachia* infecting cassava whiteflies using standard primers and protocols (Baldo *et al*., 2006). Variability in the *Wolbachia* surface protein (*wsp*) gene was also used for characterisation. Amplification of these genes was carried out in 25 µl volumes containing 2 µl DNA lysate as template, 0.4 µM of each primer, 0.15 mM of dNTPs, 1 x DreamTaq Green buffer and 0.5 unit DreamTaq Green DNA polymerase (Thermo Scientific Ltd., UK). Amplifications consisted of 94 °C for 3 minutes followed by 38 cycles of 94 °C for 30 seconds, annealing for 45 seconds (Table 3-2) and 72 °C for 1.5 minutes and final extension for 7 minutes at 72 °C. PCR products were visualised on 1% agarose gels containing RedSafe nucleic acid staining solution (Intron Biotechnology, Korea). PCR products were purified and submitted for Sanger sequencing (Source Bioscience, UK) using both forward and reverse primer for each sample, and five samples were sequenced for each location. Endosymbionts were also detected and sequences from two laboratory whitefly strains (Table 3-1). Sequences were compared to known sequences in nucleotide databases using the BLAST algorithm in NCBI. The nucleotide sequences of the endosymbionts and mtCO1 sequences of the sampled whiteflies were submitted to the NCBI nucleotide database.

3.2.3 Developing a simple diagnostic tool kit for cassava whiteflies

The mtCOI fragments from five whiteflies per location were sequenced and aligned with reference sequences (Legg *et al*., 2013) for the identification of consensus groupings. The whitefly mtCOI sequences generated were analysed to identify unique restriction

endonuclease sites using the software package NEBcutter (http://tools.neb.com/NEBcutter2). Three enzymes *Bgl* II (A/GATCT), *Apo* I (R/AATTY) and *Dde* I (C/TNAG) were found to produce unique patterns across SSA populations. The mtCOI fragments were re-amplified from 20 adults for each cassava whitefly population using 3 µl of DNA template and 1 unit of DreamTaq DNA polymerase in 30 µl volume reactions (40 cycles) for higher yields with same PCR cycling conditions.

Previously extracted DNA from four SSA2 samples were used in this assay as reference samples (Maruthi *et al*., 2001). The RFLP was carried out in a two-step procedure. First, 15 µl of PCR products were digested with 5 units of *Bgl* II. Second, the remaining 15 µl of PCR products were digested with 5 units each of *Apo* I and *Dde* I at 37 °C for 1.5 hours. The fragment sizes obtained were analysed by electrophoresis on 2% agarose gels.

3.2.4 Phylogenetic and statistical analysis

The mtCOI sequences from the whitefly, the 16S or 23S rDNA sequences from the endosymbionts and the MLST sequences from *Wolbachia* were aligned separately using ClustalW of MEGA 5.2 (Tamura *et al*., 2011). Phylogenetic trees were constructed by the maximum-likelihood method using MEGA 5.2. Different nucleotide substitution models were used based on the lowest Bayesian information criterion scores obtained. Phylogenetic trees for mtCOI and *Wolbachia* were generated using the T93+G+I substitution model, the HKY+G substitution model for *Arsenophonus*, the K2+G substitution model for *Rickettsia* and the K2 substitution model for *Cardinium* (Posada, 2003). The robustness of the clades was assessed by 1000 bootstrap replicates.

All statistical analysis and graphs were done using the R software environment (R Core Team, 2011). The probabilities of bacterial infections in cassava whitefly populations were predicted using simple binomial logistic regression. Each bacterium was used as the dependent variable and the whitefly populations as independent variables. Differences in infection patterns among groups were evaluated by Tukey's HSD test using the glht function from multcomp package of R (Hothorn *et al*., 2008).

Table 3-1: Collection sites of whitefly samples from cassava fields in Africa.

Country	District (Locations)	Date collected	Number of whiteflies tested
Tanzania	Mtwara district (Mtiniko, Namaleche)	November, 2012	10
		January, 2014	10
Tanzania	Masasi district (Napupa, Mailisita, Mnolela, Lindi)	November, 2012	15
Malawi	Thyolo district (Kasonyo, Likwakwanda)	January, 2014	15
Malawi	Mulanje district (Matipwili)	January, 2014	8
Malawi	Lilongwe district (Chitedze)	January, 2014	10
Malawi	Zomba town	January, 2014	3
Malawi	Salima district (Chitala)	November, 2013	10
Uganda	Masaka district (Masaka)	October, 2012	47
Uganda	Wakiso district (Wakiso)	October, 2012	51
Nigeria	Oyo state (Ibadan, Kajode, Ajibode, Mokola)	September, 2012	43
Nigeria	Imo state (Egbu)	October, 2012	10
Nigeria	Abia state (Umuahia)	October, 2012	15
Uganda	Namulonge (Laboratory population)	1997	----
Tanzania	Dar-es-Salaam (Laboratory population)	2010	----

Table 3-2: Primer sequences and annealing temperatures used for PCR amplification.

Target gene	Primer Name	Sequence (5'→3')	Reference	Amplicon length	Annealing temp
B. tabaci mtCOI	MTCO10	TTGATTTTTTGGTCATCCAGAAGT	Frohlich *et al*., 1999	870 bp	50℃
	MTCO12	TCCAATGCACTAATCTGCCATATTA			
Portiera 16S rDNA	28F	TGCAAGTCGAGCGGCATCAT	Zchori-Fein and Brown, 2002	1050 bp	58℃
	1098R	AAAGTTCCCGCCTTATGCGT			
Arsenophonus 23S rDNA	Ars23S-1	CGTTTGATGAATTCATAGTCAAA	Chiel *et al*., 2007	750 bp	58℃
	Ars23S-2	GGTCCTCCAGTTAGTGTTACCCAAC			
Rickettsia 16S rDNA	Rb-F	GCTCAGAACGAACGCTATC	Gottlieb *et al*., 2006	960 bp	58℃
	Rb-R	GAAGGAAAGCATCTCTGC			
Wolbachia 16S rDNA	Wol16S-F	CGGGGGAAAAATTTATTGCT	Heddi, *et al*., 1999 This study	730 bp	58℃
	Wol16S-R	CCCCATCCCTTCGAATAGGTAT			
Wolbachia wsp gene	81F	TGGTCCAATAAGTGATGAAGAAAC	Zhou., *et al*. 1998	600 bp	53℃
	471R	AAAAATTAAACGCTACTCCA			
Cardinium 16S rDNA	Card-F	TAGACACACACGAAAGTTCATGT	This study	650 bp	57℃
	Card-R	GCATGCAATCTACTTTACACTGG			
Hamiltonella 16S rDNA	Hb-F	TGAGTAAAGTCTGGGAATCTGG	Gueguen *et al*., 2010	730 bp	58℃
	Hb-R	AGTTCAAGACCGCAACCTC			
Fritschea 23S rDNA	Frit-F	GAGTTTGATCATGGCTCAGATTG	Gueguen *et al*., 2010	630 bp	62℃
	Frit-R	GCTCGCGTACCACTTTAAATGGCG			

3.3 Results

3.3.1 RFLP for molecular typing of cassava whiteflies

The mtCOI locus has been the most commonly used marker for genotyping whiteflies but the cost and time involved in gene sequencing and analysis are a limiting factor for routine diagnosis and processing large number of samples in epidemiological studies. We therefore developed a quick and cost-effective RFLP technique as an alternative to type SSA cassava whiteflies used in this study that efficiently identified the different populations. The RFLP was carried out in two steps. In the first step, digesting mtCOI products with *Bgl* II cleaved SSA2 into two fragments of size 615 and 252 bp but did not cleave mtCOI loci from other populations (Figure 3-1a). In the second step, digesting mtCOI products from SSA1 and SSA3 with *Apo* I and *Dde* I produced 2 to 5 fragments of distinctive sizes (Figure 3-1b). SSA1-SG1 and SSA3 were distinguished by the presence of fragments 122 and 213 bp, respectively. SSA1-SG2, SSA1-SG3 and SSA1-SG5 were identified by the presence of bigger fragments of 493, 402 and 344 bp, respectively (Figure 3-1b). These patterns were obtained consistently on 120 SSA1-SG1, 80 SSA1-SG2, 300 SSA1-SG3, 70 SSA2 and 24 SSA3 samples digested for each population. Fragments below 100 bp size were not visualised reliably on agarose gels, and were therefore not taken into consideration for the analysis.

3.3.2 Cassava whitefly diversity and detection

The mtCOI locus of cassava whiteflies indicated the predominance of SSA1 populations in the countries sampled, the only other group present was SSA3 in Nigeria. All *B. tabaci* samples analysed from Tanzania (35 out of 35) belonged to SSA1-SG3 type. In Malawi, about 89.1% (41/46) whiteflies were SSA1-SG3 and the remaining 10.8% (5/46) were SSA1-SG2. In Uganda, 69.4% (68/98) were SSA1-SG1 and 30.6% (30/98) were SSA1-SG2 (Figure 3-2). The Nigerian (Ibadan) populations clustered with the SSA1 group in the phylogenetic analysis but did not cluster with any of the known four sub-groups. They clustered separately with sequences from Ghana from the database; they are therefore referred to as SSA1-SG5 (Figure 3-3). In Nigeria, 60.3% (41/68) were SSA1-SG5, 35.3% (24/68) SSA3 and 4.4% (3/68) were SSA1-SG1 type (Figure 3-2). SSA2 and SSA1-SG4 were not found in our study.

3.3.3 Prevalence of bacterial endosymbionts

The primary endosymbiont *Portiera* was detected in all the samples as expected. The secondary symbionts were found in 77.3% (191 whiteflies infected out of 247 tested) of the insects and their prevalence varied significantly across the different whitefly populations (Figure 3-4). The overall infection frequencies of *Wolbachia*, *Arsenophonus*, *Rickettsia* and *Cardinium* in the cassava whiteflies were 49.4% (122/247), 40.5% (100/247), 22.3% (55/247) and 0.8% (2/247), respectively. *Hamiltonella* and *Fritschea* were not detected in any of the whiteflies tested.

Highest and lowest rates of infection by *Arsenophonus* were seen in SSA1-SG3 (64.5%, 49/76) and SSA1-SG2 (17.1%, 6/35), respectively (Appendix A). *Arsenophonus* was present mostly as double infections, with *Wolbachia* in SSA1-SG1 (17%) and SSA1-SG2 (11%), and with *Rickettsia* in SSA1-SG3 (28%). *Arsenophonus* was present in SSA1-SG5 and SSA3 mainly as single infections (Figure 3-5).

Rickettsia was absent in SSA1-SG5 but most abundant in SSA1-SG3 (53.9%, 41/76) followed by SSA1-SG2 (20%, 7/35). Its infection levels in other populations were negligible. *Cardinium* was the least prevalent endosymbiont, detected only in 2 out of the 76 SSA1-SG3 (2.6%) but not in other populations.

Wolbachia was the most abundant secondary symbiont and was the commonest symbiont in SSA1-SG1 and SSA1-SG2 populations, mostly as single infections (Figure 3-5). It was nearly fixed in SSA1-SG2 (97.1%, 34/35), and was at much higher prevalence compared to all other populations (Appendix A).

A high percentage of whiteflies were completely free of secondary symbionts in SSA1-SG1 (38%) followed by SSA1-SG5 (29.2%), SSA3 (25%), SSA1-SG3 (13.1%), and only 2.8% in SSA1-SG2 (Figure 3-5). Cassava whiteflies predominantly were singly infected by a symbiont (59.1%, 113/191), mostly by *Wolbachia* (34.0%, 65/191) whereas only 36.6% (70/191) and 4.1% (8/191) had double and triple infections, respectively. Co-infections were commonest in SSA1-SG3 (54.5%, 36/66) (Figure 3-5).

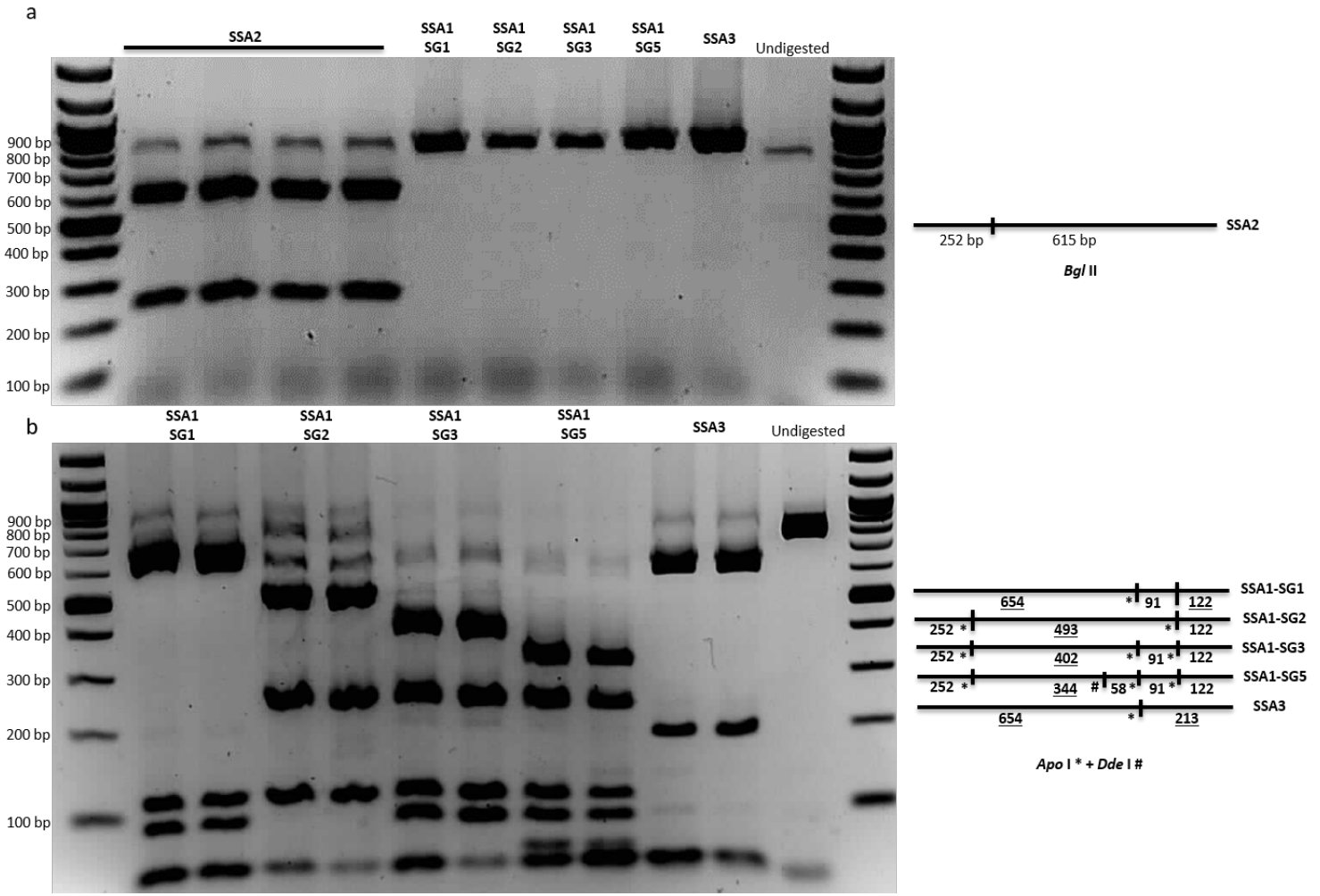

Figure 3-1: Detection of cassava whitefly populations based on RFLP profiles for high throughput screening. a: Detecting SSA2 by digestion with *Bgl* II, b: Detecting SSA1 and SSA3 by *Apo* I and *Dde* I. Underlined values represent the diagnostic fragments for the respective whitefly populations.

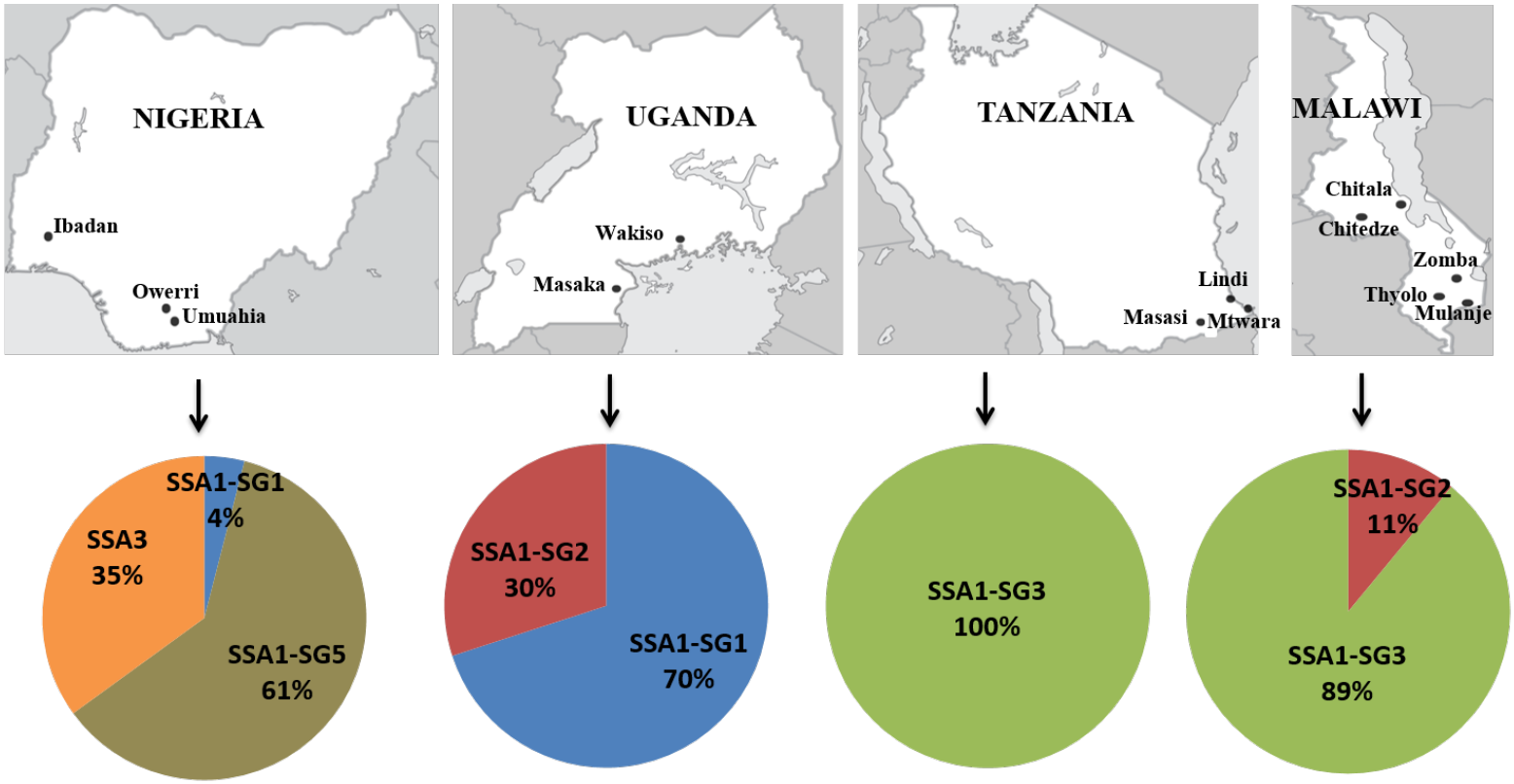

Figure 3-2: Frequency of *B. tabaci* populations in the four sampled countries.

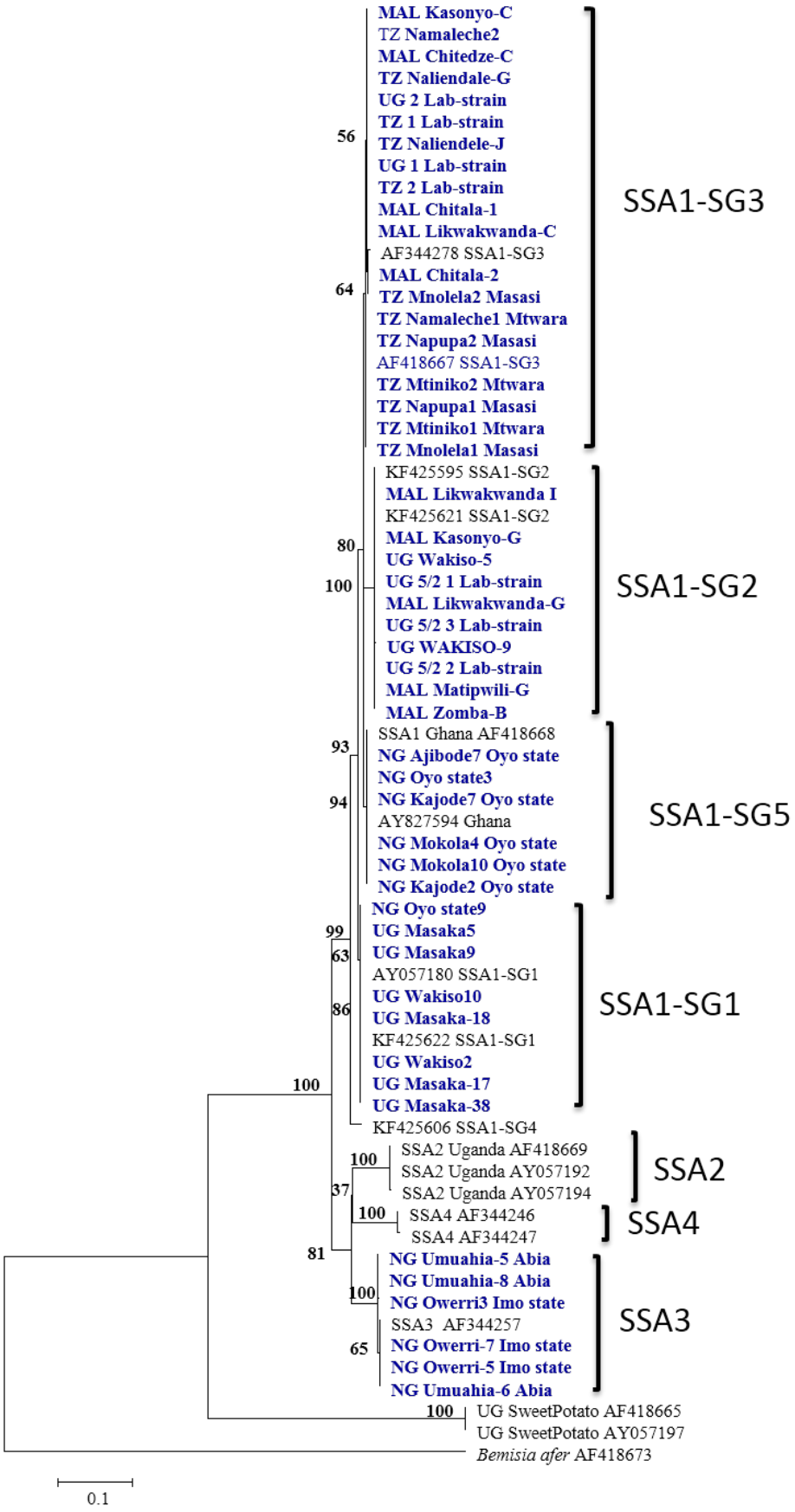

Figure 3-3: Phylogeny of mtCO1 nucleotide sequences (697 bp) of *B. tabaci* colonising cassava in SSA (blue) together with reference sequence from Genbank. Genbank accession numbers for the submitted sequences are KM377899-KM377952 and KM407138-KM407141.

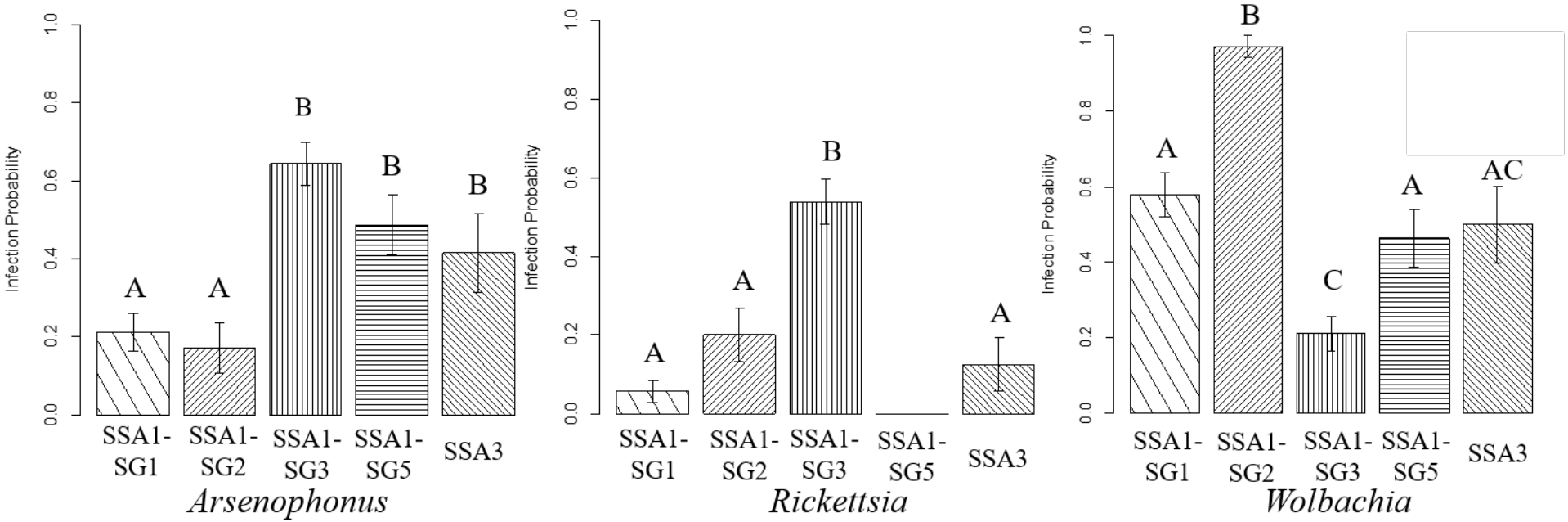

Figure 3-4: Mean infection of symbionts in the five cassava whitefly populations as determined by simple binomial logistic regression. Mean infections of a symbiont within the populations was compared by Tukey's HSD test and significant difference ($P \leq 0.5$) is indicated by different letters.

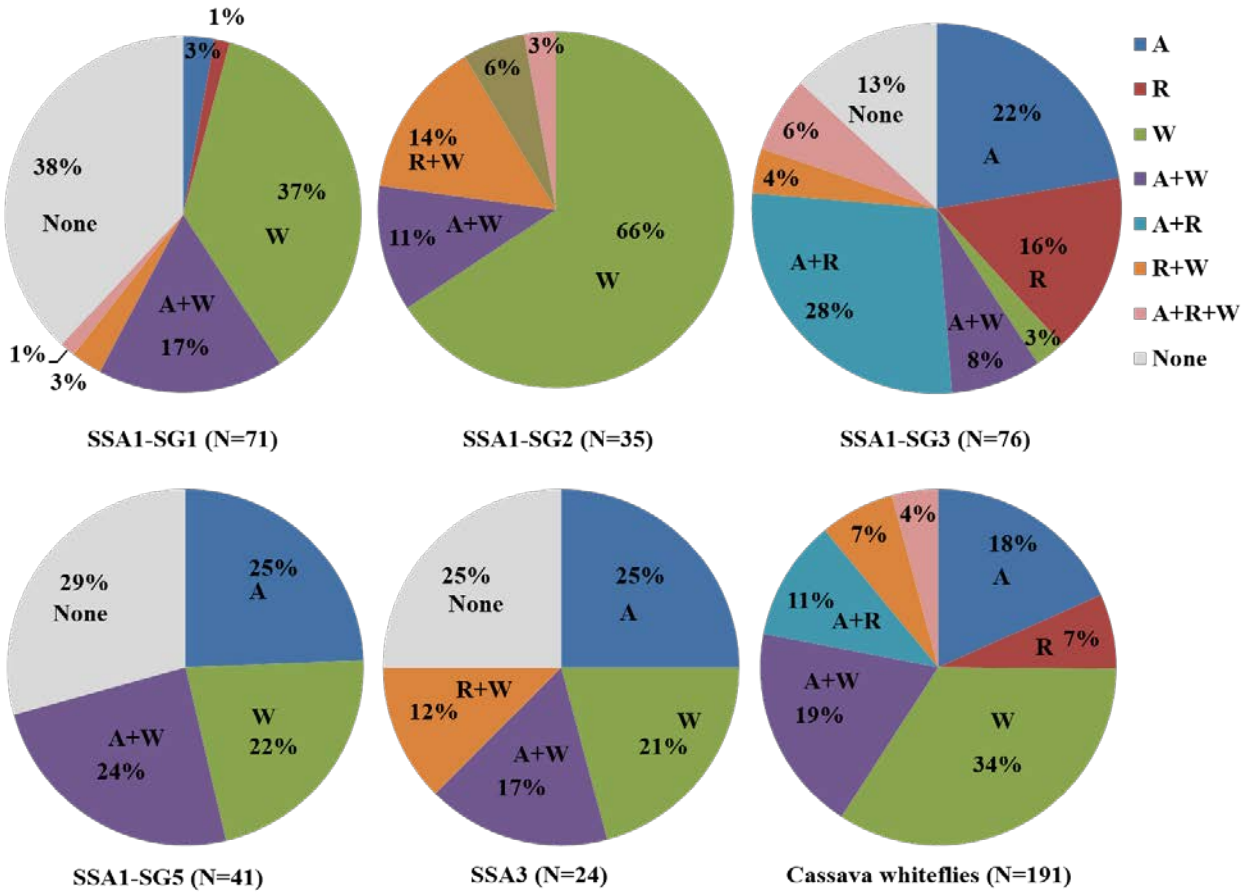

Figure 3-5: Pattern of infections of symbionts in different whitefly populations. Letters represent infection by each symbiont, A= *Arsenophonus*, R= *Rickettsia*, W= *Wolbachia*, C= *Cardinium*, None= free of S-endosymbionts.

3.3.4 Genetic diversity of endosymbionts

All five MLST fragments were amplified from *Wolbachia* infections from SSA1-SG2 and SSA1-SG3 from East African *B. tabaci* populations. However, only *coxA* was amplified from SSA1-SG5 and none from SSA3 from Nigeria despite exhaustive efforts. For SSA1-SG1, only *coxA*, *ftsZ*, *gatB* and *hcpA* were amplified except for one sample for which all five MLST markers were amplified.

Four unique *Wolbachia* sequence types were identified in this study, which were all submitted to the *Wolbachia* pubMLST database (Table 3-3). SSA1-SG1 and SSA1-SG2 were infected with identical *Wolbachia* based on five MLST alleles. These were unique to African cassava whiteflies as they shared no allele from other reported *Wolbachia* from *B. tabaci* but shared four common alleles with *Eretmocerus* sp. (parasitoid of whitefly), butterflies and *Spodoptera exempta* from wide geographical distances in the USA, Japan, India and Tanzania (Table 3-3). In contrast, SSA1-SG3 was infected with two different *Wolbachia*, but they shared three alleles (*coxA*=88, *hcpA*=106, *fbpA*=9) with *B. tabaci* from China and USA. Phylogeny of the concatenated MLST sequences of *Wolbachia* from whiteflies clustered into three sub-clades, W1, W2 and W3 (Figure 3-6). W1 sequences were from SSA1-SG1 and SSA1-SG2, and these were closely related (≥ 99.9% identical, Table 3-4) to *Culex* and butterfly species (*Hypolimnus*, *Cepora* and *Telicada*). W2 isolates were present in SSA1-SG3, and were closer to *Wolbachia* from *B. tabaci* from other geographical regions and host plants. W3 consisted of isolates from *B. tabaci* from Asia and *Bemisia afer* from Nigeria. Phylogenetic analysis of the *wsp* gene for *Wolbachia* also showed similar pattern as the SSA1-SG1 and SSA1-SG2 were clustered together and separately from SSA1-SG3 (Figure 3-7). Comparison of MLST sequences of *Wolbachia* strains showed that W1 isolates differed by a minimum of 4.5% nucleotides from W2 and W3 isolates, and W2 and W3 isolates differed by a minimum of 1% (Table 3-4).

The 23S rDNA sequences of *Arsenophonus* from cassava whiteflies clustered into three sub clades A1, A2, A3 with bootstrap scores of >70% (Figure 3-8). A3 isolates differed by 5.8% from A1, and 9.4% from A2 isolates (Table 3-5). These were incongruent with the evolution of the whitefly host based on mtCOI phylogeny. The samples belonging to clade A3 had additional 160 bp sequences and closely related (99.5% identity, Table 3-5) to sequences from *Arsenophonus nasoniae*, a male killing endosymbiont in the parasitic wasp, *Nasonia vitripennis*. Each SSA1-SG2 and SSA1-SG3 had one sample infected by both A2 and A3 strains of *Arsenophonus*.

The *Rickettsia* 16S rDNA sequences grouped into two clusters, R1 and R2 (Figure 3-9) with more than 8.5% nucleotide distances between them (Table 3-6). R1 strains, which were closer to strains from *Rickettsia* sp. nr *Bellii* were detected only in SSA1-SG3 and SSA1-SG2 populations. This strain is abundant in the invasive MEAM1 and MED species. R2 strains were identical to other strains of *Rickettsia* from native whiteflies from India and China.

Cardinium was detected only in SSA1-SG3 and the sequences were identical to the strains infecting Indian whiteflies (Figure 3-10).

Table 3-3: Comparison of MLST profile of *Wolbachia* from cassava *B. tabaci* with those from the pubMLST database, specimens in bold were generated in this study.

Host	Super group	Country	*coxA*	*fbpA*	*ftsZ*	*gatB*	*hcpA*	Sequence Type
***B. tabaci* (SSA1-SG1)**	B	Uganda	14	4	73	4	3	423*
***B. tabaci* (SSA1-SG2)**	B	Malawi, Uganda	14	4	73	4	3	423*
***B. tabaci* (SSA1-SG3)**	B	Tanzania, Malawi	88	9	105	9	13	424*
***B. tabaci* (SSA1-SG3)**	B	Tanzania	88	404*	105	9	13	425*
***B. tabaci* (SSA1-SG5)**	B	Nigeria	88	----	-----	-----	----	
B. afer	B	Nigeria	88	89	198*	105	106	427*
B. tabaci (MED)	B	USA	88	165	7	105	106	166
B. tabaci (China I)	B	China	88	9	170	207	13	377
B. tabaci (Asia II 1)	B	China	88	390	170	207	234	391
B. tabaci (China 1)	B	China	88	9	170	105	13	379
B. tabaci (Asia II 7)	B	China	88	387	7	105	106	378
B. tabaci (Asia 1)	B	China	88	387	182	207	106	395
B. tabaci (Australia)	B	Australia	88	9	170	207	221	380
B. tabaci (Asia II 9)	B	China	88	386	170	207	13	384
Eretmocerus sp. nr. emiratus	B	USA	14	4	73	105	3	161
Hypolimnus bolina	B	Japan	14	4	73	4	40	125
Telicada nyseus	B	India	14	4	73	4	40	125
Spodoptera exempta	B	Tanzania	14	4	73	4	40	125
Cepora nerissa	B	India	14	4	36	4	3	145

‘*’ New additions of *Wolbachia* sequence types to the database by this study, and ‘----’ failure to amplify genes in PCR amplifications.

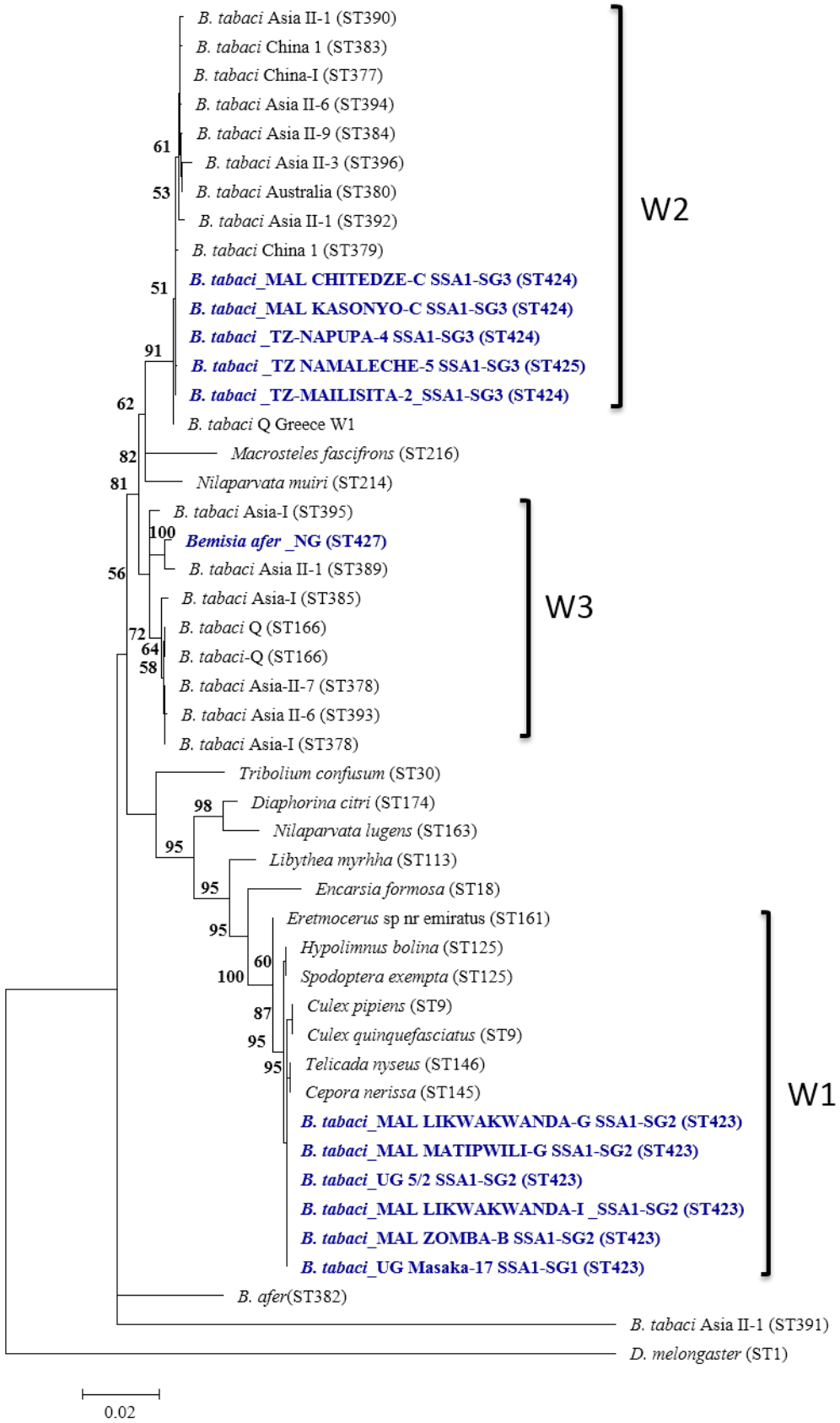

Figure 3-6: Phylogeny of concatenated MLST nucleotide sequences (2079 bp) of *Wolbachia* infecting whiteflies and other insect species. Strain names in the parentheses indicate the various *Wolbachia* sequence types. *Wolbachia* (sub-group A) from *Drosophila melanogaster* was used as an outgroup.

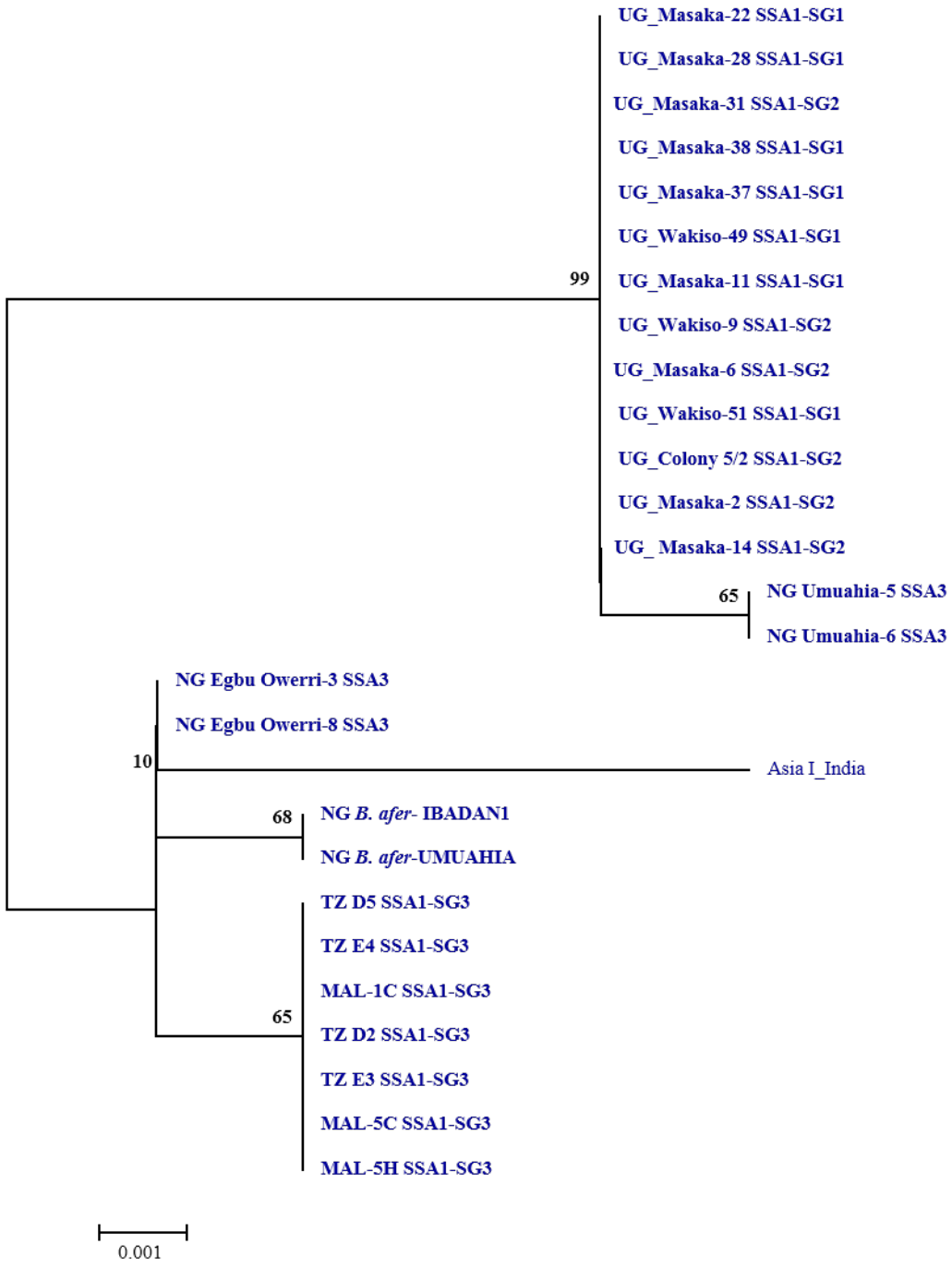

Figure 3-7: Phylogeny of *Wolbachia wsp* (596 bp) nucleotide sequences infecting cassava whiteflies in sub-Saharan Africa. Genbank accession numbers for the submitted sequences are KP208705-KP208733.

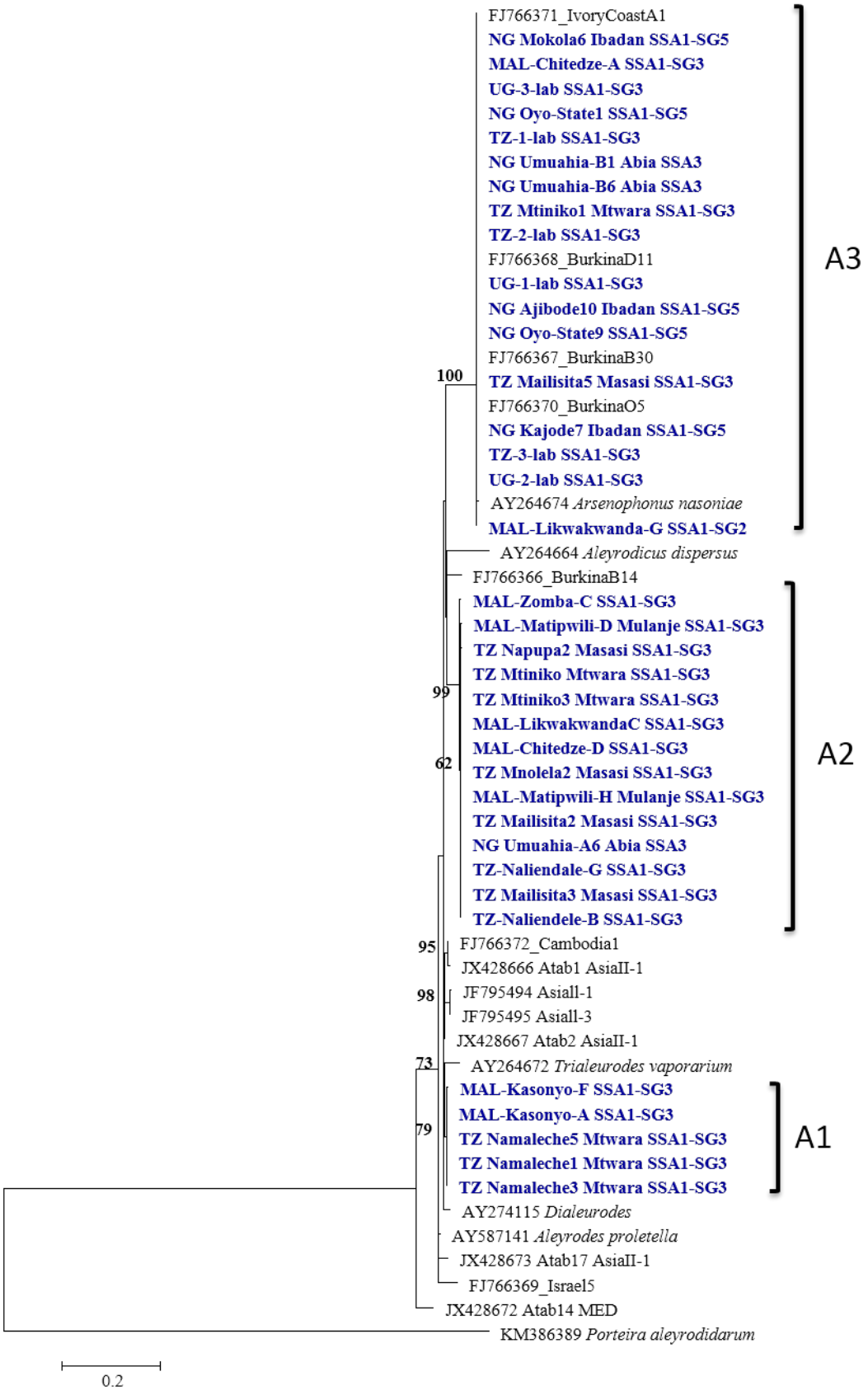

Figure 3-8: Phylogeny of *Arsenophonus* infecting whitefly species based on 23S rDNA (401 bp) nucleotide sequences. Genbank accession numbers for submitted sequences are KP377863-KP377898.

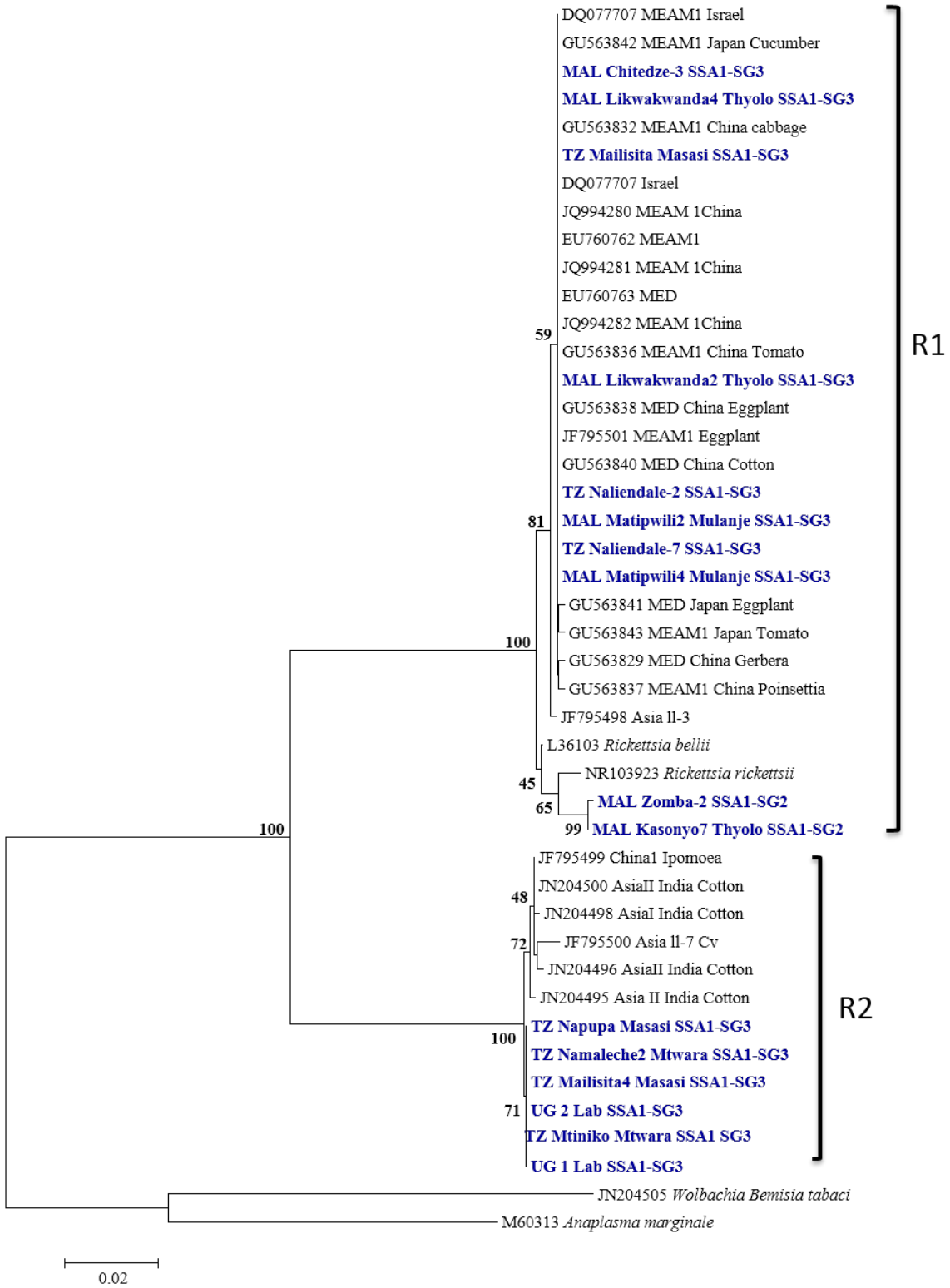

Figure 3-9: Phylogeny of whitefly-infecting *Rickettsia* 16S rDNA (859bp) nucleotide sequences. Genbank accession numbers of submitted sequences are KM38672-KM38687.

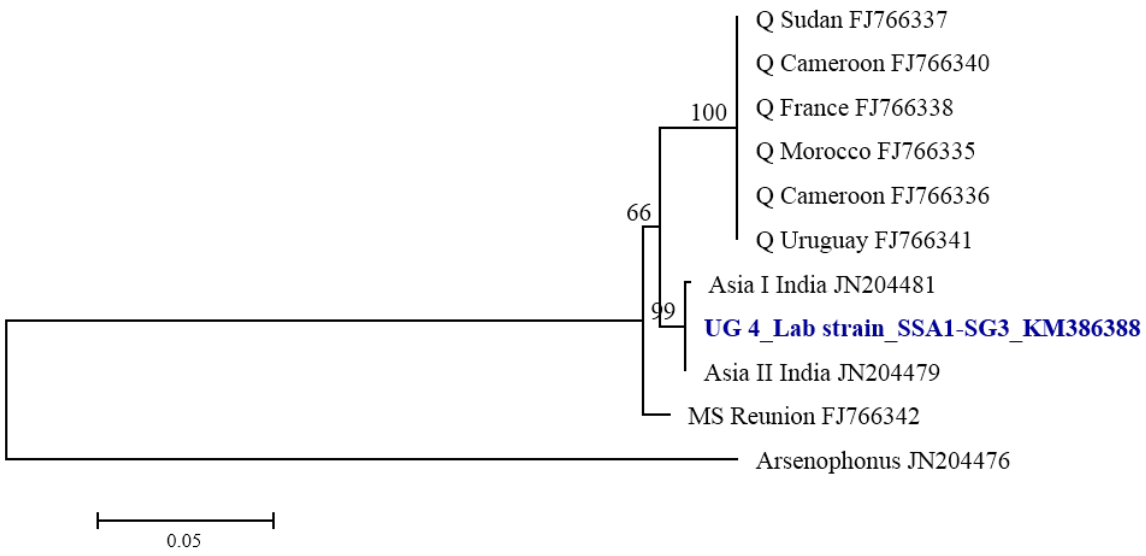

Figure 3-10 Phylogeny of *Cardinium* based on the 16S rDNA nucleotide sequences, infecting whiteflies around the world.

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Table 3-4: Percentage nucleotide identities of selected *Wolbachia* strains based on concatenated MLST sequences (p-distances were calculated using MEGA 5.0)

UG-5/2_SSA1-SG2 (423)																		
MAL_SSA1-SG2 (423)	100																	
UG_SSA1-SG1 (423)	100	100																
H. bolina (125)	99.95	99.95	99.95															
*C. pipiens*_(9)	99.90	99.90	99.90	99.86														
Eretmocerus(161)	99.71	99.71	99.71	99.66	99.61													
MAL_SSA1-SG3 (424)	95.56	95.56	95.56	95.61	95.47	95.85												
TZ_SSA1-SG3 (424)	95.56	95.56	95.56	95.61	95.47	95.85	100											
TZ_SSA1-SG3 (425)	95.51	95.51	95.51	95.56	95.42	95.80	99.95	99.95										
China-I (377)	95.47	95.47	95.47	95.51	95.37	95.75	99.90	99.90	99.86									
Asia_II-3 (396)	95.27	95.27	95.27	95.32	95.18	95.56	99.61	99.61	99.57	99.71								
Asia_II-9 (384)	95.42	95.42	95.42	95.47	95.32	95.71	99.86	99.86	99.81	99.95	99.76							
Australia (380)	95.47	95.47	95.47	95.51	95.37	95.75	99.86	99.86	99.81	99.95	99.76	99.90						
***B. afer*_Nigeria (427)**	96.53	96.53	96.53	96.58	96.43	96.82	98.22	98.22	98.17	98.12	97.88	98.07	98.07					
MED (166)	96.19	96.19	96.19	96.24	96.09	96.48	98.99	98.99	98.94	98.89	98.65	98.84	98.84	99.23				
Asia_II-1 (389)	96.24	96.24	96.24	96.29	96.14	96.53	98.22	98.22	98.17	98.31	98.02	98.26	98.26	99.61	99.04			
	W3																	
Asia_II-6 (393)	96.09	96.09	96.09	96.14	96.00	96.38	98.99	98.99	98.94	98.89	98.65	98.84	98.84	99.13	99.90	98.94		
Asia-II-7 (378)	96.14	96.14	96.14	96.19	96.04	96.43	99.04	99.04	98.99	98.94	98.70	98.89	98.89	99.18	99.95	98.99	99.95	
B. afer (382)	95.18	95.18	95.18	95.22	95.18	95.47	96.62	96.62	96.58	96.53	96.29	96.48	96.48	96.33	95.90	96.04	95.80	95.85
D. melongaster (1)	88.08	88.08	88.08	88.13	87.99	88.23	89.10	89.10	89.05	89.10	88.95	89.05	89.05	88.47	88.86	88.47	88.86	88.90
	88.95																	

Table 3-5: Percentage nucleotide identities of 23S rDNA sequences of *Arsenophonus* strains (p-distances were calculated using MEGA 5.0).

TZ_Namaleche1_SSA1-SG3															
TZ_Namaleche3_SSA1-SG3	100			**A1**											
MAL-Kasonyo-F_SSA1-SG3	99.8	99.8													
TZ-Naliendale-B_SSA1-SG3	95.4	95.4	95.2												
NG_Umuahia-A6_SSA3	95.4	95.4	95.2	100			**A2**								
MAL-Zomba-C_SSA1-SG3	95.2	95.2	94.9	99.8	99.8										
TZ-1-lab_SSA1-SG3	94.2	94.2	94.0	90.6	90.6	90.8									
MAL-Chitedze-A_SSA1-SG3	94.2	94.2	94.0	90.6	90.6	90.8	100				**A3**				
NG_Oyo-State1_SSA1-SG5	94.2	94.2	94.0	90.6	90.6	90.8	100	100							
AY264674_*A_nasoniae*	94.2	94.2	94.0	90.6	90.6	90.8	99.5	99.5	99.5						
FJ766366_ASL_Burkina_Faso	95.7	95.7	95.4	96.4	96.4	96.1	91.1	91.1	91.1	91.1					
FJ766370_MED_Burkina_Faso	94.2	94.2	94.0	90.6	90.6	90.8	100	100	100	99.5	91.1				
JX428666_AsiaII_1	98.1	98.1	97.8	95.9	95.9	95.7	92.8	92.8	92.8	92.8	95.7	92.8			
JF795495_Asiall_3	98.3	98.3	98.1	95.2	95.2	94.9	93.2	93.2	93.2	93.2	95.4	93.2	97.8		
FJ766369_MED_Israel	94.7	94.7	94.4	92.3	92.3	92.0	89.6	89.6	89.6	89.6	92.3	89.6	93.7	94.0	
JX428672_MED_China	91.5	91.5	91.3	91.3	91.3	91.1	87.0	87.0	87.0	87.0	90.6	87.0	91.5	91.8	89.4

Table 3-6: Percentage nucleotide identities of 16S rDNA sequences of *Rickettsia* strains (p-distances were calculated using MEGA 5.0).

TZ_Mailisita-2_SSA1-SG3																		
TZ_Naliendale-7_SSA1-SG3	100																	
MAL_Chitedze-3_SSA1-SG3	100	100																
MAL_Zomba-2_SSA1-SG2	98.4	98.4	98.4															
MAL_Kasonyo-7_SSA1-SG2	98.5	98.5	98.5	99.9							**R1**							
DQ077707_MEAM1_Israel	100	100	100	98.4	98.5													
JQ994281_MEAM1_China	100	100	100	98.4	98.5	100												
EU760763_MED	100	100	100	98.4	98.5	100	100											
L36103_*Rickettsia_bellii*	99.5	99.5	99.5	98.8	99.0	99.5	99.5	99.5										
NR103923_*R_rickettsii*	98.5	98.5	98.5	99.0	99.1	98.5	98.5	98.5	99									
JN204498_Asia_I_India	91.5	91.5	91.5	90.6	90.8	91.5	91.5	91.5	91.5	91.2								
JN204495_Asia_II_India	91.6	91.6	91.6	90.8	90.9	91.6	91.6	91.6	91.6	91.3	99.8							
JF795498_Asia_ll_3	99.9	99.9	99.9	98.3	98.4	99.9	99.9	99.9	99.4	98.4	91.6	91.7						
JF795500_Asia_ll_7	91.1	91.1	91.1	90.3	90.4	91.1	91.1	91.1	91.1	90.9	99.4	99.4	91.2					
JF795499_China1	91.5	91.5	91.5	90.6	90.8	91.5	91.5	91.5	91.5	91.2	99.8	99.8	91.6	99.4				**R2**
UG2_Lab_strain_SSA1-SG3	91.5	91.5	91.5	90.9	91.0	91.5	91.5	91.5	91.5	91.5	99.5	99.5	91.6	99.2	99.8			
TZ_Mtiniko_SSA1-SG3	91.5	91.5	91.5	90.9	91.0	91.5	91.5	91.5	91.5	91.5	99.5	99.5	91.6	99.2	99.8	100		
TZ_Namaleche-2_SSA1-SG3	91.3	91.3	91.3	90.8	90.9	91.3	91.3	91.3	91.3	91.3	99.4	99.4	91.5	99.1	99.7	99.9	99.9	
TZ_Napupa_SSA1-SG3	90.9	90.9	90.9	90.3	90.4	90.9	90.9	90.9	90.9	90.9	98.7	98.7	91.0	98.4	99.0	99.2	99.2	99.1

3.4 Discussion

The main aim of this study was to determine the prevalence and genetic diversity of secondary endosymbionts infecting cassava whiteflies in SSA. Whiteflies harbour multiple bacterial symbionts that play essential roles on insect biology, evolution and virus transmission. Understanding cassava whitefly diversity and the bacterial communities co-existing, within the cassava ecosystem is essential to understand the near extinction of some cassava populations in recent years, or the development of superabundant populations and the resultant epidemics of CMD and CBSD in Eastern and Central African countries in recent years (Legg *et al*., 2006; Maruthi *et al*., 2001; Sseruwagi *et al*., 2006).

At first, the genetic diversity of cassava whiteflies from Uganda, Tanzania, Malawi and Nigeria was studied by analysing the mtCOI sequences. This was done to understand the pattern of prevalence of symbionts in different whitefly populations. Cassava in these countries was colonised by five genetically different whitefly populations. SSA1 and its various sub-groups was predominant in the countries sampled, the only other group present was SSA3 in Nigeria, while SSA2 was not detected. SSA1-SG3 was found to be the predominant population in coastal Tanzania and Malawi while SSA1-SG1 was the dominant population in Uganda (Figure 3-2). Based on mtCOI phylogeny, a new population was found in Nigeria, which we referred to as SSA1-SG5 (Figure 3-3). The newly defined population SSA1-SG5 was predominant in Nigeria, followed by SSA3 (35.3%) and a very few individuals of SSA1-SG1. Overall, these results are concurrent with the previous studies that have also shown high levels of genetic diversity amongst the cassava whitefly populations in SSA (Berry *et al*., 2004; Sseruwagi *et al*., 2005, 2006; Tajebe *et al*., 2015a).

The threat of the two cassava virus disease pandemics spread by the superabundant *B. tabaci* populations requires simpler monitoring system for effective disease management. As seen above and in previous studies, mtCOI is shown to be a reliable marker for separating whitefly species and sub-populations. However, using this as a marker requires sequencing and thus incurs high costs and time. In addition, we therefore developed a robust RFLP method for typing cassava whiteflies quicker than conventional methods relatively. Using the two-step method and three restriction enzymes described in this study, we were able to reliably assign whiteflies to phylogenetic groups and subgroups found in this study, and thus saving costs as well as time.

Typing the various bacteria infecting these whiteflies, however, proved far more challenging as some of the methods and primers described in the literature did not work initially on cassava whitefly endosymbionts. This was probably due to the high genetic diversity resulting in nucleotide sequence variations as seen in both cassava whiteflies and the various bacteria that infected them. New primers were therefore developed where necessary and the DNA extraction methods and PCR conditions were optimised. Detecting bacteria confidently was a pre-requisite to understand the genetic diversity of bacteria infecting cassava whiteflies. Using the above methods, genetically diverse bacteria were found to infect cassava whiteflies in SSA

Rickettsia, Arsenophonus, Wolbachia and *Cardinium* were detected in cassava whiteflies, but not *Hamiltonella* and *Fritschea*. *Hamiltonella* was also absent in other native whitefly populations in India and China (Bing *et al*., 2013a; Singh *et al*., 2012), but was reported to be present in SSA1 cassava whiteflies from Tanzania (Tajebe *et al*., 2015b). This is contrasting to our study, and we cannot clearly explain the differences between the two studies at this time. Some of the possible explanations, however, include high site to site variation seen in endosymbiont prevalence in cassava whiteflies within a country (Tajebe L., pers comm), and that our samples may have been collected coincidentally from *Hamiltonella*-free sites. Other reasons include the low titre of the bacteria in our samples which would be below the limits of PCR detection, or primer mismatch in PCR reactions. We did obtain unspecific amplification of *Arsenophonus* with the used *Hamiltonella*-specific primers in initial studies, which indicated primer mismatch. The *Hamiltonella*-specific primers, therefore, should be used with care in future studies, while the *Hamiltonella* quandary between Tajebe *et al*., (2015b) and this study remains to be resolved. We used MLST to characterise *Wolbachia*. All five MLST alleles were amplified from all our populations except SSA1-SG5 where only *coxA* was amplified and none from SSA3 after exhaustive efforts. Difficulties in amplification of MLST alleles have been reported previously, and could be due to high variability of these genes or low titres of the symbiont (Augustinos *et al*., 2011; Bing *et al*., 2014). The surface protein *wsp* was therefore used as an alternative marker and it also confirmed the high diversity of *Wolbachia* infecting cassava whiteflies.

Overall, about 77.3% of cassava whiteflies were infected with at least one secondary symbiont, while the remaining 22.7% were completely free of the tested bacteria. These results were similar to the incidences of secondary symbionts seen in other *B. tabaci*, which

ranged from 78% to 100% depending on the study (Bing *et al*., 2013a; Chiel *et al*., 2007; Gueguen *et al*., 2010; Skaljac *et al*., 2010). Interestingly, a high percentage of the superabundant SSA1-SG1 from the CMD pandemic areas (Tajebe *et al*., 2015b) and SSA1-SG5 whiteflies (Gnankine *et al*., 2013) were also reported to be free of secondary symbionts. Thus whether the intracellular bacteria infecting cassava whiteflies are beneficial for its host remains unknown. Further studies comparing the fecundity and life cycle of bacteria-infected and uninfected cassava whiteflies is essential to understand the reasons behind the development of superabundant whiteflies, and the role of the symbionts.

Single infections of bacteria were more prevalent (59% of total infections) in cassava whiteflies than double (37%) and triple (4%) infections. This was slightly different than other studies in which co-infections were more common (> 60%) than single infections (Chiel *et al*., 2007; Gueguen *et al*., 2010; Tajebe *et al*., 2015b). The reasons or the implications of this is unknown but could be due to competition for space and resources among the symbionts (Vautrin and Vavre, 2009) or the tolerance of the host to harbour many bacterial communities (Skaljac *et al*., 2010). Specific associations between bacterial strains and whitefly populations were observed. For example, SSA1-SG1 and SSA1-SG2 were both infected with similar strains of *Wolbachia*, previously detected in butterflies and mosquitoes, whereas SSA1-SG3 was infected with a different *Wolbachia*. Infection levels of *Rickettsia* were highest in SSA1-SG3 (54%), which was also similar to the invasive *Rickettsia* sp. nr *Bellii* strain that invaded the whitefly population MEAM1 in the USA with fitness benefits to the infected host (Himler *et al*., 2011). However, infection with the same strain of *Rickettsia* in MEAM1 populations from Israel had no selective advantage to the host (Chiel *et al*., 2009) and this further indicates specific interaction between symbiont and host genotype or the environment. When and how the *Rickettsia* invaded cassava whiteflies is unknown, but it remains to be seen if they also provide fitness benefits or not on cassava plants. Another puzzle in the jigsaw of whitefly-bacterial interactions was the detection of three different strains of *Arsenophonus* in cassava whiteflies. Strain A3 in particular was highly divergent, 7% nucleotide differences, compared to other *Arsenophonus* infecting *B. tabaci* across the world. A3 is closely related to the male killing *Arsenophonus nasoniae* (Gherna *et al*., 1991), which again might influence the population dynamics and remains the focus of our future investigations. In summary, our findings provide insights to the diverse bacterial species infecting cassava whiteflies in African countries, and that these should be

considered in future studies aiming to better understand the changing population dynamics in African cassava fields.

4 ISOLATION OF WHITEFLY ISO-FEMALE LINES AND LOCALISATION OF ENDOSYMBIONTS IN NYMPHS AND ADULTS USING FLUORESCENT IN SITU HYBRIDISATION

4.1 Introduction

Results in chapter 3 indicated the prevalence and high diversity of S-endosymbionts infecting cassava whiteflies in different agro-ecologies of SSA. It is well documented that these vertically transmitted micro-organisms greatly influence insect phenotypes. However, their effect on the biology of cassava whitefly was not known. Most studies were conducted on the invasive Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) whitefly species, which are very different to the native African cassava species.

A major challenge, however, for studying the effect of each symbiont on insect biology has been the isolation of genetically identical lines of whitefly species with single and multiple infections with different symbionts. Each symbiont can be expected to produce different phenotypes in different genetic environments, therefore investigations on the variability of phenotypes should be carried out on genetically identical individuals (David *et al*., 2005). Use of isofemale lines (progeny of a single wild female) has been a central and reliable technique to investigate a variety of biological questions related to ecology and evolution especially when studying phenotypic variability in populations (David *et al*., 2005). Phenotypes associated with an isofemale line can be confidently used to define the mean characteristics of a natural population (David *et al*., 2005). We therefore tried generating isofemale lines of different cassava whitefly species with single or multiple infections and with S-symbionts as control, to investigate the effect of symbionts on whitefly biology.

Curing insects from infections of S-endosymbiotic bacteria by feeding antibiotics is one of the commonly practiced technique for generating isofemale lines with varied symbionts. The success of antibiotics in eliminating S-endosymbionts, however depend upon the endosymbiont and host species. Rifampicin was best for eliminating *Arsenophonus* across all *B. tabaci* species, and tetracycline was most efficient for eliminating *Wolbachia* (Ahmed *et al*., 2010a).

Understanding the localisation of endosymbionts in insect bodies is key to understand the mechanism of transfer of these bacteria through the progenies, their possible biological

functions and symbiont-symbiont interactions. P and S-symbionts in whiteflies uniquely occupy the same cell types called bacteriocytes. They are physically separated in other insects species such as aphids and sharpshooters (Gottlieb *et al*., 2008). The bacteriocytes aggregate into two sac-like structures called bacteriomes during egg and larval development (Baumann *et al*., 2006). They disintegrate into cell clusters in adults for efficient vertical transmission of the bacteriocyte to each oocyte during the last stages of oogenesis (Costa *et al*., 1996; Szklarzewicz and Moskal, 2001). Occasionally, S-symbionts are also known to infect cells other than the bacteriocytes with wide distribution throughout the host body.

Fluorescent in situ hybridisation (FISH) is a diagnostic tool based on DNA probes labelled with fluorescent reporter molecule which when annealed to specific target sequences of a sample DNA, confirms the presence or absence of the target when viewed under a fluorescent microscope (Bishop, 2010). FISH has been used for the localisation of *B. tabaci* endosymbionts in many studies, but not in SSA cassava whiteflies (Gottlieb *et al*., 2006; Gottlieb *et al*., 2008; Skaljac *et al*., 2010; Brumin *et al*., 2012; Priya *et al*., 2012). We therefore conducted FISH to identify the location of P and S- endosymbionts particularly infecting our isofemale lines of African cassava whiteflies.

4.2 Materials and Methods

4.2.1 Generation of isofemale lines

We tried two strategies to generate whitefly isofemale lines with different infections of S-symbionts. Strategy one was based on random isolation of individual female and male and allowing them to mate and develop isofemale colonies. This strategy is based on the findings that some individuals within an insect colony are completely free of S-symbiont, or with specific single or multiple infections. The second strategy was by curing specific bacterial infections by feeding whiteflies with artificial diets containing antibiotics to obtain desired combination of symbionts. Both strategies have been used successfully on many insect species.

Whiteflies used in this study for generating isofemale lines were collected from colonies originated from Uganda or Tanzania (Maruthi *et al*., 2001) and maintained on cassava plants in the NRI insectary (27± 5°C, 60% relative humidity and L12:D12). They were then anesthetized using carbon dioxide for 5 seconds and separated into males and females using

a stereo-binocular microscope. Single male and female were set up in 25 mm diameter clip cages on cassava leaves for laying eggs (Figure 4-1). The clip cages were removed after 10 days and the leaves with nymphs and eggs were enclosed in perforated bread bags. F1 progenies from the crosses were transferred to new cassava plants for establishing isofemale colonies.

4.2.2 Detecting endosymbionts in isofemale lines

The individual whitefly lines from the successfully established isofemale lines were screened for the presence or absence of symbionts by PCR and the genetic group of the isofemale line was determined using mtCO1 PCR-RFLP as previously described (Section 3.2.3, Chapter 3). Isofemale lines with unique combination of symbionts were propagated while the remaining lines were discarded.

4.2.3 Antibiotic curing of whiteflies from symbionts

Antibiotic treatment of isofemale lines for curing specific symbionts was done by direct feeding of newly emerged adults on an artificial diet solution (25% sucrose w/v, containing 0.5 mM Potassium Phosphate, pH = 7) containing different antibiotics (Ruan *et al*., 2006). A plastic tube (36 mm in diameter and 12 cm long, open at both ends) was covered at the top by a stretched sheet of parafilm membrane (Figure 4-2A). About 0.5 ml of diet solution containing rifampicin, tetracycline, rifampicin + tetracycline, ampicillin or doxycycline at three different concentrations (100 µg/ml, 250 µg/ml or 500 µg/ml) was then placed on the outer surface of the parafilm. The diet solution was covered with another layer of stretched parafilm. Approximately 50 newly emerged adults were introduced into the tube for feeding (Figure 4-2B). The adults were fed for 48 hours at 27±5 ^{0}C, 60% humidity and photoperiod of L12:D12, and then released onto fresh cassava plants for colony establishment.

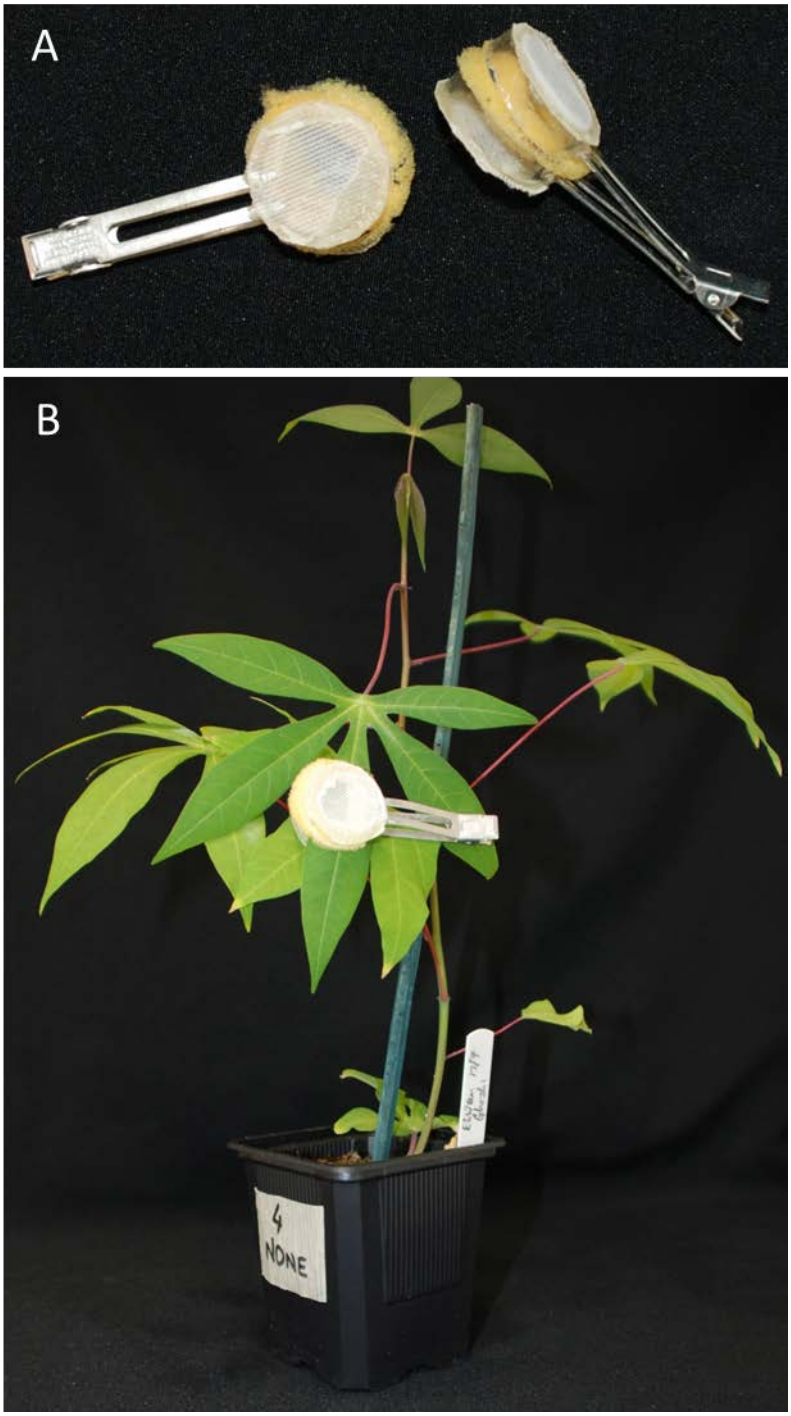


Figure 4-1: A) 25 mm diameter clip cages. B) Clip cage with single female and male whitefly attached to a cassava leaf.

4.2.3 Quantification of S-symbionts

To investigate the effectiveness of antibiotic treatment, newly emerged adults from the isofemale line SSA1-SG2 infected with *Wolbachia* were fed on artificial diet with 250 µg/ml rifampicin+tetracycline for 48 hours. *Wolbachia* was quantified by qPCR on individual whiteflies after 5 and 10 days of feeding on the antibiotic supplemented diet and compared with the SSA1-SG2 flies on control diet (no antibiotics). The qPCR was performed with Eppendorf Mastercycler Realplex^2 PCR instrument with 2X DyNAmo Flash SYBR green PCR kit (Thermo Scientific, UK). *Wolbachia* was amplified with primers designed in this study targeting 16S rDNA, and the whitefly β actin gene was used as a reference gene. Amplifications were performed in 20 µl reactions containing 10 µl of 2X SYBR green PCR mix, 0.4 µM of each primer (Table 4-1) and 1 µl of DNA lysate. Amplifications consisted of 95 °C for 7 minutes followed by 40 cycles of 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds. The mean normalised relative quantities of *Wolbachia* were calculated by comparing the quantification cycle (Cq) value of *Wolbachia* to that of the whitefly β actin gene according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The mean relative quantities of *Wolbachia* of the different treatments were compared pairwise by Tukey's HSD test.

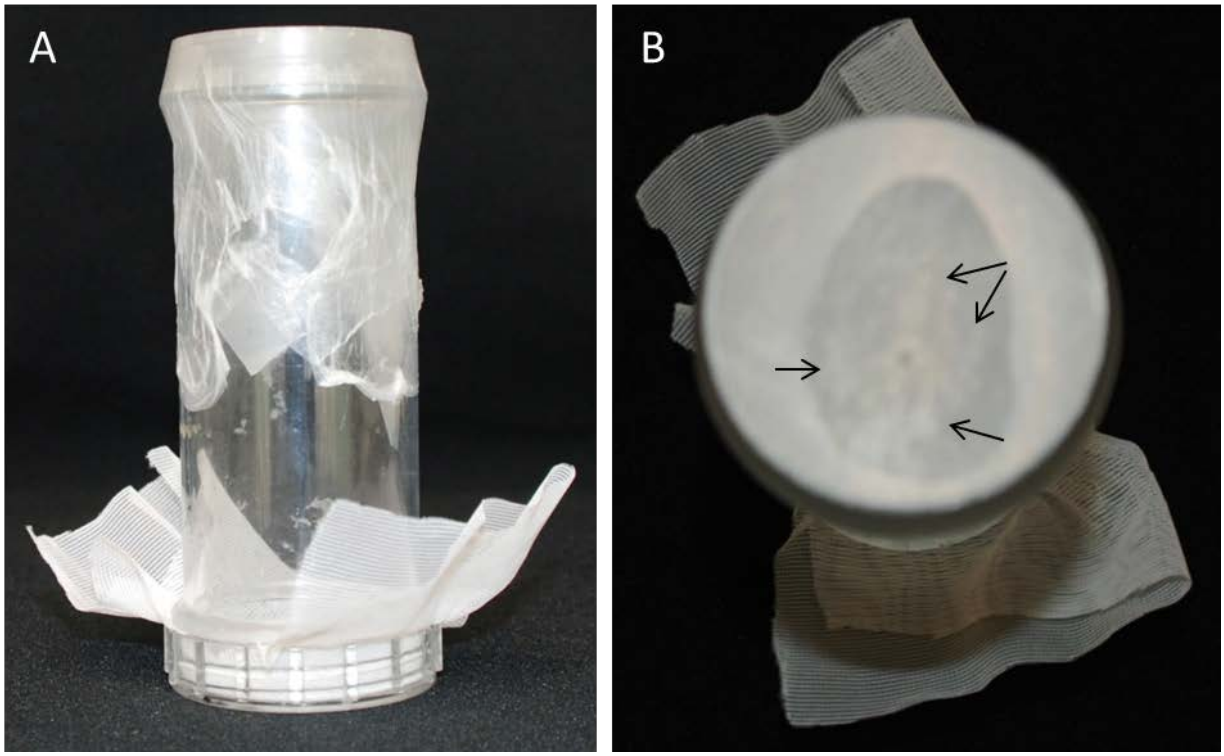


Figure 4-2: A) Plastic tube setup for feeding of whiteflies with artificial diet. B) Whiteflies (arrows) feeding on sucrose solution augmented with antibiotics.

Table 4-1: PCR primers used for relative quantification of *Wolbachia*

Target gene	**Primer name**	**Sequence (5'→3')**	**Amplicon length**	**Reference**
Wolbachia 16S rDNA	WF	GGGGAGTTTACTTTCTGTATTAC	190 bp	This study
	WR	CCCCATCCCTTCGAATAGGTAT		
B. tabaci Actin	Act-F	TCTTCCAGCCATCCTTCTTG	130 bp	Ghanim and Kontsedalov, 2009
	Act-R	CGGTGATTTCCTTCTGCATT		

4.2.4 *Localisation of symbionts in nymphs and adults by FISH*

Localisation of symbionts in cassava whitefly nymphs and adults using FISH was done as previously described (Skaljac *et al*., 2010). Whitefly adults were collected from isofemale lines known to harbour specific symbionts by PCR tests. Nymphs were picked from mature leaves of the colonies using 0.3 mm insect pins under a stereo binocular microscope. The adults and nymphs were immediately fixed in freshly prepared Carnoy's fixative (chloroform: ethanol: glacial acetic acid, 6:3:1, v/v) in 1.5 ml centrifuge tubes and left overnight at room temperature. The fixative was removed, the samples were washed with 100% ethanol and decolourised using 6% hydrogen peroxide (H_2O_2) solution in ethanol for 2 hours at room temperature to quench auto-fluorescence. The H_2O_2 solution was removed and the samples were hybridised overnight at 37 °C using hybridisation buffer (20 mM Tris HCl, pH=8, 0.9 mM NaCl, 0.0.1% [w/v] sodium dodecyl sulphate, 30% [v/v] formamide) containing 10 pmoles/ml of endosymbiont specific probes (Table 4-2) labelled with cy3 or cy5 reporter dyes for co-localisation. Isofemale lines without S-symbionts were used as negative control and the MED population from Israel with triple infections with *Arsenophonus*, *Rickettsia* and *Wolbachia* (obtained from Dr. Murad Ghanim, ARO, Volcani centre, Israel) were used as a positive control. Whole samples were mounted on microscopic slides in hybridisation buffer contained with a liquid blocker, covered with a cover slip, sealed with nail polish and visualised under a confocal microscope (IX-81 Olympus FluoView 500 confocal microscope, Olympus Optical Co, Tokyo, Japan). Twenty-five individuals each of nymphs and adults from the different isofemale lines were tested for the location of symbionts in whitefly bodies.

Table 4-2 Symbiont specific probes used for FISH

Target symbiont	**Probe name**	**Sequence (5'→ 3')**	**Reporter dye**	**Reference**
Portiera	BTP1	TGTCAGTGTCAGCCCAGAAG	Cy3	Gottlieb *et al*., 2006
Wolbachia	W1	CTTCTGTGAGTACCGTCATTATC	Cy5	Skaljac *et al*., 2010
Arsenophonus	Ars2	TCATGACCACAACCTCCAAA	Cy5	Gottlieb *et al*., 2008
Rickettsia	Rb1	TCCACGTCGCCGTCTTGC	Cy5/Cy3	Gottlieb *et al*., 2006

4.3 Results

4.3.1 Generating isofemale lines-

A total of five isofemale lines were successfully isolated with unique combination of symbionts (Table 4-3). Two isofemale lines of SSA1-SG3 with and without symbionts, one isofemale line of SSA1-SG2 with 100% *Wolbachia* infection and three isofemale lines of SSA2 genetic background (Figure 4-4) with and without *Wolbachia* and with *Arsenophonus* only were generated. The generated lines were transferred to cages and multiplied for at least six months for colony propagation before using them in experiments.

4.3.2 Antibiotic curing of whiteflies from symbiont infections

All whitefly adults that fed on the artificial diet containing 500 µg/ml antibiotics died within 48 hours of feeding. Only those fed on 100 µg/ml and 250 µg/ml antibiotic survived after 48 hours. The titres of *Wolbachia* were reduced significantly in adults fed with antibiotics (tetracycline or rifampicin or rifampicin + tetracycline), however, their concentrations restored in the F1 generation to the levels seen in the wild type isofemale lines (Figure 4-5) (Appendix B). We were therefore unable to cure *Wolbachia* from adult whiteflies with any of the antibiotics used.

Similarly, *Arsenophonus* infections in whiteflies were not removed by any of the antibiotic treatments (Table 4-4). Only *Rickettsia* infection (R2 strain, Section 3.3.4) was successfully cured from the adult whiteflies and subsequent generations by treatment with rifampicin at a dosage of 100 µg/ml diet (Figure 4-6; Table 4-4). Another isofemale line with single infections with *Arsenophonus* was generated using this method by curing *Rickettsia* from SSA1-SG3 populations doubly infected with *Arsenophonus* and *Rickettsia*.

Table 4-3 Isofemale lines of cassava whiteflies with unique symbiont combinations

Symbionts	**Genetic background**	**Colony symbol**	**Method used**
Arsenophonus + *Rickettsia*	SSA1-SG3	SSA1-SG3 AR+	Manual isolation
No secondary symbiont	SSA1-SG3	SSA1-SG3 AR-	Manual isolation
Wolbachia	SSA1-SG2	SSA1-SG2 W+	Manual isolation
Wolbachia	SSA2	SSA2 W+	Manual isolation
Arsenophonus	SSA2	SSA2 A+	Manual isolation
No secondary symbiont	SSA2	SSA2 -	Antibiotic treatment of SSA2 A+

Table 4-4: Antibiotic treatments for curing symbiont infections from cassava whiteflies

Antibiotic treatment	**Dosage (µg/ml)**	**Whitefly**	**Target**	**Symbiont removal**
Tetracycline/rifampicin	100/250	SSA1-SG3	*Arsenophonus*	No
Rifampicin	100	SSA1-SG3	*Rickettsia*	Yes
Tetracycline	100	SSA2	*Arsenophonus*	Yes
Tetracycline/rifampicin	100/250	SSA1-SG2	*Wolbachia*	No
Tetracycline+rifampicin	100/250	SSA1-SG2	*Wolbachia*	No
Tetracycline+rifampicin	100/250	SSA2	*Wolbachia*	No

4.3.3 Localisation of symbionts in cassava whitefly nymphs and adults

Arsenophonus (A3 strain, Section 3.3.4), *Rickettsia* (R2 strain, Section 3.3.4) (Figure 4-7), *Wolbachia* (W1 strain, Section 3.3.4) (Figure 4-8) and *Portiera* (Figure 4-9) were detected in the bacteriocytes of adult cassava whiteflies. These were not detected in any other organs in the nymph and adults. All bacteria were mostly found localised to the periphery of bacteriocyte giant cells.

All results were as expected and in agreement with qPCR results for the detection of S-symbionts in the isofemale lines. *Portiera* was the only bacteria detected in SSA1-SG3 AR- population that was known to be free of S-symbionts (Table 4-3, Figure 4-9 and Figure 4-10). Only *Wolbachia* and *Portiera* were detected in SSA1-SG2 W+ isofemale population (Figure 4-8, Figure 4-11) and both *Arsenophonus* and *Rickettsia* were co-localised in SSA1-SG3 AR+ population doubly infected with *Arsenophonus* and *Rickettsia* (Figure 4-7) with confirmed absence of *Wolbachia* (Figure 4-12).

Rickettsia was found scattered in the adult abdomen and in nymphs of MED (Israel) whiteflies used as a positive control in this study; whereas, *Arsenophonus* was only present inside the bacteriocytes (Figure 4-13).

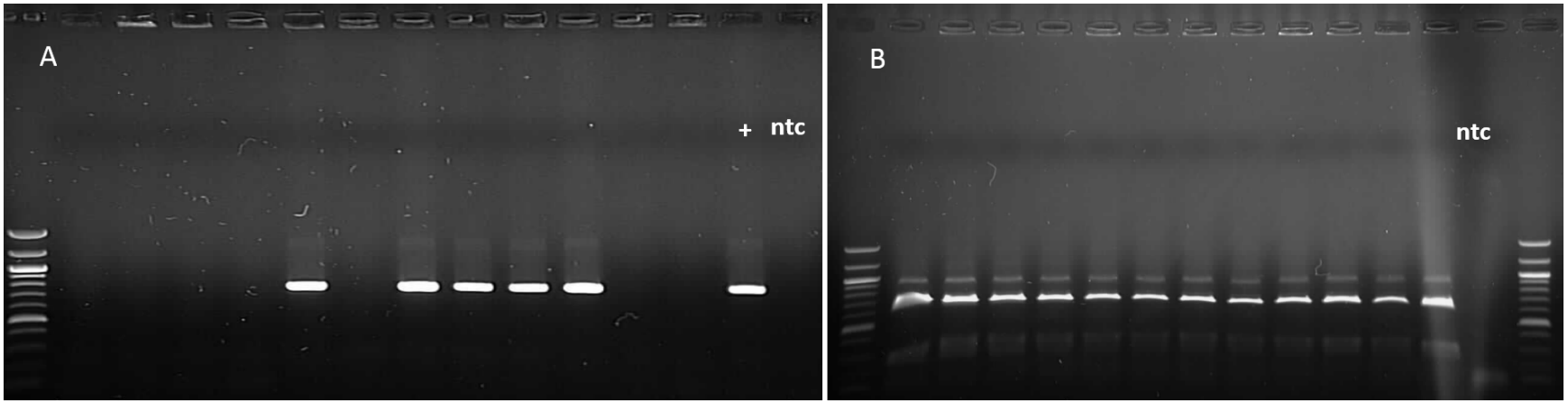


Figure 4-3: (A) PCR screening of SSA2 isofemale lines for presence of *Wolbachia* (750 bp product, 100 bp ladder, ntc= No template control). (B) Confirmation of SSA2 genetic background by digestion of mtCO1 PCR products with *Bgl* II restriction endonuclease (100 bp ladder).

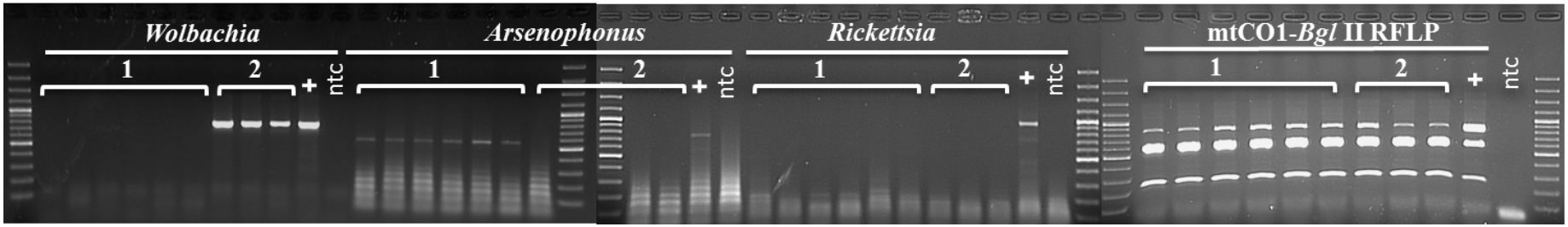


Figure 4-4: Two isofemale lines (1, 2) of SSA2 whiteflies with *Wolbachia* (750 bp) and *Arsenophonus* (550bp) infections and confirmation of SSA2 genetic background by RFLP of mtCO1 with *Bgl* II (100 bp ladder, ntc= No template control).

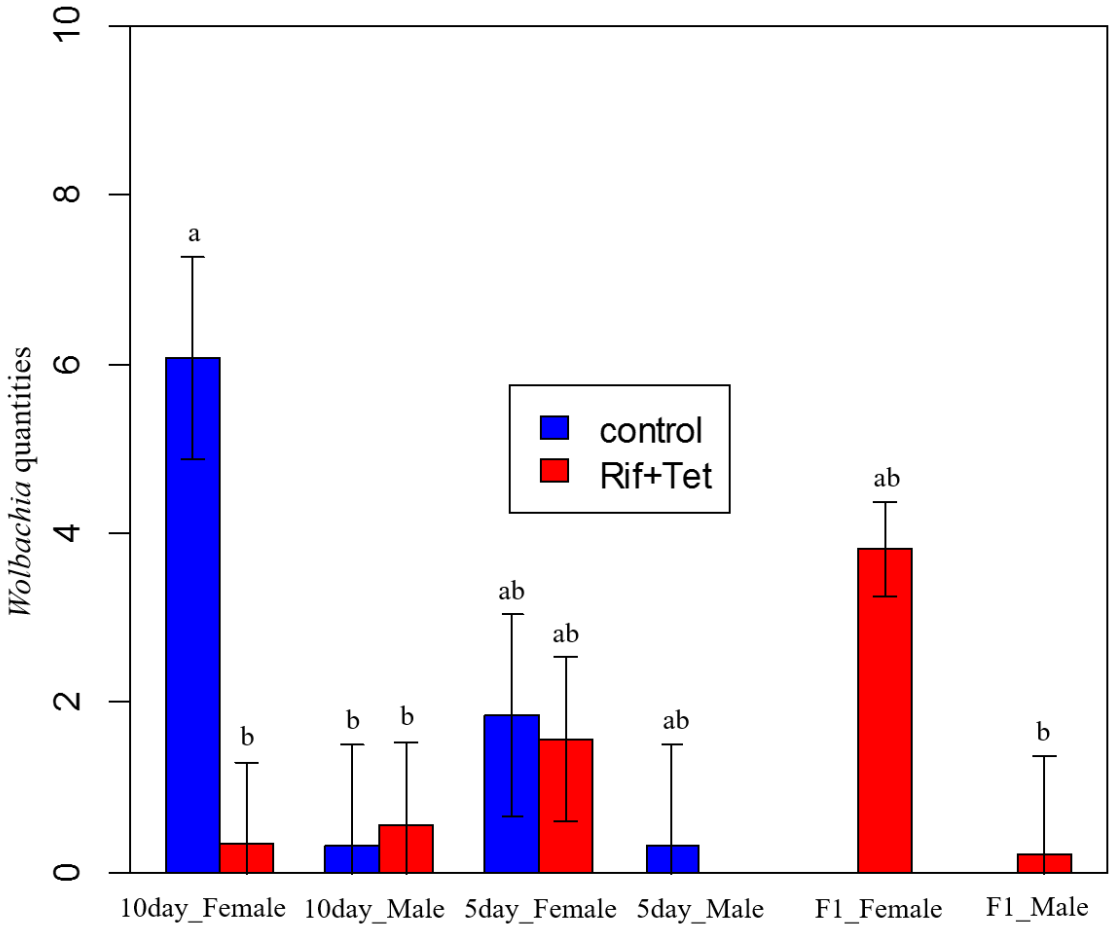


Figure 4-5: Relative quantities of *Wolbachia*: 5 and 10 days after feeding on diet with rifampicin+tetracycline (250µg/ml) in SSA1-SG2 W+ adults and in F1 progenies. Different letters indicate significant differences at $P \leq 0.5$. SSA1-SG2 W+ thus could not be cured of *Wolbachia*.

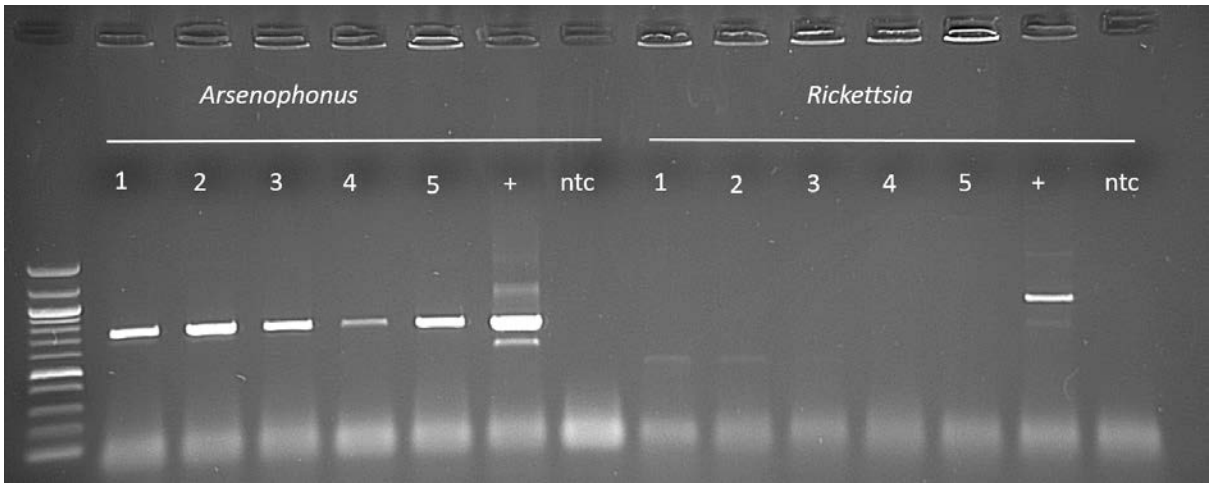


Figure 4-6: Selective curing of *Rickettsia* from SSA1-SG3 isofemale lines doubly infected with *Arsenophonus* and *Rickettsia* by rifampicin (100 bp ladder, ntc: no template control).

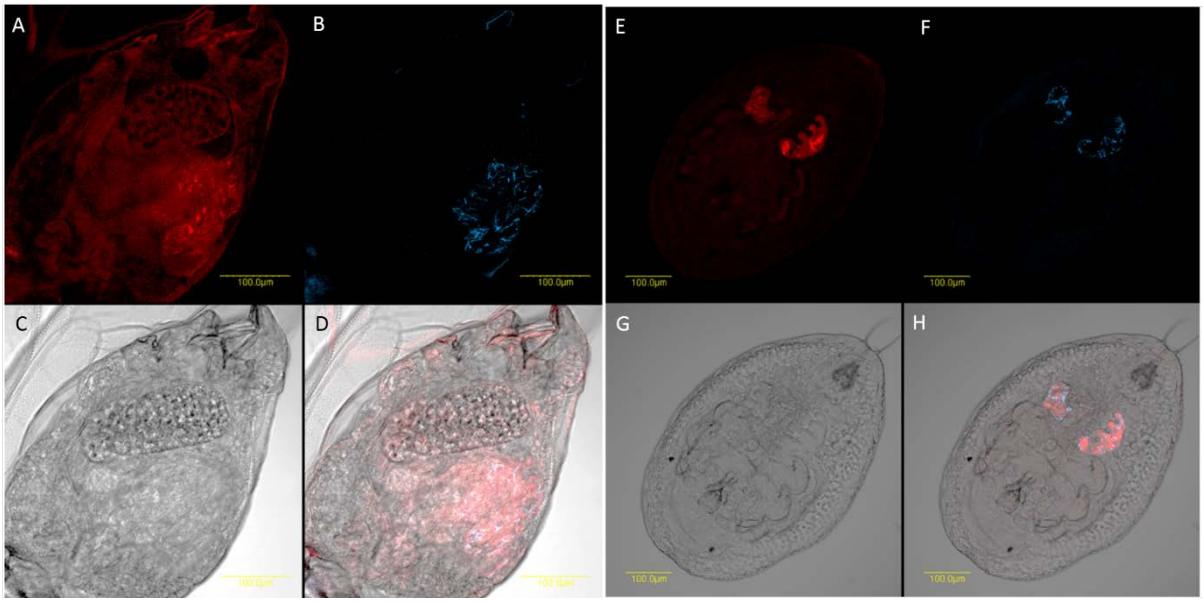


Figure 4-7: Localisation of *Rickettsia* and *Arsenophonus* in adults and nymphs of SSA1-SG3 AR+. *Rickettsia* (red) in adult abdomen (A) and nymph (E) in dark field; *Arsenophonus* (Blue) in adult abdomen (B) and nymph (F) in dark field; adult abdomen (C) and nymph (G) in bright field; Overlay of *Rickettsia* and *Arsenophonus* in adult abdomen (D) and nymph (H) in bright field. *Rickettsia* and *Arsenophonus* was co-localised only in inside the bacteriocytes in adults and bacteriome in nymphs.

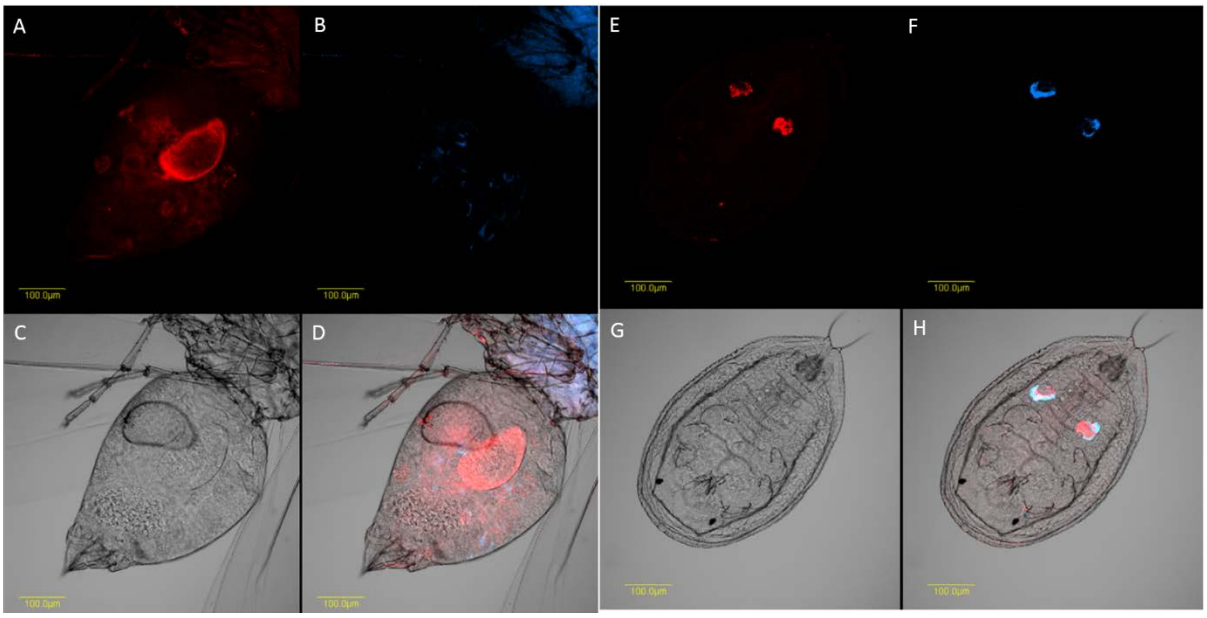


Figure 4-8 Localisation of *Portiera* and *Wolbachia* in adults and nymphs of SSA1-SG2 W+. *Portiera* (red) in adult abdomen (A) and nymph (E) in dark field; *Wolbachia* (blue) in adult abdomen (B) and nymph (F) in dark field; adult abdomen (C) and nymph (G) in bright field; Overlay of *Portiera* and *Wolbachia* in adult abdomen (D) and nymph (H) in bright field. *Portiera* (red) and *Wolbachia* (blue) were co-localised only in the bacteriocytes in adults and bacteriomes in nymphs.

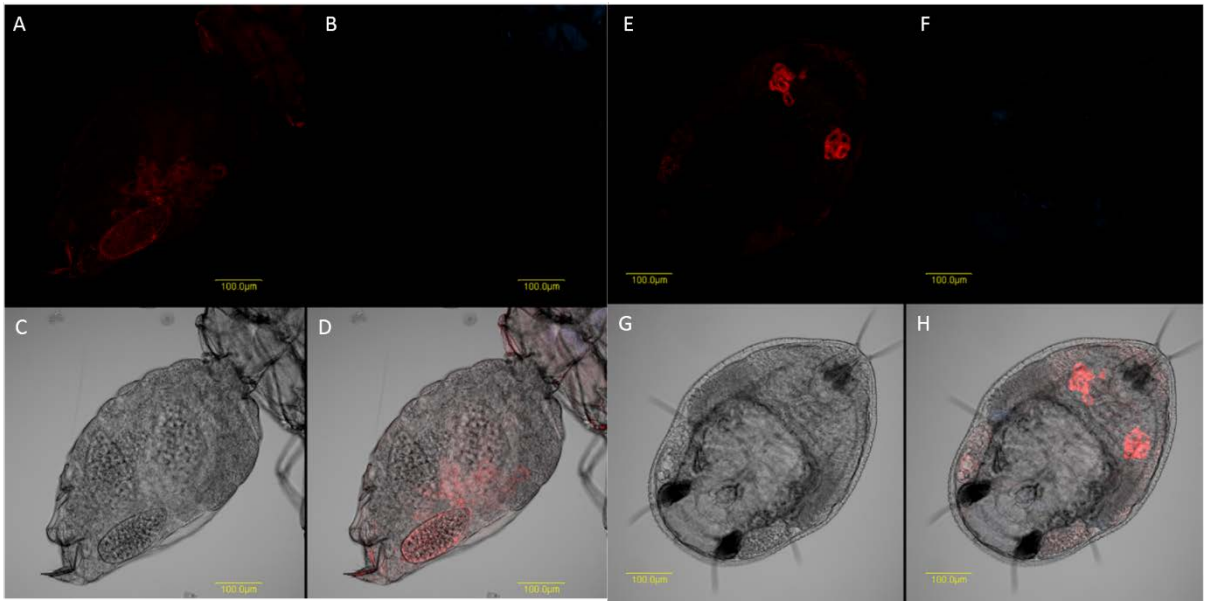


Figure 4-9: Localisation of *Portiera* and *Wolbachia* in adults and nymphs of SSA1-SG3 AR-. *Portiera* (red) in adult abdomen (A) and nymph (E) in dark field; *Wolbachia* (Blue) in adult abdomen (B) and nymph (F) in dark field; adult abdomen (C) and nymph (G) in bright field channel; Overlay of *Portiera* and *Wolbachia* in adult abdomen (D) and nymph (H) in bright field. Only *Portiera* (red) was detected and *Wolbachia* was confirmed absent.

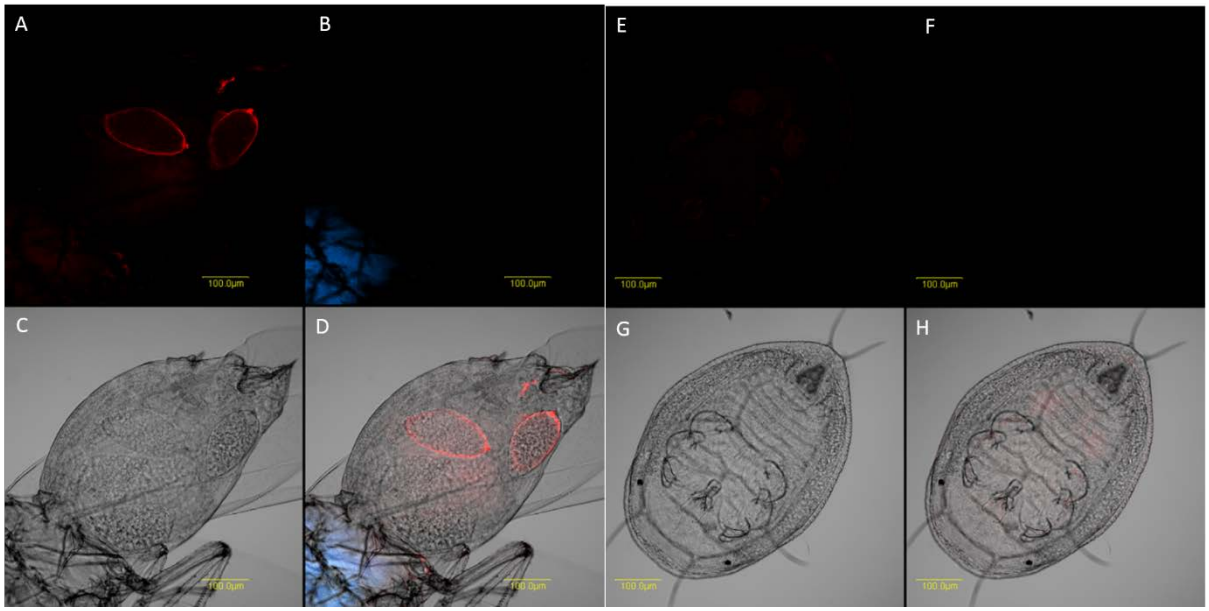


Figure 4-10: Localisation of *Rickettsia* and *Arsenophonus* in adults and nymphs of SSA1-SG3 AR-. *Rickettsia* (red) in adult abdomen (A) and nymph (E) dark field; *Arsenophonus* (blue) in adult abdomen (B) and nymph (F) in dark field; adult abdomen (C) and nymph (G) in bright field; Overlay of *Rickettsia* and *Arsenophonus* in adult abdomen (D) and nymph (H) in bright field. *Arsenophonus* (red) and *Rickettsia* (blue) signals could not be detected and confirmed absent. Red signals in A and D were auto fluorescence from egg cuticle.

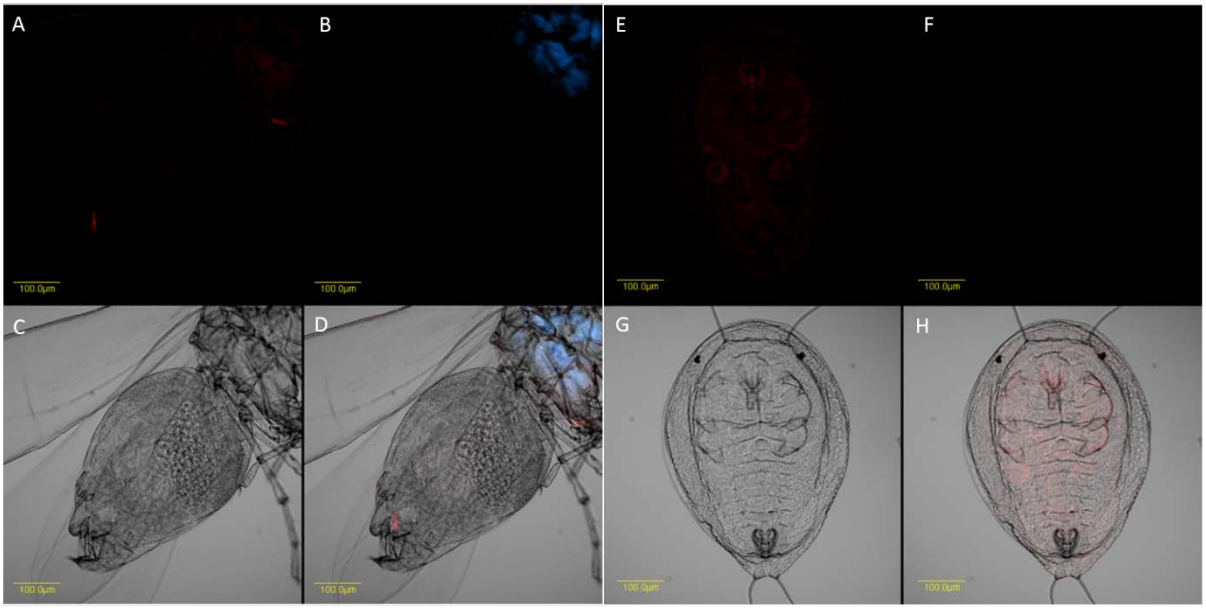


Figure 4-11: Localisation of *Rickettsia* and *Arsenophonus* in adults and nymphs of SSA1-SG2 W+. *Rickettsia* (red) in adult abdomen (A) and nymph (E) in dark field; *Arsenophonus* (blue) in adult abdomen (B) and nymph (F) in dark field; adult abdomen (C) and nymph (G) in bright field channel; Overlay of *Rickettsia* and *Arsenophonus* in adult abdomen (D) and nymph (H) in bright field. *Rickettsia* and *Arsenophonus* signals could not be detected and was confirmed absent.

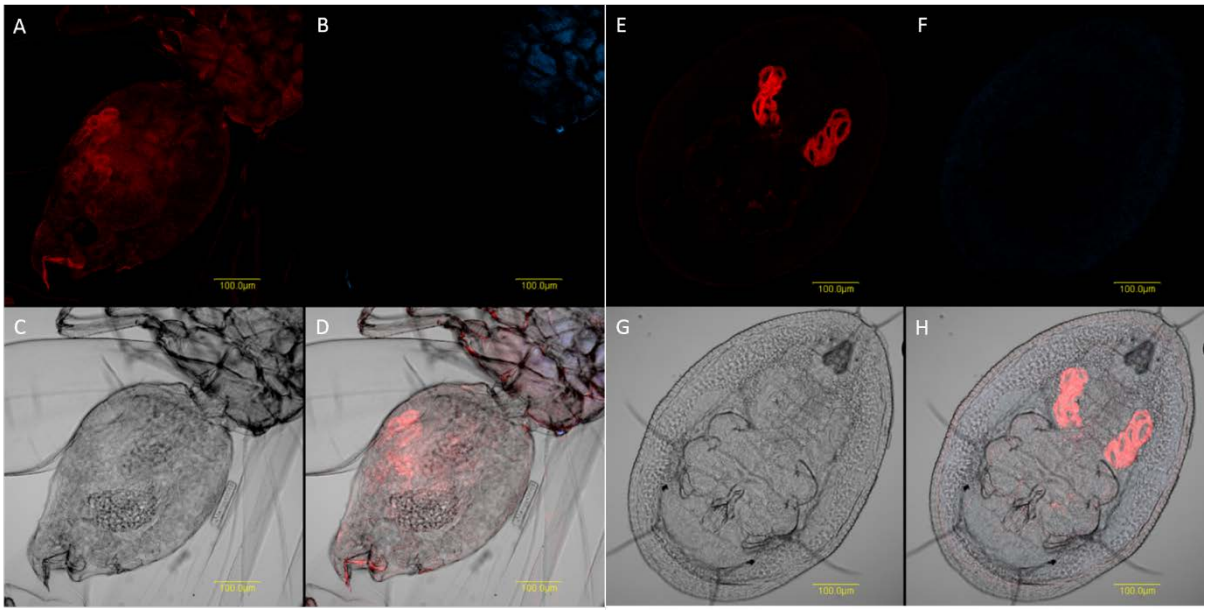


Figure 4-12: Localisation of *Portiera* and *Wolbachia* in adults and nymphs of SSA1-SG3 AR+. *Portiera* (red) in adult abdomen (A) and nymph (E) in dark field; *Wolbachia* (blue) in adult abdomen (B) and nymph (F) in dark field; adult abdomen (C) and nymph (G) in bright field; Overlay of *Portiera* and *Wolbachia* in adult abdomen (D) and nymph (H) in bright field. Only *Portiera* (red) was detected and *Wolbachia* (blue) was confirmed absent.

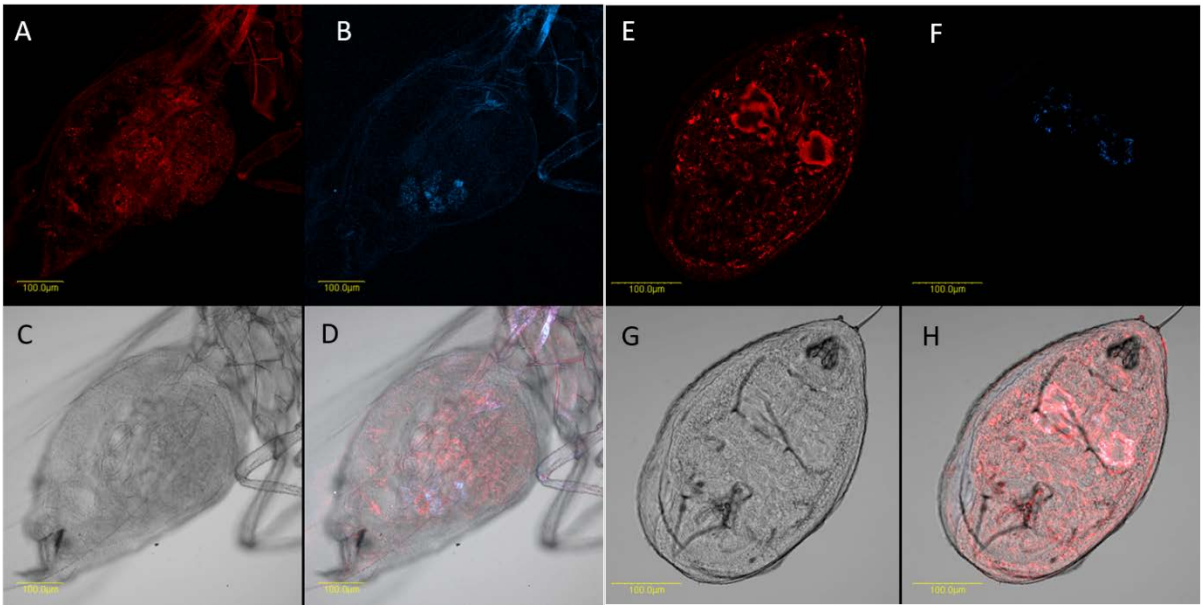


Figure 4-13: Localisation of *Rickettsia* and *Arsenophonus* in adult and nymphs of MED (Israel). *Rickettsia* (red) in adult abdomen (A) and nymph (E) in dark field; *Arsenophonus* (blue) in in adult abdomen (B) and nymph (F) in dark field; adult abdomen (c) and nymph in bright field; overlay of *Rickettsia* and *Arsenophonus* in adult abdomen (D) and nymph (H) in bright field. *Rickettsia* was found scattered inside the nymph and adult.

4.4 Discussion

Generating isofemale lines of cassava whiteflies with single or multiple infections of S-symbionts was a tedious and difficult job, and thus has been a major bottleneck for characterising different phenotypic traits of the symbionts. The failure of antibiotics to permanently cure whiteflies from infections of symbionts (except *Rickettsia*) makes the task even more difficult. Additional factors such as handling of the insects, low fecundity, slow development time, only male progenies in the F1 generation and potential contaminations from other whiteflies presented additional challenges. Generation of a successful cassava whitefly isofemale colony requires a minimum of 6-7 months. Isofemale lines are important in assessing the nature and range of phenotypic variation in natural populations especially for traits involved in determining the distribution and abundance of organisms (Hoffmann and Parsons, 1988). Six isofemale lines with different endosymbiont infections were generated in this study and they were crucial for studying the effect of S-endosymbionts on the biology of cassava whiteflies such as mating barriers, virus transmission and fecundity.

Arsenophonus, Rickettsia, Wolbachia as well as *Portiera* were detected in the bacteriocytes of cassava whitefly nymphs and adults. They were not scattered throughout the body as was seen in some other species (Gottlieb *et al*., 2006; Bing *et al*., 2014; Marubayashi *et al*., 2014).

All known reproductive manipulators such as *Wolbachia*, *Rickettsia*, and *Cardinium* are known to have a scattered phenotype in whiteflies (Gottlieb *et al*., 2006; Marubayashi *et al*., 2014; Skaljac *et al*., 2010). The confined phenotype of all tested symbionts in this study may indicate that these symbionts may not be involved in reproductive manipulation of cassava whitefly as it requires infection of reproductive organs (Gottlieb *et al*., 2008). Their role in cassava whiteflies therefore remains to be determined.

Both scattered and confined phenotypes of *Rickettsia* have been described in different individuals of the same whitefly populations (Caspi-Fluger *et al*., 2011; Gottlieb *et al*., 2008; Gottlieb *et al*., 2006). This variation in *Rickettsia* localisation could be the result of host factors which regulate symbiont mobility outside the bacteriocyte (Caspi-Fluger *et al*., 2011). In this study, *Rickettsia* R2 strain was found to be of confined phenotype. The R1 strain is mostly found in the invasive MEAM1 and MED populations but also detected by PCR in few cassava whiteflies from field collections (section 3.3.4) and can have a confined or scattered phenotype. Localisation of R1 in cassava whiteflies was not done but necessary

in future studies as it facilitates virus transmission. The *Rickettsia* with scattered phenotype (R1 strain) was also co-localised on the insect guts along with *Tomato yellow leaf curl virus* (TYLCV) in MEAM1 and this increased TYLCV transmission (Kliot *et al*., 2014).

Wolbachia has variable locations inside whiteflies; it is found confined within bacteriocytes in nymphs, but either have a scattered or confined phenotype (Bing *et al*., 2014; Gottlieb *et al*., 2008) or only confined phenotype within individual adult whitefly abdomen (Skaljac *et al*., 2010). The strict confinement of *Wolbachia* inside the bacteriocytes both in cassava whitefly nymphs and adults in this study may indicate their limited role in the reproduction of cassava whiteflies.

Co-infection of P- and S-symbionts inside a common host derived cell such as bacteriocytes specially meant to accommodate symbionts offers several advantages to the tenants (Gottlieb *et al*., 2008). Escape of symbionts from the bacteriome to the body cavity triggers wide immune reactions in the host (Anselme *et al*., 2008; Reynolds and Rolff, 2008), while expression of immune genes inside the bacteriome are minimal (Anselme *et al*., 2008). Thus sharing of space inside the bacteriome could be an adaptation by the symbionts to evade the whitefly immune system. Colonisation of the bacteriocytes by both P- and S- symbionts also allows efficient vertical transmission of symbionts through easy migration of bacteriocytes to the ovaries and entry inside the eggs during oogenesis (Gottlieb *et al*., 2008). However, sharing common space has its downfall. This creates competition for space and nutrition (Skaljac *et al*., 2010; Vautrin and Vavre, 2009), resulting in lower density of P-symbiont which is detrimental to host fitness (Gottlieb *et al*., 2008), better known as a phenomenon called ‘tragedy of commons’ (Hardin, 1968). Results on the prevalence of symbionts (Chapter 3) show that single infections of S-symbionts (59% of total infections) were more prevalent than double (37%) and triple infections (4%) in cassava whiteflies. This could be due to competition for space inside the bacteriocyte whereas, in invasive MEAM1 and MED populations where scattered phenotypes of symbionts were also found (Gottlieb *et al*., 2006, Skaljac *et al*., 2010; Marubayashi *et al*., 2014), the frequency of multiple infections are high (Chiel *et al*., 2007; Gueguen *et al*., 2010).

Hamiltonella and *Arsenophonus* are restricted to the periphery of the bacteriocytes and have never been shown to coexist in the same individual in any of the population tested (Chiel *et al*., 2007). This suggests that they have had competing interests in the ancestral whitefly and possibly explains their mutually exclusive existence (Gottlieb *et al*., 2008). We could not

detect *Hamiltonella* in any of our cassava whitefly populations and this could be due to a high proportion of insects (40.5%) harbouring *Arsenophonus* (See section 3.3.3). The varied prevalence of S-symbionts among the different cassava whitefly populations and the fact that all of them share the same cell suggests varied stringency in regulation of the symbiont densities across the populations.

5 FITNESS COSTS ASSOCIATED WITH *ARSENOPHONUS* AND *RICKETTSIA* INFECTIONS IN SSA CASSAVA WHITEFLIES

5.1 Introduction

Microorganisms colonising insects contribute up to 10% of the insect's biomass and are often beneficial to its host (Douglas, 2015). However, endosymbiotic relationships between insects and microorganisms are mostly unequal, in which one associate usually takes more than the other (Bourtzis and Miller, 2003).

Diversity of S-symbionts infecting *B. tabaci* is dependent on host population, geographical location and host plants (Chiel *et al*., 2007; Gueguen *et al*., 2010; Pan *et al*., 2012; Bing *et al*., 2013a; Tajebe *et al*., 2015b). The nature of the association between the S-symbionts and *B. tabaci* also vary with the symbiont, host population and geographical location. For example, *Rickettsia* infections in Middle East-Asia Minor 1 (MEAM1) in U.S.A provide fitness benefits with higher adult progenies, female numbers and faster development time (Himler *et al*., 2011), but similar infections in Israel provided no clear benefits (Chiel *et al*., 2009). *Hamiltonella* infections of Mediterranean (MED) in China have similar benefits with higher fecundity, nymph survival and faster development time by suppressing plant defences (Su *et al*., 2013a, 2015a) and mitigate nutritional stress (Su *et al*., 2014). In contrast, *Arsenophounus* and *Cardinium* are detrimental on host biology. *Arsenophonus* infections of Asia II-1 populations in India reduced fecundity, nymph survival and adult life span (Raina *et al*., 2015). *Cardinium* infections of MED populations in China reduced fecundity, nymph survival and increased developmental time (Fang *et al*., 2014).

Virus infections of plants are also known to affect the biology of *B. tabaci* in both positive and negative ways depending on the virus species and whitefly population. Feeding of *B. tabaci* on plants infected with begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl China virus* (TYLCCV), *Tobacco curly shoot virus* (TbCSV) and Uganda variant of *East African cassava mosaic virus* (EACMV-UG) enhance the performance of MEAM1 and SSA populations, with increased fecundity, adult numbers and adult life span (Colvin *et al*., 2004, 2006; Jiu *et al*., 2007; Guo, *et al*, 2010). In contrast, some of these viruses had minimal or negative impact on MED and Asia II-2 populations in other studies (Jiu *et al*., 2007; Liu *et al*., 2009; Guo *et al*., 2010; Su *et al*., 2015b).

S-endosymbionts are also known to alter vector capabilities of *B. tabaci* by releasing a bacterial chaperonin, GroEL which is hypothesised to protect virions inside the hostile haemolymph environments (Gottlieb *et al*., 2010; Rana *et al*., 2012). *Hamiltonella* and *Rickettsia* are known to increase virus acquisition, retention and transmission efficiency of *B. tabaci* populations possibly due to the effect of GroEL protein (Su *et al*., 2013b; Kliot *et al*., 2014).

Sap sucking insects such as aphids and whiteflies lack important immune system genes and pathways (Gerardo *et al*., 2010; Zhang *et al*., 2014) but S-symbionts contribute to complement its host defences by protecting it against abiotic and biotic stresses. *Rickettsia* infections in *B. tabaci* are known to provide conditional benefits such as thermal tolerance (Brumin *et al*, 2011) and defence against entomopathogenic bacteria (Hendry *et al*., 2014). Similar effects are also shown in aphids (Chen *et al*., 2000; Montllor *et al*., 2002; Łukasik *et al*., 2013). *Hamiltonella* helps to mitigate nutritional stress in *B. tabaci* (Su *et al*., 2014) and provides defence against parasitoids by APSE- (*Acyrthosiphon pisum* secondary endosymbiont phage) encoded toxins (Degnan and Moran, 2008a, 2008b; Oliver *et al*., 2009; Oliver *et al*., 2003) in pea aphids. Interestingly, APSE is also abundant in *Arsenophonus* infecting whiteflies (Duron, 2014) and accounted for defence against parasitism in psyllids (Hansen *et al*., 2007). Acquisition of begomoviruses also triggers whitefly immune response to play an important role in degradation of virions inside the vector (Luan *et al*., 2011; Götz *et al*., 2012).

Understanding the role of endosymbionts and the effect of immune response they induce on the biology of cassava whiteflies and their interactions with virus is important to understand the recent upsurge in whitefly populations on cassava in SSA. Therefore, the aim of this study was to investigate the effect of the S-endosymbionts *Arsenophonus* and *Rickettsia* on the biology of cassava whitefly as well as EACMV-UG acquisition and retention. We also investigated whitefly immune response and associated fitness costs upon *Arsenophonus* and *Rickettsia* infections.

5.2 Materials and methods

5.2.1 Whitefly cultures and cassava plants

Two isofemale lines of the cassava whitefly species SSA1-SG3 with identical genetic background, but differing S-endosymbiont infection were used. One colony was dually infected with *Arsenophonus* and *Rickettsia* (AR+) and the other was free of S-symbionts (AR-). The isofemale lines were maintained in the insectary at 27 ± 3˚C, 60% relative humidity and photoperiod of L12:D12.

Cassava plants variety Ebwanateraka infected with EACMV-UG were grown in the NRI quarantine glasshouse. Two month old plants were used for the whitefly biology experiments. Infection by EACMV-UG only was confirmed by PCR diagnostics tests as described before (Zhou *et al*., 1997; Alabi *et al*., 2008) (Fig. 5-1). Plants were grown in 10 x 10 cm plastic pots containing John Innes no. 2 compost and soil in equal mixture at 28 ± 5 °C and 50-60% relative humidity.

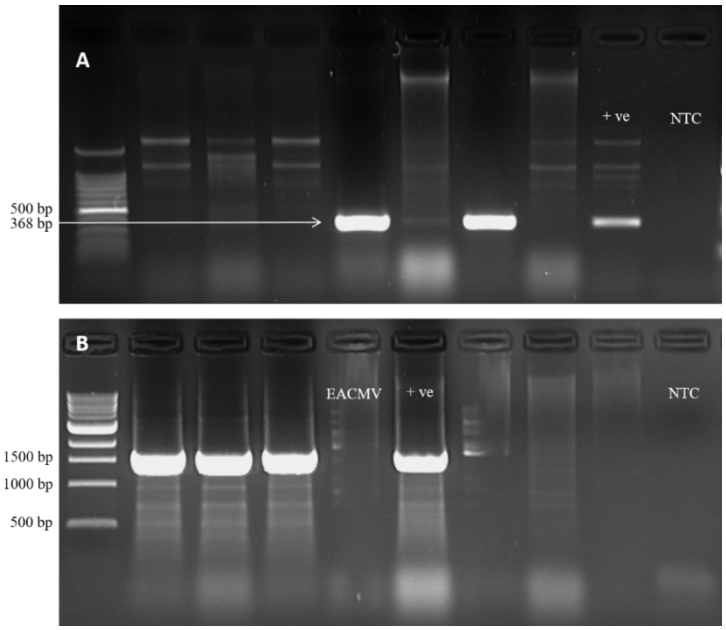


Figure 5-1: Screening for CMBs in cassava varieties in NRI quarantine glasshouse.

A) Screening for ACMV using CMB Rep/F and ACMVRep/R primers (368 bp) (Alabi *et al*., 2008). Lane (1= 100 bp ladder, 2-4= EACMV-UG, 5, 7= ACMV, 6= EACMV, 7= Healthy cassava, 9= ACMV positive control), NTC= no template control.
B) Screening for EACMV-UG using UV-AL/F1 and ACMV CP/R3 (1300 bp) (Zhou *et al*., 1997). Lane (1= 1 kb ladder, 2-4= EACMV-UG, 5= EACMV, 6= positive EACMV-UG, 7, 8= ACMV, 9= healthy cassava), NTC= no template control.

5.2.2 Whitefly fitness assays

All adult whiteflies were removed from two cassava plants from each of AR+ and AR- colonies containing many pupae ready to emerge. Newly emerged adults were collected after two days and anesthetized using carbon dioxide for 5 seconds. Insects were placed on a pre-chilled black coloured Whatman filter paper on a petri plate on an ice box for separating males from females using a stereo-binocular microscope. One female and two male adult whiteflies were confined on the underside of a cassava leaf of two months old Ebwanateraka plants in 25 mm diameter clip cages for oviposition. Each plant was set up with 4-9 clip cages depending upon the number of leaves on the plants. The experiment was set up with 30 virus infected and healthy plants each for AR+ and AR- whiteflies. The clip cages were removed after 12 days and the eggs and nymphs were counted using a stereo binocular microscope.

The leaves were enclosed in perforated bread bags and the number of nymphs developed was monitored every three days under a microscope. The average number of eggs hatching was recorded as total number of nymphs/total number of eggs. Emerged adults were collected every three days, separated into males and females and recorded. The total emergence of adults from nymphs were recorded every three days by counting empty pupal cases under microscope. The mean adult emergence (total number of adults emerged/total number of nymphs developed) and mean adult development time (from day 1 to final adult emergence) were also recorded for each colony.

5.2.3 Statistical analysis

All statistical analysis were done using the R software (R Development Core Team, 2011). Mean fecundity of the adult whiteflies were analysed using a generalised linear model with negative binomial errors with log link function. Fecundity of the adult *B. tabaci* was used as the dependent variable and the presence or absence of S-endosymbionts, virus infection of plants and their interactions were used as independent variables. Statistical inference was based on the resulting analysis of deviance and estimated standard errors. The mean proportion of nymphs developed was analysed using a generalised linear model with quasibinomial errors with logit link function.

Similarly, the mean proportion of adult emergence of the F1 generations were analysed using a generalised linear model with binomial errors with logit link function. The differences in

means of adult emergence among the different variables were compared by Tukey's HSD test using the glht function from the multcomp package of R (Hothorn *et al*., 2008). The mean adult development time of F1 generations were analysed using a simple linear model. The mean differences in adult development time were compared by Tukey's HSD test.

5.2.4 Acquisition and retention of EACMV-UG

Adult AR+ and AR- whiteflies were given acquisition access period (AAP) for 48 hours on a three month old EACMV-UG infected plant var. Ebwanateraka in a large cage. Twenty-five AR+ or AR- viruliferous whiteflies were then given inoculation access period (IAP) of 48 hours on each of two months old healthy cassava plants. The experiment had three replications and a total of thirty healthy plants were inoculated. The AR+ and AR- whiteflies were collected after 48 hours AAP and IAP from all plants of the three replications for the detection and quantification of virus titres in single whiteflies.

Total DNA from each whitefly female from AAP and IAP experiments was extracted separately using 20% Chelex as described in section 3.2.2. Quantitative real time PCR was performed with CFX96 Real time PCR detection system (Bio-Rad) with EXPRESS qPCR Supermix (ThermoFisher Scientific, UK). The whitefly tubulin gene was used as the reference for relative quantification. Primers and probe were designed in this study using whitefly tubulin sequence from NCBI database (KC161212). Multiplex detection of EACMV-UG and the whitefly tubulin gene in a single reaction with the hydrolysis probes (Table 5-1) was done to quantify virus titres in individual whitefly. The assay was standardized by using primers and probes in different concentrations. Singleplex reactions were tested for the detection of whitefly tubulin and EACMV-UG for comparison with the multiplex assay. The standardized assay was conducted in 20 µl reactions containing 10 µl of 2X qPCR super mix, 300 nM of tubulin primers, 500 nM of EACMV primers, 100 nM of each probe and 3 µl of DNA extract from individual whiteflies. PCR conditions of 95 °C for 2 minutes followed by 40 cycles of 94 °C for 15 seconds, annealing at 54 °C for 20 seconds and extension at 60 °C for 30 seconds were used for the assay. Each sample was tested in duplicates. The Cq values were determined by single threshold method and relative EACMV-UG quantities were calculated using whitefly β tubulin gene as a reference gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A common AR+ sample was used across the plates as the quantification calibrator sample.

Table 5-1: Primers used for quantifying EACMV-UG in cassava whitefly.

Target	Primer/ probe sequence (5'→3')	Product	Reference
α- Tubulin	F- TGTACCGAGGAGATGTTGTG	113 bp	This study
B. tabaci	R- GATACCGACCTTGAAACCAG		
	Probe- Cy5- ATGCTGCCATCGCCACCATC- BHQ3		
EACMV/ EACMV-UG	CMBRep/F- CRTCAATGACGTTGTACCA	113 bp	Otti *et al*., 2016
	Neweac-alt/R- CATGGAGACCGATCAGTATTGTTC		
	Probe- FAM- TCTTKGGAG/		
	ZEN/ACAGATCCAGGTGTCCACAT-IABkFQ		

The mean quantities of EACMV-UG for AAP and IAP in AR+ and AR- whiteflies were calculated using a simple linear model with $\log_e$ transformed data to fit parametric analysis. Relative virus quantities were used as the dependent variable and the two whitefly populations as independent variables. Statistical inference was based on the resulting one-way analysis of variance (ANOVA), the means and standard errors were extracted from the ANOVA model and de-transformed to produce natural values. Statistical significance of the frequency of AR+ and AR- whiteflies carrying EACMV-UG after 48 hours each of AAP and IAP was estimated in contingency tables using Fisher's exact test at $P < 0.05$.

5.2.5 EACMV-UG transmission efficiency by AR+ and AR- whiteflies

Virus inoculated plants were kept in cages for 60 days post inoculation for expression of symptoms. The inoculated leaf was removed from the plants 20 days after IAP. Total nucleic acids were extracted from 100 mg of fresh cassava leaf tissue using the CTAB extraction method as previously described (Maruthi *et al*., 2002). Two µl of the DNA lysate was used for detection of EACMV-UG using the previously described primer pairs (Table 5-1) and PCR conditions. Statistical significance of the transmission efficiency by AR+ and AR- whiteflies was estimated in contingency table using Fisher's exact test at $P < 0.05$.

5.2.6 Quantification of whitefly immune genes by qPCR

Total RNA was extracted from pooled samples of 15 individuals (10 females and 5 males) in twenty replicates using TRIzol® reagent (ThermoFisher Scientific, UK) as described previously (Pakkianathan *et al*., 2015). The extracted samples were treated with DNase I (ThermoFisher Scientific, UK) to remove genomic DNA according to manufacturer's

instructions. RNA yields were quantified using NanoDrop 2000 (ThermoFisher Scientific, UK). A total of 150 ng of whitefly RNA was used as template for first strand cDNA synthesis using RevertAid H Minus First strand cDNA synthesis kit (ThermoFisher Scientific, UK). Whitefly immune response genes (Table 5-2) were amplified using 1 µl of cDNA in qPCR using DyNAmo Flash SYBR green qPCR kit (ThermoFisher Scientific, UK). Whitefly α-tubulin gene was used as the reference gene and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Mean relative expression of immune response genes in AR+ and AR- whiteflies were determined using simple linear model with log transformed data to allow parametric analysis. Relative expression of immune response genes was used as the dependent variable and the two whitefly populations as independent variable. Statistical inference was based on the resulting one-way analysis of variance (ANOVA), the means and standard errors were extracted from the ANOVA model and de-transformed to produce natural values.

Table 5-2: Primers used for quantifying whitefly immune genes.

Target gene	**Primer sequence (5'→3')**	**Product size**	**Function**	**Reference**
α- Tubulin *B. tabaci*	F- TGTACCGAGGAGATGTTGTG R- GATACCGACCTTGAAACCAG	113 bp	Structural protein	This study
Knottin 1 (K1)	F- TCCTGGACATTGCACAACCA R- TTTCGGAGGGATTGGAATGA	81 bp	Antimicrobial peptide	Mahadav *et al*., 2009
Knottin 2 (K2)	F-CTGTTCCAAGCCAAAACCGA R-GATCATGAAGGCGGCCACTA	81 bp	Antimicrobial peptide	Mahadav *et al*., 2008
Knottin 3 (K3)	F- CATGGTCGCTGTCAACGTCT R- TTGCAACTGGCACCTTTGG	81 bp	Antimicrobial peptide	Mahadav *et al*., 2009
atg-9	F- AGGGTTCCTGGTTCACGC R-TTGCCATCATTAACTTTCTGCT		Autophagy	Luan *et al*., 2011

5.3 Results

5.3.1 Fecundity and nymph development

A total of 89 and 63 clip cages with healthy and EACMV-UG-infected plants, respectively of SSA1-SG3 AR+ and 83 and 50 clip cages of SSA1-SG3 AR- had live females after 12 days. Only these were used for the analysis of fitness assays. Infection of whiteflies by the endosymbionts, or infection of cassava plants by EACMV-UG had no significant effect on the fecundity of cassava whiteflies. AR+ whiteflies had slightly higher mean oviposition

(27.9 ± 2.63 eggs) on virus infected plants compared to healthy plants (23 ± 1.84 eggs). AR- whiteflies laid almost identical number of eggs both on virus-infected (22.4 ± 1.84 eggs) and healthy (22.1 ± 2.3 eggs) plants (Fig. 5-2A) and thus were not statistically significant (Table 5-3). The mean proportion of nymphs developed by AR+ and AR- whiteflies was also similar on healthy plants, but slightly higher (P = 0.04) on virus-infected plants (Fig. 5-2B, Table 5-4).

Table 5-3: Analysis of deviance on the fecundity of AR+ and AR- whiteflies on healthy and EACMV-UG infected cassava plants

	df	**Deviance**	**Residual df**	**Residual deviance**	***P* value (Chi)**
symbiont	1	1.70	284	305.88	0.19
virus	1	1.21	283	304.66	0.27
symbiont:virus	1	1.28	282	303.38	0.25

Table 5-4: ANOVA on mean proportions of nymphs developed for AR+ and AR- whiteflies on healthy and EACMV-UG infected cassava plants.

	df	**Deviance**	**Residual df**	**Residual deviance**	***F* value**	***P* value**
symbiont	1	0.003	284	562.28	0.002	0.96
virus	1	7.02	283	555.26	4.17	0.04*
symbiont:virus	1	0.25	282	555.00	0.15	0.69

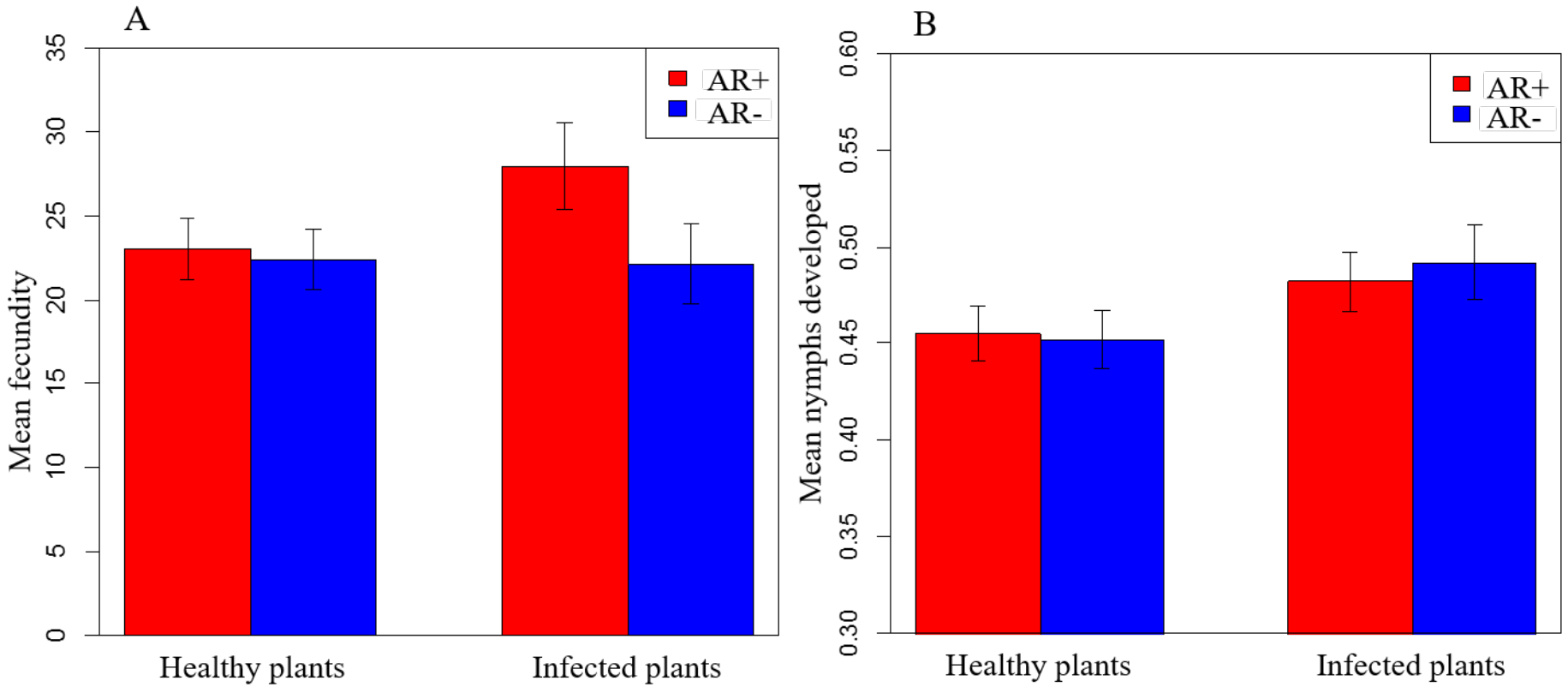


Figure 5-2: Mean fecundity (A) and proportion of nymphs developed (B) by AR+ and AR- whiteflies on healthy and EACMV-UG infected cassava plants. There was no significant difference in fecundity and nymph development between AR+ and AR-.

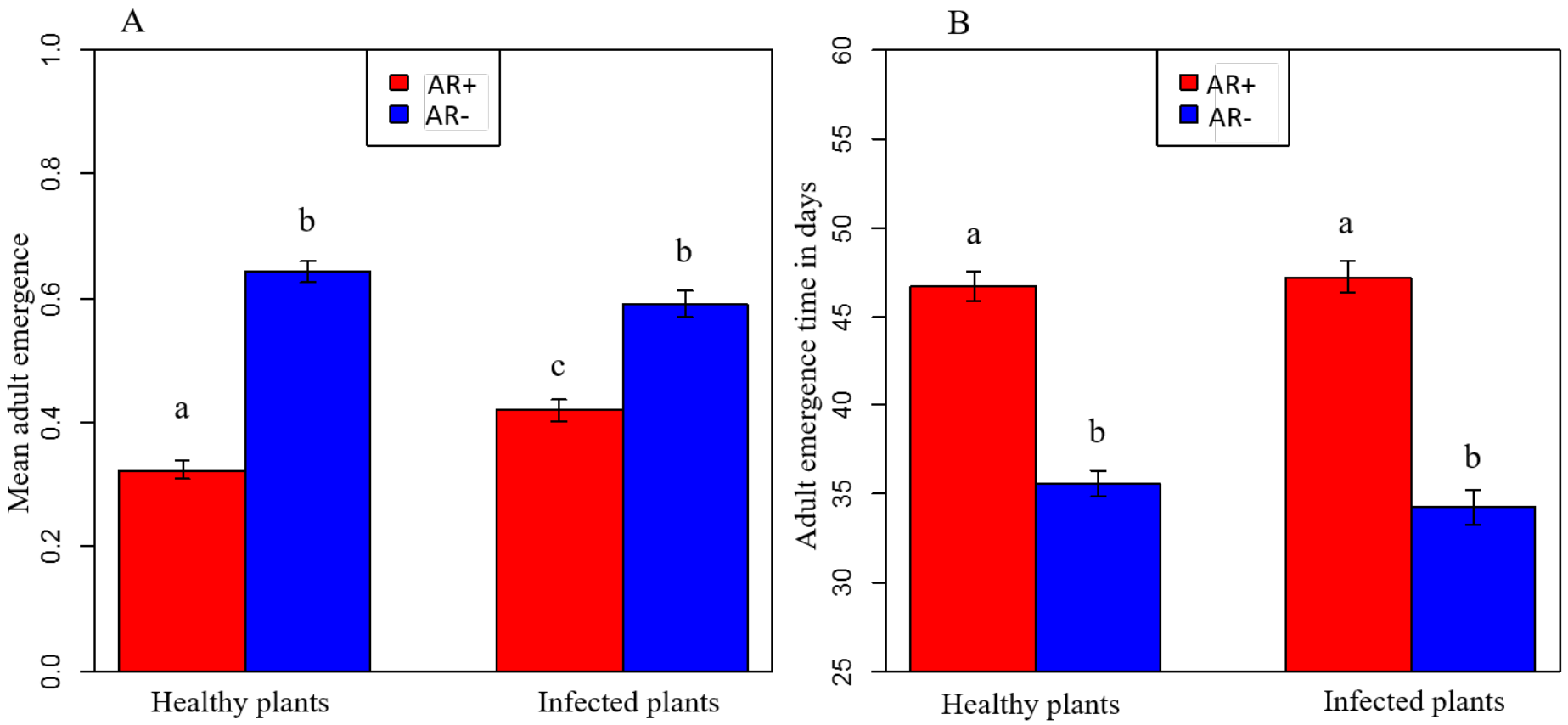


Figure 5-3: Mean proportion of adult emergence (A) and adult development time (B) in AR+ and AR- whiteflies on healthy and EACMV-UG infected cassava plants. Adult emergence was higher and quicker in AR- than AR+. Thus whiteflies free of symbionts form higher number of adults and multiply quicker.

5.3.2 Adult emergence and development time

Mean proportion of adults emerging from AR- whiteflies on both healthy (0.64) and virus-infected plants (0.59) was significantly higher ($P < 0.001$) than AR+ on healthy (0.32) and virus-infected (0.42) plants (Fig. 5-3A, Appendix C).

Mean adult development time was significantly shorter for AR- whiteflies on both healthy (35.5 days) and virus-infected (34.2 days) plants compared to AR+ whiteflies (46.7 and 47.2 days, respectively) (Fig. 5-3B, Appendix D).

5.3.3 Detection and quantification of EACMV-UG in single cassava whiteflies

The amplification of whitefly tubulin gene with the newly designed primers and probe in this study was efficient (R^2= 0.996, Appendix E), which gave us the confidence to use this as a reference gene in rest of the study. The Cq values for both EACMV-UG and whitefly tubulin were in optimal range for quantification in both singleplex and multiplex reactions (Table 5-5). EACMV-UG primers at 500 nM concentration was chosen for the assay as they produced the best Cq and ΔCq value, while the Cq for tubulin still within 30 cycles (Table 5-5). Using 100 nM of probes was equally efficient in detecting both whitefly and virus targets with similar Cq values (Table 5-5). Increasing probe concentration to 200Nm did not increase reaction efficiency.

The average number of whiteflies acquiring EACMV-UG after 48 hours of AAP was significantly higher ($P = 0.02$, Fisher's exact test) for AR- than AR+ whiteflies. The virus was detected in 91.8% (45/49) of AR- whiteflies after AAP compared to 71.8% (28/39) of AR+ (Fig. 5-4A). The mean relative quantities of virus acquired by AR- was also higher than AR+ whiteflies (Fig. 5-4B, Table 5-6) but was statistically not significant ($F = 2.23$, $P = 0.14$, Appendix F). The percentage of AR- (87.65%, 71/81) whiteflies retaining EACMV-UG after IAP was similarly higher ($P = 0.0002$, Fisher's exact test) than AR+ (61.2%, 41/67) whiteflies (Fig. 5-4C). AR- also retained higher titres (~9 folds) of EACMV-UG than AR+ whiteflies ($F = 14.59$, $P = 0.0002$) (Fig. 5-4D, Table 5-6, Appendix G).

Table 5-5: Comparison of Cq values with different primer/probe concentrations in single and multiplex reactions for detecting EACMV-UG in single cassava whiteflies

Treatment		Tubulin	Tubulin	EACMV-UG	EACMV-UG	Δ Cq
Tubulin	EACMV-UG	Cq	(multiplex - singleplex) Cq	Cq	(multiplex – singleplex) Cq	
300 nM	-----	27.66 ± 0.05	-----	-----	-----	-----
-----	300 nM	-----	-----	26.25 ± 0.01	-----	-1.41
300 nM	300 nM	28.23 ± 0.04	0.57	24.36 ± 0.06	-1.89	-3.87
300 nM	400 nM	29.08 ± 0.05	1.42	23.92 ± 0.03	-2.33	-5.16
300 nM	500 nM	29.26 ± 0.06	1.6	23.35 ± 0.03	-2.9	-5.91
300 nM	600 nM	30.44 ± 0.2	2.78	23.28 ± 0.12	-2.97	-7.16
300 nM (100 nM probe)	300 nM (100 nM probe)	28.51 ± 0.03	0.85	24.4 ± 0.15	-1.85	-4.11

5.3.4 EACMV-UG transmission efficiency by AR+ and AR- whiteflies

AR- were more efficient in transmitting EACMV-UG to healthy cassava plants than AR+ whiteflies. AR- transmitted the virus to 37.1% plants (13/35) compared to 17.2% of plants (5/29) by AR+ whiteflies. The differences in transmission were however not statistically significant (P = 0.09, Fisher's exact test). No symptoms could be observed in any of the transmitted plants and thus the results were only based upon PCR detection. Infection of the plants by EACMV-UG was confirmed by qPCR tests, although the Cq values of virus in plants was above 35 cycles, which is in the borderline of detection limit. This indictaed that the virus was transmitted by whiteflies but was not multiplying in plants.

5.3.5 Relative expression of immune genes

All the three antimicrobial peptides Knottin1 (F = 14.52, $P \leq 0.001$, Appendix H), Knottin2 (F = 6.47, P = 0.015, Appendix I) and Knottin3 (F = 184, $P \leq 0.001$, Appendix J) were over expressed by minimum two folds in AR+ compared to AR- populations (Fig. 5-5). The

autophagy related (*atg-9*) gene was also upregulated in AR+ populations (F = 184, $P \leq$ 0.001, Appendix K) (Fig 5-5).

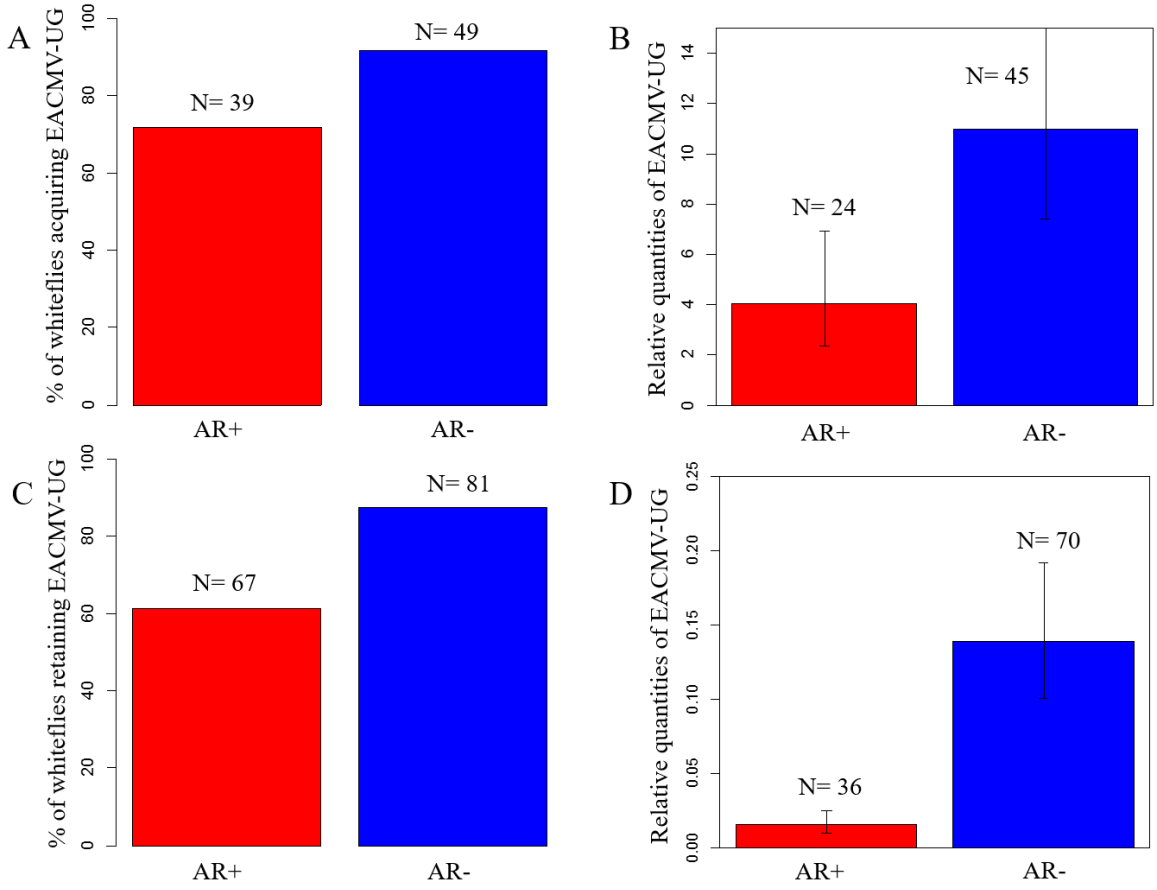


Figure 5-4: A) Percentage of SSA1-SG3 AR+ and AR- whiteflies acquiring EACMV-UG after 48 hours AAP. B) Relative quantities of EACMV-UG in AR+ and AR- after the AAP. C) Percentage of AR+ and AR- whiteflies retaining EACMV-UG after 48 hours IAP. D) Relative quantities of EACMV-UG in AR+ and AR- after the IAP. Higher proportion of AR- acquired and retained EACMV-UG than AR+. Quantities of virus acquired and retained was also higher in AR-.

Table 5-6: Mean relative quantities of EACMV-UG in single whiteflies after 48 hours AAP and IAP in AR+ and AR-

	Mean ± SE of EACMV-UG quantity	
	AAP	IAP
SSA1-SG3 AR-	10.97 ± 4.43	0.13 ± 0.045
SSA1-SG3 AR+	4.04 ± 2.29	0.015 ± 0.007

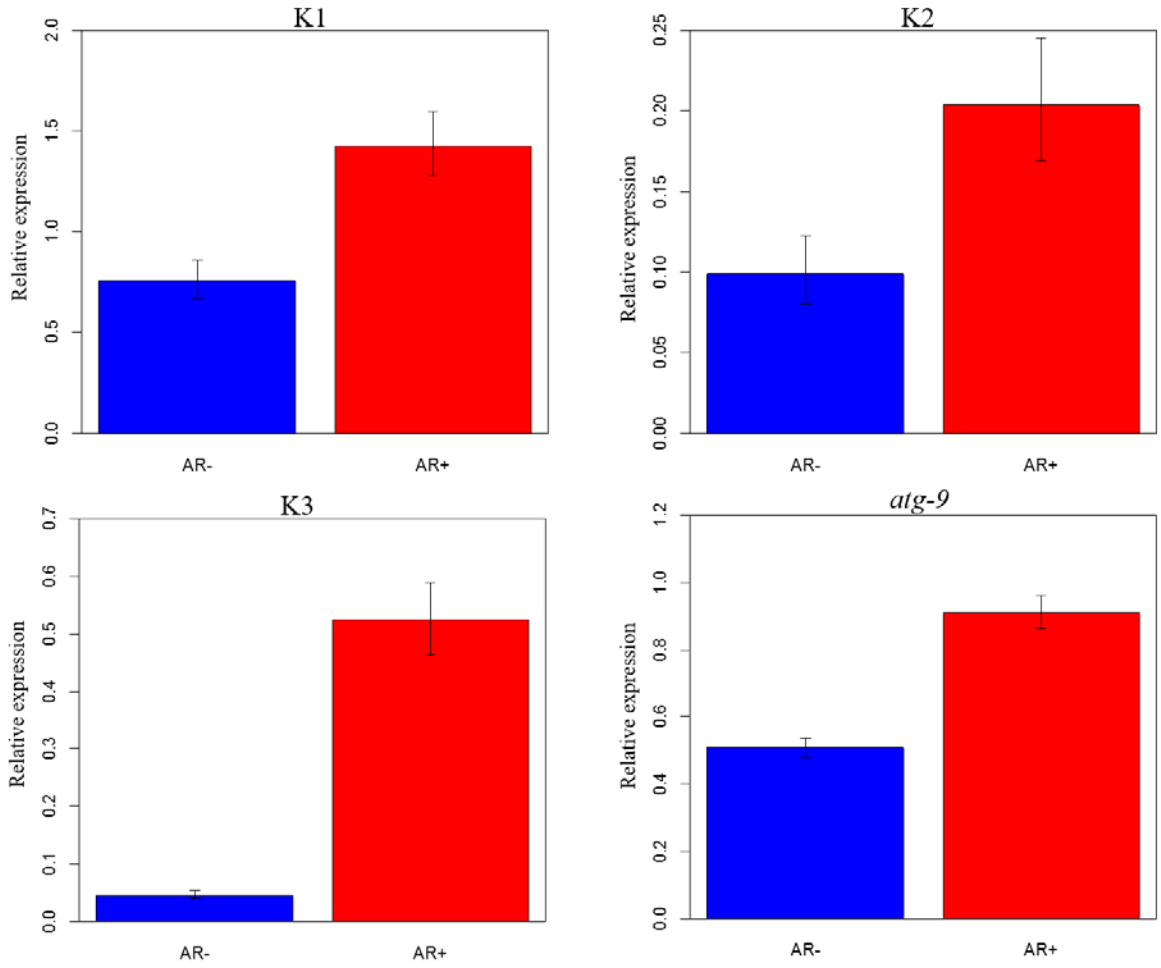


Figure 5-5: Relative expression of immune genes in SSA1-SG3 AR+ and AR- whiteflies. Expression of antimicrobial peptides and autophagy protein was higher in AR+ than AR-. Thus AR+ induce higher immune response in SSA1-SG3 whiteflies.

5.4 Discussion

We investigated fitness costs associated with *Arsenophonus* and *Rickettsia* infections of SSA1-SG3 to understand the implications of endosymbiont infections on cassava whiteflies. Under controlled laboratory conditions, AR+ infections had no effect on whitefly fecundity and nymph development. However, they had significant negative effect on adult development and life cycle (generation time). AR+ infections caused 50% reduction in the number of nymphs developing into adults and adult development time was delayed by 10 days compared to endosymbiont free AR- whiteflies. Both these parameters, if operational in cassava fields, will have significant negative impact on whitefly population development,

and thus the development of superabundant population. SSA1-SG3 has been only found in coastal East Africa (Mugerwa *et al*., 2012; Legg *et al*., 2013; Tajebe *et al*., 2015a), a region where whiteflies on cassava are very low. In a related study, 50% reduction in population was seen in mosquitoes infected with *Wolbachia* in *Drosophila* and mosquitoes (Min and Benzer, 1997; McMeniman and O'Neill, 2010). High numbers (84%) of SSA1-SG3 harbour *Arsenophonus* and/or *Rickettsia* (Chapter3, Figure 3-5). It is thus possible that the negative effects of *Arsenophonus* and *Rickettsia* infections have kept SSA1-SG3 populations under control in coastal areas. We also investigated the interaction between endosymbiont and EACMV-UG infections on whitefly biology. Virus infections of cassava increased the emergence of whiteflies infected with endosymbionts. However, EACMV-UG infections of plants, in contrast to the previous reports (Colvin *et al*., 2006) provided marginal benefits to cassava whitefly development and only slightly enhanced (3-4%) nymph emergence from eggs. This indicates that if mutually beneficial relationships exist between whiteflies and viruses, it will be specific to the super abundant populations because such an effect was not seen on the coastal SSA1-SG3 populations in this study. Further, investigations on virus acquisition and retention showed that a higher proportion of AR- flies (91.8%) acquired and retained EACMV-UG than AR+ (71.8%). AR- also retained higher titres of virus after 48 hours of IAP. These results are similar to those seen on mosquitoes in which *Wolbachia* infections caused significant reductions in the acquisition and transmission of mosquito-transmitted dengue virus (Osborne *et al*., 2009). This has led to using certain strains of *Wolbachia* as biocontrol agents to reduce dengue transmission in Australia, Vietnam and Brazil. Our results, albeit early and should be confirmed on other cassava whitefly species, however, suggest the possibility of using endosymbionts as potential biocontrol agents to reduce CMD and CBSD infections in Africa.

Symbionts must provide fitness benefits to host or skew progeny sex ratio to promote their spread from mother to offspring (Bull, 1983). However, AR+ infections in this study had negative impact on its host. Given the widespread occurrence of both *Arsenophonus* and *Rickettsia* (64.5% and 53.9% respectively) in SSA1-SG3 populations, it was surprising to find a negative relationship between AR+ and its insect host. Similar lack of fitness benefits (Chiel *et al*., 2009; Cass *et al*., 2015;) or even detrimental effects (Ruan *et al*., 2006; Fang *et al*., 2014; Raina *et al*., 2015) of symbionts has been reported in *B. tabaci* previously. Such negative association between *Rickettsia* and its host is also seen in pea aphid (Sakurai *et al*., 2005; Vorburger and Gouskov, 2011).

In the absence of direct benefits, however, endosymbionts of *B. tabaci* and other sap sucking insects must provide conditional benefits to its host under stress from nutrition, heat, pathogenic infections, parasitism (Chiel *et al*., 2009; Gerardo *et al*., 2010; Eleftherianos *et al*., 2013). To investigate this, we compared the expression of four innate immune response genes to understand the putative defensive role of these symbionts. Insects solely rely on innate/natural immune responses including cellular mechanisms such as phagocytosis, encapsulation, coagulation, melanisation and humoral mechanisms like systemic production of antimicrobial peptides (AMPs) and other proteolytic and hydrolytic enzymes (Schmid-Hempel, 2005; Jiravanichpaisal *et al*., 2006; Lemaitre and Hoffmann, 2007). Immune response genes synthesising AMPs (Knottins) and autophagy related protein (Atg-9) were expressed in higher proportions in AR+ populations. Knottins are constitutively produced antimicrobial peptides abundant in *B. tabaci* (Shatters *et al*., 2008) which are overexpressed after acquisition of begomoviruses, other pathogenic infections, parasitization or heat stress (Mahadav *et al*., 2008, 2009; Luan *et al*., 2011; Zhang *et al*., 2014). Atg-9 is transmembrane protein critically required for phagophore assembly along with other autophagy related proteins and is a major innate immune response in *B. tabaci* (Luan *et al*., 2011) and other insects such as *Drosophila melanogaster* against pathogenic infection from bacteria and virus (Yano *et al*., 2008; Shelly *et al*., 2009). The difference in expression levels were however, much lower than in other studies (Zhang *et al*., 2014), when infected with pathogenic bacteria. This suggests that AR+ are not pathogens to the cassava whitefly. Although AR+ have fitness costs on cassava whiteflies, our results have shown that elevated immune gene expression could have compensating beneficial effects as seen in other insect species.

The innate immune responses in insects are nevertheless maintained at metabolic and physiological costs (Freitak *et al*., 2003, 2007; Ardia *et al*., 2012). Evolution of higher levels of immune defence may compromise fitness traits due to additional energy demands and owing to pleiotropic responsibilities of the same resistance genes (Schmid-Hempel, 2005). For example, Toll pathway in *Drosophila* provide protection against bacterial and fungal infection (Lemaitre *et al*., 1996; Lau *et al*., 2003) but is also essential for larval development (Halfon *et al*., 1995; Qiu *et al*., 1998). Such compromises in life-history traits in exchange of resistance to biotic stress is common in insects (Rothenbuhler and Thompson, 1956; Sutter *et al*., 1968; Boots and Begon, 1993; Ferdig *et al*., 1993; Fellowes *et al*., 1999). Increased fitness costs due to higher innate immune responses is thus a crucial life-history

trade-off between current reproduction success and future expected survival of progenies (Schmid-Hempel, 2003). Key immune system genes and pathways are found lacking in hemipteran insects like pea aphids and whiteflies (Gerardo *et al*., 2010; Zhang *et al*., 2014). Only Toll and JAK/STAT pathways are identified as the main stay for defence system of hemipteran insects (Gerardo *et al*., 2010), but both pathways are also involved in the developmental processes of the insects (Arbouzova and Zeidler, 2006; Valanne *et al*., 2011). Antimicrobial protection by S-endosymbionts is believed to have evolved to compensate the reduced immune defences of hosts (Altincicek *et al*., 2008). High incidence of *Arsenophonus* and *Rickettsia* in SSA1-SG3 populations could thus be explained as an evolutionary cost for greater defence against lethal pathogens at the expense of life-history traits. Similar trade-offs between resistance to pathogens and life parameters with S-symbiont infection are commonly seen in aphids. Symbionts such as *Hamiltonella* and *Rickettsia* increase aphid resistance to parasitoid infection (Oliver *et al*., 2003; Ferrari *et al*., 2004) but are also associated with fitness costs (Gwynn *et al*., 2005; Oliver *et al*., 2006; Vorburger and Gouskov, 2011; Vorburger *et al*., 2013).

The upsurge in whitefly populations on cassava has been a key factor in driving the CMD and CBSD pandemics in SSA (Legg *et al*., 2011; Jeremiah *et al*., 2015). An invasive whitefly specie, SSA2 was associated with the spread of the CMD pandemic in the late 1990s (Legg *et al*., 2002) but since then has been displaced by SSA1-SG1 as the most abundant population (Legg *et al*., 2013). S-endosymbionts were completely absent in large numbers (38%) of SSA1-SG1 populations. Why such high numbers of superabundant whiteflies are free of S-symbionts is unknown. Therefore, determination of the nature of relationship between SSA1-SG1 and its endosymbionts would be important to understand whether absence of symbionts is a contributing factor to the associated high populations.

EACMV-UG infections of cassava increases the concentration of free amino acids in phloem sap which proposedly contributed to whitefly population build-up in cassava fields (Colvin *et al*., 2004, 2006). EACMV-UG infections of cassava plants provided no benefits to the AR- flies in the current study, although it increased the performance of AR+ whiteflies slightly. Diseased plants were generated by propagation of infected cuttings and thus had pronounced symptoms. In Colvin *et al* (2006), diseased plants were generated by whitefly inoculations and thus the symptoms were presumed to be less severe, which could possibly explain the differences between the two studies.

Nevertheless, fewer AR+ flies acquired and retained EACMV-UG than AR- populations. EACMV-UG detected after 48 hours IAP is expected to have crossed the gut epithelium and thus circulative in the haemocoel of the insects (Ghanim *et al*., 2001). Passage and retention of higher titres of virus in the haemocoel is of great importance as they can solely be transmitted by the vector (Storey, 1938) and emphasises the importance of symbionts in the epidemiology of the disease. The reason for the lower retention of virus in AR+ whiteflies is unknown although autophagy/lysosome degradation has been previously shown to be important for the degradation of begomovirus both in the whitefly and plant host (Luan *et al*., 2011; Miozzi *et al*., 2014; Gorovits *et al*., 2014). Silencing a*tg-9* gene in *B. tabaci* increased begomovirus load and transmission efficiency indicating its role in the degradation of virus inside the whitefly body (Wang *et al*., 2016). Overexpression of a*tg-9* in AR+ whiteflies thus could possibly result in greater degradation of EACMV-UG and could be the main cause of low virus retention. Better fitness traits of AR- whiteflies and generally being healthier could also be the reason for its better virus retention and acquisition abilities. In contrast to the above results, endosymbionts of MEAM1 and MED species facilitate begomovirus transmission (Su *et al*., 2013; Kliot *et al*., 2014) by protecting virions against the hostile proteolytic haemolymph environment while transit from the gut wall to the salivary glands (Kunik *et al*., 1998; Morin *et al*., 1999; Ohnesorge and Bejarano, 2009; Gottlieb *et al*., 2010; Rana *et al*., 2012). These results indicate the complexity of whitefly-endosymbiont-virus interactions, and the importance of studying them to better understand cassava disease pandemics.

In conclusion, our results provide the additional evidence of the three way interactions between the whitefly host, endosymbionts and plant viruses. Absence of S-endosymbionts in cassava whitefly populations had positive effects on its fitness and vector abilities. High numbers of the currently superabundant populations are also free of S-endosymbionts, thus indicating the possible role of endosymbionts on keeping a check on cassava whitefly populations. This gives us the opportunity to use bacterial endosymbionts as potential biocontrol agents on cassava whiteflies and virus diseases.

6 PUTATIVE ROLE OF *Wolbachia* ON CASSAVA WHITEFLY POPULATION DYNAMICS IN THE CMD PANDEMIC ZONES

6.1 Introduction

A pandemic of cassava mosaic disease (CMD) with unusually severe symptoms spread southwards from north of Uganda in the early 1990s and into north-west Tanzania and western Kenya by the late 1990s (Legg, 1999; Otim-Nape *et al*., 1997). The pandemic severely impeded cassava production in Uganda with annual losses worth 60 million USD per year and forced many farmers to relinquish cassava cultivation (Legg and Thresh, 2000). The pandemic was associated with a number of factors including the presence of a recombinant Uganda variant of *East African cassava mosaic virus* (EACMV-UG) (Zhou *et al*., 1997), unusually high numbers of whiteflies (Legg and Ogwal, 1998) and predominance of a genetically distinct and more fecund group of *B. tabaci* designated as SSA2 (Legg *et al*., 2002). However, recent genetic diversity studies on whitefly populations could not associate a specific whitefly group with pandemic and non-pandemic areas of Uganda and reported gene flow between the two populations (Maruthi *et al*., 2001). The sudden rise in whitefly numbers during the pandemic was hypothesized to be driven by a mutually beneficial relationship between the EACMV-UG infected plants and African cassava *B. tabaci* (Colvin *et al*., 2004). Cassava supported low numbers of whitefly throughout the year, usually 1-2 per leaf prior to the pandemic (Fishpool and Burban, 1994). None of the above studies however, provided conclusive evidence for the presence of high numbers of whitefly on cassava. Recent surveys across the pandemic-affected areas in Uganda and Tanzania have confirmed the absence of SSA2 population (Mugerwa *et al*., 2012; Legg *et al*., 2013; Tajebe *et al*., 2015a). A spatio-temporal transition of whitefly populations in the pandemic zone has resulted in the rapid expansion of SSA1-SG1, the new superabundant population. The upsurging SSA2 population of 1990s has been replaced by exceptionally high numbers of SSA1-SG1 in the 2000s causing rapid spread of two cassava pandemics, CMD and cassava brown streak disease (CBSD) (Legg *et al*., 2013). Similarly, another population, SSA1-SG2 has also declined with the rise of SSA1-SG1 resulting in the overall decline of whitefly genetic diversity in the CMD pandemic region (Legg *et al*., 2013). The reasons for the temporal shift of whitefly populations from SSA2 to SSA1-SG1 are unknown.

Endosymbionts infecting specific whitefly populations can have both beneficial or detrimental effect on the biology of its host (Himler *et al*., 2011; Cass *et al*., 2015; Chiel *et al*., 2009; Su *et al*., 2013a; Fang *et al*., 2014; Raina *et al*., 2015). They also facilitate transmission of circulative viruses (Su *et al*., 2013b; Kliot *et al*., 2014; Pinheiro *et al*., 2015) and thus foster spread of virus diseases. Understanding the symbiont composition of the pre- and post- pandemic whiteflies and the effect on cassava whitefly population dynamics is essential to understand the spread of the cassava virus disease pandemics.

We investigated the endosymbionts infecting whiteflies from the pandemic and non-pandemic areas of Uganda in the 1990s. Our results show that both the declining SSA2 and SSA1-SG2 were mostly infected with *Wolbachia*. In cage experiments, SSA1-SG2 infected with *Wolbachia* were replaced by SSA1-SG3 free of *Wolbachia* within three months of co-existence in a cage, which indicated poor fitness of SSA1-SG2. *Wolbachia* titres were more than ten folds higher in SSA1-SG2 and SSA2 than in other populations tested. Virus transmission capabilities and innate immune response were also compared in SSA2 whiteflies with and without S-symbiont infections.

6.2 Materials and Methods

6.2.1 Whitefly samples and colonies studied

Preserved whitefly samples (kept frozen at -80 ºC), sampled in 2000 from whitefly colonies, which were originally collected in 1997 from Uganda from four CMD pandemic and three non-pandemic sites (Fig. 6.1, Table 6.1, Maruthi *et al*. 2001) were used to investigate endosymbiont diversity. *Wolbachia* positive samples from these populations and those used in chapter 3 (Table 3.1) were used for relative quantification of *Wolbachia* and *Portiera*. Diversity of *Wolbachia* and *Arsenophonus* infecting SSA2 populations were investigated using samples from the 1997 collections and the isofemale cassava lines generated (Chapter 4, Table 4.3) in this study. Virus retention and gene expression of antimicrobial peptides were also quantified on these isofemale SSA2 populations.

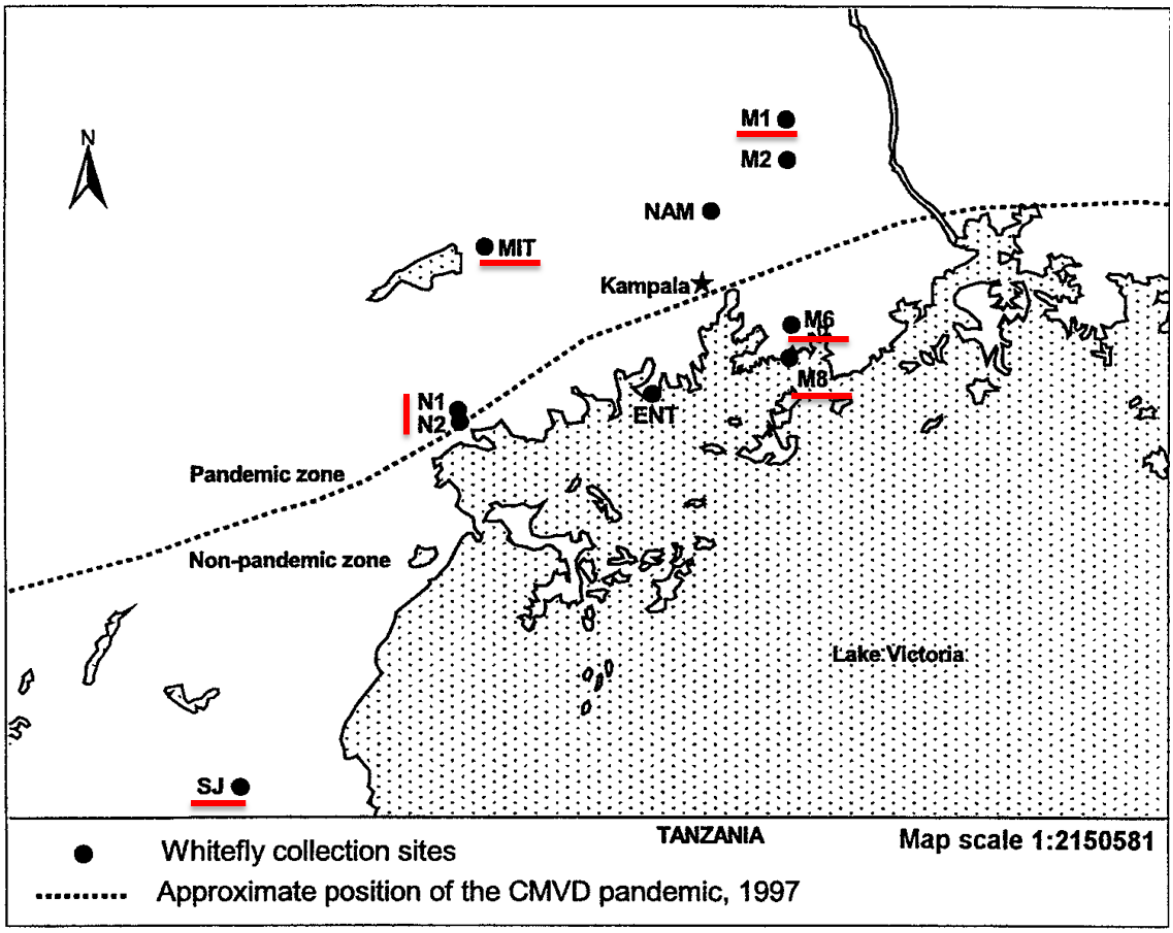


Figure 6-1: Collection sites for cassava whitefly samples from the CMD pandemic and non-pandemic zones in 1997 (Maruthi *et al*., 2001). Whitefly samples from sites underlined in red were analysed for endosymbionts diversity. Figure modified from Maruthi *et al*. (2001).

Table 6-1: Whitefly collection sites from the CMD pandemic and non-pandemic zones in 1997 analysed in this study

Location	Zone	Number of individuals tested
Mityana (MIT)	Pandemic	17
Mukono site 1 (M1)	Pandemic	14
Nkosi site 1 (N1)	Pandemic	8
Nkosi site 2 (N2)	Pandemic	15
Mukono site 6 (M6)	Non-pandemic	10
Mukono site 8 (M8)	Non-pandemic	25
Ssanji (SJ)	Non-pandemic	2

6.2.2 Detection of endosymbionts and molecular characterisation of **Wolbachia**

Total DNA was extracted from individual adult whiteflies using the Chelex method as described in section 3.2.2. The whitefly samples were genotyped by mtCO1 sequencing or PCR-RFLP (section 3.2.3). Prevalence of endosymbionts and *Wolbachia* diversity using MLST was determined (section 3.2.2). The mtCO1 and the concatenated *Wolbachia* MLST sequences were aligned using ClustalW of MEGA 5.2 (Tamura *et al*., 2011) and phylogenetic trees were constructed by the maximum-likelihood method using MEGA 5.2 using the T93+G+I substitution model. The robustness of the clades was assessed by 1000 bootstrap replicates. The MLST sequences were submitted to *Wolbachia* pubMLST database.

The distribution of whitefly populations in the pandemic and non-pandemic zones were formulated into a contingency table and their independence was tested using Fisher's exact test. The association of symbionts with the two zones were also tested using Fisher's exact test.

6.2.3 Quantification of **Wolbachia** *by qPCR*

Relative quantification of *Wolbachia* was estimated by quantitative PCR in all whitefly samples previously found to be infected with any bacteria by the end point PCR (Table 3.1 and 6.1). *Wolbachia* 16S rDNA was amplified with primers designed in this study (Table 6-2). *Portiera* amplification was used as an endogenous control to determine DNA sample quality and the whitefly β actin gene was used as a reference gene for quantification (Table 6-2). Amplifications were performed in an Eppendorf Mastercycler Realplex2 PCR instrument in 20 µl reactions containing 10 µl of 2X SYBR green PCR mix (DyNamo Flash SYBR green PCR kit, Thermo Scientific, UK), 0.4 µM of each primer (Table 6-2) and 1 µl of DNA sample. Amplifications consisted of 95 °C for 7 minutes followed by 40 cycles of 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and extension at 72°C for 30 seconds. A melting ramp from 60 ºC to 99 ºC with 0.5 ºC rise at each step was used to check the specificity of the primers. The mean normalised relative quantities of *Wolbachia* were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 6-2: Primers used for relative quantification of *Wolbachia* and *Portiera*

Target gene	**Primer name**	**Sequence (5'→3')**	**Amplicon length**	**Reference**
Wolbachia 16S rDNA	WF	GGGGAGTTTACTTTCTGTATTAC	190 bp	This study
	WR	CCCCATCCCTTCGAATAGGTAT		
Portiera 16S rDNA	Port-F	TAGTCCACGCTGTAAACG	229 bp	Pan *et al*., 2013
	Port-R	AGGCACCCTTCCATCT		
B. tabaci Actin	Act-F	TCTTCCAGCCATCCTTCTTG	130 bp	Ghanim and Kontsedalov, 2009
	Act-R	CGGTGATTTCCTTCTGCATT		

Table 6-3: Primers used for relative quantification of *Arsenophonus*

Target gene	**Sequence (5'→3')**	**Amplicon length**	**Reference**
Arsenophonus 23S rDNA	F- AAGGCTAAATACTCCTGACTGA	113 bp	This study
	R- ACTGCTTGTACGTACACGGTTT		
Tubulin (*B. tabaci*)	F- TGTACCGAGGAGATGTTGTG	113 bp	This study
	R- GATACCGACCTTGAAACCAG		

6.2.4 Retention of EACMV-UG by SSA2

Three SSA2 isofemale lines infected with either *Arsenophonus* (SSA2 A+), *Wolbachia* (SSA2 W+) or no S-endosymbionts (SSA2 -) were given acquisition access period (AAP) of 48 hours on three months old EACMV-UG infected cassava var. Ebwanateraka plant in a large cage. Twenty-five SSA2 A+, SSA2 W+ and SSA2 - viruliferous whiteflies were then inoculated to two months old healthy cassava plants of var. Ebwanateraka for 48 hours (inoculation access period-IAP). The experiment had three replications with 20 healthy plants for AAP and IAP, each. The SSA2 A+, SSA2 W+ and SSA2 - whiteflies were collected after AAP and IAP from all three replications for the detection and quantification of virus titres in individual whiteflies. Number of whiteflies retaining the virus after IAP was statistically analysed using Fisher's exact test at $P \leq 0.05$. Mean relative quantities of EACMV-UG in the SSA2 lines after IAP was determined (Section 5.2.4) and compared by Tukey's HSD test at $P \leq 0.05$ using the glht function from multcomp package of the R software (Hothorn *et al*., 2008).

6.2.5 Expression of antimicrobial peptides in SSA2 lines

Total RNA was extracted from a pool of 15 individuals (10 females and 5 males) for each SSA2 A+, SSA2 W+ and SSA2 – as described before (Section 5.2.6). Twenty such samples were prepared for each isofemale line and converted to cDNA as previously described (Section 5.2.6). The expression of three antimicrobial peptides Knottin 1 (K1), Knottin 2 (K2) and a*tg-9* genes were estimated by qPCR (Section 5.2.6). Mean expression of the antimicrobial peptides were compared using Tukey's HSD test.

6.2.6 Comparison of **Arsenophonus** *quantities in SSA1-SG3 and SSA2*

Symbiont associated phenotypes are dependent on their concentrations inside their host. *Arsenophonus* titres in SSA1-SG3 AR+ and SSA2 A+ were therefore quantified by qPCR. *Arsenophonus* 23S rDNA was amplified using primers designed in this study (Table 6-3). Whitefly tubulin gene was used as a reference gene for quantification (Table 6-3). Amplifications consisted of 95 °C for 7 minutes followed by 40 cycles of 95 °C for 15 seconds, annealing at 60 °C for 20 seconds and extension at 72 °C for 30 seconds. A melting ramp from 60 ºC to 99 ºC with 0.5 ºC rise at each step was used to check the specificity of the primers. Mean *Arsenophonus* quantities were calculated using a simple linear model

with $\log_e$ transformed data to fit parametric analysis. Statistical inference was based on the ANOVA and extracted standard errors.

6.2.7 Simulation experiments on whitefly population dynamics

The dynamics of SSA1-SG1, SSA1-SG2 and SSA2 infected with different S-symbiotic bacteria has varied markedly in East Africa since the CMD pandemic. For investigating these interactions, field simulations experiments were conducted by introducing different whiteflies infected with different bacteria in cages to monitor population development. Sixty adults (50% male and 50% female) each of SSA1-SG2 infected with *Wolbachia* (SSA1-SG2 W+) and SSA1-SG3 free of any S-endosymbionts (SSA1-SG3 AR-) were released into a cage containing two cassava plants var. Columbian (2 months old). The experiment was replicated in two additional cages with similar treatments. The cages were maintained at 27 ± 5 ºC, 60% RH, L12:D12 in the NRI insectary. Approximately 30 whiteflies were collected from each cage at every 30 days interval for up to 120 days from the beginning of the experiment. Total DNA was extracted from individual whiteflies to determine the relative proportion of each whitefly population by PCR-RFLP of the mtCOI gene (Section 3.2.3). The samples were also screened for the presence of *Wolbachia* to determine the horizontal transfer of *Wolbachia* between the two whitefly populations.

Similar experiment was conducted with SSA1-SG2 W+ and SSA1-SG3 AR+.

6.3 Results

6.3.1 Whitefly diversity in the pandemic and non-pandemic zones

All the samples tested from both the pandemic and non-pandemic zones belonged to the SSA1-SG1 and SSA2 species based on the mtCO1 sequences (Figure 6-2). However, the distribution of these two populations in the pandemic and non-pandemic zone was significantly different ($P = 0.018$, Fisher's exact test). SSA2 were abundant in the pandemic zone (66.6%) than SSA1-SG1 (33.3%) (Table 6-4). Contrastingly, numbers of SSA1-SG1 was higher (59.4%) than SSA2 (40.5%) in the non-pandemic zone (Table 6-4).

6.3.2 Endosymbiont diversity in the pandemic and non-pandemic zones

Only *Wolbachia*, *Rickettsia* and *Arsenophonus* were detected in whiteflies collected from the 1997 pandemic and non-pandemic zones (Table 6-4). *Wolbachia* was the most abundant endosymbiont symbiont detected in 43.9% (40/91) of the whitefly samples tested. However, there was no significant difference in *Wolbachia* incidence in whiteflies from the pandemic and non-pandemic zones (P = 0.66, Fisher's exact test). Similarly, infections of *Rickettsia* or the number of flies free of S-endosymbionts was also not different between the pandemic and non-pandemic zones (Fisher's exact test).

A high proportion of SSA2 were mostly infected (62.7%, 32/51) with *Wolbachia*, followed by *Rickettsia* (15.7%, 8/51) and 33.3% (17/51) were completely free of any S-endosymbionts. Higher proportions of SSA2 harboured *Wolbachia* (73.3%, P = 0.36) in the non-pandemic zone than in the pandemic (58.3%), however, was not statistically significant (P = 0.36). Fewer SSA2 whiteflies were free of symbionts in the non-pandemic zone (13.3%) than in the pandemic (41.66%, P = 0.06).

In contrast, high numbers of SSA1-SG1 were free of S-endosymbionts (70.0%, 28/40) and only 20.0% and 17.5% harboured *Wolbachia* and *Rickettsia*, respectively.

6.3.3 Genetic diversity of symbionts harboured by SSA2

Arsenophonus infecting the SSA2 populations (GenBank accession numbers: KX090270-71) were similar to the previously described A2 isolates and yielded a DNA fragment size of 550 bp (150 bp shorter than the predominant A3 isolates) by PCR amplification of the 23S rDNA (Figure 6-3).

All five MLST fragments were amplified from the *Wolbachia* infected SSA2. Phylogenetic analysis showed that SSA2 were infected with two strains of *Wolbachia* that differ only by the *fbpA* allele (Table 6-5). One of the strains (ST424) was previously found in SSA1-SG3 populations (Chapter 3, Table 3-3) and the other strain was a new sequence type (ST426) which is submitted to the *Wolbachia* pubMLST database. Phylogeny of the concatenated *Wolbachia* MLST sequences (Figure 6-4) clustered them into the previously described W3 cluster (ST424) and a new W4 cluster (ST426).

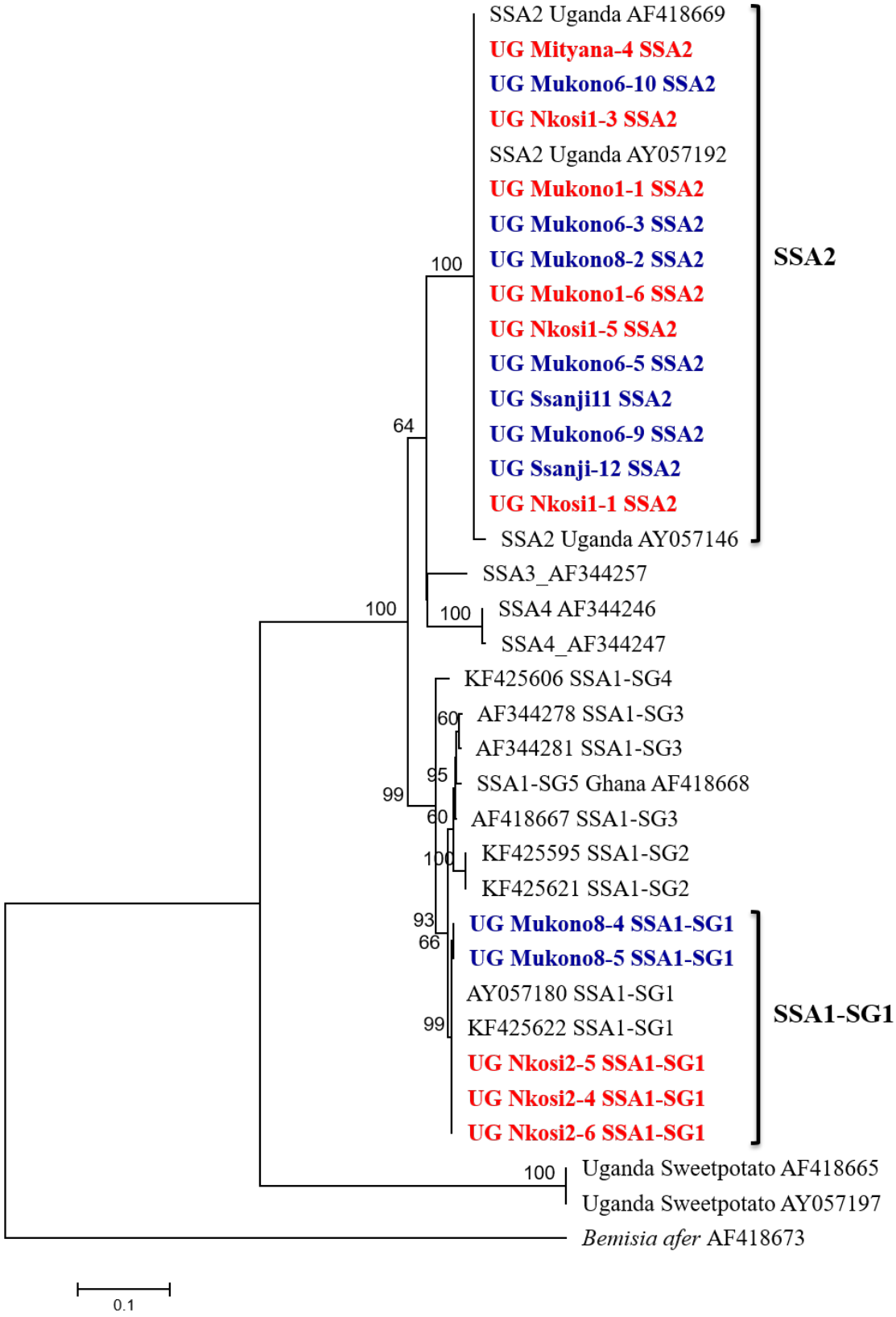


Figure 6-2: Phylogeny of mtCO1 nucleotide sequences (697 bp) of the sampled whiteflies from the 1997 CMD pandemic (red) and non-pandemic zones (blue) in Uganda.

Table 6-4: Distribution of whitefly populations and their S-endosymbionts in the sampled sites of the pandemic and non-pandemic zones in 1997 in Uganda.

	Pandemic zone					**Non-pandemic zone**				
	Quantity (proportion)	*Arsenophonus*	*Rickettsia*	*Wolbachia*	No S-symbionts*	Quantity (proportion)	*Arsenophonus*	*Rickettsia*	*Wolbachia*	No S-symbionts
SSA1-SG1	18 (33.3%)	0	5 (27.8%)	4 (22.22%)	11 (61.11%)	22 (59.46%)	1 (4.5%)	2 (9.09%)	4 (18.18%)	17 (77.27%)
SSA2	36 (66.6%)	0	4 (11.1%)	21 (58.33%)	15 (41.66%)	15 (40.54%)	0	4 (26.66%)	11 (73.33%)	2 (13.33%)
Total	54	0	9 (16.66%)	25 (46.29%)	26 (48.14%)	37	1 (2.7%)	6 (16.21%)	15 (40.54%)	19 (51.35%)

Only SSA1-SG1 and SSA2 were detected in pandemic and non-pandemic zones.

Proportions of SSA2 was significantly higher in pandemic zone.

Higher proportion of SSA1-SG1 was free of S-symbionts. Higher proportion of SSA2 harboured *Wolbachia* in pandemic than non-pandemic zone.

*Tested for *Arsenophonus*, *Cardinium*, *Hamiltonella*, *Fritschea*, *Rickettsia* and *Wolbachia*. None of the symbionts could be detected.

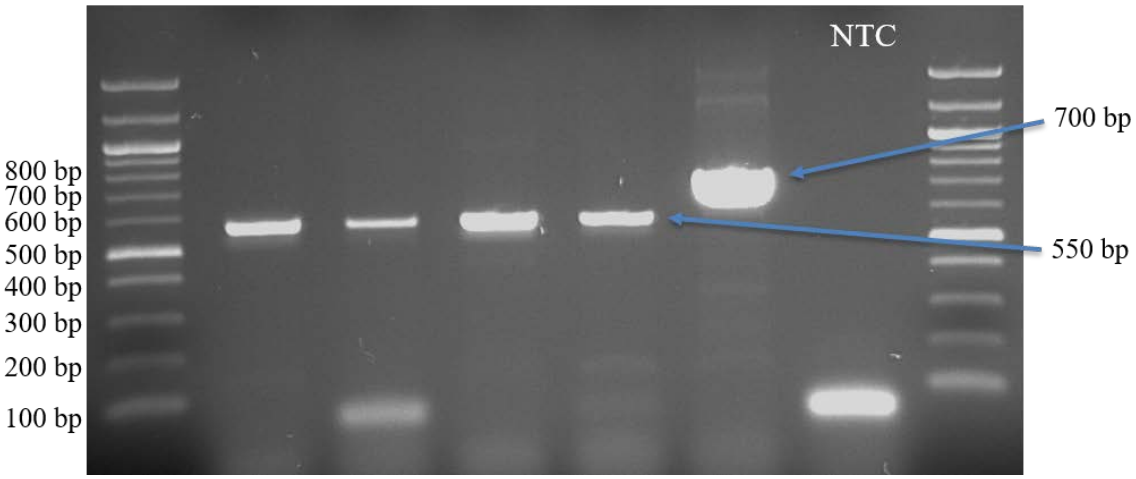


Figure 6-3: PCR diagnosis of 23S rDNA of *Arsenophonus*. Lane 1 and 8 = 100 bp DNA ladder, Lane 2, 3, 4, 5 = *Arsenophonus* infecting SSA2 populations, Lane 6 = A3 isolate of *Arsenophonus*, Lane 7 = No template control. *Arsenophonus* infecting SSA2 were similar to the A2 strain.

Table 6-5: MLST profiles of *Wolbachia* infecting SSA2 whiteflies in SSA

Host	**Super group**	***coxA***	***fbpA***	***ftsZ***	***gatB***	***hcpA***	**Sequence Type**
***B. tabaci* (SSA2)**	B	88	9	105	9	13	424
***B. tabaci* (SSA2)**	B	88	405*	105	9	13	426*

*New additions to *Wolbachia* pubMLST database.

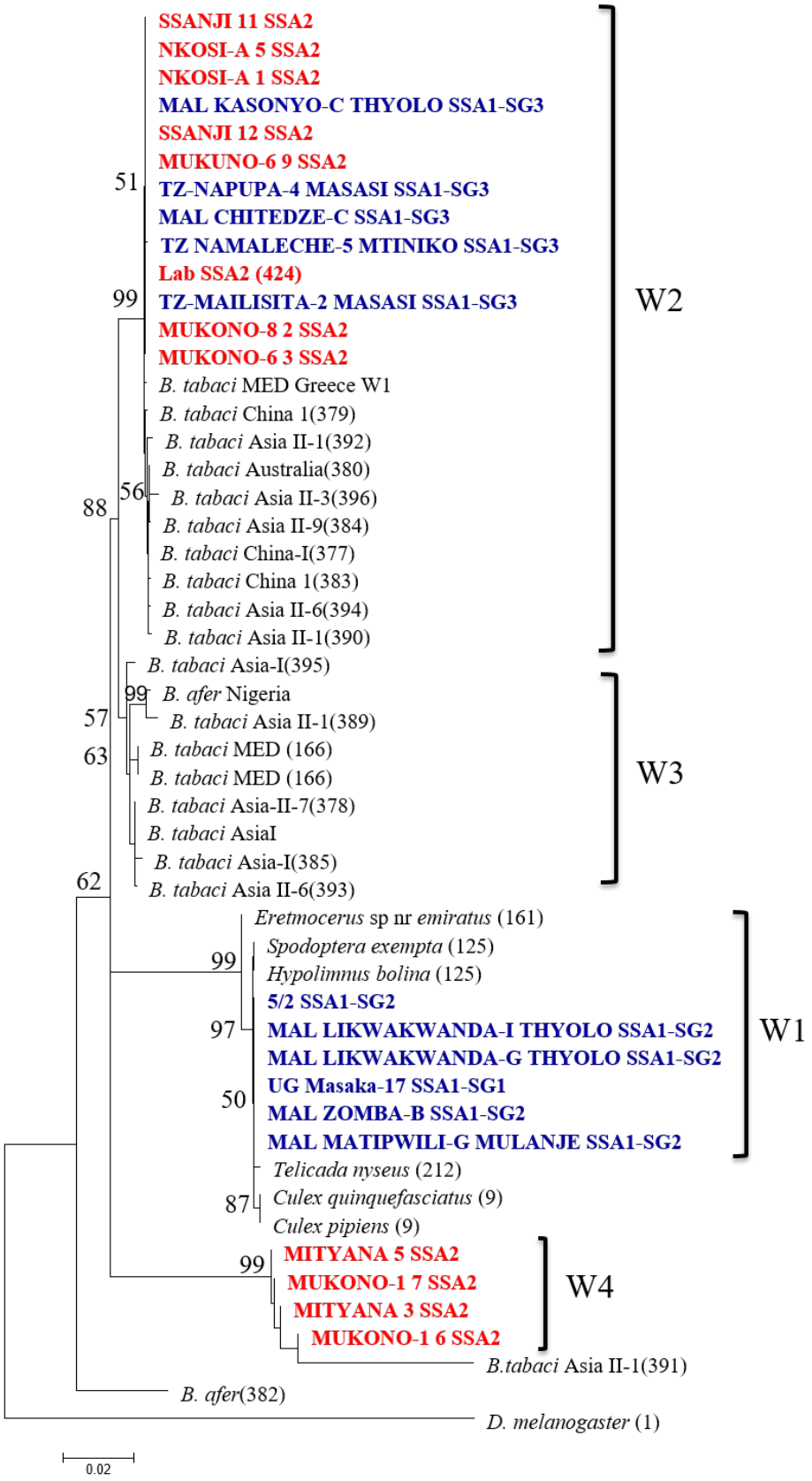


Figure 6-4: Phylogeny of concatenated MLST nucleotide sequences (2079 bp) of *Wolbachia* including the isolates infecting the SSA2 (red) and other cassava whitefly populations (blue). Similar strains of *Wolbachia* infect SSA1-SG3 and SSA2 whiteflies.

6.3.4 Quantification of **Wolbachia** *in different cassava whitefly population by qPCR*

Relative amounts of *Portiera*, the P-endosymbiont were not different across the different cassava whitefly populations tested (Figure 6-5A, Appendix L). However, the relative quantities of *Wolbachia* varied across the whitefly populations (Figure 6-5B, Appendix M). *Wolbachia* titres in SSA1-SG2 and SSA2 were significantly higher (>11 folds) than in other populations tested, and were lowest in SSA1-SG1, SSA1-SG5 and SSA3.

6.3.5 Retention of EACMV-UG by SSA2 populations

The proportions of whiteflies retaining the virus after 48 hours of IAP did not vary significantly ($P = 0.91$, Fisher's exact test) between the SSA2 A+ (67.5%, 50/74), SSA2 W+ (72.2%, 52/72) and SSA2 - (64.6%, 53/82) (Figure 6-6A). However, virus titres retained in whiteflies after the IAP varied significantly with S-endosymbiont infections. SSA2 A+ retained five folds higher quantities of EACMV-UG than SSA2 W+ and SSA2 -, and no significant difference were seen between the latter two whiteflies (Figure 6-6B, Appendix N).

6.3.6 Immune gene expression v/s symbiont infection in SSA2

The antimicrobial protein K1 was significantly overexpressed (> 2.5 folds) in SSA2 W+ than in SSA2 A+ and SSA2 - populations (Table 6-6, Figure 6-7A, Appendix O). Relative expression of K2 was also significantly higher (2 folds) in SSA2 W+ than SSA2 A+, but varied in SSA2 - populations (Table 6-6, Figure 6-7B, Appendix P). Expression of *atg-9* was similar in all three populations (Table 6-6, $P = 0.06$, Appendix Q).

6.3.7 Comparison of quantities of **Arsenophonus** *in SSA1-SG3 and SSA2*

Arsenophonus primers designed were efficient (Appendix R) and amplified the specific expected product. *Arsenophonus* titres in SSA1-SG3 A+ were significantly higher (39.7 folds) than SSA2 A+ ($F= 42$, $P \leq 0.001$, Appendix S) (Figure 6-8).

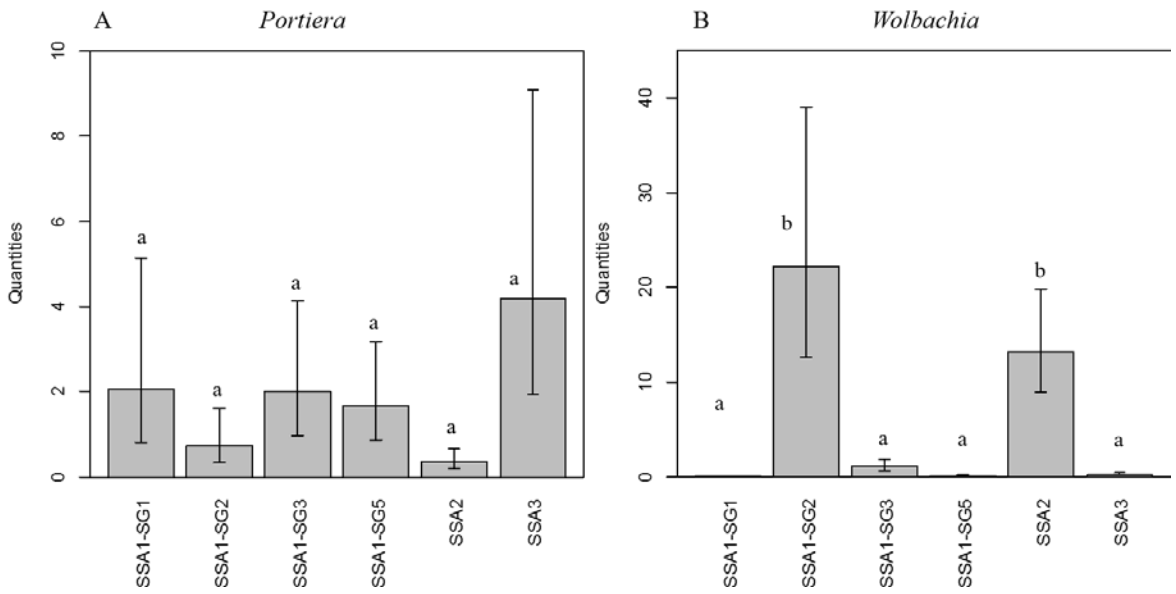


Figure 6-5: Mean quantities of *Portiera* (A) and *Wolbachia* (B) in cassava whitefly populations relative to the whitefly actin gene. Post hoc comparisons between the symbiont quantities were done by Tukey's HSD test. Different letters indicate statistically significant difference at $P < 0.05$.

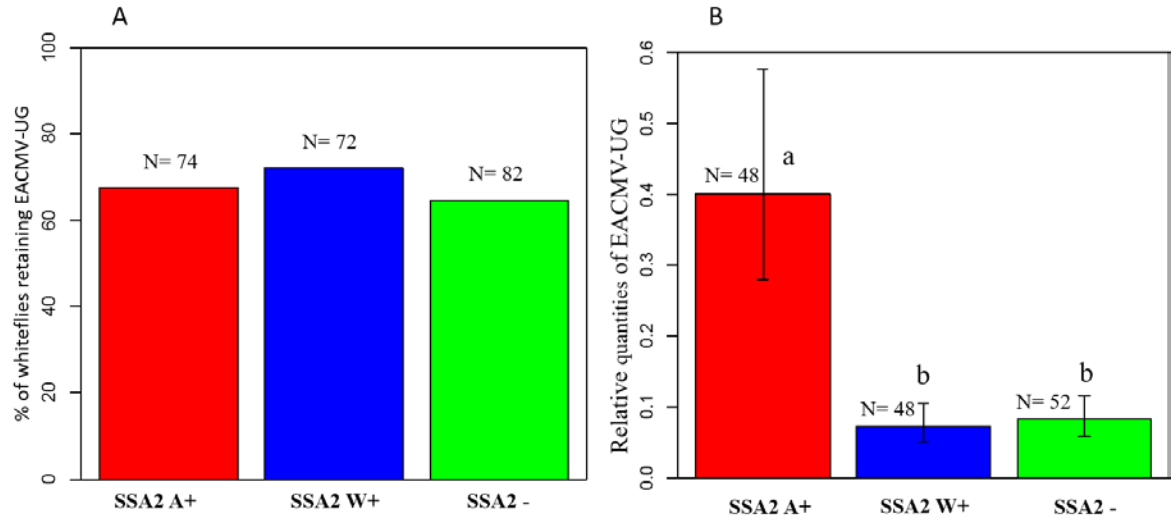


Figure 6-6: Retention of EACMV-UG by SSA2 lines. A= percentage of SSA2 A+, SSA2 W+ and SSA2 - whiteflies retaining EACMV-UG after 48 hours IAP, and B= Quantities of EACMV-UG retained after 48 hours of IAP relative to the whitefly tubulin gene. Different letters indicate statistically significant difference at $P < 0.05$.

Table 6-6: Mean relative expression of K1, K2 and *atg-9* in SSA2 A+, SSA2 W+ and SSA2 - populations

	Mean ± SE		
	K1	**K2**	***atg-9***
SSA2 A+	0.82 ± 0.14	0.77 ± 0.13	0.90 ± 0.05
SSA2 W+	2.70 ± 0.44	1.59 ± 0.25	0.74 ± 0.04
SSA2 -	0.65 ± 0.1	1.02 ± 0.18	0.85 ± 0.05

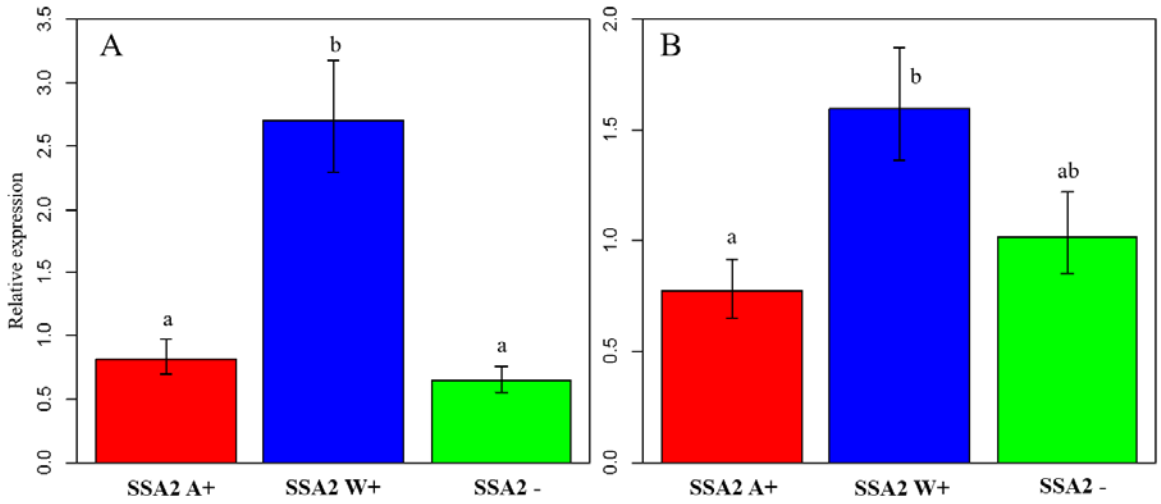


Figure 6-7: Relative expression of Knottin 1 (A) and Knottin 2 (B) in SSA2 A+, SSA2 W+ and SSA2 - populations. Expression of the antimicrobial knottin peptides are higher in SSA2 W+ than in SSA2 A+ and SSA2 Different letters indicate statistically significant difference at $P < 0.05$.

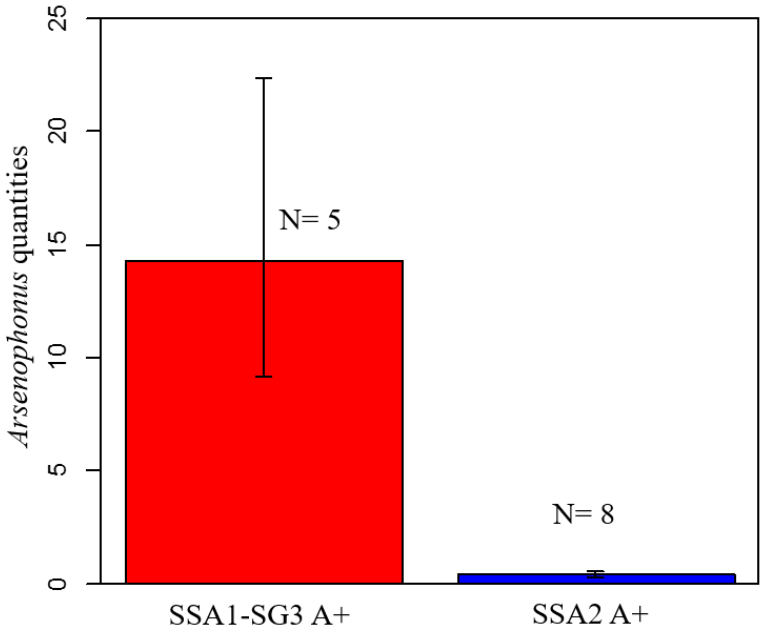


Figure 6-8: Relative quantities of *Arsenophonus* in SSA1-SG3 AR+ and SSA2 A+ whiteflies.

6.3.8 Simulation experiments on whitefly population dynamics

Proportions of SSA1-SG2 W+ and SSA1-SG3 AR- whiteflies with different symbionts varied after four months in field simulation experiments. SSA1-SG3 AR- quickly outcompeted SSA1-SG2 W+ (Figure 6-9A). SSA1-SG2 W+ were detected in all three replicate cages but in very low numbers up to 60 days and were almost completely replaced by SSA1-SG3 AR- whiteflies within 90 days. However, there was no evidence of horizontal transfer of symbionts between the two populations. Only *Wolbachia* was detected from SSA1-SG2, but not from SSA1-SG3 during the entire experiment.

In another experiment, SSA1-SG2 W+ was also replaced by SSA1-SG3 AR+ within 90 days of co-existence (Figure 6-9B). Similarly, *Wolbachia* was only present in SSA1-SG2 W+ indicating the lack of horizontal bacterial transfer between the two whiteflies.

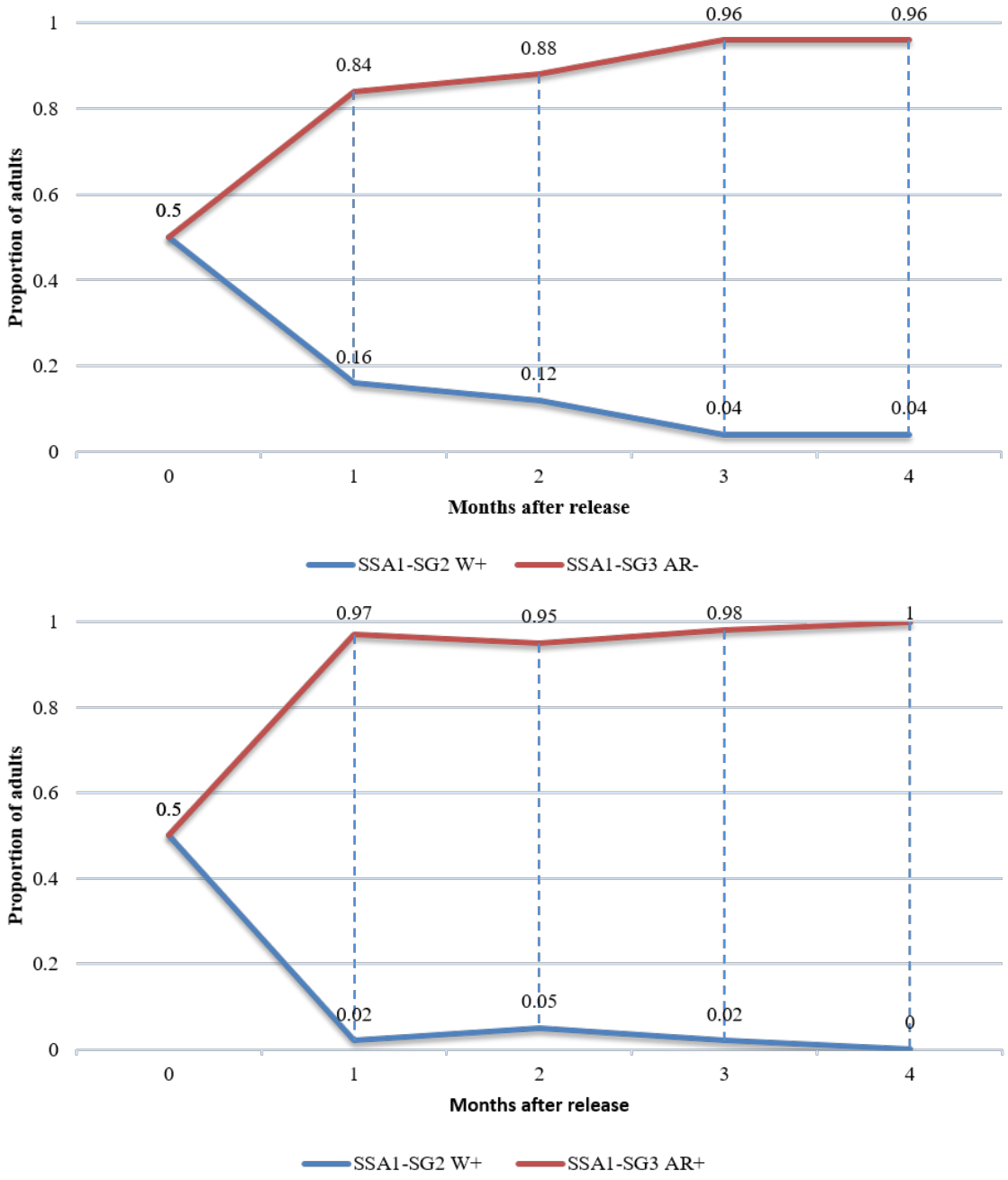


Figure 6-9: Proportion of SSA1-SG2 and SSA1-SG3 adult whiteflies in simulation cage experiments. A= 60 adults (30 males + 30 females) each of SSA1-SG2 W+ and SSA1-SG3 AR-, B= 60 adults (30 males + 30 females) of SSA1-SG2 W+ and SSA1-SG3 AR+.

6.4 Discussion

A major objective of this study was to investigate the endosymbiont diversity of cassava whiteflies collected in 1997 from the CMD pandemic and non-pandemic zones of Uganda and correlate the effect of certain symbionts on post-pandemic populations. Cassava whitefly samples collected in Uganda in 1997 from the pandemic and non-pandemic zones have been previously compared for genetic diversity, fecundity, mating capabilities and ability to transmit CMBs (Maruthi *et al*., 2001, 2002, 2004; Colvin *et al*., 2006). These studies demonstrated no significant differences between the pandemic and non-pandemic whiteflies. However, the reasons for whitefly superabundance and spread of the pandemic remained to be understood. The SSA2, was previously proposed to be an invader species (Ug2) as it was consistently associated with high whitefly numbers generally within the pandemic zones (Legg *et al*., 2002), although it was later identified in the non-pandemic areas (Colvin *et al*., 2006). Our data confirm both these findings.

Our results also show that *Wolbachia* was the most predominant symbiont (43.9%) infecting cassava whiteflies during the CMD pandemic in 1997. *Wolbachia* continues to be the most prevalent S-endosymbiont in cassava whiteflies (Section 3.3.3). Incidence of *Wolbachia* was higher in SSA2 (62.7%) than SSA1-SG1 (20%) whiteflies during the pandemic. Our findings therefore confirm high prevalence of *Wolbachia* in the SSA1-SG2 and SSA2 whiteflies, which were declining, in Uganda by 1997. In contrast, the current predominant cassava infesting populations in East and Central Africa, SSA1-SG1 and SSA1-SG3 has lower *Wolbachia* (<11 folds) titres. High incidence and proliferation rates of *Wolbachia* in SSA1-SG2 and SSA2 populations is indicative of its detrimental effects on the host biology and could be a major contributory factor for the rapid decline of the two populations. Strains of *Wolbachia* with high titres have negative effects on *Drosophila* and mosquito biology (McGraw *et al*., 2002; Chrostek *et al*., 2013; Martinez *et al*., 2015), and a determinant of reproductive impairments such as cytoplasmic incompatibility (CI) (Boyle *et al*., 1993; Noda *et al*., 2001).

The decline of *Wolbachia*-infected populations could not be demonstrated in this study as the ideal isofemale colonies SSA2 W+ and SSA2 – were not obtained in time for comparisons. However, this was demonstrated using another pandemic whitefly population, SSA1-SG2, for which the correct combinations were obtained. SSA1-SG2 infected with *Wolbachia* was almost replaced within 90 days of co-existence by the *Wolbachia*-free SSA1-SG3. These results indicate that the *Wolbachia* infection negatively affect the fitness of

SSA1-SG2, and thus possibly have contributed to their decline in African cassava fields. Aggressive strains of *Wolbachia* have been widely used for biological control of mosquitoes and their transmitted diseases in Australia (Iturbe-Ormaetxe *et al*., 2011). Thus, use of *Wolbachia* as a biological control agent to manage the superabundant whitefly numbers and the two disease epidemics remains an option to consider in future endeavours.

This study also demonstrated specific interaction between whitefly species and bacterial strains. SSA1-SG3 and SSA2 populations are infected by the same strain of *Wolbachia*, however, this strain has different rates of multiplication in these hosts. Similarly, SSA1-SG1 and SSA1-SG2 are also infected by a common strain but have different *Wolbachia* densities in the insect body. Factors determining the titres of *Wolbachia* in insects like *Drosophila*, mosquitoes and bean beetles are known to be dependent on both the bacterial strain and host genetic background (Boyle *et al*., 1993; McGraw *et al*., 2002; Kondo *et al*., 2005; Chrostek *et al*., 2013). *Wolbachia* multiplication in cassava whiteflies is thus regulated differentially in different populations. High numbers of SSA1-SG1 (70%) was free of S-endosymbionts in the 1997 collections, which has decreased to 38% in our recent collections (Section 3.3.3, Tajebe *et al*., 2015b). A major fraction (58%) of the present superabundant SSA1-SG1 is also infected with *Wolbachia* (Figure 3-5) but in very low densities in comparison to SSA1-SG2 and SSA2 whiteflies. Similarly, *Arsenophonus* titres in SSA2 were almost 40 folds lower than that of SSA1-SG3 whiteflies. Thus stringent regulation of symbiont multiplication by SSA1-SG1 populations could be a possible reason for its dominance in the Ugandan cassava fields.

Another biotic factor in the CMD pandemic is the virus, the possible interaction of which with some endosymbionts was investigated in this study. SSA2 whiteflies with *Arsenophonus* infections (SSA2 A+) retained five folds higher titres of EACMV-UG than SSA2 W+ and SSA2 - whiteflies. These results indicate the complex interactions between the whitefly, its endosymbionts and the virus. SSA1 and SSA2 are equally efficient in transmitting CMBs (Maruthi *et al*., 2002). Incidence of *Wolbachia* was more prevalent (73.3%) in the non-pandemic zone in comparison to the pandemic zone (58.3%), although the difference was not statistically significant. This was possibly due to analysis of fewer (15 individuals) samples from the non-pandemic zone.

Several key genes and pathways such as peptidoglycan receptor protein, IMD signal pathway and lysozymes contributing to the immune system of other arthropods are absent

in pea aphids and whiteflies (Gerardo *et al*., 2010; Zhang *et al*., 2014). Only Toll and JAK/STAT are proposed to be the main functional immune system pathways in aphids (Gerardo *et al*., 2010) and they are also crucial for insect development (Arbouzova and Zeidler, 2006; Valanne *et al*., 2011). S-endosymbionts of aphids and whiteflies provide protection against pathogenic bacterial infections and is thus postulated to be a reason for lack of key immune system pathways in these insects. Phagocytosis and antimicrobial peptide production are important for whitefly defence against bacterial infections. Comparisons of the gene expression of the antimicrobial peptides in SSA2 lines showed higher expression of Knottins in SSA2 W+ than SSA2 A+ or SSA2 –. Thus *Wolbachia* infections in SSA2 trigger higher immune response which could be essential for protection against other pathogenic infections. Thus protection by *Wolbachia* against pathogens could be an investment by SSA2 whiteflies for survival under adverse conditions which are compensated by reduced fitness parameters.

In conclusions, it was evident that the two declining whitefly populations SSA2 and SSA1-SG2 were both infected with *Wolbachia* in high proportions and titres during the CMD pandemic, indicating that *Wolbachia* may have contributed to their rapid decline in cassava fields. When simulation experiments were conducted to confirm this hypothesis, *Wolbachia*-infected SSA1-SG2 was almost extinct within three generations when caged together with *Wolbachia*-free whiteflies. Our results have also shown that such phenotypes can be both whitefly- and bacteria-specific and complex, however, they are clearly indicating the potential of using bacteria as biocontrol agents, which should be explored in future studies.

7 GENERAL DISCUSSION

Cassava is the source of carbohydrates for over 800 million people in the tropical and sub-tropical world (Howeler *et al*., 2013). In sub-Saharan Africa (SSA), cassava is the principal source of energy and generates incomes for the majority of rural households (Dixon *et al*., 2003). Cassava is known as Africa's food insurance as it requires low inputs, withstands drought, provides food security when other food sources are scarce and has a great potential to adapt to climate change (Dixon *et al*., 2003; Legg and Hillocks, 2003; Jarvis *et al*., 2012). However, the food security of many farmers in SSA is constrained by two severe viral diseases; cassava mosaic virus disease (CMD) and cassava brown streak disease (CBSD). Viruses that cause CMD and CBSD are transmitted by the whitefly vector, *Bemisia tabaci* (Gennadius). Cassava producing areas affected by these two diseases are expanding in epidemic forms causing heavy economic damage which are driven by increase in the abundance of, *B. tabaci* populations (Legg *et al*., 2011).

B. tabaci cryptic species complex is known to harbour seven S-endosymbionts; *Arsenophonus*, *Cardinium*, *Fritshchea*, *Hamiltonella*, *Hemipteriphilus*, *Rickettsia* and *Wolbachia* (Everett *et al*., 2005; Gueguen *et al*., 2010; Bing *et al*., 2013b). The diversity and infection frequency of these symbionts in *B. tabaci* vary with host genetic background, host plants and geographical location. Interaction of these symbionts with their insect host is highly specific. *Rickettsia* infections of Middle East-Asia Minor 1 (MEAM1) populations provide fitness benefits in USA (Himler *et al*., 2011) but neutral in Israel (Chiel *et al*., 2009; Cass *et al*., 2015). Symbiont infections can therefore be beneficial, neutral with conditional benefits or detrimental to its whitefly host. Symbionts such as *Hamiltonella*, *Rickettsia* and *Arsenophonus* are also known to interact with plant viruses to facilitate their transmission. Diversity and effect of symbionts infecting the whiteflies colonizing cassava in SSA were unknown. Aim of this study was therefore, to address this gap in our knowledge on the prevalence and genetic diversity of S-endosymbionts infecting major cassava whitefly populations in eastern Africa and to describe their putative role on population dynamics and development of cassava diseases.

Five genetically different whitefly populations were identified colonizing cassava in the four sampled countries of SSA; Uganda, Tanzania, Malawi and Nigeria. Sub-Saharan Africa 1

sub-group 1 (SSA1-SG1) and SSA1-SG2 in Uganda; SSA1-SG3 in Tanzania and Malawi; and SSA1-SG5 and SSA3 in Nigeria were the predominant populations. This was similar to previous reports of high genetic diversity among cassava whiteflies in eastern Africa (Berry *et al*., 2004; Sseruwagi *et al*., 2006; Legg *et al*., 2013; Tajebe *et al*., 2015a), which supports the suggestions that eastern Africa could indeed the centre of species origin. Surveillance and monitoring of the changing population dynamics of cassava whiteflies in the CMD pandemic region is essential for early disease forewarning to farmers (Legg *et al*., 2013). A rapid and cost effective PCR-RFLP based diagnostic was therefore developed for precise and high throughput identification of these populations. The RFLP technique developed would also ensure easier genetic typing of the described cassava whitefly species in many research studies.

More than three quarters of cassava whitefly samples of the five species tested were found to harbour at least one of the four symbionts; *Wolbachia*, *Arsenophonus*, *Rickettsia* and *Cardinium* in the decreasing order of prevalence. *Wolbachia* followed by *Arsenophonus* are the predominant symbionts in indigenous *B. tabaci* species around the world (Pan *et al*., 2012; Bing *et al*., 2013a; Bing *et al*., 2014; Jing *et al*., 2014; Singh *et al*., 2012), but has been found rarely in the invasive MEAM1 and MED species (Chiel *et al*., 2007; Gueguen *et al*., 2010; Skaljac *et al*., 2010; Bing *et al*., 2014; Marubayashi *et al*., 2014). Similarly, *Rickettsia* and *Hamiltonella* are the predominant symbionts in the invasive species while the latter is absent in the indigenous species (Singh *et al*., 2012; Bing *et al*., 2013a; Jing *et al*., 2014). However, the reasons for such associations of symbionts with specific species of *B. tabaci* (indigenous v/s invasive) are unknown but warrant future investigation.

A previous study (Tajebe *et al*., 2015b), however, detected *Hamiltonella* infections especially in SSA1-SG3 populations but not found in this study. It was later confirmed that the detection of *Hamiltonella* by Tajebe *et al*. (2015b) was indeed false positive as the results could not be reconfirmed independently (H. Delatte, personal communication). This suggests that currently *Hamiltonella* are not infecting the African cassava whiteflies. This has significant epidemiological importance because *Hamiltonella* is crucial for begomovirus transmission in other whitefly species (Gottlieb *et al*., 2010; Su *et al*., 2013b). Its absence in African cassava whiteflies thus raises questions on the protective role of symbionts for transmission of CMBs .

Majority (~60%) of the SSA cassava whiteflies harbouring symbionts were singly infected with a symbiont, which was slightly contrasting to other studies wherein more double infections (65%) were reported. This is possibly indicating competition for space and resources among the symbionts in cassava whiteflies, and the resulting fitness costs associated with the bacterial infections. The prevalence of symbionts varied significantly between the different cassava whitefly populations. *Arsenophonus* and *Rickettsia* were most abundant in SSA1-SG3 while absent in SSA1-SG5. *Wolbachia* was nearly fixed in SSA1-SG2 populations. Such specific symbiont infections with different whitefly populations are reported previously (Chiel *et al*., 2007; Gueguen *et al*., 2010; Pan *et al*., 2012; Bing *et al*., 2013a; Gnankine *et al*., 2013). Although the reasons for such associations are unknown but the phenotypic effects of these symbionts on their hosts could play a major role in the population dynamics of their host. For example, high numbers (38%) of SSA1-SG1, which is the current superabundant population in the CMD pandemic zone was free of S-endosymbionts, whereas most (>85%) of the less abundant SSA1-SG2 and SSA1-SG3 were infected with at least one symbiont. Comparison of symbiont infections from Uganda CMD pandemic in 1997 showed SSA2 with >97% infections but SSA1-SG1 with only 30%. This we believe was the main reason for the rapid decline of SSA2 and concurrent raise of SSA1-SG1 as a superabundant population.

Establishing isofemale lines of cassava whiteflies with different symbiont infections was essential for studying such symbiont phenotypes on host biology, which was done in this study. Isolation of genetically identical isofemale lines with single and multiple infections with different symbionts has been a major challenge, however. Two strategies were tried for generation of the isofemale lines in this study; first, random selection of single adult male and female to isolate progenies with specific symbiont combinations by chance, and second, selective curing of whiteflies by antibiotic treatment. Use of high doses of antibiotics failed to cure most symbionts. This was associated with problems like low fecundity, slow development time and lack of female progenies in the F1 generation. However, we succeeded in developing five unique isofemale lines of SSA1-SG3 and SSA2 with different S-endosymbiont combinations by the first random selection and one line of SSA2 by antibiotic curing. Comparison was made of biological parameters such as fecundity, adult emergence and development time and virus transmission with and without S-symbionts under same genetic background to understand the nature of whitefly-symbiont relationship and their role in the superabundance phenomenon.

Studies on the localization of endosymbionts inside their whitefly host can also help understand their interaction with their host. For example, presence of *Wolbachia* in the reproductive organs of *Drosophila* (Bressac and Rousset, 1993; Dobson *et al*., 1999; Clark *et al*., 2002) and tsetse flies (Cheng *et al*., 2000) manipulate the reproduction of its host, whereas the presence of *Rickettsia* inside the whitefly gut (Kliot *et al*., 2014) enhanced virus transmission. S-symbionts in whiteflies are mostly confined in the bacteriocytes (Gottlieb *et al*., 2008; Skaljac *et al*., 2010; Marubayashi *et al*., 2014) but some like *Rickettsia*, *Wolbachia* and *Cardinium* can be found scattered inside the insect body (Gottlieb *et al*., 2006; Skaljac *et al*., 2010; Bing *et al*., 2014; Marubayashi *et al*., 2014). In this study, *Portiera* and the S-symbionts *Arsenophonus*, *Rickettsia* and *Wolbachia* were only detected in the bacteriocytes of the nymph and adult cassava whiteflies. Strict confinement of the symbionts inside the bacteriocytes indicated no direct influence of symbionts on its host reproduction but influence indirectly on whitefly biology. Sharing of the common bacteriocytes by both the primary and secondary symbionts allows efficient vertical transmission of the symbionts. However, sharing of space have fitness costs to the whiteflies as it reduces the density of the primary symbiont (Gottlieb *et al*., 2008) due to competition for space and nutrition (Vautrin and Vavre, 2009). As previously mentioned, frequency of double and triple infections were much lower than single infections with symbionts in cassava whiteflies which also indicate the existent costs with shared habitats of symbionts inside their host.

Comparison of life cycle parameters of cassava whiteflies with and without S-symbiont infection under controlled environmental conditions was necessary to establish the costs and benefits of symbiont infections. Herein, the lifecycle parameters such as fecundity, nymph formation, adult development and emergence time were compared for SSA1-SG3 whiteflies with dual infections of *Arsenophonus* and *Rickettsia* (AR+) and free of S-symbionts (AR-) on healthy and EACMV-UG-infected cassava plants. Results of this study demonstrated that the fitness costs are associated with AR+ infections to cassava whiteflies. The endosymbiont infections had no effect on the fecundity and nymph development, but almost halved the rate of adult emergence from nymphs. AR+ infections also extended adult emergence time by about 10 days thus increasing the number of days required to complete the life cycle. This implies that whiteflies free of S-symbionts have shorter life cycle and produce more adults. Thus, S-symbiont infections, by their negative impact on whitefly life parameters can inhibit the development of superabundant whitefly populations. A high proportion (38%) of the current superabundant population SSA1-SG1 near the Lake Victoria region in Kenya and

Tanzania were free of S-symbionts(Mugerwa *et al*., 2012; Legg *et al*., 2013). Contrastingly, a high proportion (84%) the coastal SSA1-SG3 populations, which have always been low in numbers (Jeremiah *et al*., 2015), were infected with *Arsenophonus* and/or *Rickettsia*. The results are pointing to the negative effect of S-symbiont infections on SSA1-SG3, and positive effect to SSA1-SG1 in the absence of S-symbionts. Such whitefly-bacteria interactions may determine the whether or not a whitefly species can develop into a superabundant population or will be under check.

The ability of the bacteria to survive in whiteflies despite their negative effects is a key parameter in their interaction with the host. Vertically transmitted symbionts are expected to either benefit or manipulate host reproduction for survival (Wernegreen, 2004). Thus, a highly prevalent symbiont in a population should be either conferring fitness benefits or causing female bias in the progenies to maintain its infection frequency. The reduced progeny numbers and development time of its whitefly host by *Arsenophonus* and *Rickettsia* in SSA1-SG3 is, however, contradictory to this hypothesis. However, it is possible that these symbionts contribute to the fitness of its whitefly host under adverse conditions such as nutritional and thermal stress or pathogenic and parasitic infections found in other whitefly species (Chiel *et al*., 2009). Major innate immune genes (antimicrobial and autophagy proteins) were overexpressed in the AR+ than in AR- populations, indicating that these bacteria are likely to protect SSA1-SG3 from infections of other pathogens. This is what may have happened in African cassava populations as hemipterans such as aphids and whiteflies are deficient in several key immune genes and pathways (Gerardo *et al*., 2010; Zhang *et al*., 2014). Thus symbiont infections could be maintained to protect the insect-host by complementing its immune system (Altincicek *et al*., 2008). Innate immune responses like phagocytosis, synthesis of antimicrobial proteins, melanisation are the backbone of insect immune defence system (Lemaitre and Hoffmann, 2007). Overexpression of immune response genes in our whiteflies provides an indirect evidence for the defence of whitefly against pathogens by AR+ infections. Such selective benefits to whitefly hosts by its symbionts under stress conditions is reported on other whitefly species (Brumin *et al*., 2011; Hendry *et al*., 2014; Su *et al*., 2014).

Constitutively primed immune defences against pathogenesis in insects are maintained by additional metabolomics and physiological demands (Freitak *et al*., 2003, 2007; Ardia *et al*., 2012). The physiology in such organisms is evolved to sustain an efficient defence system at the expense of some other important fitness trait (Schmid-Hempel, 2005). Apart from

additional metabolic requirements, some costs also result due to pleiotropic roles of the same gene or pathways in both insect defence and development (Schmid-Hempel, 2003, 2005). For example, Toll pathway in *Drosophila* provide protection against bacterial and fungal infection (Lemaitre *et al*., 1996; Lau *et al*., 2003; Valanne *et al*., 2011) but is also essential for larval development (Halfon *et al*., 1995; Qiu *et al*., 1998). Trade-offs between current reproductive success (life history traits) and future survival of progenies (immunity, resistance) with endosymbiont infections of aphids are well established (Gwynn *et al*., 2005; Oliver *et al*., 2006, 2008; Vorburger and Gouskov, 2011; Vorburger *et al*., 2013). AR+ infections in SSA1-SG3 could thus be evolved to maintain an efficient immune system to provide defence against lethal pathogens as a trade-off to outweigh the observed fitness costs. This could explain the possible reasons for high prevalence of *Arsenophonus* and *Rickettsia* in SSA1-SG3, despite fitness costs.

In addition to their role on the apparent balancing of fitness costs v/s benefits, endosymbionts are also known to interact with viruses inside whitefly bodies. *Rickettsia* and *Hamiltonella* infection in *B. tabaci* release GroEL proteins which protect virions inside the whitefly haemocoel and facilitate virus transmission. Rate of virus acquisition and frequency of viruliferous whiteflies are important factors determining the rate of spread of cassava mosaic disease (Holt *et al*., 1997; Jeger *et al*., 2004). Retention of higher quantities of virus in whiteflies could effectively prolong the infectious period of the whiteflies enabling transmission for longer period. However, AR+ infections of cassava whiteflies had negative effect on the acquisition and retention of EACMV-UG. Frequency of AR- acquiring and retaining EACMV-UG (91.8%, 87.6%) was higher than AR+ populations (71.8%, 61.2%). AR- also retained higher titres of EACMV-UG than AR+ populations after 48 hours of IAP, indicating that GroEL was probably not involved in virion protection in cassava whiteflies. The reasons for the lower retention of virus in AR+ whiteflies could be due to immune responses such as autophagy which plays crucial role in the degradation of viruses in whitefly and plant host (Luan *et al*., 2011; Gorovits *et al*., 2014; Wang *et al*., 2016). This was supported when higher expression of the autophagy related Knottin proteins was observed in AR+ whiteflies, which can lead to greater degradation of the acquired virus. Similar reduction in dengue virus titres inside mosquitoes and transmission was associated with *Wolbachia* infections (Osborne *et al*., 2009; Walker *et al*., 2011; Frentiu *et al*., 2014). These results are therefore indicating the possible role of S-endosymbiont infections to reduce whitefly numbers as well as to reduce their virus transmission capabilities.

The CMD pandemic in Uganda in the 1990s was proposed to be invaded by a highly fecund SSA2 population and the rapid spread of the disease was due to a mutually beneficial interaction between virus infected-plants and whitefly populations. EACMV-UG infections however offered minimal benefits to AR+ or AR- whiteflies in this study. Endosymbionts are also believed to have a role in whitefly population dynamics. To verify this, cassava whiteflies from the Uganda CMD pandemic and non-pandemic zones, collected in 1997, were analysed for endosymbiont diversity for the first time. Symbiont infections were found independent of the pandemic zones but this could be due to low number of samples (15) that we were able to access from the non-pandemic sites. Nevertheless, *Wolbachia* was the most prevalent symbiont (~44%) infecting cassava whiteflies. Frequency of *Wolbachia* was much higher in SSA2 (62.7%) than in SSA1-SG1 (20.0%). *Wolbachia* was also found nearly fixed in SSA1-SG2, which is also in decline in cassava fields. As stated above, these observations also indicate that *Wolbachia* infections may have caused the decline of SSA1-SG2 and SSA2. However, these interactions seem to be species-specific as different strains of *Wolbachia* infected SSA1-SG2 and SSA2 but caused similar phenotypic effect. We have limited knowledge about gene flow between these populations and thus the reasons for the existence of host specific *Wolbachia* strains remains to be understood. However, the multiplication rates of *Wolbachia* were highly influenced by the host genetic background as both SSA1-SG2 and SSA2 supported high titres of *Wolbachia*. Contrastingly, SSA1-SG1 and SSA1-SG3 (Legg *et al*., 2013), which are currently the predominant population in East and central Africa had lower densities of *Wolbachia*, indicating the critical role of host genetic background in the developed phenotype. Efficient regulation over symbiont densities by SSA1-SG1 populations could be a key reason for its dominance in the cassava fields.

This study also provided further evidence of the association of SSA2 populations with the CMD pandemic in the 1990s. The superabundant SSA2 populations in the pandemic zones have since declined to very low levels in the recent surveys (Sseruwagi *et al*., 2006; Mugerwa *et al*., 2012; Legg *et al*., 2013; Tajebe *et al*., 2015a). While we could not use SSA2 populations to investigate this, as we could not use generate correct combinations bacterial infections, however, we used SSA1-SG2 W+ and SSA1-SG3 W- populations to establish the role of *Wolbachia* in the decline of SSA1-SG2. *Wolbachia*-infected SSA1-SG2 was quickly outcompeted and completely replaced by *Wolbachia* free SSA1-SG3 within 90 days of co-existence. This result firmly confirms that the population bottleneck encountered by

SSA1-SG2 whiteflies was due to the negative impact of *Wolbachia* infections on its biology and perhaps and had limited or no effect of agricultural practices or climate change.

We also investigated the effect of S-endosymbionts on the vector abilities of cassava whiteflies. In contrast to SSA1-SG3 AR+, SSA2 A+ retained five times higher titres of EACMV-UG than SSA2 W+ or with no S-symbionts (SSA2 -). *Arsenophonus* titre in SSA2 A+ were much lower (39.7 times) than in SSA1-SG3 AR+. Such low concentrations of *Arsenophonus* in SSA2 may not have triggered high immune response (Knottins) and thus had no effect on the negative effect on EACMV-UG concentrations in insect the body. SSA2 - was completely free of any detectable S-symbionts and thus the lack of immune response has led to the accumulation of high virus concentrations. Association of the highly abundant *Wolbachia* with lower retention of EACMV-UG is promising and could have wider implications for use as a bioncontrol agent in the management of CMD. In summary, this study demonstrated the negative effects of S-endosymbiont infections (both AR+ and W+ infections) on the biology and lifecycle of cassava whiteflies. The two populations that have rapidly declined in recent years (SSA2 and SSA1-SG2) were both infected with high prevalence and titres of bacterial infections. In simulation experiments, endosymbiont-infected SSA1-SG2 declined rapidly in the presence of endo-free whiteflies, suggesting that endosymbionts may have contributed to their decline in cassava fields. Endosymbiont infections generally contributed to heightened immune response, which lead to the decreased virus acquisition and retention and thus decreased vectoring abilities of African cassava whiteflies. These results thus raise the possibility of using S-endosymbionts as biocontrol agents for whiteflies and whitefly-transmitted viruses. Our, results also show that endosymbiont-whitefly interactions are specific to a species or even up to populations and thus cannot be generalised to all cassava whiteflies. Future studies to understand the role of symbionts on other whitefly populations (SSA1-SG1, SSA1-SG2, SSA2) are urgently warranted. Further studies on whitefly transcriptomic and proteomics are required to gain greater insights into the mechanisms of whitefly, endosymbiont and virus interactions.

7.1 Key findings of this study

- At least five genetically distinct whitefly populations were found to infect cassava in SSA.

- A high throughput and cost effective PCR-RFLP test based on mtCO1 gene was developed for easy identification of the five whitefly types used in this study.
- Diverse facultative endosymbiotic bacteria are harboured by African cassava whiteflies.
- *Wolbachia, Arsenophonus, Rickettsia* and *Cardinium* are the S-endosymbionts in decreasing order of prevalence in cassava whiteflies in SSA.
- Majority of the cassava whiteflies were singly infected with a symbiont.
- Prevalence of S-endosymbionts varied significantly with different cassava whitefly populations.
- Frequency and quantities of *Wolbachia* are higher in the declining SSA1-SG2 and SSA2 populations.
- SSA1-SG2 whiteflies infected *Wolbachia* were out competed by SSA1-SG3 infected with *Arsenophonus* + *Rickettsia* and SSA1-SG3 free of symbionts in separate experiments. SSA1-SG2 was completely replaced within 90 days of co-existence.
- *Arsenophonus, Rickettsia* and *Wolbachia* in cassava whiteflies are present only inside the bacteriocytes of cassava whitefly nymphs and adults but absent in the haemocoel.
- *Arsenophonus* and *Rickettsia* infections have fitness costs on SSA1-SG3 populations by negatively impacting adult development and duration of lifecycle.
- Symbionts also negatively affect vector capabilities of cassava whiteflies. Higher numbers of SSA1-SG3 free of symbionts whiteflies acquired, retained EACMV-UG virus than SSA1-SG3 infected with *Arsenophonus* + *Rickettsia*. The former also retained higher titres of EACMV-UG.
- *Arsenophonus* and *Rickettsia* infections trigger higher immune response and thus could play vital role in providing protection to whiteflies against other pathogens but may have also contributed to reduction in EACMV-UG concentrations in the process.
- SSA2 were more abundant in the CMD pandemic Uganda in 1997.
- The results overall show that secondary symbionts greatly affect cassava whitefly biology and vector abilities.

8 REFERENCE

Ahmed, M. Z., Ren, S., Xue, X., Li, X. X., Jin, G., Qiu, B. L. (2010a). Prevalence of endosymbionts in *Bemisia tabaci* populations and their in vivo sensitivity to antibiotics. *Current Microbiology* 61(4), pp. 322–8.

Ahmed, M. Z., Ren, S. X., Mandour, N. S., Greeff, J. M., Qiu, B. L. (2010b). Prevalence of *Wolbachia* supergroups A and B in *Bemisia tabaci* (Hemiptera: Aleyrodidae) and some of its natural enemies. *Journal of Economic Entomology* 103(5), pp. 1848–1859.

Ahmed, M. Z., De Barro, P. J., Ren, S. X., Greeff, J. M., Qiu, B. L. (2013). Evidence for horizontal transmission of secondary endosymbionts in the *Bemisia tabaci* cryptic species complex. *Plos One* 8(1), p. e53084.

Alabi, O. J., Kumar, P. L., Naidu, R. A. (2008). Multiplex PCR for the detection of *African cassava mosaic virus* and *East African cassava mosaic Cameroon virus* in cassava. *Journal of Virological Methods* 154(1), pp. 111–120.

Alicai, T., Omongo, C. A., Maruthi, M. N., Hillocks, R. J., Baguma, Y., Kawuki, R., Bua, A., Otim-Nape, G. W., Colvin, J. (2007). Re-emergence of cassava brown streak disease in Uganda. *Plant Disease* 91(1), pp. 24–29.

Anselme, C., Pérez-Brocal, V., Vallier, A., Vincent-Monegat, C., Charif, D., Latorre, A., Moya, A. and Heddi, A. (2008). Identification of the weevil immune genes and their expression in the bacteriome tissue. *BMC Biology*, 6(1), p. 43.

Antignus, Y., Mor, N., Joseph, R. Ben, Lapidot, M., Cohen, S. (1996). Ultraviolet-absorbing plastic sheets protect crops from insect pests and from virus diseases vectored by insects. *Environmental Entomology* 25(5), pp. 919–924.

Antignus, Y., Lapidot, M., Hadar, D., Messika, Y., Cohen, S. (1998). Ultraviolet-absorbing screens serve as optical barriers to protect crops from virus and insect pests. *Journal of Economic Entomology* 91(6), pp. 1401–1405.

Antignus, Y., Lachman, O., Pearlsman, M., Koren, A., Matan, E., Tregerman, M., Ucko, O., Messika, Y., Omer, S., Unis, H. (2004). Development of an IPM system to reduce the damage of squash leaf curl begomovirus in zucchini squash crops. Abstract Compendium, In *2nd European Whitefly Symposium, Cavtat, Croatia*.

Antignus, Y., Lachman, O., Pearlsman, M. (2005). Light manipulation by soil mulches protects crops from the spread of Begomoviruses. In *IX International Plant Virus Epidemiology Symposium, Lima, Peru*.

Arbouzova, N. I., Zeidler, M. P. (2006). JAK/STAT signalling in *Drosophila*: insights into conserved regulatory and cellular functions. *Development* 133(14), pp. 2605–2616.

Ardia, D. R., Gantz, J. E., Brent, C., Strebel, S. (2012). Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Functional Ecology* 26(3), pp. 732–739.

Asiimwe, P., Ecaat, J. S., Guershon, M., Kyamanywa, S., Gerling, D., Legg, J. P. (2007a). Evaluation of Serangium n. sp.(Col., Coccinellidae), a predator of *Bemisia tabaci* (Hom., Aleyrodidae) on cassava. *Journal of Applied Entomology* 131(2), pp. 76–80.

Asiimwe, P., Ecaat, J. S., Otim, M., Gerling, D., Kyamanywa, S., Legg, J. P. (2007b). Life-table analysis of mortality factors affecting populations of *Bemisia tabaci* on cassava in Uganda. *Entomologia Experimentalis et Applicata* 122(1), pp. 37–44.

Augustinos, A. A., Santos-Garcia, D., Dionyssopoulou, E., Moreira, M., Papapanagiotou, A., Scarvelakis, M., Doudoumis, V., Ramos, S., Aguiar, A. F., Borges, P. A. V (2011). Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? *Plos One* 6(12), p. e28695.

Ausher, R. (1997). Implementation of integrated pest management in Israel. *Phytoparasitica* 25(2), pp. 119–141.

Baldo, L., Hotopp, J. C. D., Jolley, K. A., Bordenstein, S. R., Biber, S. A., Choudhury, R. R., Hayashi, C., Maiden, M. C. J., Tettelin, H., Werren, J. H. (2006). Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology* 72(11), pp. 7098–7110.

Baldo, L., Werren, J. H. (2007). Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. *Current Microbiology*, 55(1), pp. 81–87.

Basu, A. N. (1995). *Bemisia tabaci (Gennadius): crop pest and principal whitefly vector of plant viruses*. Westview Press Boulder, CO.

Baumann, P., Moran, N. A., Baumann, L., Dworkin, M. (2006). Bacteriocyte-associated endosymbionts of insects. *The Prokaryotes*, Springer, 1, pp. 403–438.

Bayhan, E., Ulusoy, M. R., Brown, J. K. (2006). Host range, distribution, and natural enemies of *Bemisia tabaci* 'B biotype'(Hemiptera: Aleyrodidae) in Turkey. *Journal of Pest Science* 79(4), pp. 233–240.

Bedford, I. D., Pinner, M., Liu, S., Markham, P. G. (1994). *Bemisia tabaci*-Potential infestation, phytotoxicity and virus transmission within European agriculture. In *Proceedings-Brighton Crop Protection Conference, Pests and Diseases, Brighton, UK, 21-24 November ases, 1994, vol. 2.*, British Crop Protection Council, BCPC Publications, pp. 911–916.

Bellotti, A., van Schoonhoven, A. (1978). Mite and insect pests of cassava. *Annual Review of Entomology* 23(1), pp. 39–67.

Berry, S. D., Fondong, V. N., Rey, C., Rogan, D., Fauquet, C. M., Brown, J. K. (2004). Molecular evidence for five distinct *Bemisia tabaci* (Homoptera: Aleyrodidae) geographic haplotypes associated with cassava plants in sub-Saharan Africa. *Annals of the Entomological Society of America* 97(4), pp. 852–859.

Bing, X., Ruan, Y., Rao, Q., Wang, X., Liu, S. (2013a). Diversity of secondary endosymbionts among different putative species of the whitefly *Bemisia tabaci*. *Insect Science* 20(2), pp. 194–206.

Bing, X. L., Yang, J., Zchori-Fein, E., Wang, X. W., Liu, S. S. (2013b). Characterization of a newly discovered symbiont of the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Applied and Environmental Microbiology* 79(2), pp. 569–75.

Bing, X. L., Xia, W. Q., Gui, J. D., Yan, G. H., Wang, X. W., Liu, S. S. (2014). Diversity and evolution of the *Wolbachia* endosymbionts of *Bemisia* (Hemiptera: Aleyrodidae) whiteflies. *Ecology and Evolution 4*(13), 2714-2737.

Bishop, R. (2010). Applications of fluorescence in situ hybridization (FISH) in detecting genetic aberrations of medical significance. *Bioscience Horizons 3*(1), 85-95.

Boots, M., Begon, M. (1993). Trade-offs with resistance to a granulosis virus in the Indian meal moth, examined by a laboratory evolution experiment. *Functional Ecology* pp. 528–534.

Bourtzis, K., Miller, T. A. (2003). *Insect symbiosis*. CRC Press.

Boykin, L. M., Shatters, R. G., Rosell, R. C., McKenzie, C. L., Bagnall, R. A., De Barro, P., Frohlich, D. R. (2007). Global relationships of *Bemisia tabaci* (Hemiptera: Aleyrodidae) revealed using Bayesian analysis of mitochondrial COI DNA sequences. *Molecular Phylogenetics and Evolution* 44(3), pp. 1306–1319.

Boykin, L. M., Bell, C. D., Evans, G., Small, I., & De Barro, P. J. (2013). Is agriculture driving the diversification of the *Bemisia tabaci* species complex (Hemiptera: Sternorrhyncha: Aleyrodidae)?: Dating, diversification and biogeographic evidence revealed. *BMC Evolutionary Biology*, 13(1), 1.

Boykin, L. M., De Barro, P. J. (2014). A practical guide to identifying members of the *Bemisia tabaci* species complex: and other morphologically identical species. *Frontiers in Ecology and Evolution* 2, p. 45.

Boyle, L., O'Neill, S. L., Robertson, H. M., Karr, T. L. (1993). Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*, *Science* 260(5115), pp. 1796–1799.

Braig, H. R., Zhou, W., Dobson, S. L., O'Neill, S. L. (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology* 180(9), pp. 2373–2378.

Brelsfoard, C. L., Dobson, S. L. (2009). *Wolbachia*-based strategies to control insect pests and disease vectors. *Asia Pacific Journal of Molecular Biology and Biotechnology* 17(3), pp. 55–63.

Bressac, C., Rousset, F. (1993). The reproductive incompatibility system in *Drosophila simulans*: DAPI-staining analysis of the *Wolbachia* symbionts in sperm cysts. *Journal of Invertebrate Pathology* 61(3), pp. 226–230.

Brown, J. K., Bird, J. (1992). Whitefly-transmitted geminiviruses and associated disorders in the Americas and the Caribbean Basin. *Plant Disease* 76(3), pp. 220–225.

Brown, J. K., Frohlich, D. R., Rosell, R. C. (1995). The sweetpotato or silverleaf whiteflies: biotypes of *Bemisia tabaci* or a species complex? *Annual Review of Entomology* 40(1), pp. 511–534.

Brown, J. K. (2010). Phylogenetic biology of the *Bemisia tabaci* sibling species group. In *Bemisia: Bionomics and Management of a Global Pest* pp. 31–67.

Brumin, M., Kontsedalov, S., Ghanim, M. (2011). *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. *Insect Science* 18(1), pp. 57–66.

Brumin, M., Levy, M., Ghanim, M. (2012). Transovarial transmission of *Rickettsia* spp. and organ-specific infection of the whitefly *Bemisia tabaci*. *Applied and Environmental Microbiology* 78(16), pp. 5565–74.

Buchner, P. (1965). Endosymbiosis of Animals with Plant Microorganims, John Wiley and sons.

Bull, J. J. (1983). *Evolution of sex determining mechanisms.* The Benjamin/Cummings Publishing Company, Inc.

Butter, N. S., Vir, B. K. (1989). Morphological basis of resistance in cotton to the whitefly *Bemisia tabaci*. *Phytoparasitica* 17(4), pp. 251–261.

Byrne, D. N., Bellows Jr, T. S. (1991). Whitefly biology. *Annual Review of Entomology* 36(1), pp. 431–457.

Calvert, L. A., Thresh, J. M. (2002). The viruses and virus diseases of cassava. *Cassava: biology, production and utilization*, CABI Publishing: Oxon, UK, pp. 237–260.

Calvo, J., Bolckmans, K., Stansly, P. A., Urbaneja, A. (2009). Predation by *Nesidiocoris tenuis* on *Bemisia tabaci* and injury to tomato. *Biocontrol* 54(2), pp. 237–246.

Calvo, F. J., Bolckmans, K., Belda, J. E. (2011). Control of *Bemisia tabaci* and *Frankliniella occidentalis* in cucumber by *Amblyseius swirskii*. *Biocontrol* 56(2), pp. 185–192.

Campbell, B. C., Steffen-Campbell, J. D., Gill, R. J. (1996). Origin and radiation of whiteflies: an initial molecular phylogenetic assessment. *Bemisia: 1995, taxonomy, biology, damage, control and management*, Andover, Hants, UK: Intercept, 1996.

Caspi-Fluger, A., Inbar, M., Mozes-Daube, N., Mouton, L., Hunter, M. S., Zchori-Fein, E. (2011). *Rickettsia* ‘in’and ‘out’: two different localization patterns of a bacterial symbiont in the same insect species. *Plos One*, 6(6).

Cass, B. N., Himler, A. G., Bondy, E. C., Bergen, J. E., Fung, S. K., Kelly, S. E., Hunter, M. S. (2015). Conditional fitness benefits of the *Rickettsia* bacterial symbiont in an insect pest. *Oecologia*, pp. 1–11.

Ceballos, H., Kulakow, P., Hershey, C. (2012). Cassava breeding: current status, bottlenecks and the potential of biotechnology tools. *Tropical Plant Biology* 5(1), pp. 73–87.

Channarayappa, C., Shivashankar, G., Muniyappa, V., Frist, R. H. (1992). Resistance of *Lycopersicon* species to *Bemisia tabaci*, a tomato leaf curl virus vector. *Canadian Journal of Botany* 70(11), pp. 2184–2192.

Chen, D., Montllor, C. B., Purcell, A. H. (2000). Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. *Entomologia Experimentalis et Applicata* 95(3), pp. 315–323.

Cheng, Q., Ruel, T. D., Zhou, W., Moloo, S. K., Majiwa, P., O’neill, S. L., Aksoy, S. (2000)

Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies. *Glossina* spp., *Medical and Veterinary Entomology* 14(1), pp. 44–50.

Chiel, E., Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Katzir, N., Inbar, M., Ghanim, M. (2007). Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of Entomological Research* 97(4), pp. 407–13.

Chiel, E., Inbar, M., Mozes-Daube, N., White, J. A., Hunter, M. S., Zchori-Fein, E. (2009). Assessments of fitness effects by the facultative symbiont *Rickettsia* in the sweetpotato whitefly (Hemiptera: Aleyrodidae). *Annals of the Entomological Society of America* 102(3), pp. 413–418.

Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. A., Martinez, J., Jiggins, F. M., Teixeira, L. (2013). *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis. *Plos Genetics* 9(12), p. e1003896.

Clark, M. E., Veneti, Z., Bourtzis, K., Karr, T. L. (2002). The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. *Mechanisms of Development* 111(1), pp. 3–15.

Clark, E. L., Karley, A. J., Hubbard, S. F. (2010). Insect endosymbionts: manipulators of insect herbivore trophic interactions? *Protoplasma* 244(1-4), pp. 25–51.

Colvin, J., Omongo, C. A., Maruthi, M. N., Otim-Nape, G. W., Thresh, J. M. (2004). Dual begomovirus infections and high *Bemisia tabaci* populations: two factors driving the spread of a cassava mosaic disease pandemic. *Plant Pathology* 53(5), pp. 577–584.

Colvin, J., Omongo, C. A., Govindappa, M. R., Stevenson, P. C., Maruthi, M. N., Gibson, G., Seal, S. E., Muniyappa, V. (2006). Host-plant viral infection effects on arthropod-vector population growth, development and behaviour: Management and epidemiological implications. *Advances in Virus Research* 67, pp. 419–452.

Cook, P. E., McMeniman, C. J., O'Neill, S. L. (2008). Modifying insect population age structure to control vector-borne disease. In *Transgenesis and the management of vector-borne disease*, Springer New York, pp. 126–140.

Costa, H. S., Ullman, D. E., Johnson, M. W., Tabashnik, B. E. (1993a). Association between *Bemisia tabaci* density and reduced growth, yellowing, and stem blanching of lettuce and kai choy. *Plant Disease* 77(10), pp. 969–972.

Costa, H. S., Westcot, D. M., Ullman, D. E., Johnson, M. W. (1993b). Ultrastructure of the endosymbionts of the whitefly, *Bemisia tabaci* and *Trialeurodes vaporariorum*. *Protoplasma* 176(3-4), pp. 106–115.

Costa, H. S., Toscano, N. C., Henneberry, T. J. (1996). Mycetocyte inclusion in the oocytes of *Bemisia argentifolii* (Homoptera: Aleyrodidae). *Annals of the Entomological Society of America* 89(5), pp. 694–699.

Costa, H. S., Enneberry, T. J., Toscano, N. C. (1997). Effects of antibacterial materials on *Bemisia argentifolii* (Homoptera: Aleyrodidae) oviposition, growth, survival, and sex ratio. *Journal of Economic Entomology* 90(2), pp. 333–339.

Cuthbertson, A. G. S. (2013). Update on the status of *Bemisia tabaci* in the UK and the use of entomopathogenic fungi within eradication programmes. *Insects* 4(2), pp. 198–205.

Czosnek, H., Laterrot, H. (1997). A worldwide survey of *Tomato yellow leaf curl viruses*. *Archives of Virology* 142(7), pp. 1391–1406.

Czosnek, H., Ghanim, M. (2012). Back to basics: are begomoviruses whitefly pathogens? *Journal of Integrative Agriculture 11*(2), 225-234.

David, J. R., Gibert, P., Legout, H., Petavy, G., Capy, P., Moreteau, B. (2005) Isofemale lines in *Drosophila*: an empirical approach to quantitative trait analysis in natural populations. *Heredity* 94(1), pp. 3–12.

De Barro, P. J. (1995). *Bemisia tabaci* biotype B: a review of its biology, distribution and control. *CSIRO Australia Division of Entomology Technical Paper*, CSIRO Division of Entomology, (36).

De Barro, P. J., Hart, P. J. (2000) Mating interactions between two biotypes of the whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae) in Australia. *Bulletin of Entomological Research, Cambridge University Press*, 90(02), pp. 103–112.

De Barro, P. J., Liu, S. S., Boykin, L. M., Dinsdale, A. B. (2011). *Bemisia tabaci*: a statement of species status. *Annual Review of Entomology* 56, pp. 1–19.

De Ponti, O. M. B., Romanow, L. R., Berlinger, M. J. (1990). Whitefly-plant relationships: plant resistance, *Whiteflies: their bionomics, pest status and management. Andover, UK: Intercept*, pp. 91–106.

Degnan, P. H., Moran, N. A. (2008a). Diverse phage-encoded toxins in a protective insect endosymbiont. *Applied and Environmental Microbiology*, 74(21), pp. 6782–6791.

Degnan, P. H., Moran, N. A. (2008b). Evolutionary genetics of a defensive facultative symbiont of insects: exchange of toxin-encoding bacteriophage. *Molecular Ecology*, 17(3), pp. 916–929.

Dinsdale, A., Cook, L., Riginos, C., Buckley, Y. M., De Barro, P. (2010). Refined Global Analysis of *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae) mitochondrial cytochrome oxidase 1 to identify species level genetic boundaries. *Annals of the Entomological Society of America* 103(2), pp. 196–208.

Dixon, A. G. O., Bandyopadhyay, R., Coyne, D., Ferguson, M., Ferris, R. S. B., Hanna, R., Hughes, J., Ingelbrecht, I., Legg, J., Mahungu, N. (2003). Cassava: From poor farmers' crop to pacesetter of African rural development. *Chronica Horticulturae* 43(4), pp. 8–15.

Dobson, S. L., Bourtzis, K., Braig, H. R., Jones, B. F., Zhou, W., Rousset, F., O'Neill, S. L. (1999). *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology* 29(2), pp. 153–160.

Douglas, A. E., Francois, C., Minto, L. B. (2006). Facultative 'secondary'bacterial symbionts and the nutrition of the pea aphid, *Acyrthosiphon pisum*. *Physiological Entomology* 31(3), pp. 262–269.

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident

microorganisms. *Annual Review of Entomology*, 60, pp. 17–34.

Dubern, J. (1994). Transmission of African cassava mosaic geminivirus by the whitefly (*Bemisia tabaci*). *Tropical Science* 34(1), pp. 82–91.

Duffus, J. E., Larsen, R. C., Liu, H. Y. (1986). *Lettuce infectious yellows virus*: a new type of whitefly-transmitted virus. *Phytopathology 76*(1), pp. 97-100.

Duron, O., Bernard, C., Unal, S., Berthomieu, A., Berticat, C. and Weill, M. (2006). Tracking factors modulating cytoplasmic incompatibilities in the mosquito *Culex pipiens*. *Molecular Ecology* 15(10), pp. 3061–3071.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., Hurst, G. D. (2008). The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology* 6(1), p. 27.

Duron, O. (2014). *Arsenophonus* insect symbionts are commonly infected with APSE, a bacteriophage involved in protective symbiosis. *FEMS Microbiology Ecology* 90(1), pp. 184–194.

Eleftherianos, I., Atri, J., Accetta, J., Castillo, J. C. (2013) Endosymbiotic bacteria in insects: guardians of the immune system. *Frontiers in Physiology* 4(46), pp. 1–10.

Engelstädter, J., Hurst, G. D. D. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology, Evolution, and Systematics*, 40, pp. 127–149.

Engelstädter, J., Telschow, A. (2009). Cytoplasmic incompatibility and host population structure. *Heredity* 103(3), pp. 196–207.

Everett, K. D. E., Thao, M., Horn, M., Dyszynski, G. E., Baumann, P. (2005). Novel chlamydiae in whiteflies and scale insects: endosymbionts "*Candidatus* Fritschea bemisiae" strain Falk and "*Candidatus* Fritschea eriococci" strain Elm. *International Journal of Systematic and Evolutionary Microbiology* 55(Pt 4), pp. 1581–7.

Fang, Y. W., Liu, L. Y., Zhang, H. L., Jiang, D. F., Chu, D. (2014). Competitive ability and fitness differences between two introduced populations of the invasive whitefly *Bemisia tabaci* Q in China. *Plos One*, 9(6), e100423.

FAOSTAT, Data (2014). Food and agricultural commodities production, *FAO: Rome, Italy* (2012).

Fauquet, C., Fargette, D. (1990). *African cassava mosaic virus*: etiology, epidemiology and control. *Plant Disease*, 74(6), pp. 404–411.

Fellowes, M. D. E., Kraaijeveld, A. R., Godfray, H. C. J. (1999). Cross-resistance following artificial selection for increased defense against parasitoids in *Drosophila melanogaster*. *Evolution*, pp. 966–972.

Ferdig, M. T., Beerntsen, B. T., Spray, F. J., Li, J., Christensen, B. M. (1993). Reproductive costs associated with resistance in a mosquito-filarial worm system. *The American Journal of Tropical Medicine and Hygiene* 49(6), pp. 756–762.

Ferrari, J., Darby, A. C., Daniell, T. J., Godfray, H. C. J., Douglas, A. E. (2004). Linking the

bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology* 29(1), pp. 60–65.

Firdaus, S., Vosman, B., Hidayati, N., Supena, J., Darmo, E., GF Visser, R., van Fishpool, L. D. C., Burban, C. (1994). *Bemisia tabaci*: the whitefly vector of African cassava mosaic geminivirus. *Tropical Science* 34(1), pp. 55–72.

Flint, H. M., Naranjo, S. E., Leggett, J. E., Henneberry, T. J. (1996). Cotton water stress, arthropod dynamics, and management of *Bemisia tabaci* (Homoptera: Aleyrodidae). *Journal of Economic Entomology* 89(5), pp. 1288–1300.

Freitak, D., Ots, I., Vanatoa, A., Hörak, P. (2003). Immune response is energetically costly in white cabbage butterfly pupae. *Proceedings of the Royal Society of London B: Biological Sciences* 270(2), pp. S220–S222.

Freitak, D., Wheat, C. W., Heckel, D. G., Vogel, H. (2007). Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. *BMC Biology* 5(1), p. 56.

Frentiu, F. D., Zakir, T., Walker, T., Popovici, J., Pyke, A. T., van den Hurk, A., McGraw, E. A., O'Neill, S. L. (2014). Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. *Plos Neglected Tropical Diseases* 8(2), p. e2688.

Frohlich, D., Torres-Jerez, I., Bedford, I., Markham, P., Brown, J. (1999). A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. *Molecular Ecology* 8(10), pp. 1683–91.

Gennadius, P. (1889). Disease of tobacco plantations in the Trikonia. The aleurodid of tobacco. *Ellenike Georgia* 5, pp. 1–3.

Gerardo, N. M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S. M., De Vos, M., Duncan, E. J., Evans, J. D., Gabaldón, T., Ghanim, M. (2010). Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. *Genome Biology* 11(2), p. R21.

Gerling, D., Horowitz, A. R., Baumgaertner, J. (1986). Autecology of *Bemisia tabaci*. *Agriculture, Ecosystems and Environment* 17(1), pp. 5–19.

Gerling, D., Alomar, Ò., Arnò, J. (2001). Biological control of *Bemisia tabaci* using predators and parasitoids. *Crop Protection* 20(9), pp. 779–799.

Ghanim, M., Morin, S., Czosnek, H. (2001). Rate of *Tomato yellow leaf curl virus* translocation in the circulative transmission pathway of its vector, the whitefly *Bemisia tabaci*. *Phytopathology* 91(2), pp. 188–196.

Ghanim, M., Kontsedalov, S. (2009). Susceptibility to insecticides in the Q biotype of *Bemisia tabaci* is correlated with bacterial symbiont densities. *Pest Management Science* 65(9), pp. 939–942.

Gherna, R. L., Werren, J. H., Weisburg, W., Cote, R., Woese, C. R., Mandelco, L., Brenner, D. J. (1991). NOTES: *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. *Interntional Journal of Systematic*

Bacteriology, 41(4), pp. 563–565.

Glaser, R. L., Meola, M. A. (2010). The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *Plos One* 5(8), p. e11977.

Gnankine, O., Mouton, L., Henri, H., Terraz, G., Houndete, T., Martin, T., Vavre, F., Fleury, F. (2013). Distribution of *Bemisia tabaci* (Homoptera: Aleyrodidae) biotypes and their associated symbiotic bacteria on host plants in West Africa. *Insect Conservation and Diversity* 6(3), pp. 411–421.

Gorovits, R., Moshe, A., Ghanim, M., Czosnek, H. (2014). Degradation mechanisms of the *Tomato yellow leaf curl virus* coat protein following inoculation of tomato plants by the whitefly *Bemisia tabaci*. *Pest Management Science* 70(10), pp. 1632–1639.

Gottlieb, Y., Ghanim, M., Chiel, E., Gerling, D., Portnoy, V., Steinberg, S., Tzuri, G., Horowitz, A. R., Belausov, E., Mozes-daube, N., Kontsedalov, S., Gershon, M., Gal, S., Katzir, N., Zchori-fein, E. (2006). Identification and Localization of a *Rickettsia* sp . in *Bemisia tabaci* (Homoptera : Aleyrodidae). *Applied and Environmental Microbiology* 72(5), pp. 3646–3652.

Gottlieb, Y., Ghanim, M., Gueguen, G., Kontsedalov, S., Vavre, F., Fleury, F., Zchori-Fein, E. (2008). Inherited intracellular ecosystem: symbiotic bacteria share bacteriocytes in whiteflies. *The FASEB Journal* 22(7), pp. 2591–2599.

Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Kontsedalov, S., Skaljac, M., Brumin, M., Sobol, I., Czosnek, H., Vavre, F., Fleury, F. (2010). The transmission efficiency of *Tomato yellow leaf curl virus* by the whitefly *Bemisia tabaci* is correlated with the presence of a specific symbiotic bacterium species. *Journal of Virology* 84(18), pp. 9310–9317.

Gotz, M., Popovski, S., Kollenberg, M., Gorovits, R., Brown, J. K., Cicero, J. M., Czosnek, H., Winter, S., Ghanim, M. (2012). Implication of *Bemisia tabaci* heat shock protein 70 in begomovirus-whitefly interactions. *Journal of Virology* 86(24), pp. 13241-13252.

Gueguen, G., Vavre, F., Gnankine, O., Peterschmitt, M., Charif, D., Chiel, E., Gottlieb, Y., Ghanim, M., Zchori-Fein, E., Fleury, F. (2010). Endosymbiont metacommunities, mtDNA diversity and the evolution of the *Bemisia tabaci* (Hemiptera: Aleyrodidae) species complex. *Molecular Ecology* 19(19), pp. 4365–4378.

Guo, J. Y., Ye, G. Y., Dong, S. Z., Liu, S. S. (2010). An invasive whitefly feeding on a virus-infected plant increased its egg production and realized fecundity. *Plos One* 5(7), e11713.

Guo, J., Cong, L., Wan, F. (2013). Multiple generation effects of high temperature on the development and fecundity of *Bemisia tabaci* (Gennadius)(Hemiptera: Aleyrodidae) biotype B. *Insect Science* 20(4), pp. 541–549.

Gwynn, D. M., Callaghan, A., Gorham, J., Walters, K. F. A., Fellowes, M. D. E. (2005). Resistance is costly: trade-offs between immunity, fecundity and survival in the pea aphid. *Proceedings of the Royal Society of London B: Biological Sciences*, 272(1574), pp. 1803–1808.

Halfon, M. S., Hashimoto, C., Keshishian, H. (1995). The *Drosophila* toll gene functions

zygotically and is necessary for proper motoneuron and muscle development. *Developmental Biology* 169(1), pp. 151–167.

Hansen, A. K., Jeong, G., Paine, T. D., Stouthamer, R. (2007). Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. *Applied and Environmental Microbiology* 73(23), pp. 7531–7535.

Hardin, G. (1968) The tragedy of the commons, S*cience*, 162(3859), pp. 1243–1248.

Heddi, A., Grenier, A. M., Khatchadourian, C., Charles, H., Nardon, P. (1999). Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proceedings of the National Academy of Sciences* 96(12), pp. 6814–6819.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science* 322(5902), p. 702.

Hendry, T. A., Hunter, M. S., Baltrus, D. A. (2014). The facultative symbiont *Rickettsia* protects an invasive whitefly against entomopathogenic *Pseudomonas syringae* strains. *Applied and Environmental Microbiology* 80(23), pp. 7161–7168.

Hilje, L., Costa, H. S., Stansly, P. A. (2001). Cultural practices for managing *Bemisia tabaci* and associated viral diseases. *Crop Protection* 20(9), pp. 801–812.

Hillocks, R. J., Jennings, D. L. (2003). Cassava brown streak disease: a review of present knowledge and research needs. *International Journal of Pest Management* 49(3), pp. 225–234.

Himler, A. G., Adachi-Hagimori, T., Bergen, J. E., Kozuch, A., Kelly, S. E., Tabashnik, B. E., Chiel, E., Duckworth, V. E., Dennehy, T. J., Zchori-Fein, E., Hunter, M. S. (2011). Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* 332(6026), pp. 254–6.

Hoffmann, A. A., Parsons, P. A. (1988). The analysis of quantitative variation in natural populations with isofemale strains. *Genetics Selection Evolution* 20(1), pp. 87–98.

Holt, J., Jeger, M. J., Thresh, J. M., Otim-Nape, G. W. (1997). An epidemiological model incorporating vector population dynamics applied to African cassava mosaic virus disease. *Journal of Applied Ecology* pp. 793–806.

Hornett, E. A., Duplouy, A. M. R., Davies, N., Roderick, G. K., Wedell, N., Hurst, G. D. D., Charlat, S. (2008). You can't keep a good parasite down: evolution of a male-killer suppressor uncovers cytoplasmic incompatibility. *Evolution* 62(5), pp. 1258–1263.

Horowitz, A. R., Podoler, H., Gerling, D. (1984). Life table analysis of the tobacco whitefly *Bemisia tabaci* (Gennadius) in cotton fields in Israel. *Acta Oecologia Applicata*, 5(3), pp. 221–233.

Horowitz, A. R. (1986). Population dynamics of *Bemisia tabaci* (Gennadius): with special emphasis on cotton fields. *Agriculture, Ecosystems and Environment* 17(1), pp. 37–47.

Hothorn, T., Bretz, F., Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal 50*(3), pp. 346-363.

Howeler, R. H., Lutaladio, N., Thomas, G. (2013). *Save and Grow: Cassava: a guide to sustainable production intensification*. Food and Agriculture Organization of the United Nations, No. FAO 633.6828 S266). FAO, Rome, Italy.

Heusden, A. W. (2013). The *Bemisia tabaci* species complex: additions from different parts of the world. *Insect Science* 20(6), pp. 723–733.

Hunter, M. S., Perlman, S. J., Kelly, S. E. (2003). A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella. Proceedings of the Royal Society of London B: Biological Sciences* 270(1529), pp. 2185–2190.

Hurst, G. D. D., Jiggins, F. M., von der Schulenburg, J. H. G., Bertrand, D., West, S. A., Goriacheva, I. I., Zakharov, I. A., Werren, J. H., Stouthamer, R., Majerus, M. E. N. (1999). Male–killing *Wolbachia* in two species of insect. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 266(1420), pp. 735–740.

Hurst, G. D. D., Jiggins, F. M., Majerus, M. E. N. (2003). Inherited microorganisms that selectively kill male hosts: the hidden players of insect evolution. *Insect Symbiosis* pp. 177–197.

Ishii, Y., Matsuura, Y., Kakizawa, S., Nikoh, N., Fukatsu, T. (2013). Diversity of bacterial endosymbionts associated with *Macrosteles* leafhoppers vectoring phytopathogenic phytoplasmas. *Applied and Environmental Microbiology* 79(16), pp. 5013–5022.

Iturbe-Ormaetxe, I., Walker, T., O'Neill, S. L. (2011). *Wolbachia* and the biological control of mosquito-borne disease. *EMBO Reports* 12(6), pp. 508–518.

Jarvis, A., Ramirez-Villegas, J., Campo, B. V. H., Navarro-Racines, C. (2012). Is cassava the answer to African climate change adaptation? *Tropical Plant Biology* 5(1), pp. 9–29.

Jauset, A. M., Sarasua, M. J., Avilla, J., Albajes, R. (2000). Effect of nitrogen fertilization level applied to tomato on the greenhouse whitefly. *Crop Protection* 19(4), pp. 255–261.

Jeger, M. J., Holt, J., Van Den Bosch, F., Madden, L. V (2004). Epidemiology of insect-transmitted plant viruses: Modelling disease dynamics and control interventions. *Physiological Entomology* 29(3), pp. 291–304.

Jeremiah, S. C., Ndyetabula, I. L., Mkamilo, G. S., Haji, S., Muhanna, M. M., Chuwa, C., Kasele, S., Bouwmeester, H., Ijumba, J. N., Legg, J. P. (2015). The dynamics and environmental influence on interactions between cassava brown streak disease and the whitefly, *Bemisia tabaci*. *Phytopathology 105*(5), 646-655.

Jeyaprakash, A., Hoy, M. A. (2000). Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Molecular Biology* 9(4), pp. 393–405.

Jing, X., Wong, A. C., Chaston, J. M., Colvin, J., McKenzie, C. L., Douglas, A. E. (2014). The bacterial communities in plant phloem-sap-feeding insects. *Molecular Ecology* 23(6), pp. 1433–1444.

Jiravanichpaisal, P., Lee, B. L., Söderhäll, K. (2006). Cell-mediated immunity in arthropods:

hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* 211(4), pp. 213–236.

Jiu, M., Zhou, X. P., Tong, L., Xu, J., Yang, X., Wan, F. H., Liu, S. S. (2007). Vector-virus mutualism accelerates population increase of an invasive whitefly. *Plos One*, 2(1), p. e182.

Jones, D. R. (2003) Plant viruses transmitted by whiteflies. *European Journal of Plant Pathology*, *109*(3), pp. 195–219.

Kikuchi, Y. (2009) Endosymbiotic bacteria in insects: Their diversity and culturability. *Microbes and Environments* 24(3), pp. 195–204.

Kliot, A., Ghanim, M. (2013). The role of bacterial chaperones in the circulative transmission of plant viruses by insect vectors. *Viruses* 5, pp. 1516-35.

Kliot, A., Cilia, M., Czosnek, H., Ghanim, M. (2014). Implication of the bacterial endosymbiont *Rickettsia* spp. in interactions of the whitefly *Bemisia tabaci* with *Tomato yellow leaf curl virus*. *Journal of Virology* 88(10), pp. 5652–5660.

Koga, R., Tsuchida, T., Fukatsu, T. (2009). Quenching autofluorescence of insect tissues for in situ detection of endosymbionts. *Applied Entomology and Zoology* 44(2), pp. 281–291.

Kondo, N., Shimada, M., Fukatsu, T. (2005). Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. *Biology Letters*, 1(4), pp. 488–491.

Kontsedalov, S., Zchori-Fein, E., Chiel, E., Gottlieb, Y., Inbar, M., Ghanim, M. (2008). The presence of *Rickettsia* is associated with increased susceptibility of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides. *Pest Management Science* 64(8), pp. 789–792.

Kunik, T., Palanichelvam, K., Czosnek, H., Citovsky, V., Gafni, Y. (1998). Nuclear import of a geminivirus capsid protein in plant and insect cells: Implications for the viral nuclear entry. *Plant Journal* 13(98), pp. 121–129.

Lau, G. W., Goumnerov, B. C., Walendziewicz, C. L., Hewitson, J., Xiao, W., Mahajan-Miklos, S., Tompkins, R. G., Perkins, L. A., Rahme, L. G. (2003). The *Drosophila melanogaster* toll pathway participates in resistance to infection by the gram-negative human pathogen *Pseudomonas aeruginosa*. *Infection and Immunity* 71(7), pp. 4059–4066.

Laven, H. (1956). Cytoplasmic Inheritance in Culex. *Nature* 177(4499), pp. 141–142.

Legg, J. P., Ogwal, S. (1998). Changes in the incidence of African cassava mosaic virus disease and the abundance of its whitefly vector along south–north transects in Uganda. *Journal of Applied Entomology* 122(1-5), pp. 169–178.

Legg, J. P., Raya, M. D. (1998). Survey of cassava virus diseases in Tanzania. *International Journal of Pest Management* 44(1), pp. 17–23.

Legg, J. P. (1999). Emergence, spread and strategies for controlling the pandemic of cassava mosaic virus disease in east and central Africa. *Crop Protection* 18(10), pp. 627–637.

Legg, J. P., Thresh, J. M. (2000). Cassava mosaic virus disease in East Africa: a dynamic disease in a changing environment. *Virus Research* 71(1), pp. 135–149.

Legg, J. P., French, R., Rogan, D., Okao-Okuja, G., Brown, J. K. (2002). A distinct *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodidae) genotype cluster is

associated with the epidemic of severe cassava mosaic virus disease in Uganda., *Molecular Ecology* 11(7), pp. 1219–29.

Legg, J. P., Hillocks, R. J. (2003). Cassava Brown Streak Virus Disease: Past, present and future. *Proceedings of an International Workshop*, Mombasa, Kenya, 27-30 October, 2002.

Legg, J. P., Fauquet, C. M. (2004). Cassava mosaic geminiviruses in Africa. *Plant Molecular Biology* 56(4), pp. 585–599.

Legg, J. P., Owor, B., Sseruwagi, P., Ndunguru, J. (2006). Cassava mosaic virus disease in East and Central Africa: epidemiology and management of a regional pandemic. *Advances in Virus Research* 67, pp. 355–418.

Legg, J. P., Jeremiah, S. C., Obiero, H. M., Maruthi, M. N., Ndyetabula, I., Okao-Okuja, G., Bouwmeester, H., Bigirimana, S., Tata-Hangy, W., Gashaka, G. (2011). Comparing the regional epidemiology of the cassava mosaic and cassava brown streak virus pandemics in Africa. *Virus Research* 159(2), pp. 161–170.

Legg, J. P., Sseruwagi, P., Boniface, S., Okao-Okuja, G., Shirima, R., Bigirimana, S., Gashaka, G., Herrmann, H.-W., Jeremiah, S., Obiero, H., Ndyetabula, I., Tata-Hangy, W., Masembe, C., Brown, J. K. (2013). Spatio-temporal patterns of genetic change amongst populations of cassava *Bemisia tabaci* whiteflies driving virus pandemics in East and Central Africa. *Virus Research* 186, pp. 61–75.

Legg, J. P., Shirima, R., Tajebe, L. S., Guastella, D., Boniface, S., Jeremiah, S., Nsami, E., Chikoti, P., Rapisarda, C. (2014). Biology and management of *Bemisia* whitefly vectors of cassava virus pandemics in Africa. *Pest Management Science* 70(10), pp. 1446–1453.

Legg, J. P., Kumar, P. L., Makeshkumar, T., Tripathi, L., Ferguson, M., Kanju, E., Ntawuruhunga, P., Cuellar, W. (2015). Chapter Four-Cassava Virus Diseases: Biology, Epidemiology, and Management. *Advances in Virus Research* 91, pp. 85–142.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86(6), pp. 973–983.

Lemaitre, B., Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annual Review of Immunology.* 25, pp. 697–743.

Levsky, J. M., Singer, R. H. (2003). Fluorescence in situ hybridization: past, present and future. *Journal of Cell Science* 116(14), pp. 2833–2838.

Li, T. Y., Vinson, S. B., Gerling, D. (1989). Courtship and mating behavior of *Bemisia tabaci* (Homoptera: Aleyrodidae). *Environmental Entomology* 18(5), pp. 800–806.

Li, Z. X., Lin, H. Z., Guo, X. P. (2007). Prevalence of *Wolbachia* infection in *Bemisia tabaci*. *Current Microbiology* 54(6), pp. 467–471.

Liu, J., Zhao, H., Jiang, K., Zhou, X., Liu, S. (2009). Differential indirect effects of two plant viruses on an invasive and an indigenous whitefly vector: implications for competitive displacement. *Annals of Applied Biology* 155(3), pp. 439–448.

Liu, S., Colvin, J., De Barro, P. J. (2012). Species concepts as applied to the whitefly *Bemisia*

tabaci systematics: how many species are there? *Journal of Integrative Agriculture* 11(2), pp. 176–186.

Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25(4), pp. 402–8.

Lloyd, L. L. (1922). The control of the greenhouse whitefly (*Asterochiton vaporariorum*) with notes on its biology. *Annals of Applied Biology* 9(1), pp. 1–32.

Luan, J., Li, J., Wang, Y., Li, F., Bao, Y., Zhang, C., Liu, S., Wang, X. (2011). Global analysis of the transcriptional response of whitefly to *Tomato yellow leaf curl China virus* reveals the relationship of coevolved adaptations. *Journal of Virology* 85(7), pp. 3330–3340.

Łukasik, P., Guo, H., Asch, M., Ferrari, J., Godfray, H. C. J. (2013). Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. *Journal of Evolutionary Biology* 26(12), pp. 2654–2661.

Mahadav, A., Gerling, D., Gottlieb, Y., Czosnek, H., Ghanim, M. (2008). Parasitization by the wasp *Eretmocerus mundus* induces transcription of genes related to immune response and symbiotic bacteria proliferation in the whitefly *Bemisia tabaci*. *BMC Genomics* 9, p. 342.

Mahadav, A., Kontsedalov, S., Czosnek, H., Ghanim, M. (2009). Thermotolerance and gene expression following heat stress in the whitefly *Bemisia tabaci* B and Q biotypes. *Insect Biochemistry and Molecular Biology* 39(10), pp. 668–676.

Maiden, M. C. J. (2006). Multilocus sequence typing of bacteria. *Annual Review of Microbiology* 60, pp. 561–588.

Mansoor, S., Khan, S. H., Bashir, A., Saeed, M., Zafar, Y., Malik, K. A., Briddon, R., Stanley, J., Markham, P. G. (1999). Identification of a novel circular single-stranded DNA associated with cotton leaf curl disease in Pakistan. *Virology* 259(1), pp. 190–199.

Martinez, J., Ok, S., Smith, S., Snoeck, K., Day, J. P., Jiggins, F. M. (2015). Should symbionts be nice or selfish? Antiviral effects of *Wolbachia* are costly but reproductive parasitism is not. *Plos Pathogens* 11(7), p. e1005021.

Marubayashi, J. M., Kliot, A., Yuki, V. A., Rezende, J. A. M., Krause-Sakate, R., Pavan, M. A., Ghanim, M. (2014). Diversity and localization of bacterial endosymbionts from whitefly species collected in Brazil. *Plos One* 9(9), p. e108363.

Maruthi, M. N., Colvin, J., Seal, S. (2001). Mating compatibility, life-history traits, and RAPD-PCR variation in *Bemisia tabaci* associated with the cassava mosaic disease pandemic in East Africa. *Entomologia Experimentalis et Applicata* 99(1), pp. 13–23.

Maruthi, M. N., Colvin, J., Seal, S., Gibson, G., Cooper, J. (2002). Co-adaptation between cassava mosaic geminiviruses and their local vector populations. *Virus Research* 86(1), pp. 71–85.

Maruthi, M. N., Colvin, J., Thwaites, R. M., Banks, G. K., Gibson, G., Seal, S. E. (2004).

Reproductive incompatibility and cytochrome oxidase I gene sequence variability amongst host-adapted and geographically separate *Bemisia tabaci* populations (Hemiptera: Aleyrodidae). *Systematic Entomology*, 29(4), pp. 560–568.

Maruthi, M. N., Hillocks, R. J., Mtunda, K., Raya, M. D., Muhanna, M., Kiozia, H., Rekha, A. R., Colvin, J., Thresh, J. M. (2005). Transmission of *Cassava brown streak virus* by *Bemisia tabaci* (Gennadius). *Journal of Phytopathology* 153(5), pp. 307–312.

Mascarin, G. M., Kobori, N. N., Quintela, E. D., Delalibera, I. (2013). The virulence of entomopathogenic fungi against *Bemisia tabaci* biotype B (Hemiptera: Aleyrodidae) and their conidial production using solid substrate fermentation. *Biological Control* 66(3), pp. 209–218.

Mbanzibwa, D. R., Tian, Y. P., Tugume, A. K., Mukasa, S. B., Tairo, F., Kyamanywa, S., Kullaya, A., Valkonen, J. P. T. (2009). Genetically distinct strains of *Cassava brown streak virus* in the Lake Victoria basin and the Indian Ocean coastal area of East Africa. *Archives of Virology* 154(2), pp. 353–359.

McGraw, E. A., Merritt, D. J., Droller, J. N., O'Neill, S. L. (2002). *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proceedings of the National Academy of Sciences* 99(5), pp. 2918–2923.

McKenzie, C. L., Hodges, G., Osborne, L. S., Byrne, F. J., Shatters Jr, R. G. (2009). Distribution of *Bemisia tabaci* (Hemiptera: Aleyrodidae) biotypes in Florida-investigating the Q invasion. *Journal of Economic Entomology* 102(2), pp. 670–676.

McKenzie, C. L., Kumar, V., Palmer, C. L., Oetting, R. D., Osborne, L. S. (2014). Chemical class rotations for control of *Bemisia tabaci* (Hemiptera: Aleyrodidae) on poinsettia and their effect on cryptic species population composition. *Pest Management Science* 70(10), pp. 1573–1587.

McMeniman, C. J., O'Neill, S. L. (2010). A virulent *Wolbachia* infection decreases the viability of the dengue vector *Aedes aegypti* during periods of embryonic quiescence. *Plos Neglected Tropical Diseases* 4(7), p. e748.

Min, K. T., Benzer, S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proceedings of the National Academy of Sciences* 94(20), pp. 10792–10796.

Miozzi, L., Napoli, C., Sardo, L., Accotto, G. P. (2014). Transcriptomics of the interaction between the monopartite phloem-limited geminivirus *Tomato yellow leaf curl Sardinia virus* and *Solanum lycopersicum* highlights a role for plant hormones, autophagy and plant immune system fine tuning during infection. *Plos One* 9(2), p. e89951.

Mohanty, A. K., Basu, A. N. (1987). Biology of the whitefly vector, *Bemisia tabaci* Genn. on four host plants throughout the year. *Journal of Entomological Research* 11, pp. 15-18.

Monger, W. A., Seal, S., Isaac, A. M., Foster, G. D. (2001). Molecular characterization of the Cassava brown streak virus coat protein. *Plant Pathology* 50(4), pp. 527–534.

Montllor, C. B., Maxmen, A., Purcell, A. H. (2002). Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecological Entomology* 27(2), pp.

189–195.

Morales, F. J., Anderson, P. K. (2001). The emergence and dissemination of whitefly-transmitted geminiviruses in Latin America. *Archives of Virology* 146(3), pp. 415–441.

Moran, N. A. (1996). Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proceedings of the National Academy of Sciences* 93(7), pp. 2873–2878.

Moran, N. A., Baumann, P. (2000). Bacterial endosymbionts in animals. *Current Opinion in Microbiology* 3(3), pp. 270–275.

Moran, N. A., Mira, A. (2001). The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. *Genome Biology* 2(12), pp. 1–54.

Moran, N. A., Dale, C., Dunbar, H., Smith, W. A., Ochman, H. (2003). Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. *Environmental Microbiology* 5(2), pp. 116–126.

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., Rocha, B. C., Hall-Mendelin, S., Day, A., Riegler, M. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* 139(7), pp. 1268–1278.

Morin, S., Ghanim, M., Zeidan, M., Czosnek, H., Verbeek, M., van den Heuvel, J. F. J. M. (1999). A GroEL homologue from endosymbiotic bacteria of the whitefly *Bemisia tabaci* is implicated in the circulative transmission of *Tomato yellow leaf curl virus*. *Virology* 256(1), pp. 75–84.

Mound, L. A. (1962). Studies on the olfaction and colour sensitivity of *Bemisia tabaci* (Genn.)(Homoptera, Aleyrodidae). *Entomologia Experimentalis et Applicata* 5(2), pp. 99–104.

Mound, L. A. (1963). Host-correlated variation in *Bemisia tabaci* (Gennadius)(Homoptera: Aleyrodidae), In *Proceedings of the Royal Entomological Society of London. Series A, General Entomology* pp. 171–180.

Mound, L. A., Halsey, S. H. (1978). *Whitefly of the world. A systematic catalogue of the Aleyrodidae (Homoptera) with host plant and natural enemy data.* John Wiley and Sons, New York, p. 340.

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

Mware, B. O., Ateka, E. M., Songa, J. M., Narla, R. D., Olubayo, F., Amata, R. (2009). Transmission and distribution of cassava brown streak virus disease in cassava growing areas of Kenya. *Journal of Applied Biosciences* 16, pp. 864–870.

Nair, N. G., Daniel, R. S. (1983). Preference of *Bemisia tabaci* Gen. to cassava varieties and their reaction to cassava mosaic disease. *Journal of Root Crops* 9(1-2), pp. 45–49.

Noda, H., Koizumi, Y., Zhang, Q., Deng, K. (2001). Infection density of *Wolbachia* and

incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. *Insect Biochemistry and Molecular Biology* 31(6), pp. 727–737.

Nombela, G., Williamson, V. M., Muñiz, M. (2003). The root-knot nematode resistance gene Mi-1.2 of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Molecular Plant-Microbe Interactions* 16(7), pp. 645–649.

O'Neill, S. L., Giordano, R., Colbert, A. M., Karr, T. L., Robertson, H. M. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences* 89(7), pp. 2699–2702.

O'Neill, S. L., Gooding, R. O. N. H., Aksoy, S. (1993). Phylogenetically distant symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. *Medical and Veterinary Entomology* 7(4), pp. 377–383.

Ohnesorge, S., Bejarano, E. R. (2009). Begomovirus coat protein interacts with a small heat-shock protein of its transmission vector (*Bemisia tabaci*), *Insect Molecular Biology* 18(6), pp. 693–703.

Oliveira, M. R. V, Henneberry, T. J., Anderson, P. (2001). History, current status, and collaborative research projects for *Bemisia tabaci*. *Crop Protection* 20(9), pp. 709–723.

Oliver, K. M., Russell, J. A., Moran, N. A., Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences* 100(4), pp. 1803–1807.

Oliver, K. M., Moran, N. A., Hunter, M. S. (2006). Costs and benefits of a superinfection of facultative symbionts in aphids, *Proceedings of the Royal Society of London B: Biological Sciences* 273(1591), pp. 1273–1280.

Oliver, K. M., Campos, J., Moran, N. A., Hunter, M. S. (2008). Population dynamics of defensive symbionts in aphids, *Proceedings of the Royal Society of London B: Biological Sciences* 275(1632), pp. 293–299.

Oliver, K. M., Degnan, P. H., Hunter, M. S., Moran, N. A. (2009). Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* 325(5943), pp. 992–994.

Omongo, C. A., Kawuki, R., Bellotti, A. C., Alicai, T., Baguma, Y., Maruthi, M. N., Bua, A., Colvin, J. (2012). African cassava whitefly, *Bemisia tabaci*, resistance in African and South American cassava genotypes. *Journal of Integrative Agriculture* 11(2), pp. 327–336.

Osborne, S. E., San Leong, Y., O'Neill, S. L., Johnson, K. N. (2009). Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *Plos Pathogens* 5(11), p. e1000656.

Otim, M., Kyalo, G., Kyamanywa, S., Asiimwe, P., Legg, J. P., Guershon, M., Gerling, D. (2008) Parasitism of *Bemisia tabaci* (Homoptera: Aleyrodidae) by *Eretmocerus mundus* (Hymenoptera: Aphelinidae) on cassava. *International Journal of Tropical Insect Science* 28(3), p. 158.

Otim-Nape, G. W., Bua, A., Thresh, J. M., Baguma, Y., Ogwal, S., Semakula, G. N., Acola,

G., Byabakama, B., Martin, A. (1997). *Cassava mosaic virus disease in Uganda: the current pandemic and approaches to control.* Natural Resources Institute (NRI).

Otti, G., Bouvaine, S., Kimata, B., Mkamillo, G., Kumar, P., Tomlins, K., Maruthi, M. N. (2016). High throughput multiplex real time PCR assay for the simultaneous quantification of DNA and RNA viruses infecting cassava plants. *Journal of Applied Microbiology* Wiley Online Library.

Pakkianathan, B. C., Kontsedalov, S., Lebedev, G., Mahadav, A., Zeidan, M., Czosnek, H., Ghanim, M. (2015). Replication of *Tomato yellow leaf curl virus* in its whitefly vector, *Bemisia tabaci*. *Journal of Virology* 89(19), pp. 9791–9803.

Palumbo, J. C., Horowitz, A. R., Prabhaker, N. (2001). Insecticidal control and resistance management for *Bemisia tabaci*. *Crop Protection* 20(9), pp. 739–765.

Pan, H., Chu, D., Ge, D., Wang, S., Wu, Q., Xie, W., Jiao, X., Liu, B., Yang, X., Yang, N. (2011). Further spread of and domination by *Bemisia tabaci* (Hemiptera: Aleyrodidae) biotype Q on field crops in China. *Journal of Economic Entomology* 104(3), pp. 978–985.

Pan, H., Li, X., Ge, D., Wang, S., Wu, Q., Xie, W., Jiao, X., Chu, D., Liu, B., Xu, B., Zhang, Y. (2012). Factors affecting population dynamics of maternally transmitted endosymbionts in *Bemisia tabaci*. *Plos One* 7(2), p. e30760.

Pan, H. P., Chu, D., Liu, B. M., Xie, W., Wang, S. L., Wu, Q. J., Xu, B. Y., Zhang, Y. J. (2013). Relative amount of symbionts in insect hosts changes with host-plant adaptation and insecticide resistance. *Environmental Entomology* 42(1), pp. 74–78.

Parsa, S., Medina, C., Rodríguez, V. (2015). Sources of pest resistance in cassava. *Crop Protection* 68, pp. 79–84.

Perlman, S. J., Hunter, M. S., Zchori-Fein, E. (2006). The emerging diversity of *Rickettsia. Proceedings of the Royal Society B: Biological Sciences* 273(1598), pp. 2097–2106.

Pinheiro, P. V., Kliot, A., Ghanim, M., Cilia, M. (2015). Is there a role for symbiotic bacteria in plant virus transmission by insects? *Current Opinion in Insect Science* 8, pp. 69–78.

Pollard, D. G. (1955). Feeding habits of the cotton whitefly, *Bemisia tabaci* Genn.(homoptera: aleyrodidae). *Annals of Applied Biology* 43(4), pp. 664–671.

Polston, J. E., De Barro, P., Boykin, L. M. (2014). Transmission specificities of plant viruses with the newly identified species of the *Bemisia tabaci* species complex. *Pest Management Science 70*(10), 1547-1552.

Posada, D. (2003). Selecting models of evolution, *The phylogenetic handbook. A practical approach to DNA and protein phylogeny. Cambridge University Press, Cambridge*, pp. 256–282.

Priya, N. G., Pandey, N., Rajagopal, R. (2012). LNA probes substantially improve the detection of bacterial endosymbionts in whole mount of insects by fluorescent in-situ hybridization. *BMC Microbiology* 12(1), p. 81.

Qiu, P., Pan, P. C., Govind, S. (1998). A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development* 125(10), pp. 1909–1920.

Quintela, E. D., Abreu, A. G., Lima, J. F. dos S., Mascarin, G. M., dos Santos, J. B., Brown, J. K. (2016). Reproduction of the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) B biotype in maize fields (Zea mays L.) in Brazil. *Pest Management Science*, Wiley Online Library.

R Development Core Team, R. (2011). R: A language and environment for statistical computing, Team, R. D. C. (ed.), *R Foundation for Statistical Computing*, *R Foundation for Statistical Computing*, R Foundation for Statistical Computing, p. 409.

Raina, H. S., Rawal, V., Singh, S., Daimei, G., Shakarad, M., Rajagopal, R. (2015). Elimination of *Arsenophonus* and decrease in the bacterial symbionts diversity by antibiotic treatment leads to increase in fitness of whitefly, *Bemisia tabaci*. *Infection, Genetics and Evolution* 32, pp. 224–230.

Rana, V. S., Singh, S. T., Priya, N. G., Kumar, J., Rajagopal, R. (2012). *Arsenophonus* GroEL interacts with CLCuV and is localized in midgut and salivary gland of whitefly *B. tabaci*. *Plos One* 7(8), p. e42168.

Rao, Q., Rollat-Farnier, P. A., Zhu, D. T., Santos-Garcia, D., Silva, F. J., Moya, A., Latorre, A., Klein, C. C., Vavre, F., Sagot, M. F. (2015). Genome reduction and potential metabolic complementation of the dual endosymbionts in the whitefly *Bemisia tabaci*. *BMC Genomics* 16(1), p. 226.

Reynolds, S., Rolff, J. (2008). Immune function keeps endosymbionts under control. *Journal of Biology* 7(8), p. 28.

Ros, V. I. D., Breeuwer, J. A. J. (2009). The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobia sarothamni*. *Heredity* 102(4), pp. 413–422.

Rosell, R. C., Blackmer, J. L., Czosnek, H., Inbar, M. (2010). Mutualistic and dependent relationships with other organisms. In *Bemisia: Bionomics and Management of a Global Pest* pp. 161–183.

Rothenbuhler, W. C., Thompson, V. C. (1956). Resistance to American foulbrood in honey bees. I. Differential survival of larvae of different genetic lines. *Journal of Economic Entomology* 49(4), pp. 470–475.

Ruan, Y., Xu, J., Liu, S. (2006). Effects of antibiotics on fitness of the B biotype and a non-B biotype of the whitefly *Bemisia tabaci*. *Entomologia Experimentalis et Applicata* 121(2), pp. 159–166.

Rubinstein, G., Czosnek, H. (1997). Long-term association of tomato yellow leaf curl virus with its whitefly vector *Bemisia tabaci*: effect on the insect transmission capacity, longevity and fecundity. *Journal of General Virology 78*(10), 2683-2689.

Saikia, A. K., Muniyappa, V. (1989). Epidemiology and control of *Tomato leaf curl virus* in Southern India. *Tropical Agriculture* 66(4), pp. 350–354.

Sakurai, M., Koga, R., Tsuchida, T., Meng, X. Y., Fukatsu, T. (2005). *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. *Applied and Environmental Microbiology*

71(7), pp. 4069–4075.

Santos-Garcia, D., Farnier, P. A., Beitia, F., Zchori-Fein, E., Vavre, F., Mouton, L., Moya, A., Latorre, A., Silva, F. J. (2012). Complete genome sequence of "*Candidatus* Portiera aleyrodidarum" BT-QVLC, an obligate symbiont that supplies amino acids and carotenoids to *Bemisia tabaci*. *Journal of Bacteriology* 194(23), pp. 6654–6655.

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. *Annual Review of Entomology* 50, pp. 529–551.

Schmid-Hempel, P. (2003). Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London B: Biological Sciences* 270(1513), pp. 357–366.

Schuster, D. J., Mueller, T. F., Kring, J. B., Price, J. F. (1990). Relationship of the sweetpotato whitefly to a new tomato fruit disorder in Florida. *HortScience* 25(12), pp. 1618–1620.

Segarra-Carmona, A. E., Bird, I., Escudero, J., Formaris-Rullán, G., Franqui, R. A. (1990). Silvering of *Cucurbita moschata* (Duchesne) Poir associated with *Bemisia tabaci* Genn.(Homoptera: Aleyrodidae) in Puerto Rico. *Journal of Agriculture of the University of Puerto Rico* 74(4), pp. 477–478.

Sharaf, N., Batta, Y. (1985). Effect of some factors on the relationship between the whitefly *Bemisia tabaci* Genn.(Homopt., Aleyrodidea) and the parasitoid *Eretmocerus mundus* Mercet (Hymenopt., Aphelinidae). *Zeitschrift für Angewandte Entomologie* 99(1-5), pp. 267–276.

Shatters Jr, R. G., McKenzie, C. L., Boykin, L. M., Gazit, S., Sinisterra, X., Weathersbee III, A. A., Brown, J. K., Czosnek, H. (2008). A knottin-like putative antimicrobial gene family in the whitefly *Bemisia tabaci* biotype B: Cloning and transcript regulation. *Journal of Insect Science* 8.

Shelly, S., Lukinova, N., Bambina, S., Berman, A., Cherry, S. (2009). Autophagy is an essential component of *Drosophila* immunity against vesicular stomatitis virus. *Immunity* 30(4), pp. 588–598.

Simón, B., Cenis, J. L., De La Rúa, P. (2007). Distribution patterns of the Q and B biotypes of *Bemisia tabaci* in the Mediterranean Basin based on microsatellite variation, *Entomologia Experimentalis et Applicata* 124(3), pp. 327–336.

Singh, J., Sohi, A. S., Brar, D. S., Denholm, I., Russel, D., Briddon, R. (1999). Management of cotton leaf curl viral disease in India. *Proceeding of the ICAC-CCRI Regional Consultation, Insecticide Resistance Management in Cotton. Central Cotton Res. Instit, Multan, Pakistan*, pp. 277–278.

Singh, S. T., Priya, N. G., Kumar, J., Rana, V. S., Ellango, R., Joshi, A., Priyadarshini, G., Asokan, R., Rajagopal, R. (2012). Diversity and phylogenetic analysis of endosymbiotic bacteria from field caught *Bemisia tabaci* from different locations of North India based on 16S rDNA library screening. *Infection, Genetics and Evolution*, 12(2), pp. 411–419.

Skaljac, M., Zanic, K., Ban, S. G., Kontsedalov, S., Ghanim, M. (2010). Co-infection and

localization of secondary symbionts in two whitefly species. *BMC Microbiology* 10, p. 142.

Snyder, A. K., Rio, R. V. M. (2013). Interwoven biology of the tsetse holobiont. *Journal of Bacteriology* 195(19), pp. 4322–4330.

Sseruwagi, P., Legg, J. P., Maruthi, M. N., Colvin, J., Rey, M. E. C., Brown, J. K. (2005). 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. *Annals of Applied Biology* 147(3), pp. 253–265.

Sseruwagi, P., Maruthi, M. N., Colvin, J., Rey, M. E. C., Brown, J. K., Legg, J. P. (2006). Colonization of non-cassava plant species by cassava whiteflies (*Bemisia tabaci*) in Uganda. *Entomologia experimentalis et Applicata* 119(2), pp. 145–153.

Stansly, P. A., Naranjo, S. E. (2010). *Bemisia: bionomics and management of a global pest*. Springer, p. 528.

Storey, H. H. (1938). Investigations of the mechanism of the transmission of plant viruses by insect vectors. II. The part played by puncture in transmission. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 125(841), pp. 455–477.

Su, Q., Oliver, K. M., Pan, H., Jiao, X., Liu, B., Xie, W., Wang, S., Wu, Q., Xu, B., White, J. A. (2013a) Facultative symbiont *Hamiltonella* confers benefits to *Bemisia tabaci* (Hemiptera: Aleyrodidae), an invasive agricultural pest worldwide. *Environmental Entomology* 42(6), pp. 1265–1271.

Su, Q., Pan, H., Liu, B., Chu, D., Xie, W., Wu, Q., Wang, S., Xu, B., Zhang, Y. (2013b). Insect symbiont facilitates vector acquisition, retention, and transmission of plant virus. *Scientific Reports* 3, p. 1367.

Su, Q., Oliver, K. M., Xie, W., Wu, Q., Wang, S., Zhang, Y. (2015a). The whitefly-associated facultative symbiont *Hamiltonella defensa* suppresses induced plant defences in tomato. *Functional Ecology 29*(8), 1007-1018.

Su, Q., Preisser, E. L., Zhou, X. M., Xie, W., Liu, B. M., Wang, S. L., Wu, Q. J., Zhang, Y. J. (2015b). Manipulation of host quality and defense by a plant virus improves performance of whitefly vectors. *Journal of Economic Entomology* 108(1), pp. 11–19.

Su, Q., Xie, W., Wang, S., Wu, Q., Liu, B., Fang, Y., Xu, B., Zhang, Y. (2014). The endosymbiont *Hamiltonella* increases the growth rate of its host *Bemisia tabaci* during periods of nutritional stress. *Plos One* 9(2), e89002.

Subandiyah, S., Nikoh, N., Tsuyumu, S., Somowiyarjo, S., Fukatsu, T. (2000) Complex endosymbiotic microbiota of the citrus psyllid *Diaphorina citri* (Homoptera: Psylloidea). *Zoological Science* 17(7), pp. 983–989.

Sutter, G. R., Rothenbuhler, W. C., Raun, E. S. (1968). Resistance to American foulbrood in honey bees: VII. Growth of resistant and susceptible larvae. *Journal of Invertebrate Pathology* 12(1), pp. 25–28.

Szklarzewicz, T., Moskal, A. (2001). Ultrastructure, distribution, and transmission of endosymbionts in the whitefly *Aleurochiton aceris* Modeer (Insecta, Hemiptera,

Aleyrodinea). *Protoplasma* 218(1-2), pp. 45–53.

Tajebe, L. S., Boni, S. B., Guastella, D., Cavalieri, V., Lund, O. S., Rugumamu, C. P., Rapisarda, C., Legg, J. P. (2015a) Abundance, diversity and geographic distribution of cassava mosaic disease pandemic-associated *Bemisia tabaci* in Tanzania. *Journal of Applied Entomology* 139(8), pp. 627–637.

Tajebe, L. S., Guastella, D., Cavalieri, V., Kelly, S. E., Hunter, M. S., Lund, O. S., Legg, J. P., Rapisarda, C. (2015b). Diversity of symbiotic bacteria associated with Bemisia tabaci (Homoptera: Aleyrodidae) in cassava mosaic disease pandemic areas of Tanzania. *Annals of Applied Biology* 166(2), pp. 297–310.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28(10), pp. 2731–9.

Teixeira, L., Ferreira, A., Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *Plos Biology* 6(12), p. e2.

Thao, M. L., Baumann, L., Hess, J. M., Falk, B. W., Ng, J. C. K., Gullan, P. J., Baumann, P. (2003) Phylogenetic evidence for two new insect-associated Chlamydia of the family Simkaniaceae. *Current Microbiology* 47(1), pp. 46–50.

Thao, M. L., Baumann, P. (2004). Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Applied and Environmental Microbiology*, 70(6), 3401-3406.

Thresh, J. M., Otim-Nape, G. W., Legg, J. P., Fargette, D., Thro, A. M., Akoroda, M. O. (1997). African cassava mosaic virus disease: the magnitude of the problem. In *African Journal of Root and Tuber Crops* International Society for Tropical Root Crops-Africa Branch (ISTRC-AB), pp. 13–19.

Thresh, J. M., Cooter, R. J. (2005). Strategies for controlling cassava mosaic virus disease in Africa, *Plant Pathology* 54(5), pp. 587–614.

Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T., Fukatsu, T. (2002). Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. *Molecular Ecology* 11(10), pp. 2123–2135.

Valanne, S., Wang, J. H., Rämet, M. (2011). The *Drosophila* toll signaling pathway. *The Journal of Immunology* 186(2), pp. 649–656.

Varma, A., Dhar, A. K., Mandal, B. (1992). MYMV transmission and control in India, In *Mungbean Yellow Mosaic Disease: Proceedings of an international workshop*, Asian Vegetable Research and Development Center, Taipei, pp. 8–27.

Vautrin, E., Vavre, F. (2009). Interactions between vertically transmitted symbionts: cooperation or conflict? *Trends in Microbiology* 17(3), pp. 95–99.

Von Dohlen, C. D., Kohler, S., Alsop, S. T., McManus, W. R. (2001). Mealybug β-proteobacterial endosymbionts contain γ-proteobacterial symbionts. *Nature* 412(6845), pp.

433–436.

Verma, A. K., Ghatak, S. S., Mukhopadhyay, S. (1990). Effect of temperature on development of whitefly (*Bemisia tabaci*)(Homoptera: Aleyrodidae) in West Bengal. *Indian Journal of Agricultural Sciences* 60(5), pp. 332–336.

Vorburger, C., Gouskov, A. (2011). Only helpful when required: a longevity cost of harbouring defensive symbionts. *Journal of Evolutionary Biology* 24(7), pp. 1611–1617.

Vorburger, C., Ganesanandamoorthy, P., Kwiatkowski, M. (2013). Comparing constitutive and induced costs of symbiont-conferred resistance to parasitoids in aphids. *Ecology and Evolution* 3(3), pp. 706–713.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., Leong, Y. S., Dong, Y., Axford, J., Kriesner, P. (2011). The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476(7361), pp. 450–453.

Walsh, P. S., Metzger, D. A., Higuchi, R. (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10(4), pp. 506–513.

Wang, J., Weiss, B. L., Aksoy, S. (2013). Tsetse fly microbiota: form and function. *Frontiers in Cellular and Infection Microbiology* Frontiers Media SA, 3.

Wang, X. R., Wang, L. L., Liu, S. S., Wang, X. W. (2016). The role of autophagy in the interactions between *Bemisia tabaci* and *Tomato yellow leaf curl virus*. In Seruwagi, P., Legg, J., Njuguna, C., Wosula, E. (ed.), *2nd International whitefly symposium*, Arusha, Tanzania, p. 71.

Weeks, A. R., Marec, F., Breeuwer, J. A. J. (2001). A mite species that consists entirely of haploid females. *Science* 292(5526), pp. 2479–2482.

Wernegreen, J. J. (2004). Endosymbiosis: lessons in conflict resolution. *PLoS Biology* 2(3).

Werren, J. H., Zhang, W., Guo, L. R. (1995). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 261(1360), pp. 55–63.

Werren, J. H. (1997). *Wolbachia* run amok. *Proceedings of the National Academy of Sciences* 94(21), pp. 11154–11155.

Werren, J. H., Windsor, D. M. (2000). *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proceedings of the Royal Society of London. Series B: Biological Sciences* 267(1450), pp. 1277–1285.

Werren, J. H., Baldo, L., Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 6(10), pp. 741–51.

Williams, W. G., Kennedy, G. G., Yamamoto, R. T., Thacker, J. D., Bordner, J. (1980). 2-Tridecanone: a naturally occurring insecticide from the wild tomato *Lycopersicon hirsutum* f. *glabratum*. *Science* 207(4433), pp. 888–889.

Winter, S., Koerbler, M., Stein, B., Pietruszka, A., Paape, M., Butgereitt, A. (2010). Analysis

of *cassava brown streak viruses* reveals the presence of distinct virus species causing cassava brown streak disease in East Africa. *Journal of General Virology* 91(5), pp. 1365–1372.

Wraight, S. P., Carruthers, R., Bradley, C. A., Jaronski, S. T., Lacey, L. A., Wood, P., Galaini-Wraight, S. (1998). Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against the silverleaf whitefly, *Bemisia argentifolii*. *Journal of Invertebrate Pathology* 71(3), pp. 217–226.

Wu, D., Daugherty, S. C., Van Aken, S. E., Pai, G. H., Watkins, K. L., Khouri, H., Tallon, L. J., Zaborsky, J. M., Dunbar, H. E., Tran, P. L. (2006). Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biology* 4(6), p. e188.

Xie, W., Meng, Q., Wu, Q., Wang, S., Yang, X., Yang, N., Li, R., Jiao, X., Pan, H., Liu, B., Su, Q., Xu, B., Hu, S., Zhou, X., Zhang, Y. (2012). Pyrosequencing the *Bemisia tabaci* transcriptome reveals a highly diverse bacterial community and a robust system for insecticide resistance. *Plos One* 7(4), p. e35181.

Xu, J., De Barro, P. J., Liu, S. S. (2010). Reproductive incompatibility among genetic groups of *Bemisia tabaci* supports the proposition that the whitefly is a cryptic species complex. *Bulletin of Entomological Research* 100(03), pp. 359–366.

Xue, X., Li, S. J., Ahmed, M. Z., De Barro, P. J., Ren, S. X., Qiu, B. L. (2012). Inactivation of *Wolbachia* reveals its biological roles in whitefly host. *Plos One*, 7(10), p. e48148.

Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., Takada, H., Goldman, W. E., Fukase, K., Silverman, N. (2008). Autophagic control of listeria through intracellular innate immune recognition in *Drosophila*. *Nature immunology* 9(8), pp. 908–916.

Yokomi, R. K., Hoelmer, K. A., Osborne, L. S. (1990). Relationships between the sweetpotato whitefly. *Phytopathology* 80, pp. 895–900.

Zchori-Fein, E., Gottlieb, Y., Kelly, S. E., Brown, J. K., Wilson, J. M., Karr, T. L., Hunter, M. S. (2001). A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proceedings of the National Academy of Sciences* 98(22), pp. 12555–12560.

Zchori-Fein, E., Brown, J. K. (2002). Diversity of prokaryotes associated with *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). *Annals of the Entomological Society of America* 95(6), pp. 711–718.

Zchori-Fein, E. and Perlman, S. J. (2004). Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Ecology* 13(7), pp. 2009–2016.

Zchori-Fein, E., Bourtzis, K. (2011). *Manipulative tenants: bacteria associated with arthropods*. CRC press, p. 245.

Zhang, C. R., Zhang, S., Xia, J., Li, F. F., Xia, W. Q., Liu, S. S., Wang, X. W. (2014). The immune strategy and stress response of the Mediterranean species of the *Bemisia tabaci* complex to an orally delivered bacterial pathogen. *Plos One* 9(4), p. e94477.

Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G. W., Robinson, D. J., Harrison, B.

D. (1997). Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *Journal of General Virology* 78(8), pp. 2101–2111.

Zhou, W., Rousset, F., O'Neill, S. (1998). Phylogeny and PCR–based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 265(1395), pp. 509–515.

Zug, R., Hammerstein, P. (2012). Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *Plos One* 7(6), p. e38544.

9 APPENDICES

Appendix A: Multiple comparisons of mean infection incidence of symbionts: Tukey's HSD test
(p values ≤ 0.05, ≤ 0.01, ≤ 0.001 represented as '*', '**', '***', respectively)

Arsenophonus

Comparison		*Z* value	*P* value
SSA1-SG1	SSA1-SG2	-0.483	0.9885
SSA1-SG1	SSA1-SG3	5.078	<0.001***
SSA1-SG1	SSA1-SG5	2.972	0.0237*
SSA1-SG1	SSA3	1.939	0.2900
SSA1-SG2	SSA1-SG3	4.270	<0.001***
SSA1-SG2	SSA1-SG5	2.793	0.0402*
SSA1-SG2	SSA3	2.030	0.2453
SSA1-SG3	SSA1-SG5	-1.637	0.4657
SSA1-SG3	SSA3	-1.949	0.2847
SSA1-SG5	SSA3	-0.555	0.9808

Rickettsia

Comparison		*Z* value	*P* value
SSA1-SG1	SSA1-SG2	2.150	0.1586
SSA1-SG1	SSA1-SG3	5.279	<0.001***
SSA1-SG1	SSA1-SG5	-0.015	1.0000
SSA1-SG1	SSA3	1.086	0.7748
SSA1-SG2	SSA1-SG3	3.210	0.0080**
SSA1-SG2	SSA1-SG5	-0.017	1.0000
SSA1-SG2	SSA3	-0.748	0.9303
SSA1-SG3	SSA1-SG5	-0.018	1.0000
SSA1-SG3	SSA3	-3.194	0.0086**
SSA1-SG5	SSA3	0.016	1.0000

Wolbachia

Comparison		*Z* value	*P* value
SSA1-SG1	SSA1-SG2	3.083	0.01543*
SSA1-SG1	SSA1-SG3	-4.417	<0.001***
SSA1-SG1	SSA1-SG5	-1.163	0.75455
SSA1-SG1	SSA3	-0.659	0.96111
SSA1-SG2	SSA1-SG3	-4.605	<0.001***
SSA1-SG2	SSA1-SG5	-3.459	0.00420**
SSA1-SG2	SSA3	-3.224	0.00949**
SSA1-SG3	SSA1-SG5	2.791	0.03660*
SSA1-SG3	SSA3	2.666	0.05189
SSA1-SG5	SSA3	0.285	0.99839

Appendix B: Multiple comparisons of mean quantities of *Wolbachia* after treatment with antibiotics. (Tukey's HSD test). '*'= $P \leq 0.05$

Comparison		*P* value
Female-Rifampicin+Tetracyclin-10days	Female-Control-10days	0.029*
Male-Control-10days	Female-Control-10days	0.056
Male-Rifampicin+Tetracyclin-10day	Female-Control-10days	0.04*
Female-Control-5day	Female-Control-10days	0.286
Female-Rifampicin+Tetracyclin-5day	Female-Control-10days	0.142
Male-Control-5days	Female-Control-10days	0.056
F1-Female-Rifampicin+Tetracyclin	Female-Control-10days	0.736
F1-Male-Rifampicin+Tetracyclin	Female-Control-10days	0.048*
Male-Control-10days	Female-Rifampicin+Tetracyclin-10days	1.00
Male-Rifampicin+Tetracyclin-10day	Female-Rifampicin+Tetracyclin-10days	1.00
Female-Control-5day	Female-Rifampicin+Tetracyclin-10days	0.98
Female-Rifampicin+Tetracyclin-5day	Female-Rifampicin+Tetracyclin-10days	0.99
Male-Control-5days	Female-Rifampicin+Tetracyclin-10days	1.00

F1-Female-Rifampicin+Tetracyclin	Female-Rifampicin+Tetracyclin-10days	0.10
F1-Male-Rifampicin+Tetracyclin	Female-Rifampicin+Tetracyclin-10days	1.00
Male-Rifampicin+Tetracyclin-10day	Male-Control-10days	1.00
Female-Control-5day	Male-Control-10days	0.989
Female-Rifampicin+Tetracyclin-5day	Male-Control-10days	0.995
Male-Control-5days	Male-Control-10days	1.00
F1-Female-Rifampicin+Tetracyclin	Male-Control-10days	0.222
F1-Male-Rifampicin+Tetracyclin	Male-Control-10days	1.00
Female-Control-5day	Male-Rifampicin+Tetracyclin-10day	0.993
Female-Rifampicin+Tetracyclin-5day	Male-Rifampicin+Tetracyclin-10day	0.997
Male-Control-5days	Male-Rifampicin+Tetracyclin-10day	1.00
F1-Female-Rifampicin+Tetracyclin	Male-Rifampicin+Tetracyclin-10day	0.145
F1-Male-Rifampicin+Tetracyclin	Male-Rifampicin+Tetracyclin-10day	0.999
Female-Rifampicin+Tetracyclin-5day	Female-Control-5day	0.999
Male-Control-5days	Female-Control-5day	0.989
F1-Female-Rifampicin+Tetracyclin	Female-Control-5day	0.841
F1-Male-Rifampicin+Tetracyclin	Female-Control-5day	0.982
Male-Control-5days	Female-Rifampicin+Tetracyclin-5day	0.995
F1-Female-Rifampicin+Tetracyclin	Female-Rifampicin+Tetracyclin-5day	0.552
F1-Male-Rifampicin+Tetracyclin	Female-Rifampicin+Tetracyclin-5day	0.990
F1-Female-Rifampicin+Tetracyclin	Male-Control-5days	0.222
F1-Male-Rifampicin+Tetracyclin	Male-Control-5days	1.00
F1-Male-Rifampicin+Tetracyclin	F1-Female-Rifampicin+Tetracyclin	0.190

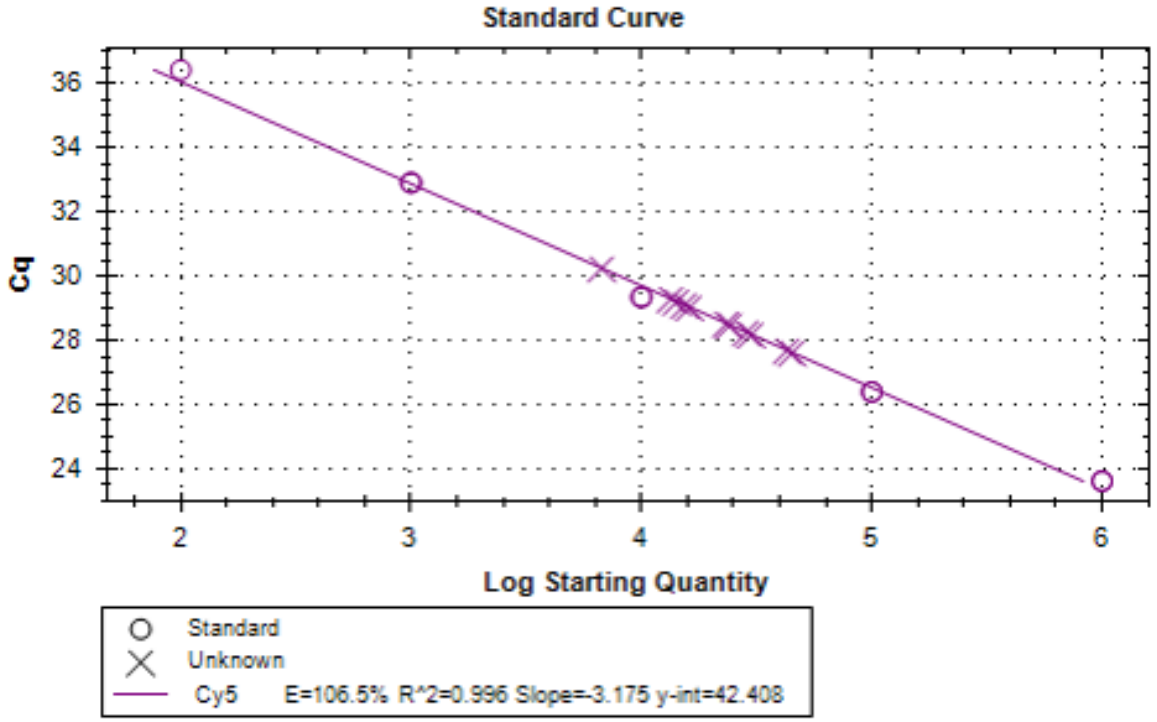
Appendix C: Multiple comparison of mean proportion of adult emergence of SSA1-SG3 AR+ and SSA1-SG3 AR-: Tukey's HSD test.

Comparison		Standard error	*Z* value	*P* value
AR- on healthy plants	AR+ on healthy plants	0.1	13.08	< 0.001**
AR+ on virus plants	AR+ on healthy plants	0.09	4.18	< 0.001**
AR- on virus plants	AR+ on healthy plants	0.1	9.87	< 0.001**
AR+ on virus plants	AR- on healthy plants	0.1	-9.05	< 0.001**
AR- on healthy plants	AR- on virus plants	0.1	-1.93	0.2
AR- on virus plants	AR+ on virus plants	0.1	6.2	< 0.001**

Appendix D: Multiple comparison of mean duration of adult emergence of SSA1-SG3 AR+ and SSA1-SG3 AR-: Tukey's HSD test.

Comparison		*P* value
AR- on healthy plants	AR+ on healthy plants	< 0.001**
AR+ on virus plants	AR+ on healthy plants	< 0.98
AR- on virus plants	AR+ on healthy plants	< 0.001**
AR+ on virus plants	AR- on healthy plants	< 0.001**
AR- on healthy plants	AR- on virus plants	0.7
AR- on virus plants	AR+ on healthy plants	< 0.001**

Appendix E: Efficiency of tubulin primers (300 nM) multiplexed with EACMV-UG (500 nM) with 100 nM of hydrolysis probes.



Appendix F: ANOVA for quantities of EACMV-UG acquired by SSA1-SG3 AR+ and SSA1-SG3 AR- after 48 hours AAP

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
Symbiont	1	15.6	15.59	2.23	0.139
Residuals	67	467.88	6.98		

Appendix G: ANOVA for quantities of EACMV-UG retained by SSA1-SG3AR+ and SSA1-SG3 AR- after 48 hours IAP

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
Symbiont	1	118.47	118.47	14.59	0.0002
Residuals	113	917.55	8.12		

Appendix H: ANOVA for expression of Knottin 1 by SSA1-SG3 AR+ and SSA1-SG3 AR-

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
symbiont	1	3.88	3.89	15.5	0.0003
residuals	40	10.00	0.25		

Appendix I: ANOVA for expression of Knottin 2 by SSA1-SG3 AR+ and SSA1-SG3 AR-

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
symbiont	1	7.099	7.099	9.4	0.0038
residuals	40	30.20	0.755		

Appendix J: ANOVA for expression of Knottin 3 in SSA1-SG3 AR+ and SSA1-SG3 AR-

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
symbiont	1	58.13	58.13	195.21	< 2.2e-16
residuals	39	11.61	0.3		

Appendix K: ANOVA for expression of *atg-9* in SSA1-SG3 AR+ and SSA1-SG3 AR-

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
symbiont	1	3.58	3.58	55.00	4.963e-09
residuals	40	2.60	0.65		

Appendix L: Multiple comparison of quantities of *Portiera* in different SSA cassava whiteflies (Tukey's HSD test).

Comparison		diff	lwr	upr	*P* value adjusted
SSA1-SG1	SSA1-SG2	-0.016	-4.70	2.67	0.97
SSA1-SG1	SSA1-SG3	-0.025	-3.61	3.56	1.00
SSA1-SG1	SSA1-SG5	-0.217	-3.66	3.23	0.99
SSA1-SG1	SSA2	-1.713	-4.99	1.566	0.67
SSA1-SG1	SSA3	0.709	-2.97	4.39	0.99
SSA1-SG2	SSA1-SG3	0.991	-2.27	4.25	0.96
SSA1-SG2	SSA1-SG5	0.799	-2.30	3.901	0.98
SSA1-SG2	SSA2	-0.697	-3.61	2.22	0.99
SSA1-SG2	SSA3	1.720	-1.64	5.09	0.69
SSA1-SG3	SSA1-SG5	-1.916	-3.18	2.79	0.99
SSA1-SG3	SSA2	-1.688	-4.47	1.10	0.51
SSA1-SG3	SSA3	0.734	-2.52	3.99	0.99
SSA1-SG5	SSA2	-1.49	-4.10	1.11	0.57
SSA1-SG5	SSA3	0.92	-2.17	4.03	0.97
SSA2	SSA3	2.42	-4.91	5.34	0.16

Appendix M: Multiple comparison of quantities of *Wolbachia* in different SSA cassava whiteflies (Tukey's HSD test).

Comparison		diff	lwr	upr	*P* value adjusted
SSA1-SG1	SSA1-SG2	6.7997621	4.1104817	9.48904256	0.0000000
SSA1-SG1	SSA1-SG3	3.8492127	1.3336212	6.36480415	0.0004184
SSA1-SG1	SSA1-SG5	1.4618982	1.0998547	4.02365122	0.5823249
SSA1-SG1	SSA2	6.2833970	3.8905922	8.67620182	0.0000000
SSA1-SG1	SSA3	2.2502707	0.4390098	4.93955107	0.1566105
SSA1-SG2	SSA1-SG3	-2.9505495	5.2139162	0.68718274	0.0037513
SSA1-SG2	SSA1-SG5	-5.3378639	7.6524280	3.02329984	0.0000001
SSA1-SG2	SSA2	-0.5163651	2.6424280	1.60969774	0.9886024
SSA1-SG2	SSA3	-4.5494915	7.0044574	2.09452558	0.0000148
SSA1-SG3	SSA1-SG5	-2.3873144	4.4975699	0.27705896	0.0173634
SSA1-SG3	SSA2	2.4341844	0.5325759	4.33579278	0.0046625
SSA1-SG3	SSA3	-1.5989420	3.8623088	0.66442472	0.3279587
SSA1-SG5	SSA2	4.8214988	2.8592316	6.78376594	0.0000000
SSA1-SG5	SSA3	0.7883724	1.5261916	3.10293647	0.9394737
SSA2	SSA3	-4.0331264	6.1591892	1.90706354	0.0000093

Appendix N: Post hoc comparisons of quantities of EACMV-UG retained by SSA2 A+, SSA2 W+ and SSA2 - (Tukey's HSD test)

Comparison		diff	lwr	upr	*P* value adjusted
SSA2 A+	SSA2 -	-1.58	-2.77	-0.39	0.0054
SSA2 A+	SSA2 W+	-1.71	-2.92	-0.49	0.0030
SSA2 W+	SSA2 -	-0.12	-1.31	1.065	0.9669

Appendix O: Post hoc comparisons of quantities of Knottin 1 in SSA2 A+, SSA2 W+ and SSA2 - (Tukey's HSD test)

Comparison		diff	lwr	upr	*P* value adjusted
SSA2 A+	SSA2 -	0.23	-0.79	0.32	0.57
SSA2 A+	SSA2 W+	1.19	0.62	1.76	<0.00001
SSA2 W+	SSA2 -	1.43	0.88	1.97	<0.00001

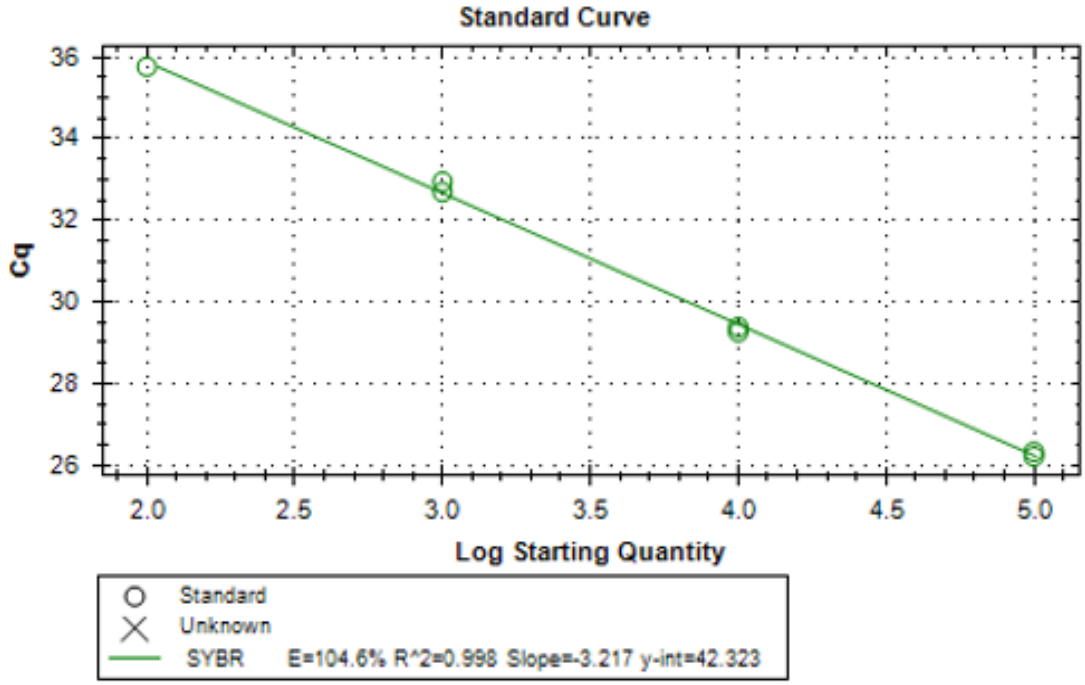
Appendix P: Post hoc comparisons of quantities of Knottin 2 in SSA2 A+, SSA2 W+ and SSA2 - (Tukey's HSD test)

Comparison		diff	lwr	upr	*P* value adjusted
SSA2 A+	SSA2 -	0.27	-0.32	0.87	0.051
SSA2 A+	SSA2 W+	0.72	0.16	1.29	0.0008
SSA2 W+	SSA2 -	0.45	-0.12	1.03	0.155

Appendix Q: ANOVA for expression of *atg-9* in SSA2 A+, SSA2 W+ and SSA2 -

	df	Sum sq.	Mean sq.	*F*	*P*
symbiont	2	0.46	0.23	2.99	0.06
residuals	64	4.91	0.07		

Appendix R: Efficiency of *Arsenophonus* primers



Appendix S: ANOVA for quantification of *Arsenophonus* in SSA1-SG3 AR+ and SSA2 A+

	df	**Sum sq.**	**Mean sq.**	***F***	***P***
Species	1	41.76	41.76	42.00	4.545e-05
Residuals	11	10.94	0.99		

Appendix T: Published paper (Chapter 3)