Assessment of incidence, severity and distribution patterns of citrus greening in Kenya and Tanzania; the role of African Citrus Triozid endosymbionts in disease epidemiology

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ABSTRACT

Infestation by insect pests and infection of disease causing pathogens are among the major biotic constraints hindering production, profitability and contribution of citrus to economic development. The African citrus triozid (ACT), *Trioza erytreae* (Del Guerico) (Hemiptera: Triozidae) and the Asian citrus psyllid (ACP) *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae), known vectors of *Candidatus* Liberibacter spp. pathogens, causal agents of the incurable and deadly citrus greening disease (CGD) are regarded as the most economically important citrus pests across all major citrus producing areas in the world. The objective of this study is to assess the incidence, severity and distribution patterns of CGD in Kenya and Tanzania, and to provide molecular characterization of potential circulating citrus greening pathogens. This study further investigates the interactions between *Candidatus* Liberibacter africanus (CLaf), causal agent of the African form of CGD and ACT, with special reference to the impact of CLaf infection on various fitness parameters and dispersal ability of the vector. This study also evaluates the diversity of ACT parasitoids and further characterizes endosymbionts associated with both *T. erytreae* and its parasitoids.

Citrus trees were randomly selected in orchards and rated for visual CGD symptoms in various geographic regions across Kenya and Tanzania, representing the low, mid and high altitudes areas. PCR and phylogenetic analysis were used for identification of the CLaf pathogen in collected leaves and insect samples. The results indicated a widespread occurrence of the CLaf subsp. clausena (CLafcl) in citrus trees throughout the surveyed regions. In contrast, only in a very low proportion of the ACT vectors (21%) could CLaf infection be detected, and none of the sampled and analysed ACP showed any signs of a CLaf or *Candidatus* Liberibacter asiaticus (CLas) infection. The level of disease incidence and severity varied across the different regions, with chronic greening situation observed in the Upper midland (UM) (1,300-1,800 meter above sea level [m.a.s.l.]) and Lower midland (LM) (800-1,300 m.a.s.l.) regions in both countries. Moreover, both *T. erytreae* and *D. citri* vectors coexisted at high elevations in the UM and LM regions, illustrating that *D. citri* was spreading fast in these regions, quickly adapting to new geographical areas and ecologies.

The findings on the effects of a CLaf infection on fitness traits of ACT established that CLaf infected (CLaf+) ACT conferred some fitness benefits, which included increased fecundity, fertility, faster egg-to-adult development time, higher female ratio and dispersal capability, all of which could potentially help to promote the spread of infection. However, the CLaf+ ACT vectors also incurred some fitness costs such as reduced nymphal and adult survival. Overall, the results suggested that CLaf infection had fitness effects on ACT, but there was no clear-cut distinction between benefits and costs of an infection, since the pathogen affected several fitness parameters of the vector in different directions, resulting in simultaneous fitness gains and costs for CLaf and ACT. These complex and subtle interactions between fitness traits and the effects of a pathogen infection could all contribute to strong ecological impacts and evolutionary pressures on the host and the pathogen, and thus ultimately on the epidemiology of disease.

Morphological and molecular characterization identified several ACT parasitoid species like *Tamarixia dryi*, *Psyllaephagus pulvinatus*, *Tetrastichus* sp, *Aphidencyrtus cassatus* and *Charipinae* sp. In addition, four eubacterial symbionts (*Wolbachia*, *Rickettsia*, *Arsenophonus* and *Candidatus* Liberibacter sp.) were detected in *T. erytreae*, while parasitoids harboured three symbionts (*Wolbachia*, *Rickettsia* and *Cardinuim*). Maximum likelihood phylogenetic inferences clustered the identified eubacterial symbionts within the α and γ proteobacteria subdivisions. Phylogenetic inferences of 16S rRNA gene sequences indicated that *Wolbachia* strains from ACT and the parasitoids did not form a single monophyletic clade; however, both clustered within Supergroup B. These identified parasitoids and endosymbionts could be explored as part of future biological and integrated pest and disease management strategies against ACT and CGD, respectively.

Bewertung von Inzidenz, Schweregrad und Verteilungsmustern der Zitrus Greening Krankheit in Kenia und Tansania; die Rolle von African Citrus Triozid Endosymbionten in der Epidemiologie der Krankheit

KURZFASSUNG

Der Befall durch Schadinsekten und die Infektion mit krankheitserregenden Pathogenen gehören zu den größten biotischen Einschränkungen, die die Produktion, Rentabilität und den Beitrag von Zitrusfrüchten zur wirtschaftlichen Entwicklung behindern. Die Afrikanische Zitrus Trizide (ACT), *Trioza erytreae* (Del Guerico) (Hemiptera: Trioidae) und die Asiatische Zitrus Psyllide (ACP) *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae), bekannte Vektoren von *Candidatus* Liberibacter spp., Erreger der unheilbaren und lethalen Citrus Greening Disease (CGD) gelten als die wirtschaftlich bedeutendsten Zitrusschädlinge in allen wichtigen Zitrusanbaugebieten. Das Ziel dieser Studie ist es, die Inzidenz, den Schweregrad und das Verteilungsmuster von CGD in Kenia und Tansania zu untersuchen und eine molekulare Charakterisierung möglicher zirkulierender Citrus Greening-Pathogene zu ermöglichen. Diese Studie untersucht des Weiteren die Interaktionen zwischen Candidatus Liberibacter africanus (CLaf), dem Erreger der afrikanischen Form von CGD und ACT, unter besonderer Berücksichtigung der Auswirkungen einer CLaf-Infektion auf verschiedene Fitnessparameter und die Ausbreitungsfähigkeit des Vektors. Diese Studie charakterisiert auch die Diversität von ACT-Parasitoiden und die der Endosymbionten von *T. erytreae* und seinen Parasitoiden.

Zufällig ausgewählt Zitrusbäume in Obstgärten wurden für visuelle CGD-Symptome in verschiedenen geographischen Regionen mit niedrigen, mittleren und hohen Lagen eingestuft. PCR und phylogenetische Analysen wurden zur Identifizierung von *Candidatus* Liberibacter Pathogenen in den gesammelten Blättern und Insektenproben durchgeführt. Die Ergebnisse weisen auf ein weit verbreitetes Vorkommen des CLaf-Subspezies *clausena* (CLafcl) Pathogens auf Zitrusbäumen in den untersuchten Regionen hin. Im Gegensatz dazu konnte nur bei einem sehr geringen Anteil der ACT-Vektoren (20%) eine CLaf-Infektion nachgewiesen werden, und keine der in der Stichprobe erhobenen und analysierten ACP zeigte Anzeichen einer CLaf- oder Liberibacter Kandidat asiaticue (CLas-) Infektion. Das Ausmaß der Erkrankungshäufigkeit und -schwere variierte in den verschiedenen Regionen, wobei chronische CGD Infektionen in beiden Ländern in mittleren (1.300-1.800 über dem Meeresspiegel [ü.d.M.]) und niederen Lagen (800-1.300 ü.d.M.) beobachtet wurden. Darüber hinaus koexistierten sowohl *T. erytreae*- als auch *D. citri*-Vektoren in höheren Lagen, was zeigt, dass sich *D. citri* in diesen Regionen rasch ausgebreitet und schnell an neue geografische Gebiete und Umweltbedingungen angepasst hat.

Die Ergebnisse bezüglich des Effekts einer CLaF-Infektion auf Fitnessmerkmale von ACT zeigen, dass CLaf-infizierte (CLaf+) ACT einige Fitnessvorteile besitzen, d.h. erhöhte Fekundität, Fertilität, schnellere Ei-zu-Adult-Entwicklungszeit, höherer weiblicher Anteil und Ausbreitungsfähigkeit. Diese könnten möglicherweise dazu beitragen, die Ausbreitung der Infektion zu fördern. Die CLaf+ ACT-Vektoren erlitten jedoch auch einige Fitnesskosten, wie ein verringertes Nymphen- und Adult-Überleben. Insgesamt deuteten die Ergebnisse darauf hin, dass die CLaF-Infektion Fitness-Effekte auf ACT hatte. Es ergab sich jedoch keine klare Unterscheidung zwischen Nutzen und Kosten der Infektion, da der Erreger mehrere Fitness-Parameter des Vektors in verschiedenen Richtungen beeinflusste, was zu gleichzeitigen Fitnessgewinnen und -kosten für CLaf und ACT führte. Diese komplexen und subtilen Interaktionen zwischen Fitnessmerkmalen und den Auswirkungen einer Pathogeninfektion könnten ökologischen Auswirkungen und evolutionären Druck auf den Wirt und den Erreger bewirken und damit letztendlich die Epidemiologie der Krankheit beeinflussen.

Mittels morphologischen und molekularen Charakterisierungen gelang es, verschiedene ACT Parasitoide wie *Tamarixia dryi*, *Psyllaephagus pulvinatus*, *Tetrastichus* sp., *Aphysidertus cassatus* und *Charipinae* sp. zu identifizierten. Darüber hinaus wurden in *T. erytreae* vier eubakterielle Symbionten (*Wolbachia*, *Rickettsia*, *Arsenophonus* und *Candidatus* Liberibacter sp.) nachgewiesen, während Parasitoide drei Symbionten (*Wolbachia*, *Rickettsia* und *Cardinuim*) beherbergten. Phylogenetische Inferenzen mit maximaler Wahrscheinlichkeit gruppierten die identifizierten eubakteriellen Symbionten innerhalb der α- und γ-Proteobakterien. Phylogenetische Inferenzen von 16S-rRNA-Gensequenzen zeigten, dass *Wolbachia*-Stämme von ACT und seinen Parasitoiden keine einheitliche monophyletische Gruppe bildeten; jedoch wurden beide Supergruppe B zugeordnet. Die identifizierten Parasitoide und Endosymbionten sollten als mögliche Komponenten zukünftiger biologischer und integrierter Schädlings- und Krankheitsmanagementstrategien gegen ACT bzw. ACGD weiter erforscht werden.

TABLE OF CONTENTS

1 Introduction 1

1.1 Background 1

1.2 Citrus greening disease 2
1.2.1 Candidatus Liberibacter pathogens 2
1.2.2 Geographic distribution 2
1.2.3 Transmission 3
1.2.4 Symptoms 3
1.2.5 Host range 4
1.2.6 Management and control 4

1.3 Natural enemies of citrus psyllids 5

1.4 Bacterial endosymbionts 8

1.5 Problem statement 9

1.6 Research aim and objectives 10

1.7 Thesis structure 10

2 Citrus greening disease, East African situation: Incidence, severity and distribution patterns 11

2.1 Abstract 11

2.2 Introduction 11

2.3 Material and methods 14
2.3.1 Study areas 14
2.3.2 Sampling strategy 15
2.3.3 Collection of plant tissues and insect samples 16
2.3.4 Tissue preparation and DNA extraction 19
2.3.5 PCR amplification, Agarose gel electrophoresis and DNA purification 19
2.3.6 Sequencing and phylogenetic analysis 20

2.4 Results 20
2.4.1 Surveys 20
2.4.2 Incidence and severity 22
2.4.3 Detection of *Candidatus* Liberibacter spp. in leaf samples 23
2.4.4 Detection of Candidatus Liberibacter spp. in psyllids 24
2.4.5 Sequence identification 25
2.4.6 Data analysis 26

2.5 Discussion 29

3 What is in it for me? Costs and benefits of a *Candidatus* Liberibacter Africanus infection in the African citrus triozid *Trioza erytreae*, vector of the African citrus greening disease 34

3.1 Abstract 34

3.2 Introduction 34
3.3 Material and methods 37
3.3.1 Maintenance of host plants and insect colonies 37
3.3.2 Establishment of experimental isofemale lines 38
3.3.3 Mating assay 38
3.3.4 Fecundity assays for fitness evaluation 38
3.3.5 Immature development and survivorship 39
3.3.6 Reproductive capacity 40
3.3.7 Adult survival 40
3.3.8 Effect of CLaf on ACT dispersal capability 40
3.3.9 Effect of alternate host plants on dispersal of CLaf+ and CLaf- ACTs 41
3.3.10 DNA extraction 41
3.3.11 Quantitative real-time PCR assays 42
3.3.12 Statistical analysis 42
3.4 Results 43
3.4.1 Effect of CLaf on ACT fitness traits 43
3.4.2 Reproductive capacity and population parameters 45
3.4.3 Relative quantification of bacterial 16sRNA CLaf gene 47
3.4.4 Effects of CLaf on ACT dispersal 48
3.5 Discussion 50
4 Characterization of African citrus triozid, their parasitoids and endosymbionts: Intergral resources for biological control success 54
4.1 Abstract 54
4.2 Introduction 54
4.3 Materials and methods 57
4.3.1 Insect collection and identification 57
4.3.2 Genomic DNA extraction 58
4.3.3 Screening and sequencing 59
4.3.4 Sequence Analysis 60
4.4 Results 60
4.4.1 Identification of *T. erytreae* and its parasitoid species 60
4.4.2 Detection of bacterial symbionts 63
4.4.3 Frequency of secondary endosymbionts 65
4.4.4 Phylogenetic analysis of 16S rRNA gene sequences 65
4.5 Discussion 70
5 Synthesis 74
6 Outlook 78
7 References 81
8 Appendices 114

LIST OF ACRONYMS AND ABBREVIATIONS

ACGD	African citrus greening disease
ACT	African citrus triozid
ACP	Asian citrus psyllid
ANOVA	Analysis of Variance
CGD	Citrus greening disease
CI	Cytoplasmic incompatibility
CL	Confidence Limit
CLaf	*Candidatus* Liberibacter africanus
CLam	*Candidatus* Liberibacter americanus
CLas	*Candidatus* Liberibacter asiaticus
COI	Cytochrome oxidase subunit I
cPCR	Conventional polymerase chain reaction
df	Degrees of freedom
DNA	Deoxyribonucleic Acid
HLB	Huanglongbing
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated pest management
m.a.s.l	meters above sea level
MEGA	Molecular Evolutionary Genetics Analysis
mt	Mitochondrial
ML	Maximum Likelihood
L	Lowlands
LH	Lower highland
LM	Lower midland
Qpcr	Quantitative polymerase chain reaction
RFC	Relative fold change
RSA	Republic of South Africa
S-	Secondary
SE	Standard error
SSA	sub-Saharan Africa
Spp.	Species
P-	Primary
PCA	Principal Component Analysis
UM	Upper midland
α	Alpha
β	Beta
γ	Gamma

1 Introduction

1.1 Background

Among the diversity of fruits cultivated in sub-Saharan Africa (SSA), citrus ranks high as both food and cash crop, as a large proportion of the population, mainly smallholders, are involved in the cultivation and marketing of the fruit (Seif 1996). Sweet oranges (*Citrus sinensis* (L.) Osbeck) are the major fruit species grown and represent approximately 70% of the citrus output (FAO 2006; FAOSTAT 2012). Other species of commercial importance include lemons (*C. limon* Burm. f.), limes (*C. aurantifolia* L.), grapefruit (*C. paradisi* Macf.) and tangerines (*C. reticulata* Blanco) all in the rutaceae family. The health benefits of citrus fruits are well documented especially in the provision of vitamin C, carotenoids and polyphenols (Sanofer 2014; Turner et al. 2013). In Kenya and Tanzania the majority of the fruits produced are consumed locally as fresh product and a smaller quantity is processed into juices, jam and marmalades (MMA 2008). However, local market demand for citrus in the two countries far outweighs the supply which in the case of Kenya is below 25% of the production potential, resulting in importation from South Africa and Egypt (Muendo and Tschirley 2004; Obukosia and Waithaka 2000).

Despite this local shortage, the major citrus growers in both countries are keen to target lucrative export markets where certain cultivars can fetch higher returns. Nonetheless, there has only been marginal improvement in yields compared to global levels. For example, yields of sweet oranges of smallholder farmers in Kenya range from 4-10 t/ha, far below the required yield capacity of 50-75 t/ha (Obukosia and Waithaka 2000; Pole et al. 2010). The low citrus yields in SSA are attributed, among others, to various insect pests and diseases, inadequate capital, inadequate planting materials and poor orchard management practices (Kilalo et al. 2009). Citrus greening disease (CGD) commonly referred to as Huanglongbing, or specifically in Africa as Africa citrus greening disease (ACGD), is reported as one of the major limitations to citrus production (Ministry of Agriculture 1982; Kilalo et al. 2009; Muendo and Tschirley 2004). The African citrus triozid (ACT), *Trioza erytreae* (Del Guerico) (Hemiptera: Triozidae), is the vector responsible for transmitting *Candidatus* Liberibacter africanus (CLaf) which causes African citrus greening disease (ACGD).

1.2 Citrus greening disease

1.2.1 Candidatus Liberibacter pathogens

Candidatus Liberibacter pathogens are fastidious α-, phloem-restricted, non-cultured, Gram-negative proteobacteria and the causative agent of Huanglongbing (HLB) (Garnier and Bove 1987; Jagoueix et al. 1996). Three strains are recognized based on 16S rDNA; the African strain *Candidatus* Liberibacter africanus (CLaf), the Asian isolate *Candidatus* Liberibacter asiaticus (CLas) and the Latin American isolate *Candidatus* Liberibacter americanus (CLam) (Bassanezi and Gottwald 2004; Garnier et al. 2000; Texeira et al. 2005). Since none of these greening pathogens can been cultured on artificial media, there remains a "*Candidatus*" designation associated with their taxonomy (Halbert and Manjunath 2004). Moreover, several subspecies of CLaf have been identified from indigenous rutaceous trees; e.g. van den Berg et al. (1992) reported CLaf subsp. clausena (CLafCl) from *Clausena anisata* (Wild) Hook. f. ex Benth., Korsten et al. (1996) detected CLaf subsp. vepridis (CLafV) from a *V*epris *lanceolata* (Lam.) G.Don while Roberts and Pietersen (2016) detected CLaf subsp. tecleae (CLafT) from *Teclea gerrardii* Verdoorn. Furthermore, widespread occurrence of CLaf subsp. capensis (CLafC) has been found on *Calodendrum capense* (L.f.) Thunb. plants (Garnier et al. 2000).

1.2.2 Geographic distribution

The Asiatic CLas strain is the most widely distributed, virulent and devastating form of HLB pathogen that has been reported from citrus crops in Asia (Garnier and Bove 1996), the Mascarene Islands (Garnier et al. 1996), South and Central America (Halbert and Manjunath 2004), Papua New Guinea (Weinert et al. 2004) and more recently in Africa (Saponari et al. 2010). The Latin American strain CLam has been found in Brazil and Texas (Bové 2006; Texeira et al. 2005) while the African strain CLaf has been described in southern and eastern Africa countries including Madagascar (Batool et al. 2007; Graca 1991; Magomere et al. 2009; Moll et al. 1978; van Vuuren 1978). The presence of the greening disease was suspected as early as 1982 in Kenya (Anon 1982). Geographically, the first reports on detection of HLB were at high altitude areas in the Thika, Kamiti and Kitale regions of Kenya (Seif and Whittle 1984). In Tanzania the wide spread presence of the *T. erytreae* vector and greening disease was revealed in Arusha and along the slopes of the Uluguru mountains in the Morogoro, Matombo, Msikitini and Langali regions (Evers and Grisoni 1991).

1.2.3 Transmission

The citrus greening pathogen is mainly transmitted from one plant to another through insect vectors, vegetative propagation or grafting of infected plant material and dodder (capoor et al. 1974; Roistacher 1996). Vegetative transmission occurs through the use of infected bud wood (Oberholzer et al. 1965). The Asian citrus psyllid *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae) and *T. erytreae* are the two species of citrus psyllids transmitting HLB. The greening pathogen is found in the sieve tubes of infected plants and can be acquired and transmitted by the fourth and fifth instar nymphs or adult psyllids during phloem feeding (Garnier and Bové 1983).

The ACT is the principle vector of the CLaf pathogen causing the African citrus greening disease (ACGD) (Mcclean 1974) and develops at temperatures of 22-25°C. The adult psyllid ingests the bacteria, which penetrates the gut during the latent period of about 21 days and proliferates in the insect hemolymph (Batool et al. 2007). The pathogen can be injected with saliva into the next plant during feeding. *Trioza erytreae* can acquire the inoculum within five days of feeding and transmits the disease in less than 60 minutes of feeding (van Vuuren and da Graça 1977). The Asian citrus psyllid can withstand temperatures above 30°C and is able to transmit CLas or CLam pathogen via salivary secretions to an uninfected plant during 15–30 minutes of phloem-feeding with a latent period of 8–12 days (Salibe and Cortez 1966). The *Candidatus* Liberibacter pathogens can also be passed vertically from infected mothers to uninfected offspring via the egg, or through venereal transmission where infected males or females transfer and infect the other sex during mating (Tabachnick 2015).

1.2.4 Symptoms

Affected trees show stunting, twig dieback, sparse yellow foliage, or severe fruit drop (CABI/EPPO 1995; Catara et al. 1988). Mottling and chlorosis are the main characteristic leaf symptoms. The asymmetric blotchy mottle pattern distinguishes it from zinc, manganese, magnesium, calcium, and iron deficiency symptoms (Graça 1991). Leaves may also become thicker, leathery and midribs and lateral veins are sometimes enlarged, swollen, and corky (Batool et al. 2007). HLB-affected fruits are under-developed, asymmetric, lopsided, with a bent fruit axis and also show “color inversion” or “red nose” (Akhtar and Ahmad 1999). In the case of ACGD, fruits remain immature with green and brownish aborted seeds (CABI/ EPPO 1995; Garnier and Bové 1983).

1.2.5 Host range

Greening disease affects almost all citrus cultivars and close relatives (Wang and Trivedi 2013). At present, there is no real resistance to HLB, but some species and cultivars show some tolerance to the infection while others are more susceptible to decline i.e. small-fruited acid lime trees have been found to be only slightly affected while grapefruits are more tolerant to infection than most of the sweet orange cultivars (Halbert and Manjunath 2004; Liu et al. 2011; Wang and Trivedi 2013).

1.2.6 Management and control

Maintenance of clean nursery stocks, internal quarantine systems, pruning and removal of infected and neglected citrus trees are essential for reducing inoculum sources and spread of HLB (Graça 1991). Thermotherapy, where graft wood is heated to 48–50°C for several minutes has also been an effective control; however, this method is impractical for large-scale use (Cheema et al. 1982; Lin 1964; Nariani and Bhagabati 1980; Schwarz and Green 1972). Chemotherapy approaches involving the use of antibiotics such as tetracycline hydrochloride through foliar spray and trunk injections can successfully suppress greening symptoms (Chiu et al. 1979; Martinez et al. 1970; Schwarz and van Vuuren 1970; Zhao 1981). For example, in India control of HLB has been achieved through immersion in penicillin-carbendazin (Cheema et al. 1986), injection with ledermycin (demeclocycline hydrochloride) and a streptomycin-chlortetracycline mixture (Nariani and Bhagabati 1980), and foliar sprays with agrimycin and carbendazin (Cheema et al. 1985; Kapur et al. 1986).

A complete eradication of the citrus psyllid vectors is, however, not feasible due to their large and wide distribution. Nonetheless, chemical and biological control agents have been used to reduce vector densities. The most effective systemic insecticide treatments for *T. erytreae* control include endosulfan and monocrotophos sprays and dimethoate applications to the soil (Milne and de Villiers 1977; Milne 1977; Pyle 1977; Wortmann and Schafer 1977). However, these insecticides can interfere with biological control of other pests; in addition, the high cost of insecticides is a major hindrance for small farmers.

1.3 Natural enemies of citrus psyllids

Parasitoids

The solitary ectoparasitoid *Tamarixia dryi* (Waterston) (Hym.: Eulophidae) and endoparasitiod *Psyllaephagus pulvinatus* (Waterston) (Hym.: Encyrtidae) are the most common and effective primary parasitoids of *T. erytreae* (van den Berg and Greenland 2000; Catling 1969; Mc Daniel and Moran 1972; Tamesse et al. 2002). *Tamarixia dryi* lays its eggs singly beneath the third, fourth or fifth instar nymphs and the larva sucks out the body contents of the nymph, then when fully grown binds the remains of its dead host with filaments to the edges of the leaf gall (McDaniel 1970). *Tamarixia dryi* has successfully been used to control *T. erytreae* populations in citrus orchards on Réunion Island and Mauritius (Aubert 1987; Aubert and Quilici 1988; Etienne and Aubert 1980).

Psyllaephagus pulvinatus is reported to have failed to establish itself in the Réunion Island, however, Tamesse et al. (2002) found *P. pulvinatus* to be more efficient than *T. dryi* in controlling *T. erytreae* in Cameroon, suggesting that the efficacy of the parasitoid may vary depending on the environment. Catling (1969) also emphasized on the importance of synchrony between these parasitoids and *T. erytreae*. He recorded a parasitism of at least 40-50% during periods of favourable synchrony but under poor synchrony parasitism of the susceptible nymph stages was reduced to below 10%.

The populations of *T. dryi* and *P. pulvinatus* are however strongly limited by hyperparasitoids, the most important of which is *Aphidencyrtus cassatus* Annecke (Hym.: Encyrtidae) (Daniel and Moran 1972; Tamesse et al. 2002). Other secondary and tertiary hyperparasitiods of *T.erytreae* and *P. pulvinatus* include; *Tetrastichus* sp. (Hym.: Eulophidae), *Coccophagus pulvinariae* Compere, *Mariella exitiosa* Compere (both Hym.: Aphelinidae), *Pachyneuron* sp. (Hym.: Pterornalidae), *Cheiloneurus cyanonotus* Waterson (Hym.: Encyrtidae) (Catling 1969; Daniel and Moran 1972; Prinsloo 1981). *Tamarixia dryi* is reported to also parasitize *Trioza eastopi* Orian, which feeds and breeds on the common weed Litsea chinensis Jacq. (Aubert and Quilici 1983). However, both *Trioza eastopi* and Litsea chinensis have Asian origin and do not occur in Africa.

On the other hand, *Tamarixia radiata* (Waterston) (Hym.: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Argarwal) (Hym: Encyrtidae) are the known primary parasitoids of *D. citri* nymphs (Tang 1990). *Tamarixia radiata* develops as idiobiont ectoparasitoids, adult females shows arrhenotokous parthenogenesis, are solitary, synovigenic and oviposits on third- to fifth-instar nymphs of *D. citri* (Chen and Stansly 2014;

Chien and Chu 1991). At favorable temperatures of 25°C - 30°C, a single female fecundity can reach around 300 eggs and through host feeding and oviposition behavior, can destroy around 500 *D. citri* nymphs during its lifetime (Chien and Chu 1991; Chien 1995; Étienne et al. 2001).

Tamarixia radiata has successfully been introduced to several countries such as Réunion Island (Aubert and Quilici 1983), Taiwan (Chien et al. 1989), Mauritius (Quilici 1986), Philippines (Gavarra et al. 1990), Saudi Arabia (Aubert 1984), Indonesia (Nurhadi 1987), Guadalupe (Étienne et al. 2001), California (Hoddle 2012), Florida (Qureshi et al. 2009; Skelley and Hoy 2004), and Texas (Flores et al. 2017). The parasitoid has also been detected in Brazil and Puerto Rico where no known releases were made (Pluke et al. 2008; Torres et al. 2006). However, varying levels of *D. citri* control by *T. radiata* is reported in different countries, for example, Husain and Nath (1924) reported as high as 95% parasitism level of *D. citri* nymphs in India. On Réunion Island, *T. radiata* is also credited with providing excellent control of *D. citri* where the parasitism rates often exceeded 90% (Aubert et al. 1996). Parasitism levels in Puerto Rico at Isabela is reported to exceed 50% and 70% on average , but were found to be more variable and averaged 38.5% at Corozál and Gurabo when psyllid populations were lower, while in Florida the parasitism rates are reported to be lower (rarely exceeding 20%) and more sporadic (Étienne et al. 2001; Michaud 2004; Pluke et al. 2008; Qureshi et al. 2009; Tsai et al. 2002). The difference in *T. radiata* efficiency could be attributed to environmental differences, interguild competition with other beneficial organisms, or genetic differences among geographical populations of the parasitoids (Barr et al. 2009). *Tamarixia radiata* has a high host specificity on *D. citri*, although *Bactericera cockerelli* Sulc is reported to be parasitized at a low level (5%) (Hoddle and Pandey 2014).

Diaphorencyrtus aligarhensis is a koinobiont endoparasitoid that preferentially parasitizes second and third instar *D. citri* nymphs (Sule et al. 2014). *Diaphorencyrtus aligarhensis* has however provided low levels of *D. citri* control in several citrus growing regions including Taiwan, Réunion Island and Saudi Arabia, but has failed to establish itself in other regions like Florida despite repeated release efforts (Chien et al. 1989; Rohrig et al. 2011). Nonetheless, it is anticipated that *D. aligarhensis* could complement *T. radiata* thereby enhancing *D. citri* biological control. Other parasitic wasps of *D. citri* include *Psyllaephagus* sp. (Lin and Tao 1979), *Chartocerus walkeri* and *Encarsia* (Tang 1988). The efficacy of both *T. radiata* and *D. aligarhensis* is limited by the activity of hyperparasitoids such as *Pachyneuron* and *Tetrastichus* (Chiu et al. 1988; Tang 1988). *Diaphorencyrtus*

aligharensis and *T. radiatus* are reported to be too specific to parasitize *T. erytreae* (Van den Berg 1984).

Predators

The green lacewing [*Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae)], lady beetles [*Chilocorus nigritus* (F.), *Adalia bipunctata* (L.), *Brumus quadripustulatus* (L.), *Cryptolaemus montrouzieri* Mulsant, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae)], predatory mites [*Leptus Latreille*, *Bochartia* sp. and *Abrolophus* (all Acari: Erythraeidae), and *Iphiseius degenerans* (Berlese) (Acari: Phytoseiidae)], *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae), syrphids (Diptera: Syrphidae), spiders (Arachnida: Araneae), and dusty wing (Neuroptera: hemeroboiidae) are recorded as predators of *T. erytreae* (Catling and Annecke 1968; Catling 1970; Etienne 1978; González-Hernández 2003; van den Berg et al. 1987; Samways 1984a; Van der Merwe 1923). However, these predators have not effectively suppressed *T. erytreae* populations and are considered to play a minor role in reducing the population dynamics of *T. erytreae* (van den Berg et al. 1992b; Catling and Annecke 1968; Catling 1970).

The most important predators of *D. citri* that can limit the nymphal population include; coccinellid ladybeetles [Olla v-nigrum Mulsant, Curinus coeruleus Mulsant, Exochomus childreni Mulsant, Harmonia axyridis Pallas, Cycloneda sanguinea (L.) and Coelophora inaequalis (F.)] followed by lacewings [(Neuroptera: Chrysopidae); Ceraeochrysa sp. and Chrysoperla sp.] and spiders [(Araneae) Hibana velox (Becker)](Michaud 2001; Michaud and Olsen 2004; Qureshi et al. 2009). Hoverflies (Diptera: Syrphidae), cockroach (Blattella asahinai Mizukubo), praying mantids, ants and mites are also often encountered as polyphagous predators of Asian citrus psyllids (Michaud 2002, 2004; Qureshi and Stansly 2008; Yang et al. 2006).

Pathogenic microorganisms

Trioza erytreae is attacked by *Cladosporium oxysporium* (Berk. and Curtis)(Samways and Grech 1986) and *Capnodium citri* (Berk. and Desm.) (Aubert 1987), and both fungi are sensitive to desiccation and are density-dependent. *Diaphorina citri* is naturally infected by several entomopathogenic fungi such as *Paecilomyces fumosoroseus* (Wize), *Hirsutella citriformis* Speare, *Cephalosphorium lecanii* Zimm (*Verticillium lecanii*), *Beauveria bassiana* (Bals.) Vuill., *Cladosporium* nr. *oxysporum* (Berk. and Curt.) and *Capnodium citri* (Berk. and Desm.) (Aubert, 1987; Étienne et al. 2001; Gavarra and Mercado 1988; Rivero-Aragon

and Grillo-Ravelo, 2000; Samson, 1974; Samways and Grech, 1986; Subandiyah et al. 2000a; Xie et al. 1988). The biological control contribution of most these entomopathogenic fungi are not well documented, however, of most interest are two Isaria fumosorosea (Ifr) (formerly Paecilomyces fumosoroseus) strains, Agricultural Research Service Entomopathogenic Fungi (ARSEF) 3581 and the Apopka 97 (American Type Culture Collection 20874), both of which are highly pathogenic toward adult and nymphal stages of *D. citri* and are easily mass-produced as blastospore formulations (Avery et al. 2009, 2011; Jackson et al. 1996; Stauderman et al. 2012). Since Ifr blastospores are desiccation tolerant and germinate quickly on suitable hosts they are ideal for use in foliar applications or auto-dissemination management strategies (Chow et al. 2016: Jackson et al. 2010; Jackson and Payne 2007; Moran et al. 2011)

1.4 Bacterial endosymbionts

Symbiosis (syn-, together and –bios, life) is defined as any association between different species (de Bary 1879). Symbiosis includes interactions ranging from commensalism (neutral), mutualism where both organisms benefit from the interaction, to parasitism where survival of one organism comes at the expense of the other (Werren and O'Neill 1997).

Most psyllids harbor the obligate or primary (P-) endosymbiont ''*Candidatus* Carsonella ruddii'' within the bacteriocytes (Baumann 2005; Subandiyah et al. 2000b; Thao et al. 2000a). Psyllids also host morphologically different bacteria within the syncytial region called facultative or secondary (S-) endosymbionts. P-endosymbionts are frequently associated with key metabolic processes such as nutrition, development and reproduction (Sloan and Moran 2012). In contrast, S-endosymbionts are not strictly required for insect survival and can be categorized as either facultative mutualists or reproductive manipulators (Moran et al. 2008).

Facultative mutualists can influence ecologically relevant fitness traits of their hosts and have diverse functions such as protection against natural enemies, host plant colonization and heat shock resistance, which can significantly impact the success of biological control (Hansen et al. 2007; Hedges et al. 2008; Oliver et al. 2010). Reproductive manipulators spread their infections by biasing the sex ratio of their hosts towards production of females (Moran et al. 2008). They manipulate host insects through induction of different reproductive alterations such as (a) induction of thelytokous parthenogenesis where infected females produce daughters without fertilization by males, observed in *Wolbachia*, *Rickettsia* spp. and

Cardinium hertigii (Hagimori et al. 2006); (b) feminization of genetic males where genetic male embryos develop phenotypically as females shown in *Wolbachia* and *C. hertigii* (Zchori-Fein and Perlman 2004); (c) male killing where male embryos are killed during development which potentially increases investment in daughters, observed in *Wolbachia*, *Arsenophonus*, *Rickettsia* spp., *Flavobacterium* spp., *Spiroplasma* spp. (Jiggins et al. 2000; von der Schulenburg et al. 2001; Werren et al. 1986); and (d) oogenesis, in which uninfected females cannot produce mature oocytes, observed in *Wolbachia* (Takano et al. 2017).

Other reproductive manipulators do not distort the host sex ratio but rather induce sterility with hosts carrying different types of cytoplasmic infection, a phenotype called cytoplasmic incompatibility. This phenomenon is expressed when an infected male mates with a female that is not infected; when male and female are both infected with two different *Wolbachia* strains; or when the male is infected with two strains and the female is infected with a single *Wolbachia* strain (Giordano et al. 1997). The outcomes from such crosses can range from severe incompatibility, resulting in very few to no offspring, a medium level of incompatibility, to no incompatibility at all (Giordano et al. 1997; Hoffmann et al. 1996; Wade and Stevens 1985). Reproductive manipulators have profound effects on host phenotypes and can be exploited to achieve applicative purposes, such as controlling pest populations or interfering with transmission of insect-borne diseases (Brelsfoard et al. 2009; Hancock et al. 2011; Iturbe-Ormaetxe et al. 2011).

1.5 Problem statement

Huanglongbing is one of the world's most devastating diseases of citrus for which there is still no known cure (Bové 2006; McClean and Schwarz 1970). In East Africa, the disease has resulted in the collapse of the Kenyan and Tanzanian citrus industries and the reported losses due to ACGD can range from 25 to 100% (Muendo and Tschirley 2004; Swai et al. 1988). However, crucial information on occurrence, distribution and the extent of damage of HLB, and in particular ACGD, are still very limited, both globally as well as in SSA, to help guide management decision and reduce disease transmission.

Furthermore, the current understanding of vector-pathogen interactions and their implications for the establishment and spread of ACGD remains limited. No studies have explored the direct effects of the CLaf pathogen on fitness-related variables of its ACT vector to understand the transmission and epidemiology of this important vector-transmitted plant disease. Moreover, the link between dispersal of infected and non-infected ACTs in the

presence and absence of citrus and alternative host plants is not yet well established to determine safe isolation distances for ACGD quarantine and eradication purposes in Africa.

In addition, considering the economic importance of ACT with regard to ACGD transmission, biological control studies using endosymbionts and parasitoids associated with *T. erytreae* are warranted. However, little is known about ACT parasitoids in East Africa, and no studies on the molecular characterization of ACT parasitoids have been undertaken. Thorough analyses of endosymbiont diversity in parasitoids and their hosts are also lacking.

1.6 Research aim and objectives

Based on these research problems, this study aims to assess the citrus greening situation in Kenya and Tanzania and further determines the role of endosymbionts in disease epidemiology. Specifically, the following objectives were pursued:

1. Assess the incidence, severity and distribution patterns of CGD in Kenya and Tanzania.
2. Assess the role of CLaf infection on ACT fitness parameters.
3. Explore the dispersal behavior of CLaf infected and non-infected ACTs in the presence and absence of citrus and alternative host plants.
4. Characterize ACT parasitoids and the diversity of endosymbionts associated with both ACT and its parasitoids.

1.7 Thesis structure

The thesis is organized into six chapters. It starts with a general introduction and includes statement of the problem, research aim and objectives of the study. Chapter 2 surveys the CGD situation in Kenya and Tanzania to come up with comprehensive data on the extent, incidence and severity of CGD. It further identifies the circulating pathogen strains in citrus plant tissues and insect vectors and establishes the spread of the disease in both countries. Chapter 3 addresses the effect of CLaf on *T. erytreae* fitness traits and dispersal capabilities, with the aim of providing more insights on the pathogen influence on population dynamics of vector and disease epidemiology. Chapter 4 explores the diversity and phylogenetic relationships among ACT parasitoids in Kenya. It also describes the main endosymbionts harbored by both ACT and its parasitoids and further compares phylogenies of the 16S rRNA gene sequences present in the host and parasitoids. Chapter 5 concludes the dissertation with a synthesis of results, highlighting important findings and Chapter 6 provides an outlook for future research.

2 Citrus greening disease, East African situation: incidence, severity and distribution patterns

2.1 Abstract

Globally, citrus greening disease (CGD) is considered the most damaging disease of citrus threatening citrus production worldwide and East Africa in particular. Our study aimed for the first time to assess the incidence, severity and distribution patterns of CGD in Kenya and Tanzania, thereby helping to guide on management decision and control strategies for reducing CGD transmission.

The level of disease incidence and severity varied across the different surveyed regions with chronic greening situation observed in the midland regions. Despite the preference of the pathogen for cooler climates, we observed presence of CGD symptoms and *Trioza erytreae* vector in very warm and humid environments of Kisumu (Kenya), indicating likelihood of this pathogen and vector invading and adapting to similar environments in the lowland regions. Both *T. erytreae* and Asian citrus psyllid *Diaphorina citri* were found to coexist in upper and lower midland regions, illustrating that *D. citri* is quickly adapting to new geographical areas and ecologies. PCR and molecular phylogeny identified CLaf subsp. clausena as the main causal agent of CGD in most of citrus plants and insect samples, but no CLaf pathogen was detected in *D. citri.*

The pathogen CLaf subsp. clausena played a major role in the spread of CGD. The association of CLaf subsp. clausena with citrus stresses the potential importance of *Clausena anisata* trees as reservoirs for CLaf. These findings provide valuable insights into understanding, predicting and controlling CGD by employing stringent and early disease detection tools to curb the spread of the disease.

2.2 Introduction

Citrus is one of the most economically important fruit crops grown by commercial and smallholders farmers worldwide (Yesuf 2013). Smallholder farmers yields in Africa often do not exceed of 4-10 t/ha, while the crop has the potential of producing up to 50-70t/ha for countries that practice integrated pest management (IPM) programmes (Kilalo et al. 2009). Huanglongbing, commonly referred to as citrus greening disease (CGD) or specifically in Africa as Africa citrus greening disease (ACGD), is one of the most serious diseases of citrus, responsible for the collapse of several citrus industries in Asia and Africa (Graça 1991; Bové 2006; Gottwald et al. 2007). In Kenya and Tanzania, CGD had the greatest impact on citrus

production in the cooler highland regions, causing yield losses of 25-100% (Pole et al. 2010). The yield of affected trees are considerably reduced by continuous fruit drop, dieback, and tree stunting, and the poor quality of fruits which are inedible (Bové 2006; Gottwald et al. 2007; Graça 1991). Moreover, production costs increase due to higher fertilizer and more frequent insecticide applications, tree removal and replanting (Farnsworth et al. 2014). This has often rendered commercial citrus production unprofitable, forcing many farmers to curtail or completely abandon citrus production (Ekesi 2015).

Citrus greening disease is caused by the gram negative bacteria *Candidatus* Liberibacter spp. belonging to the alpha subdivision of proteobacteria (Gopal et al. 2007). The disease is associated with three species i.e. *Candidatus* Liberibacter africanus (CLaf), *Candidatus* Liberibacter asiaticus (CLas) and *Candidatus* Liberibacter americanus (CLam) based on 16S rDNA (Jagoueix et al. 1994; Garnier et al. 2000; Teixeira et al. 2005). The Asiatic strain CLas is the most widely distributed, virulent and devastating form that has been reported from citrus crops in Asia (Garnier and Bove 1996), the Mascarene Islands (Garnier et al. 1996), South and Central America (Halbert and Manjunath 2004), Papua New Guinea (Weinert et al. 2004) and more recently in Africa (Saponari et al. 2010). The Latin American strain CLam has been found in Brazil and Texas (Bové 2006; Texeira et al. 2005) while the African strain CLaf has been detected in southern and eastern Africa countries including Madagascar (da Graça 1991; Magomere et al. 2009).

A number of rutaceous plants including *Clausena anisata*, *Vepris lanceolata*, *Zanthoxylum capense, Calodendrum capense, Tecleae geradii*, *Oricia bachmannii* and *Fagara* species have been found to support the development of *T. erytreae* (Halbert and Manjunath 2004; Van den Berg 1990). Moreover, subspecies of CLaf, have been identified from indigenous rutaceous trees i.e. Van den Berg et al. 1992 reported *Clausena anisata* (Wild) Hook. f. ex Benth. as an alternate host to citrus harboring CLaf subsp. clausena (CLafCl), Korsten et al. (1996) detected CLaf subsp. vepridis (CLafV) from a *V*epris *lanceolata* using dot hybridization probes and PCR, Roberts and Pietersen (2016) detected CLaf subsp. tecleae (CLafT) from *Teclea gerrardii*. Furthermore, widespread occurrence of CLaf subsp. capensis (CLafC) has been found on *Calodendrum capense* (Garnier et al. 2000), but there have been no reports of CLafC infection on citrus (Pietersen et al. 2010). It is presumed that the persistence of the disease may be due to the presence of alternate hosts of CLaf (Pietersen et al. 2010). Further identification of rutaceous species is important for determining the existence of other alternative host or a possible ancestor of CLaf and to help

fully understand the possible impact these Liberibacter may have on commercial citrus crops (Roberts and Pietersen 2016).

Research efforts therefore, endeavor to determine potential alternate hosts to citrus of CLaf amongst the Rutaceae family as a means of making disease pressure reduction more efficient (Pietersen et al. 2010). The detection of CLas in Ethiopia (Saponari et al. 2010) might signal a further spread of the Asian strain to other warmer citrus-growing areas of East Africa and beyond and into regions that so far have not been affected by CGD. The more recent reports of CLas occurrence in Uganda and Tanzania (Kalyebi et al. 2015; Shimwela et al. 2016) continue to generate debates on the identity of the pathogen (Roberts et al. 2017), highlighting the need for more intensive surveys and screening of samples to assess the overall threat and spread of citrus greening in East Africa and beyond. Moreover, the association of the CLaf subspecies with various alternative host plants warrants more in-depth investigations on the importance of these alternative hosts for the CGD dynamics.

Citrus greening is primarily spread via graft inoculation from contaminated citrus plants and by its two main vectors, the indigenous African citrus triozid vector *Trioza erytreae* (del Guerico) and the invasive Asian citrus psyllid vector *Diaphorina citri* Kuwayama (Bové 2006; Halbert and Manjunath 2004). Both CLas and CLam are vectored by *D. citri* in Asia, the Americas, the Arabian Peninsula, and several islands in the Pacific and Indian Oceans while CLaf is transmitted by *T. erytreae*, commonly found in southern and eastern Africa, the Arabian Peninsula, and several islands in the Indian Ocean (Halbert and Manjunath 2004; Li et al. 2006; Manjunath et al. 2008). More recently, Monzó et al. (2015) documented the presence of *T. erytreae* in the Canary Islands, Madeira, northern Portugal and north-western Spain. The three *Candidatus* Liberibacter species are not adapted to the same range of the temperature, i.e. CLas thrives at temperatures >30°C, while CLaf is more prevalent in cooler areas where temperatures do not exceed 30°C (Jagoueix et al. 1994), which is mirrored by the ecological preferences of the two vector species, resulting in the different altitudinal distribution of the two pathogen species. Recent findings by Rwomushana et al. (2017) and Shimwela et al. (2016), however, indicate the occurrence and rapid spread of *D. citri* in some low- and also highland regions of Tanzania and Kenya. Since both vector species have also been shown experimentally to efficiently transmit both CLas and CLaf (Aubert 1987; Lallemand et al. 1986), there is a possibility that this could also happen in the field if both pathogen and vector species are present. Moreover mixed infections of CLas and CLaf in the presence of only one of the vectors would be another

possibility (Bové 2006). Therefore, the reported presence of CLas and occurrence of both *D. citri* and *T. erytreae* in East Africa poses higher risks of invasion for neighboring countries and their citrus industries.

Management of CGD is difficult due to the nonspecific nature of the disease symptoms, prolonged latency of the disease in infected trees and probably irregular distribution of the pathogen in such trees (Manjunath et al. 2008). Environmental effects, especially of temperature, on symptom expression and possibly also on bacterial multiplication, variations in tolerance to the bacterium in both plant hosts and vectors and the fastidious nature of the bacterium further complicates the management process (Manjunath et al. 2008). When CGD becomes established and without effective control of the bacterium inoculums or its vector, the disease incidence quickly increases (Bassanezi et al. 2011). Evolution of disease severity throughout the tree canopy, especially in young trees, is very fast, greatly reducing the productive life span of the affected orchards (Gottwald et al. 2007). Since there is no cure or resistance to CGD, disease management primarily entails the continuous removal of symptomatic trees in order to reduce the amount of the pathogen inoculum and frequent insecticide applications to reduce vector populations (Bové 2006; Gottwald et al. 2007; Graca 1991). Detailed knowledge on the identity of the pathogen populations and their distribution is a prerequisite for monitoring and development of effective management strategies to combat the disease. However, the available information on occurrence, distribution and the extent of damage of CGD is still very limited in both Kenya and Tanzania. Thus, our objectives were to access incidence, severity and patterns of distribution of CGD in Kenya and Tanzania by employing molecular tools to identify the circulating strains.

2.3 Material and methods

2.3.1 Study areas

Field surveys were conducted from the period of December 2015 to November 2017 in citrus growing regions of Kenya and Tanzania. Samples were collected from orchards and backyards where citrus was grown for both commercial and subsistence purposes. A total of 105 backyards (95) and orchards (10) were surveyed in the Kisumu, Kericho, Nakuru, Machakos, Kitui, Makueni, Meru, Kirinyaga, Embu, Nyeri, Murang'a regions in Kenya, while the Morogoro and Tanga regions surveyed in Tanzania (Figure 2.1). These regions were stratified into lower highland (LH):1,800-2,400 meters above sea level (m.a.s.l.), upper

midland (UM):1,300-1,800 m.a.s.l., lower midland (LM) 800-1,300 m.a.s.l. and lowlands (L) <800 m.a.s.l. (Jaetzold and Schmidt 1983), based on the altitudinal gradient within them. Information on cultivated citrus varieties, age of citrus trees, vector and CGD presence, knowledge of the vector and greening disease, pest management practices by the farmers and source of planting material were obtained using a semi-structured questionnaire (see table S1, supplementary material). Latitude, longitude and altitude data of all surveyed sites were taken using a global positioning system device (eTrek 209, Garmin, USA).

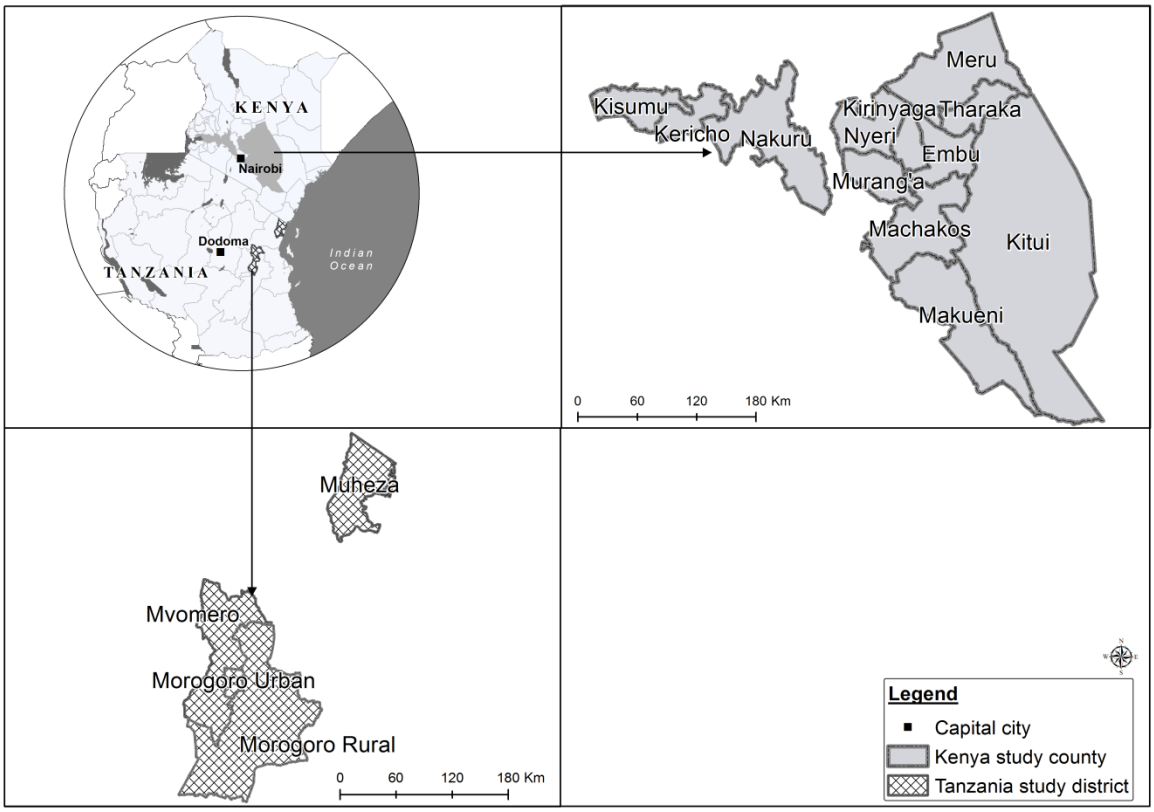


Figure 2.1 Map of Kenya and Tanzania showing regions where citrus greening surveys were conducted

2.3.2 Sampling strategy

Citrus plants in small orchards and backyards with <20 trees were individually inspected and incidence and severity assessed based on visual CGD symptoms. In orchards with >20 trees, diagonal transects were made across the field in the form of an "X". Trees were randomly selected to cover edges and midpoints of the orchard, with intervals between inspected and sampled trees determined by the size of the orchard. Incidence was calculated as the proportion of plants showing CGD symptoms, expressed as a percentage of the total number of trees in the orchard. In order to determine severity, it was originally planned to assess at

least 20 randomly selected trees from each orchard; however, very few trees were encountered in most orchards. Therefore, severity was estimated from randomly scoring a minimum of five citrus trees in each backyard or orchard. The canopy of each selected tree was divided into four quadrants along the four cardinal points and four twigs picked from each quadrant were assessed for CGD symptoms. Severity was estimated using the percentage leaf area infected based on a 1-4 scoring scale where 0 = no symptoms, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = > 75% (Gottwald et al. 2007). Data on incidence and severity were subjected to ANOVA, in case of significant F-values followed a Tukey test for multiple comparisons of means. All analyses were performed using R (2.13.1).

2.3.3 Collection of plant tissues and insect samples

To confirm CLaf or any other CGD species presence or absence, both plant tissues and insect sampled from symptomatic trees were subjected to molecular characterization. Suspected symptomatic trees that showed fruit colour inversion, lop-sidedness, yellow shoots, mottling and galling were sampled (Bové 2006; Graça et al. 2015). In Kenya these samples were taken at altitudes ranging from 1,100 to 2,316 m.a.s.l. whereas in Tanzania from 410 m.a.s.l. (in Tanga) to 1,229 m.a.s.l. (in Morogoro) (Table 2.1). For each tree sampled, at least 10 symptomatic leaves were collected, placed in sealed plastic bags, labelled and then stored at -80°C until used for DNA extraction in the laboratories of the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi, Kenya.

Young leaves and flushing shoots were inspected for adults and nymphs of the vector and insects were collected through aspiration (Hall and Hentz 2010). Samples of *T. erytreae* were obtained from different surveyed regions in Kenya and in the absence of adults or nymphs in certain areas distinct galling symptoms could be observed on older leaves, suggesting that the vector had previously occurred at these sites. Specimens of *D. citri* were collected in some LM (800-1,300 m.a.s.l.) and UM (1,300-1,800 m.a.s.l.) regions in Kisumu and Kericho (Kenya). A similar situation was observed in Tanzania, where numerous leaf galls typical of *T. erytreae* feeding were observed in several sites though nymphs and adults could only be collected from two sites. In contrast, *D. citri* nymphs and adults were collected from numerous sites in the Morogoro and Muheza regions of Tanzania. All insect samples were placed in 1.5 ml Eppendorf tubes with 95% ethanol for later DNA extraction.

Table 2.1 Regions where symptomatic leaves and vector samples were collected from backyards and orchards in Kenya and Tanzania

Table 1. Symptomatic leaves and Vector samples collected from symptomatic backyard and orchards in Kenya and Tanzania

Region	Country	Category	Elevation	GPS Coordinates	Citrus varieties	Leaf Symptoms	*T. erytreae*	*D. citri*
Machakos	Kenya	Backyard	1628	S01^{0}17'24.5"E037^{0}22'41.0"	Lemon (LE), Valencia (VA)	Vein degeneration, galls	Present	Absent
Machakos	Kenya	Orchard	1544	S01^{0}16'58.6"E037^{0}20'57.9"	LE, T,angerine,WashingtonNavel	Leaf Galls	Absent	Absent
Machakos	Kenya	Backyard	1540	S01^{0}17'11.2"E037^{0}20'09.0"	Lemon, VA,Washington avel(WN)	zinc deficiency, leaf galls	Absent	Absent
Machakos	Kenya	Orchard	1911	S01^{0}28'28.1"E037^{0}18'36.1"	W.N, LE, Tangerine (TA)	zinc deficiency, leaf galls	Absent	Absent
Machakos	Kenya	Orchard	1831	S01^{0}28'53.1"E037^{0}19'06.7"	Lemon, Valencia, Tangerine	zinc deficiency, leaf galls	Absent	Absent
Machakos	Kenya	Backyard	1778	S01^{0}29'30.7" E037^{0}19'16.7"	Lemon, Tangerine, WN,	zinc deficiency, leaf galls	Absent	Absent
Machakos	Kenya	Backyard	1815	S01^{0}27'43.8"E037^{0}18'23.8"	Valencia, Lemon, Tangerine	zinc deficiency, Leaf galls	Absent	Absent
Kitui	Kenya	Orchard	1401	S01^{0}08'55.0"E038^{0}00'18.9"	W.N, Lemon,TA,VA	Leaf galls	Absent	Absent
Kitui	Kenya	Orchard	1385	S01^{0}09'24.0"E038^{0}00'53.6"	Valencia, lemon, W.N	Leaf galls	Absent	Absent
Morogoro	Tanzania	Backyard	406	S06^{0}44'48.9"E037^{0}53'16.8"	Lemon	Zinc defiency	Absent	Present
Morogoro	Tanzania	Orchard	529	S06^{0}50'38.6"E037^{0}39'44.9"	LE, Pomelo, quarts, W.N,TA, VA	Leaf galls	Absent	Present
Morogoro	Tanzania	Backyard	663	S06^{0}51'17.6"E037^{0}40'36.6"	Valencia, lemon	Vein degeneration	Absent	Present
Morogoro	Tanzania	Orchard	262	S07^{0}02'15.8"E037^{0}48.5'51.6'	Valencia, W.N, Tangerine	Zinc defiency	Absent	Present
Morogoro	Tanzania	Backyard	552	S06^{0}54'46.3"E037^{0}34'18.3"	Lemon	Leaf galls	Absent	Present
Morogoro	Tanzania	Backyard	785	S06^{0}59'49.2"E037^{0}34'19.6"	Valencia, lemon	Vein degeneration	Absent	Absent
Morogoro	Tanzania	Backyard	1229	S07^{0}03'33.8"E037^{0}35'00.0"	Lemon	Vein degeneration	Absent	Absent
Morogor	Tanzania	Backyard	1096	S07^{0}03'26.0"E037^{0}34'38.1"	Valencia	Leaf galls	Absent	Absent
Morogoro	Tanzania	Backyard	812	S07^{0}00'09.7"E037^{0}34'13.7"	Lemon	Vein degeneration	Present	Present
Morogoro	Tanzania	Backyard	651	S06^{0}59'10.2"E037^{0}33'39.9"	W.N, Valencia. Lime, Tangerine	Vein degeneration	Absent	Present
Tanga	Tanzania	0rchard	212	S05^{0}09'12.4"E038^{0}51'28.6"	WN, Valencia,	Zinc defiency	Absent	Present
Tanga	Tanzania	Backyard	486	S05^{0}06'03.8"E038^{0}38'57.8"	Lemon, Tangerine	Vein degeneration	Absent	Absent
Tanga	Tanzania	Backyard	898	S05^{0}06'03.3"E038^{0}38'01.4"	Lemon	Leaf galls	Absent	Absent
Tanga	Tanzania	Backyard	410	S05^{0}06'04.0"E038^{0}39'56.4"	Lemon, Washington Navel	Vein deg., Blotchy mottle	Absent	Absent
Meru	Kenya	Backyard	1427	N00^{0}08'27" E037^{0}50'17.82"	Lemon,Tangerine	Vein deg. zinc deficiency	Present	Absent
Meru	Kenya	Backyard	1275	N00^{0}06'26.82"E037^{0}46'1.92"	Lemon	Vein degeneration	Present	Absent
Meru	Kenya	Backyard	1276	N00^{0}06'26.58"E037^{0}46'1.86"	Valencia, Lemon	Blotchy mottle	Present	Absent
Meru	Kenya	Backyard	1274	N00^{0}06'26.88"E037^{0}46'1.86"	Valencia, Washington navel	Vein deg. Blotchy mottle	Present	Absent
Meru	Kenya	Backyard	1298	N00^{0}07'30.36"E037^{0}49'56.04"	Lemon, W.N, Valencia	zinc deficiency	Present	Absent
Meru	Kenya	Nusery	1381	N00^{0}52'49.68"E037^{0}09'13.5"	Lemon, Tangerine, Valencia	Leaf galls	Present	Absent

Table 1(continuation). Symptomatic leaves and Vector samples collected from symptomatic backyard and orchards in Kenya and Tanzania

Region	Country	Category	Elevation	Citrus varieties	GPS Coordinates	Leaf Symptoms	*T. erytreae*	*D. citri*
Embu	Kenya	Orchard	1223	W.N.TA. Valencia,	S00^{0}32'47.9" E037^{0}35'08.6"	Blotchy mottle	Present	Absent
Kirinyaga	Kenya	Backyard	1340	Lemon, W.N	S00^{0}33'34.7" E037^{0}23'16.7"	zinc deficiency	Absent	Absent
Kirinyaga	Kenya	Backyard	1338	W.N, TA, LE, VA	S00^{0}33'47.46" E037^{0}23'3.18"	Vein deg. zinc deficiency	Present	Absent
Nyeri	Kenya	Backyard	1778	Lemon	S00^{0}27'45.8" E037^{0}05'27.3"	Vein deg., Blotchy mottle	Present	Absent
Murang'a	Kenya	Backyard	1385	Le. Valencia, W.N, TA	S00^{0}52'47.8" E037^{0}09'13.5"	Blotchy mottle	Present	Absent
Murang'a	Kenya	Orchard	1394	Tangerine, W.N, VA	S00^{0}51'47.0"E037^{0}08'14.3"	zinc deficiency	Present	Absent
Murang'a	Kenya	Orchard	1396	Lemon, W.N, VA, TA	S00^{0}51'38.8"E037^{0}07'49.5"	zinc deficiency	Present	Absent
Murang'a	Kenya	Backyard	1434	Lemon, Valencia, WN	S00^{0}51'12.5"E037^{0}07'30.8"	zinc deficiency	Present	Absent
Murang'a	Kenya	Backyard	1495	Lemon, WN	S00^{0}50'40.7"E037^{0}06'10.4"	zinc deficiency	Present	Absent
Kisumu	Kenya	Backyard	1271	Lemon, Va, W.N, TA	S 00 10'29.82"E035 05'16.26"	Blotchy mottle	Present	Present
Kisumu	Kenya	Backyard	1299	Lemon	S 00 10'1.08"E035 05'3.84"	zinc deficiency	Absent	Absent
Kisumu	Kenya	Backyard	1153	W.N, Lemon	S 00 10'43.92"E034 55'24.72"	zinc deficiency	Present	Absent
Kisumu	Kenya	Backyard	1152	W.N, Lemon, VA, TA	S00 10'58.02"E034 54'32.88"	Blotchy mottle	Present	Absent
Kericho	Kenya	Backyard	1483	W.N, Valencia, Lemon	S00 15'32.76"E035 08'48.96"	Vein degeneration	Absent	Absent
Kericho	Kenya	Backyard	1403	Valencia, W.N, LE, TA	S00 14'4.32"E035 08'26.82"	Vein deg., Rabbit ear	Present	Present
Kericho	Kenya	Backyard	1398	Valencia	S00 13'14.82" E035 11'4.5"	Vein degeneration	Present	Present
Kericho	Kenya	Backyard	1397	WN, Lemon, TA	S00 11'41.82"E035 12'9.3"	Blotchy mottle, zinc deficiency	Present	Present
Kericho	Kenya	Backyard	1375	Lemon, W.N, Valencia	S00 11'50.28"E035 12'46.14"	zinc deficiency	Present	Present
Kericho	Kenya	Backyard	1409	Lemon, Valencia, WN	S00 19'17.64"E035 03'16.8"	zinc deficiency	Present	Absent
Kericho	Kenya	Backyard	1773	W.N, Lemon, TA	S00 28'31.32"E035 03'57"	Vein degeneration	Present	Absent
Kericho	Kenya	Backyard	1830	Lemon, WN	S00 28'25.8"E035 03'47.4"	Vein degeneration	Present	Absent
Nakuru	Kenya	Backyard	2310	Le , W.N, VA	S00 11'43.38"E035 34'47.88"	zinc deficiency	Present	Absent
Nakuru	Kenya	Backyard	2304	W.N, TA	S00 10'15.84"E035 33'35.4"	Leaf galls	Present	Absent
Nakuru	Kenya	Orchard	2316	Tangerine, Valencia,	S00 09'58.92"E035 36'41.94"	Blotchy mottle, Vein	Present	Absent

Citrus varieties abbreviations: Washington Navel (WN); Tangerine (TA); Lemon (LE); Valencia (VA); Pomelo (PO); Qumquarts (Quarts)

2.3.4 Tissue preparation and DNA extraction

Collected leaf samples were washed under running tap water, dried on filter paper and midribs and petioles of plant leaves excised with a sterile razor blade and then cut into small pieces. Small chopped leaf midribs of 0.2 g were pulverized for 3 min with a Tissue lyser II (Qiagen, USA) in a 2 ml tube containing ceramic beads and lysing matrix (MP Biomedicals, Santa Ana, USA) in the presence of lysis buffer PA1(Bioline, UK) and RNaseA. Plant genomic DNA was then extracted using Isolate II plant DNA extraction kit (Bioline, UK) according to the manufacturer's protocol. Insect samples were surface-sterilized using 3% bleach, passed through 70% ethanol for a few seconds and finally rinsed three times with distilled water. Insect genomic DNA was extracted from individual adults of *T. erytreae* and *D. citri* using the Isolate II genomic DNA Kit (Bioline, UK), following the manufacturers protocol. To avoid contamination, samples from different regions were extracted separately and tests carried out in a blind fashion once the extraction numbers were assigned to them. Each batch of extractions included at least one extraction with water as a negative control to monitor any cross-contamination between the samples. The resultant DNA was eluted in a final volume of 50ul and its yield and purity confirmed by measuring OD260 nm and OD260 nm/280 nm, respectively using a Nanodrop 2000/2000c Spectrophotometer (Thermo scientific, Wilmington, USA). The plant and insect DNA samples were stored at -20°C prior to PCR screening for pathogen presence.

2.3.5 PCR amplification, Agarose gel electrophoresis and DNA purification

Plant and insect samples were screened for presence of CLaf and other pathogen species using primers OI1 (5'-GCGCGTATTTTATACGAGCGGCA-3') and OI2c (5'-GCCTCGCGACTTCGCAACCCAT-3') targeting partial regions of 16SrDNA (Jagoueix et al.1996) and A2 (5'-TATAAAGGTTGACCTTTCGAGTTT-3') and J5 (5'-ACAAAAGCAGAAATAGCACGAACAA-3') targeting rpIJ/rpIL ribosomal protein genes (Hocquellet et al. 1999). Isolated DNA was amplified in 30 µl PCR mix consisting 6 µl of 5X My Taq PCR Buffer (Bioline, UK) (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers), 1.5 µl 10 µM of each primer , 1.8 µL 25 mM $MgCl_2$ (Thermo Scientific, USA), 0.125 µL 1unit My Taq DNA polymerase (Bioline, UK) and 15 ng/uL of DNA template. This reaction was set up in the Mastercycler Nexus Gradient thermocycler (Thermo Scientific, USA) using the following conditions: initial denaturation for 2 min at 95°C, followed by 40 cycles of

denaturation at 95°C for 30 s, 45sec annealing at 58.7°C for A2/J5 or 56.9°C for OI1/OI2c and primer elongation at 72°C for 1min followed by final extension step at 72°C for 10 mins.

PCR products were resolved in 1.5% agarose gel stained with ethidium bromide (10 mg/mL) and subjected to electrophoresis at 100 volts for 1hr (Bio-Rad model 200/2-0 power supply, Bio-Rad laboratories Inc., USA). Then visualization of DNA bands on the gel was done under an ultraviolet transilluminator, photographed and documented using the KETA GL imaging system software (Wealtec Corp., USA). The successfully amplified PCR products with an expected band size of 669-703bp and 1,100bp using A2/J5 and OI1/OI2C primers, respectively, were gel excised and purified using Isolate II PCR and Gel Kit (Bioline, UK), following the manufacturers protocol. The purified products were then sent for bi-directional sequencing at Macrogen Inc., (Netherlands).

2.3.6 Sequencing and phylogenetic analysis

Sequences were assembled and edited using BioEdit v7.0.5.2 (Hall 1999). Consensus sequences from both the forward and reverse strands were generated and queried via BLASTN in the GenBank database to determine the closest sequence identities and to check for similarity with already identified organisms (Altschul et al. 1990). Multiple alignment of the assembled and trimmed sequences were done in Clustal X software version 2.1 (Thompson et al. 1997) and the aligned sequences used for phylogenetic and molecular evolutionary analyses in MEGA version 6 (Tamura et al. 2013). Neighbour-joining trees were constructed using Maximum Likelihood method to visualize divergence patterns within members of the *Candidatus* Liberibacter species from the collected plant and insect samples. The reliability of the clustering pattern in the tree was evaluated using bootstrap analysis with 1,000 replicates. Evolutionary divergence over sequence pairs between groups was estimated using the p-distance model in MEGA6.0 (Tamura et al. 2013). To further infer relationships among members of the genus *Candidatus* Liberibacter, a table of genetic distances generated by MEGA6.0 (Tamura et al. 2013) was used to create principal component plots in R (2.13.1).

2.4 Results

2.4.1 Surveys

The regions surveyed represented altitudes ranging from lows of 212 m.a.s.l. in Tanga to highs of 1,229 m.a.s.l. in Morogoro (both Tanzania) and 2,316 m.a.s.l. in Nakuru (Kenya). Citrus greening symptoms were encountered in most of the surveyed sites, with blotchy

mottling and vein degeneration of leaf veins as the most common symptoms; in areas where the disease had severely progressed also die back could be observed (Figure 2.2). Other features such as underdeveloped lopsided fruits and color inversion "red nose" were less prominent. Citrus greening symptoms were mostly conspicuous on rough lemon but were also observed on Valencia, Washington navel and tangerines.

Of the 105 farmers interviewed, 90% had farms of < 1ha, of which only a small part was used for citrus production. Intercropping was a common practice in most of the surveyed farms (89%), with citrus trees frequently intercropped with a number of cash crops such as maize, bananas, mangoes and vegetables. Around 59% of the farms visited had unhealthy citrus trees; visually disease-free citrus trees were only observed on farms where intensive management practices were employed. Most farmers (85%) grew grafted citrus seedlings obtained from nurseries along the roadsides. Many farmers (50%) occasionally treated their citrus plants with pesticides, mostly after observing pests but only 14% of the farmers used fertilizers. Farmers most often relied on their own experiences (47%) or advice from agricultural commercial retailers (40%) to manage pest and diseases; only few farmers (13%) obtained advice from government extension officers. Over 90% of the farmers could not recognize CGD symptoms or the psyllid vector.

Figure 2.2 (a) Fruits with color inversion; (b) leaves with blotchy mottle; (c) leaves showing vein degeneration; (d) leaves showing chlorotic zinc deficiency and vein degeneration; (e) leaves with galls; and (f) adults and nymphs of *Trioza erytreae*.

2.4.2 Incidence and severity

Incidence and severity levels differed significantly ($P<0.01$) across the surveyed regions. In Kenya, moderate levels of CGD incidence (27-33%) were found in the LH regions while higher mean incidence (32-100%) was observed in UM regions. We found no incidence of CGD in the LM regions of Makueni and Kitui in Kenya. In Tanzania, the disease had spread to the L regions of Mzumbe and Kidididimo, but no incidence was found in Muheza and Mkuyuni. Highest CGD severity was observed in Tanzania around LM regions of Mzumbe, Amani and Mlali and in Kenya in the LM and UM regions of Embu, Soin and Sigowet (Table 2.2).

Table 2.2 Incidence and severity of citrus greening disease in citrus growing regions of Kenya and Tanzania in 2015-2017

Region	Location	*Incidence%	*Severity (0-4)
		Mean ± SE	Mean ± SE
LH (1800-2400)			
Kenya	Londiani	33 ± 4.3^{bcd}	1.0 ± 0.25^{cd}
Kenya	Kathiani	27 ± 7.9^{cd}	0.8 ± 0.27^{cd}
UM (1300-1800)			
Kenya	Nyeri	100^{a}	1.4 ± 0.16^{abcd}
Kenya	Meru	63 ± 10.9^{ab}	1.4 ± 0.32^{bcd}
Kenya	Soin	58 ± 8.1^{abc}	1.7 ± 0.87^{abc}
Kenya	Kirinyaga	71 ± 21.7^{a}	1.0 ± 0.23^{cd}
Kenya	Sigowet	57 ± 5.2^{abc}	2.0 ± 0.27^{abc}
Kenya	Murang'a	36 ± 5.9^{bcd}	1.0 ± 0.27^{cd}
Kenya	Kangundo	32 ± 12.1^{bcd}	0.9 ± 0.37^{cd}
LM(800-1300)			
Kenya	Awasi	76 ± 9.9^{a}	1.3 ± 0.38^{bcd}
Kenya	Embu	48 ± 2.5^{abcd}	2.0 ± 1.01^{abc}
Kenya	Ahero	30 ± 10^{bcd}	0.4 ± 0.005^{cd}
Kenya	Mwingi	11 ± 7.5^{d}	0.4 ± 0.09^{cd}
Kenya	Kitui	0^{d}	0^{d}
Kenya	Makueni	0^{d}	0^{d}
LM (800-1300)			
Tanzania	Mlali	79 ± 21.2^{a}	2.3 ± 0.65^{ab}
Tanzania	Amani	67 ± 19.3^{ab}	2.4 ± 0.6^{ab}
L <800			
Tanzania	Mzumbe	90 ± 10^{a}	2.8 ± 0.42^{a}
Tanzania	Kidididimo	35 ± 25^{bcd}	1.3 ± 0.76^{bcd}
Tanzania	Muheza	0^{d}	0^{d}
Tanzania	Mkuyuni	0^{d}	0^{d}

*Incidence=Percentage of trees infected with citrus greening disease

*Severity=Citrus greening diseases symptoms assessed on a visual 0-4 rating scale

Mean values followed by the same letter are not significantly different (P>0.01, Tukey LSD)

Apart from pixie mandarin that was commonly grown in Makueni (Kenya), all other citrus varieties encountered during the survey visually appeared to be susceptible to CGD, though we noted significant differences in disease severity. Out of the 128 randomly selected trees showing visual CGD symptoms, we recorded the highest severity scores on lemons and the least on tangerines (Table 2.3).

Table 2.3 Severity scores of citrus greening disease detected on different citrus varieties in Kenya and Tanzania

Varieties	**n=128**	**Mean severity score**
Lemon	43	2.02 ± 0.02a
Valencia	30	1.78 ± 0.15a
Washington navel	32	1.25 ± 0.15b
Tangerines	23	0.52 ± 0.14c

Means followed by the same letter are not significantly different (P<0.001, Tukey SNK)

2.4.3 Detection of *Candidatus* Liberibacter spp. in leaf samples

Primers f-OI1 and r-OI2c (OI1/OI2c) for amplification of 16S rDNA, and primers f-*rpl*A2 and r-*rpl*J5 (A2/J5) for amplification of ribosomal protein genes (*rpl*) were used for screening suspected leaf samples. In both countries, A2/J5 primer gave expected band sizes of 669 bp from diseased samples (Figure 2.3) while OI1/OI2c primer amplified expected DNA fragments of 1,100 bp from diseased leaf samples collected from the different regions (Figure 2.4). No amplified bands were observed for samples collected from LM regions of Mwingi and Kitui in Kenya and L regions of Muheza and Mkuyuni in Tanzania.

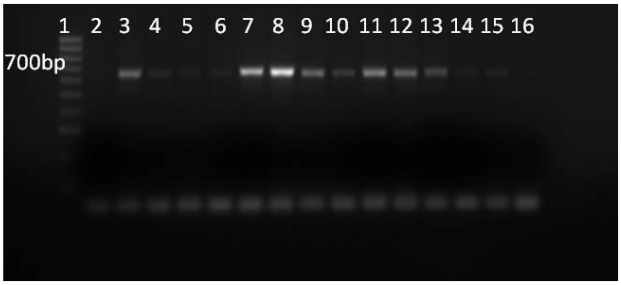


Figure 2.3 1% Agarose gel of A2/J5 ribosomal protein rDNA PCR products. **1**: 100bp DNA ladder, **2**: Negative control, **3-15**: Positive amplified bands of between 600-700bp in symptomatic plant samples, **16**: No amplification

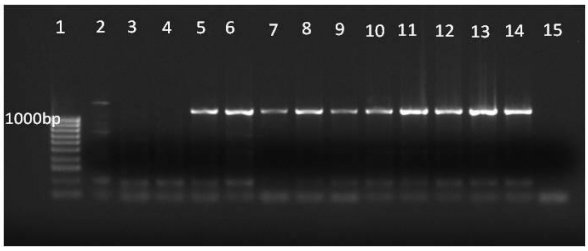


Figure 2.4 1% Agarose gel of OI1/OI2c 16S rDNA PCR products. **1**: 100bpDNA ladder, **2-4**: No amplification, **5-14**: Positive 1160bp amplified bands from symptomatic samples, **15**: Negative control

2.4.4 Detection of Candidatus Liberibacter spp. in psyllids

In Kenya, OI1/OI2c primer gave expected band size of 1,100 bp from few *T. erytreae* insect samples collected from Kangundo, Meru, Kirinyaga, Murang'a, Kericho, Londiani and Awasi regions, while samples collected from Thika and Embu tested negative for CLaf (Figure 2.5).

Figure 2.5 1% Agarose gel of OI1/OI2c 16S rDNA PCR products. **1**: 1kb DNA ladder, **3-8**: amplification from infected insect samples, **9-12**: No amplification, **16**: Negative control

D. citri samples collected from Awasi and Kericho regions in Kenya also tested negative for the pathogen. Moreover, some samples that positively tested for CLaf using OI1/OI2c primer could not produce the same positive PCR product using the A2/J5 primer (Figure 2.6); rather they gave low molecular weight products that were thought to be primer dimmer artifacts or products of mispriming. This indicated that OI1/OI2c primer was more robust in indexing for CLaf infection in insect samples since PCR reactions gave out strongly amplified bands. Both negative and positive controls consistently produced the expected results.

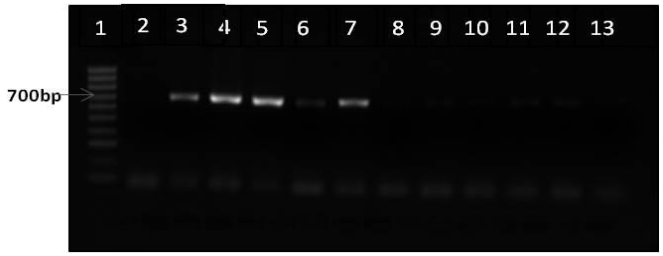


Figure 2.6 1% Agarose gel of A2/J5 ribosomal protein rDNA PCR products. **1**: 100bp ladder, **2**: Negative control, **3-7**: Amplification from infected insect samples, **8-13**; weakly amplified samples

2.4.5 Sequence identification

Plant and insect PCR products amplified from single bands using OI1/OI2C and A2/J5 primer sets were sequenced. 16S rRNA gene sequences obtained using the OI1/OI2C primer for plant samples from Kenya and Tanzania showed homology to CLaf subsp. clausena (KX770998.1) and CLaf (CP004021.1) in a BLAST search. *Trioza erytreae* samples also screened using OI1/OI2c primer showed homology to CLaf subsp. clausena (LafCl). rplJ sequences obtained using the A2/J5 primer for the same Kenya and Tanzania plant samples also showed homology (95-100%) to CLaf subsp. clausena (KX770998.1) and CLaf (CP004021.1). CLaf subsp. clausena was detected in 40 (73%) while CLaf in 15 (27%) of the sequenced samples from the different sites, indicating that CLaf subsp. clausena was widely spread in most of the sampled regions of Kenya and Tanzania (Figure 2.7).

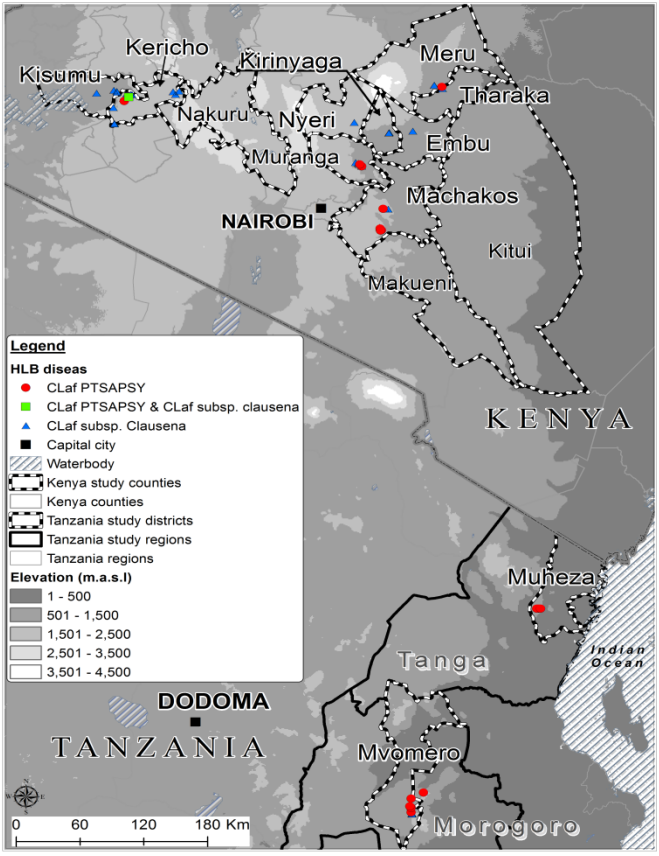


Figure 2.7 Distribution patterns of CLaf and CLaf subsp. clausena in Kenya and Tanzania

2.4.6 Data analysis

Evolutionary analysis involved 61 nucleotide sequences and the mean nucleotide frequencies were 30.06% (A), 33.29% (T/U), 10.59% (C), and 26.06% (G). The overall transition/ transversion bias was *R* = 0. 744, and the summary of nucleotide substitution matrix is shown in Table 2.4. The nucleotide frequencies were A = 25%, T/U = 25%, C = 25.00%, and G = 25% and maximum Log likelihood for this computation was -1644.392.

Table 2.4 Maximum likelihood estimates of substitution matrix from 61 infected citrus samples

	A	T	C	G
A	-	*9.91*	*3.15*	**17.26**
T	*8.95*	-	**0.79**	*7.76*
C	*8.95*	**2.5**	-	*7.76*
G	**19.91**	*9.91*	*3.15*	-

Each entry is the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

Phylogeny of all plants and *T. erytreae* samples was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The phylogenetic tree based on analysis of rplJ sequences had two distinct branches. The first branch separated into two clusters. Cluster one was occupied by plant samples collected from different regions in Kenya and Tanzania that linked to CLaf subsp. clausena of GenBank accession (KX770998.1), CLaf subsp. vepridis and CLaf subsp. capensis (Figure 2.8). The second cluster was occupied by samples that linked to CLaf (GU120044.1), including CLaf subsp. zanthoxyli (KJ197227.1) and CLaf subsp. tecleae (KU561667.1). These two clusters were distinct from those of CLam and CLas that branched separately as out groups forming sister clade relationships (Figure 2.8).

The majority of the Kenyan samples clustered together with CLaf subsp. clausena, a clear indication that it is the most widely spread subspecies.

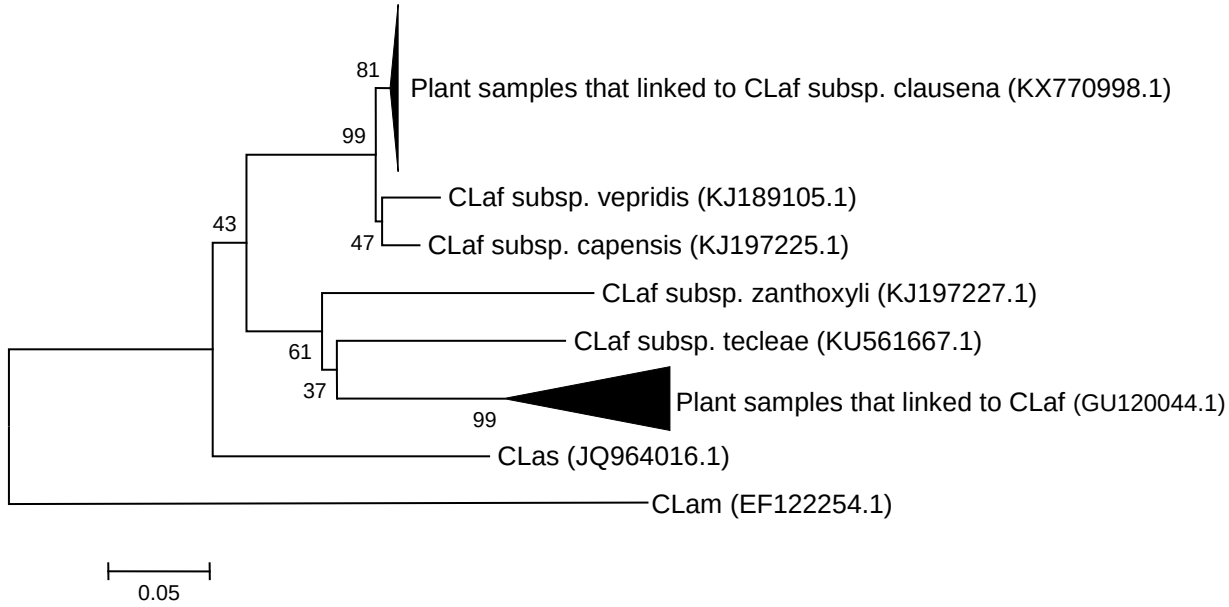


Figure 2.8 Condensed Maximum-Likelihood tree based on analysis of rplJ sequences from symptomatic citrus samples collected from Kenya and Tanzania compared to species of the genus *Candidatus* Liberibacter. Sequences linked to GenBank accessions of CLaf subsp. clausena (KX770998.1) and CLaf (GU120044.1). CLas and Clam branch out separately

Based on 16S rDNA phylogenetic analysis using OI1/OI2C primer, sequences from insect samples clustered with 16s rDNA sequences of CLaf subsp. capensis (LafC), CLaf subsp. vepridis (LafV) and CLaf subsp. clausenae (LafCl) showed homology to these three CLaf subspecies (Figure 2.9).

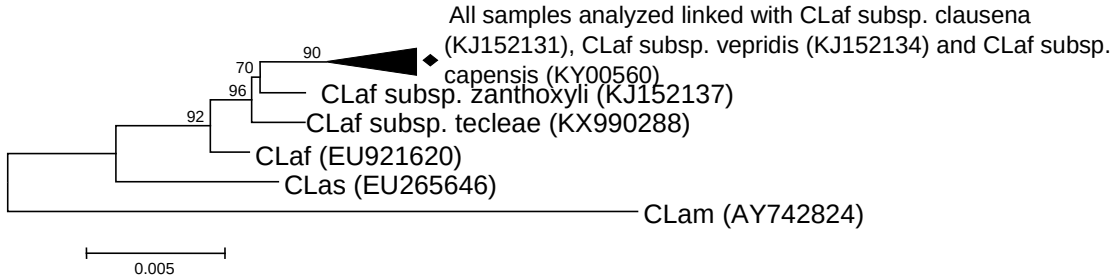


Figure 2.9 Condensed maximum-likelihood tree based on analysis of OI1/OI2C sequences from insect samples collected from Kenya compared to members of the genus *Candidatus* Liberibacter. Sequences linked to CLaf subsp. clausena (KJ152131), CLaf subsp. vepridis (KJ152134) and CLaf subsp. capensis (KY00560)

Estimates of evolutionary divergence over sequence pairs between groups were successfully generated from all sequenced samples. Numbers of base substitution are presented as a

genetic distance matrix (Table 2.5). Overall mean genetic distance of 0.011 was within the acceptable ranges of species variability and was inferred for the 61 nucleotide sequences generated from the various populations found in this study. Other members of the genus *Candidatus* Liberibacter clearly separated from CLaf with more than 0.137 genetic distances. CLaf subsp. clausena exhibited a genetic distance of 0.154 from CLaf while CLam exhibited a very distant relationship with all other members of the genus *Candidatus* Liberibacter with a genetic distance of 0.4. Other closely related CLaf subspecies included CLaf subsp. capensis and CLaf subsp. clausena with a genetic distance of 0.027. Both CLaf subsp. vepridis and CLaf subsp. zanthoxyli had the greatest genetic distance of 0.431 from CLam.

Table 2.5 Estimates of evolutionary divergence of rplJ gene region over sequence pairs between groups as determined using p-distance model in Mega 6.0 (Tamura et al. 2013)

	CLaf.1	CLaf.2	CLaf (GU120044.1)	LafCl.2	LafCl (KX770998.1)	LafC (KJ197225.1)	LafV (KJ189105.1)	CLas (KP210469.1)	CLas (JQ964016.1)	LafT (KU561667.1)	LafZ (KJ197227.1)	CLam (EF122254.1)
CLaf.1												
CLaf.2	0.007											
CLaf (GU120044.1)	0.005	0.002										
LafCl.2	0.157	0.157	0.153									
LafCl (KX770998.1)	0.154	0.154	0.151	0.002								
LafC (KJ197225.1)	0.168	0.168	0.164	0.029	0.027							
LafV (KJ189105.1)	0.164	0.164	0.161	0.037	0.034	0.037						
CLas (KP210469.1)	0.211	0.211	0.207	0.184	0.181	0.185	0.198					
CLas (JQ964016.1	0.207	0.207	0.203	0.180	0.177	0.181	0.195	0.002				
LafT (KU561667.1)	0.137	0.130	0.133	0.185	0.185	0.192	0.207	0.238	0.234			
LafZ (KJ197227.1)	0.162	0.152	0.155	0.174	0.177	0.185	0.189	0.218	0.214	0.164		
CLam (EF122254.1)	0.400	0.400	0.395	0.420	0.417	0.408	0.431	0.391	0.396	0.407	0.431	

The distance matrix was used to generate the principal component plot. The PCA clustered CLaf samples in the study with the respective GenBank accession of CLaf and those of CLaf subsp. clausena with gene accession of CLaf subsp. clausena, while CLam clustered on its own. Furthermore, CLaf subsp. tecleae and CLaf subsp. zanthoxyli were closely associated with CLaf and occupied an axis separated from other *Candidatus* Liberibacter species (Figure 2.10). CLam also separated on a different axis.

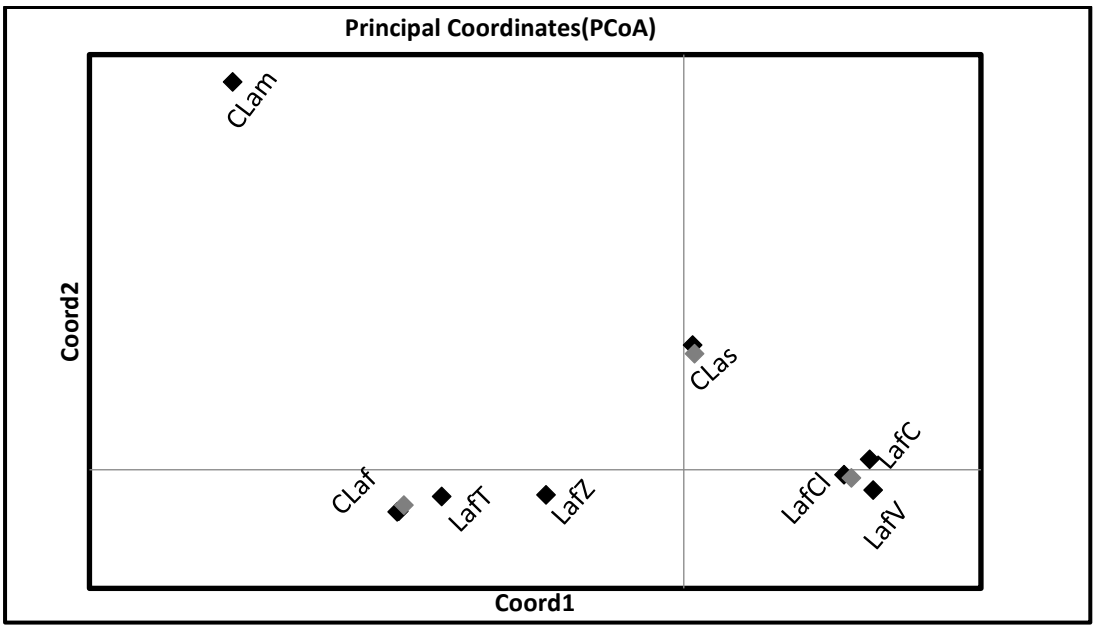


Figure 2.10 Plots of the principal component analysis (PCA) for symptomatic citrus samples and other *Candidatus* Liberibacter species calculated using R

2.5 Discussion

Globally, CGD is recognized as the worst vector-transmitted citrus disease (Halbert and Manjunath 2004). Since its discovery in East Africa, there has been a massive decline in citrus production, especially for small scale producers (Ekesi 2015). Detailed assessments of the incidence, severity and spatial distribution patterns of CGD are important to help guide on management decision and control strategies for reducing pathogen transmission. We here report on an exhaustive countrywide assessment of the greening situation in Kenya and Tanzania based on traditional symptomology (Batool et al. 2007) and PCR techniques (Das 2004) for pathogen detection and identification.

In this for the first time we could demonstrate the wide spread distribution of CLaf subsp. clausenae and its extensive association with CGD transmission. Previously CLaf subsp. clausenae was only linked to indigenous rutaceous plants (Roberts et al. 2015) but our data suggests that it can also act as alternative sources of CLaf infection in citrus orchards. These findings stress the possible economic and environmental impact that other *Candidatus* Liberibacter subspecies may have on commercial citrus crops and give further insights into the divergence of these subspecies (Roberts et al. 2016).

Most plant tissues sampled tested positive for the bacterial pathogen, in contrast to both psyllid vectors collected from the same sites. The latter might have been the result of interspecies competition for nutrition or host components required for replication and

dissemination between the pathogen and other bacterial endosymbionts (Nachappa et al. 2014). Another explanation could have been the low titers of CLaf in the insects compared to the plants, reducing the chances for detection (Manjunath et al. 2008). Finally, it could have been possible that the insect vectors were not the major source of pathogen transmission, but the disease resulted primarily from importation and movement of infected seedlings or grafting of infected plant parts.

Our study further provided the first quantitative report on CGD incidence and severity in the main citrus growing regions of Kenya and Tanzania. Previous studies by Kilalo et al. (2009) and Muendo and Tschirley (2004) listed citrus greening as one of the major constraints to citrus production in the region but did not quantify incidence or severity, though they are essential to determine the relative importance of the disease. We recorded significant differences in disease incidence across the different altitudes of the surveyed regions of both countries. Our results confirmed observations of Seif and Whittle (1984) and Anon (1982), who reported the presence of the disease in the highlands and in regions above 700 m.a.s.l. where the indigenous psyllid vector thrives. Overall, we observed a chronic greening situation in the upper and lower midland regions of Kenya and Tanzania where the disease had wiped out most citrus trees, leaving few scanty trees in backyards. Yet we also could demonstrate vector presence and disease incidence in some relatively warm and humid areas of Ahero and Awasi in the Kisumu region of Kenya, indicating the possibility of both vector and the CLaf pathogen adapting to such environmental conditions.

In Tanzania, Grison and Evers (1991) recorded CGD in Arusha and further described the greening situation on an altitudinal transect along the slopes of the Uluguru mountains in Morogoro, Matombo, Msikitini and Langali where *T. erytreae* was found to be widely present. We also observed pronounced greening symptoms in Morogoro, Msikitini and Langali; however, in our survey, *D. citri* turned out to be the predominant vector in the region, possibly due to seasonal fluctuations in temperature. Both *D. citri* and *T. erytreae* are known to have different ecological characteristics, resulting in the different altitudinal distributions of CGD. The former vector species is more heat tolerant and is commonly found in the lowland tropics and subtropics (Bové 2006), for instance in the lowland coastal regions of Muheza and Kilifi, Kenya (Rwomushana et al. 2017), while *T. erytreae* is more sensitive to heat and its abundance is thus restricted to the cooler highland areas of Africa (da Graça et al. 2015). Interestingly, we found both vector species coexisting at high elevations in the upper and lower midlands regions of Soin and Awasi in Kenya and Morogoro in Tanzania.

Although, we did not detect any CLaf infection in the sampled *D. citri*, our findings, however, show that *D. citri* is spreading fast in the region, quickly adapting to new geographical areas and ecologies. Although we did not detect CGD or its vectors in some citrus producing regions of eastern Kenya like Makueni and Kitui, because of the accumulating evidence of *D. citri*'s presence in the coastal lowlands (Rwomushana et al. 2017), chances are high that CLaf is rapidly adapting to these environments with all the inherent negative consequences for citrus production.

We could not establish a clear relationship between vector abundance and CGD presence since disease symptoms were not always matched with pathogen detection in the psyllid vectors. Seasonal and temporal variations have major effects on the population dynamics of hemipteran vectors like *T. erytreae* (Yang et al. 2013), for instance in terms of reproduction and mortality (List 1939), therefore affecting their ability to infect plants (Mcclean 1974). Furthermore, vectors like *T. erytreae* and *D. citri* can feed and complete their development on several alternative host plants in the Rutaceae family such as *Murraya koenigii* (L.) Spreng (Sétamou et al. 2016) and *Clausena anisata* (Willd.) Hook.f.ex Benth. (Van den Berg et al. 1992). Their presence could have contributed to the low vector populations in most of the visited citrus farms as the insects might have shifted to these alternate hosts in the absence of new flushes on citrus trees. Surveys conducted at more frequent intervals and accompanied by population dynamics studies could, therefore, improve our understanding of the relationship between vector populations and the spread of CGD in East Africa.

The majority of interviewed citrus growers could not recognize CGD and did not know its cause or the way it spreads. This lack of knowledge probably contributed to the observed poor disease management and most often lack of control strategies, leading to high disease incidences in the surveyed regions of Kenya and Tanzania. Yet contrary to disease incidence, the observed disease severity levels were generally rather moderate. The reasons for this are not entirely clear since few farmers employed suitable disease management strategies. Hence the observed low disease severity levels were possibly a result of partial disease tolerance of the planted citrus varieties, and may be also environmental factors such as temporal variation could have played a role in reduced CGD symptomatic expression (Gottwald 2010). The majority of the growers used lemon as their rootstock, probably because of its vigorous growth, imparting high and consistent fruit yields and its higher tolerance to most citrus diseases (Kapur et al. 1984). Yet, we recorded highest disease

severity and strongest presence of *T. erytreae* on rough lemons. Thus, they may have served as reservoirs for the bacterium and the increased psyllid activity on rough lemons might be attributed to the frequent flushing of the trees, leading to increased rate of feeding of the vectors and by consequence higher disease transmission in such orchards (Magomere et al. 2009). Other citrus varieties like Valencia were equally susceptible, but CGD severity on tangerines was significantly lower.

In conclusion, the level of disease incidence and severity varied across the different regions with chronic greening situation observed in the UM (1,300-1,800 m.a.s.l) and LM (800-1300 m.a.s.l) regions of Kenya and Tanzania. In order to rejuvenate citrus production in these regions, more intensive control strategies should be advocated. The CLaf pathogen and its vector were, however, reported absent in major citrus production regions of Makueni, Kitui in Kenya and Muheza in Tanzania. Since these areas are less prone to the vectors and the citrus greening pandemic, they could be used for establishment of insect proof nurseries for production of clean nursery stock free of CGD. The greening symptoms were more pronounced in Valencia and lemon varieties compared to tangerine. In Makueni region in Kenya, a madrin variety known as Pixie was commonly grown in most orchards and may have offered some level of resistance to the disease. Both *T. erytreae* and *D. citri* vector were found to coexist at high elevations in the UM and LM regions, illustrating that *D. citri* is spreading fast in the regions, quickly adapting to new geographical areas and ecologies, and thus has a higher chance of acquiring the CLaf pathogen in the near future. Though we did not detect any CLaf infection in the sampled *D. citri*, more surveys need to be conducted and more specimens screened to rule out the possibility of *D. citri* transmitting CLaf. We detected a widespread occurrence of CLaf subsp. clausena (CLafCl) on citrus trees across Kenya and Tanzania. The detection of circulating CGD subspecies is an important aspect of management of the disease. The association of CLafCl with citrus stresses the potential importance of *C. anisata* trees as reservoirs for CLaf. Farmers should, therefore, be advised to eliminate *C. anisata* trees in the proximity of their citrus orchards to limit the increase in the population of infected psyllids.

To completely understand the possible impact of Liberibacter subspecies and efficiently lessen the burden of disease pressure on commercial citrus crops, further research efforts need to determine in greater detail the potential reservoir hosts for CLaf within the Rutaceae family. Since CLaf has been found to be less virulent than CLas (Garnier and Bove 1996) and reports indicate more restricted host ranges of the CLaf subspecies (Garnier et al.

2000; Pietersen et al 2010; Roberts et al. 2015), genes responsible for the host range and pathogenesis should be identified and exploited to control CGD. No resistant citrus seedling trees or scion-rootstock combinations have as yet been identified (da Graça et al. 2015), though some studies have shown variations in sensitivity in different rootstocks (Boava et al. 2015). Thus production of clean planting material through tissue culture systems, breeding for CLaf resistance (Boava et al. 2015) and utilization of embryo rescued seed from healthy chimera sections of diseased fruit (Vuuren and Manicom 2009) should, therefore, be the foci of future research. Finally, farmers should also be advised on how to maintain their orchards, practice general hygiene and sanitary measures such as removal of infected trees and clearing of neglected orchards to reduce inoculum source in order to lower incidence, spread and economic impact of the disease.

3 What is in it for me? Costs and benefits of a *Candidatus* Liberibacter africanus infection in the African citrus triozid *Trioza erytreae*, vector of the African citrus greening disease

3.1 Abstract

Pathogen–insect interactions can have far-reaching consequences for the functioning and evolution of the organisms involved. However, our current understanding of these interactions and their implications for the establishment and spread of insect-borne diseases remains limited. Here, we examine fitness traits in the African citrus triozid (ACT) that are influenced by a *Candidatus* Liberibacter africanus (CLaf) infection, causal agent of the African citrus greening disease (ACGD), and explore some of the ecological and evolutionary consequences of the pathogen induced alterations in the host's phenotypes and behaviour. Our results revealed that CLaf infection of ACT (CLaf+) conferred some fitness benefits to the psyllid vector, which included increased fecundity, fertility, faster egg-to-adult development time and higher proportion of female progeny. However, survival of nymphs and adults were comparatively reduced for CLaf uninfected (CLaf-) ACT and both groups produced almost the same total number of progeny. Furthermore, CLaf+ ACT had the highest growth parameters, indicating that its overall population fitness was comparatively improved. CLaf expression levels positively correlated in qPCR analysis with all other fitness parameters except for percentage nymphal survivorship. CLaf+ ACT also exhibited increased dispersal capabilities and a subsequent net directed movement from *Citrus limon* to *Clausena anisata* and *Murraya koenigii*, the latter two ACT alternate host plants. These results suggest that CLaf infection has fitness effects on ACT, but there is no clear-cut distinction between benefits and costs of infection. These complex and subtle interactions between fitness traits and the effects of pathogen infection all contribute to strong ecological impacts and evolutionary pressures on the host and the pathogen, thus ultimately on the epidemiology of ACGD.

3.2 Introduction

Numerous pathogens are transmitted from one host to another by insect vectors. Diverse hosts including vertebrates, invertebrates and plants can be manipulated by a plethora of pathogens, including bacteria (Werren et al. 2008), viruses (Ingwell et al. 2012), acanthocephalans, cestodes, trematodes (Poulin and Thomas 1999), plasmodia and

trypanosomes (Koella et al. 1998). Apart from causing diseases, vector-pathogen interactions can alter hosts' phenotypes with substantial consequences for the hosts' population dynamics and species persistence in ecosystems (Hatcher et al. 2012; Lefèvre et al. 2009; Thomas et al. 1997). The interactions between a pathogen and its vector vary in many ways, most of which are yet to be explored and identified. One way to evaluate such interactions is by studying the effects of the tested pathogen on the vector biology and behaviour.

Vector-pathogen interactions can alter a range of physiological, behavioral and morphological traits in their hosts (Poulin and Thomas, 1999). For instance, they can instigate slight shifts in the time spent performing a given activity, i.e. biting rate in Leishmania-infected sand flies (Poulin 1994). Other pathogens can alter more than one distinct host trait, resulting in even more striking phenotypic differences between infected and uninfected hosts. For example the cestode *Schistocephalus solidus* (Pseudophyllidea, Schistocephalidae) alters the vertical distribution and body coloration of its stickleback intermediate host, leading to higher predation by birds and other fish, and sexual selection through mate choice in the stickleback (Milinski 1984; Poulin and Thomas 1999; Smith and Kramer 1987). Such pathogen-induced changes in host phenotype can be (a) an adaptive response by the host, aimed at eliminating or outlasting the pathogen, or at minimizing its effects on the host's fitness, (b) the expression of pathogen genes altering the host phenotype in ways that benefit the pathogen, that is by increasing its probability of transmission, or (c) a mere pathological consequences of an infection, not adaptive to either host or pathogen (Poulin and Thomas 1999).

Whereas most pathogens have developed mechanisms to avoid the host's immune response, the host can activate efficient defense responses against a pathogen infection, generating resistance, tolerance or avoidance (Medzhitov et al. 2012; Svensson and Råberg 2010). Avoidance behaviour reduces contact with pathogens; resistance on the other hand, minimizes the success of a pathogen by preventing its establishment or inhibiting its growth, while tolerance minimizes the negative fitness effects of a given pathogen without controlling the infection (Best et al. 2008; Graham et al. 2011; Kutzer and Armitage 2016; Råberg et al. 2009). Resistance can interfere with host-pathogen survival, reducing the pathogen prevalence in a host population, but tolerance effects are dictated by whether the host evolves mortality or fecundity tolerance (Best et al. 2008; Best et al. 2014; Graham et al. 2011; Vale and Little 2012). In mortality tolerance the infected host lives longer, prolonging the

pathogen's transmission period, and pathogen prevalence increases in a population, while fecundity tolerance is predicted to be neutral with respect to pathogen fitness; however, if fecundity tolerance comes at the cost of a reduced vector lifespan, the parasite's transmission period will be reduced (Best et al. 2008; Kutzer and Armitage 2016; Roy and Kirchner 2000).

A range of complex direct and indirect interactions exist within plant-pathogen-vector systems, i.e. (a) the pathogen depends on the vector for transmission and dispersal, (b) the pathogen may directly affect the vector because of its presence and replication within the vector, (c) the pathogen and the vector may directly compete for the same host plant as shared food source, and lastly, (d) they can both induce defense mechanisms in the host plant, and affect each other indirectly by the response of the plant (Belliure et al. 2005). These interactions can be defined by their relative effects on the fitness of the individual organisms (Nadarasah and Stavrinides 2011). Several studies have highlighted the indirect positive effects of plant pathogens on their insect vector, but others also found neutral and negative effects (DeAngelis et al. 1993; Friedli and Bacher 2001; Jensen 1959; Kluth et al. 2002; Maris et al. 2004; Moran 2001; Roca et al. 1997; Wijkamp et al. 1996; Zhang et al. 2000). Direct effects of pathogens on their vector have rarely been studied, probably because diseases are much easier to recognize and measure in plants than in insects (Purcell 1982). Furthermore, numerous vector-borne plant pathogens cannot be cultured outside a living host, and the epidemiological consequences of vector pathology are difficult to assess (Nachappa et al. 2014; Purcell 1982). Thus, few studies have demonstrated positive, negative and/ or neutral effects of infection by plant pathogens in their arthropod vectors (Medeiros et al. 2004; Miller and Coon 1964; Nachappa et al. 2012; Pelz-Stelinski and Killiny 2016; Stumpf and Kennedy 2007; Wijkamp et al. 1996).

Here, we focus on an invasive plant pest, the African Citrus Triozid (ACT) *Trioza erytreae*, (Del Guercio) (Hemiptera, Triozidae), the principal vector of *Candidatus* Liberibacter africanus (CLaf), the causal agent of African Citrus Greening Disease (ACGD), the most devastating disease of citrus in Africa and beyond (Bové 2006; Gottwald et al. 2007; Graca 1991). No studies have explored the direct effects of the CLaf pathogen on fitness-related variables of their ACT insect vector and the current understanding of the host-pathogen interactions and their implications for the establishment and spread of ACGD remains limited. Yet studies on host-pathogen interactions are key for a better understanding

of the transmission and epidemiological parameters of important plant and/or vector-borne diseases. Furthermore, unravelling such interactions can lead to the development of successful disease control and pest management strategies.

3.3 Material and methods

3.3.1 Maintenance of host plants and insect colonies

Seedlings of rough lemon, *Citrus limon* Osbeck; tangerine, *C. tangerina* Yu. Tanaka; and Valencia and Washington navel, *C. sinensis* (L.) Osbeck (all Rutaceae) were obtained from a commercial nursery in Embu, Kenya. Alternate Rutaceae host plants, i.e. *Murraya koenigii* (L.) Spreng., *Teclea nobilis* Hook.f., *Vepris undulata* (Thunb.) Verdoorn & C.A. Sm., and *Clausena anisata* (Willd.) Hook.f. ex Benth., were grown from plant cuttings in white plastic containers (35 cm height × 29 cm top diameter × 20 cm bottom diameter) containing soil and manure mix. The seedlings were fertilized twice a month with 3 pellets per plant of calcium ammonium nitrate 27% N (MEA LTD) and foliar fertilizer (Gleecobalance^{TM}) diluted at 30ml/15Liter. Additionally, the plants were watered on alternate days to enable their frequent flushing. The plants were kept in a screen house (10m x 5m x 2 m) at the Nairobi, Kenya, campus of the International Centre of Insect Physiology and Ecology (*icipe*) and leaf tissues regularly screened using conventional polymerase chain reaction (cPCR) to ensure they remained free of CLaf. Citrus seedlings showing typical symptoms of ACGD, such as blotchy mottle and leaf vein degeneration, were also obtained from a small nursery in Kisumu, Kenya and further screened for confirmation of the CLaf pathogen.

A vector colony free of a CLaf infection (CLaf-) was established from insects collected on citrus trees showing no symptoms of greening in orchards in Kericho (S00 11'41.82"; E035 12'9.3") and Meru (N00052'49.68"; E037009'13.5"), both in Kenya. The ACT adults were placed in insect proof Perspex cages (60cm x 60cm x 60 cm) containing potted healthy young flushing citrus or alternate host plant seedlings and these cages were kept in secure screen houses at ambient temperatures of around 20$\pm$5°C and a photoperiod of 12 - 12 h (light and darkness). After one month a new generation of citrus psyllids was obtained and the young adults were again transferred to healthy seedlings under the same conditions. This procedure was repeated severally for ≈14 generations. Individuals from each generation were cPCR assayed on a monthly basis to ensure that they remained CLaf-.

An infected vector colony (CLaf+) was established from disease-free specimens from the CLaf- colony by transferring the ACT on diseased citrus plants. Newly emerged ACT

individuals from each generation as well as the symptomatic plants, were also routinely and repeatedly tested for CLaf using diagnostic cPCR. CLaf+ and CLaf- colonies were maintained in separate secure screen houses to avoid any cross contaminations.

3.3.2 Establishment of experimental isofemale lines

Eggs collected from individual gravid ACT females of the CLaf+ and CLaf- colonies were used to establish isofemale lines under the same conditions mentioned before. Separate 34cm x 34cm x 61cm cages with sided white mesh polyester netting (Bioquip, Rancho Dominguez, USA) were created for each independent isofemale line. CLaf+ and CLaf- isofemales were maintained for at least four generations on infected and uninfected *C. limon* plants, respectively, and infection status of both isofemale lines were routinely assayed by cPCR.

3.3.3 Mating assay

To obtain experimental gravid isofemales for the subsequent assays, 2-3 days old isofemales were paired and caged in conical 50ml falcon tubes containing excised citrus leaves. Prior to introducing the insects, excised leaves from citrus seedlings were thoroughly washed with deionized water to remove any residues. Using a sharp clean razor blade, the terminal end of the leaf petiole was cut diagonally to create a wider area for more water absorption (Ammar et al. 2013), and each petiole was immediately inserted into a small 2ml microcentrifuge tube filled with tap water. A piece of parafilm membrane was wrapped around the top of the tube and the petiole to minimize water evaporation and to keep insects from coming into contact with water. For ventilation purposes, the bottoms of the falcon tubes were cut and sealed with a piece of fine-mesh screen cloth using masking tape. The excised leaf in the microcentrifuge tube was then inserted into the larger 50ml falcon tube and the newly emerged paired adults were introduced into the tubes before capping the bottom with their flip-top cap. These combined caging tubes were then placed on a tube rack and kept on the bench-top in the laboratory at 23 ± 1°C and insects were allowed a five-day mating period.

3.3.4 Fecundity assays for fitness evaluation

Single mated CLaf+ and CLaf- isofemales were transferred to individual separate (34cm x 34cm x 61cm) white polyester mesh insect cages (Bioquip, Rancho Dominguez, USA) containing disease-free *C. limon* seedlings with new leaf growth for a seven-day oviposition period. Experiments were conducted on *C. limon* plants being among the preferred host plant

for ACTs', because of their light yellowish color and soft leaves which regularly flush, thus providing copious, soft flush for oviposition and nymphal development (Moran and Buchan 1975). Infested citrus plants were held in the laboratory maintained at 23±1°C and a photoperiod of 12:12 (L: D) h to mimic field conditions. When a gravid isofemale could not be retrieved or was found dead before the end of the seven-day oviposition period, a new isofemale was introduced. At the end of the oviposition period, the isofemales were removed and kept in a -20°C freezer for subsequent qPCR detection of CLaf. The eggs laid on the plants were counted under a Leica MZ 125 microscope and fecundity, defined as cumulative egg production per female over the seven-day oviposition period, was recorded.

3.3.5 Immature development and survivorship

After counting of eggs, the infested citrus seedlings with eggs from the different isofemale lines (CLaf+ and CLaf-) were daily observed to record developmental time and stage-specific nymphal survivorship. The time period between oviposition and the first appearance of initial nymphal instars was considered the pre-oviposition period. Nymphal developmental time was defined as the period between the first appearance of the first nymphal instar and the beginning of adult emergence, whereas total developmental time as the period from egg laying to the onset of adult eclosion, and percentage hatching as the number of F1 nymphs that resulted from the total number of eggs deposited. The number of nymphs emerging in each instar was recorded, and nymphal instar stage was determined based on the size of the nymph and the development of the wing pads (Moran and Blowers 1967). Percentage nymphal survival was defined as the total number of F1 adults that emerged out of the total number of nymphs produced. The total number of F1 adult ACTs that emerged was counted and the sex ratio of F1 adults determined. Males and females were differentiated based on the shape of the apex of the abdomen (Blowers and Moran 1967). Four biological replicates were conducted during the periods of January and March (experiment 1), April and June (experiment 2), July and September (experiment 3), and October and December (experiment 4) of 2017. In each experiment four replicates were initiated, resulting in a total of 16 isofemale lines for each CLaf+ and CLaf- isofemale lines, respectively.

3.3.6 Reproductive capacity

After determination of the sex ratio, 40 pairs (female and male) of newly emerged ACT adults from the cohort population of CLaf+ and CLaf- nymphs were placed in separate rearing Perspex cages (10cm × 5cm × 10 cm) containing a *C. limon* flush leaf inserted in a glass vial with water and covered with parafilm. Ten cages were used for each treatment and experiments repeated four times. The paired adults were transferred to a new *C. limon* leaf after every 24 hrs and the laid eggs counted under a stereomicroscope. Daily observations of numbers of newly laid eggs were recorded until the death of all the females. Females that died within the first 24 h of an experiment or those that produced no eggs were excluded from the analysis. The bioassays were conducted under the same experimental conditions as described above.

3.3.7 Adult survival

Adult survival of CLaf+ and CLaf- was assessed under the same controlled laboratory conditions of 23±1°C and a photoperiod of 12:12 (L: D) h. Five groups of pairs (10 males and 10 females) of newly emerged CLaf+ and CLaf- ACTs were released to individual healthy potted citrus plants in separate (34cm x 34cm x 61cm), white mesh polyester cages (Bioquip, Rancho Dominguez, USA), and daily the number of dead insects were recorded, collected and sexed until all individuals were dead.

3.3.8 Effect of CLaf on ACT dispersal capability

Dispersal experiments were carried out in a screen house under ambient temperature conditions 20 ± 5°C and light condition of 12:12 (L: D) h. Mark–release–recapture method with fluorescent powder (Kobori et al. 2010) was used to examine the effect of CLaf on dispersal of ACT. A previous report indicates that there are no significant differences in mortality between marked and unmarked citrus psyllids (Nakata 2008); moreover our preliminary studies also showed that marking did not interfere with the ability of ACT to disperse or survive. Therefore, CLaf- adults were marked while the CLaf+ ones not, and both males and females were studied in this experiment. We used pinkish fluorescent dyes (1162Y luminous powder-pink, Bioquip Inc., USA) as it is best detectable for visually tracking psyllids in the field (Nakata 2008). For marking purposes, 25 newly emerged (5-7 days old) CLaf- ACTs of each sex were transferred into separate transparent plastic tubes (12 cm in

length and 3 cm in diameter) each containing 10 mg of the fluorescent dye (Nakata 2008). The tubes were gently hand-shaken for 5 seconds to coat the insects.

Fifty marked and sexed CLaf- and CLaf+ adults, respectively, were released in a cage on a potted *C. limon* plant (settling plant). After two days, insects that were not located on the settling plant were removed together with the cage and four similar *C. limon* plants (the dispersal plants) of the same size, height and number of flushes as the settling plant, were placed at distances of 100 cm, 200 cm, 300 cm and 400 cm from the settling plant. Subsequently, CLaf- and CLaf+ adult dispersal to the different plants was assessed daily for seven days by counting and recapturing individual insects that moved from the settling plants to the dispersal plants. The sex of each recaptured adult was assessed under a Leica MZ 125 microscope. All dispersal experiments were replicated four times.

3.3.9 Effect of alternate host plants on dispersal of CLaf+ and CLaf- ACTs

Dispersal of CLaf+ and CLaf- ACTs to different host plants, i.e. *C. limon*, *T. nobilis*, *M. koenigii*, *C. anisata*, and *V. undulata* was simultaneously tested. *Citrus limon* was used as the settling plant and placed centrally while the four alternate hosts as dispersing plants which were spaced equidistantly around the settling plant. The number of adult ACTs settling on each alternate host plant was recorded daily for seven consecutive days and the experiment was repeated four times while rotating the position of each plant for each replicate.

3.3.10 DNA extraction

DNA extraction was performed on individual isofemales that were retrieved after a seven-day oviposition period and stored in 70% ethanol at 4°C at the end of each experiment. The insects were surface sterilized using 3% sodium hypochlorite and rinsed once in 70% ethanol then thrice in distilled water. Genomic DNA was extracted from individual insects using the Isolate II genomic DNA kit (Bioline, London, UK), according to the manufactures protocol. The resultant DNA was eluted to a final volume of 50µl. The quality and quantity were checked using a Nanodrop 2000/2000c spectrophotometer (Thermo Scientific, Wilmington, USA) and samples were stored at -20°C until used for qPCR assays.

3.3.11 Quantitative real-time PCR assays

The extracted DNA from the individual isofemales were used to quantify 16S rDNA gene in bacteria-infected (CLaf+) and bacteria-free (CLaf-) ACTs. The qPCR reaction mix, consisting of 15ng/µl DNA template, was amplified in three independent replicates with 7.5 µL of Fast SYBR® Green master mix (Applied Biosystems, Carlsbad, USA). CLaf 16S rDNA gene region was amplified using 0.5 picomoles each of forward primer LibUF (5'-GGCAGGCCTAACACATGC-3') (Phahladira et al. 2012; Roberts et al. 2015), reverse primer HLBr (5'-GCGTTATCCCGTAGAAAAAGGTAG-3') (Li et al. 2006) and reference gene(XM_008488758.2_Fwd:5'-TTGGAGCTAAATTCTGGGA-3'and XM_008488758.2_Rev: 5'GCAGTCAGAGGGGCAAATCCA 3') designed from the *Diaphorina citri* tubulin beta chain-like gene (Khamis, unpublished data).

The reactions were carried out in a Strategene Mx3005P real time qPCR machine (Agilent Technologies, USA) according to the manufacturer's instructions. The cycling conditions for the LibUF/HLBr markers involved an initial step of 95°C for 10 min, 40 cycles of 95°C for 3 sec, 60.9°C for 45 sec, 72°C for 1 min, followed by one cycle of 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec. The set up for the reference gene was the same with LibUF/HLBr markers except the annealing temperature was at 45°C for 30 sec. Crossing threshold (Ct) values were recorded for all the sample reactions and relative expression levels calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The amplification curves for Ct determination, and melting curves for temperature estimations at the peak of the curves, were analysed using the MxPro qPCR software from Strategene (Agilent Technologies, USA).

3.3.12 Statistical analysis

Statistical analyses were done using the StataCorp. 2017 Stata Statistical Software. Data on fecundity, sex ratio and development time of CLaf+ and CLaf- ACTs were analysed by a Student t test ($p \leq 0.05$). If the data did not follow a normal distribution according to the Shapiro-Wilk normality test, a nonparametric Mann-Whitney U test ($p \leq 0.05$) was performed. For the effect of CLaf on nymphal stage specific survival, we used a mixed model with insect as the random effect because we had a panel data situation and therefore measurements were pseudo replicates. Adult survival was analysed using Kaplan–Meier and pair-wise comparisons done between the different sexes, also between CLaf+ and CLaf- treatments and

their combination, using Logrank test. Pearson's rank correlation coefficient test was performed to determine the direction and magnitude of the relationships between CLaf expression level and fitness traits. Life table parameters for CLaf+ and CLaf- ACTs were estimated using the jackknife with the formula suggested by Carey (1993), and according to the method of Birch (1948). Two-way ANOVA was used to assess the sex dependent differences on dispersal of CLaf+ and CLaf- adult ACTs. The effect of CLaf on dispersal of insects to varying distances and host plants over a seven-day period was analysed using a Poisson model for counts and a multinomial logistic model for categorical variables. In order to adjust for pseudoreplicates, i.e. repeated measurements, the mixed effects model version or, in cases of data scarcity, the cluster version of the appropriate model was chosen.

3.4 Results

3.4.1 Effect of CLaf on ACT fitness traits

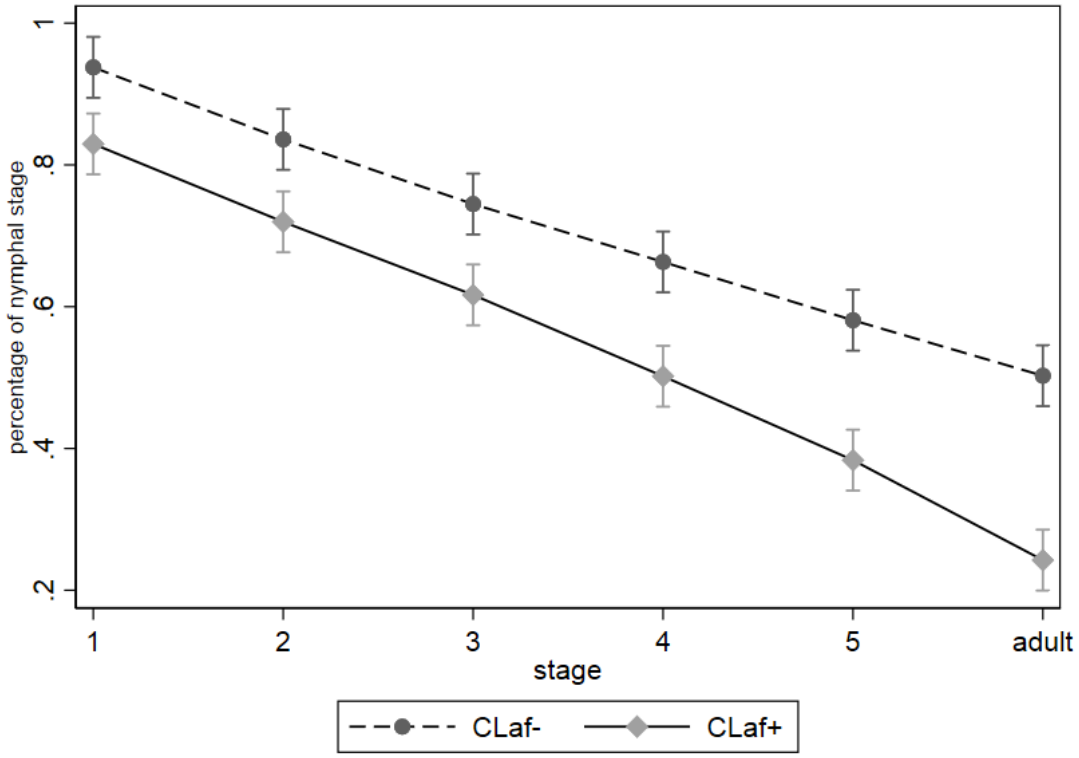
CLaf+ isofemales laid significantly ($t=5.86$, $df=19$, $P<0.0001$) more eggs (173 ± 11.6, mean ± SE) than their CLaf- counterparts (76 ± 6.9, mean ± SE), over a seven-day fecundity period. Also, CLaf infection considerably affected the development time of ACT immature stages, with egg incubation, egg to adult emergence, and duration of the third and fifth instar being significantly shorter in CLaf+ than in CLaf- ACTs (Table 3.1).

Table 3.1 Developmental duration (mean ± SE) of various developmental stages of CLaf+ and CLaf- ACT reared at 23±1°C

Developmental stage	Development time (days) Mean ± SE		
	CLaf + ACT	CLaf - ACT	P Value
Eggs	6.81 ± 0.38	7.63 ± 0.25	P=0.001
Nymphs 1st Instar	4.00 ± 0.37	4.31 ± 0.35	P=0.2
2nd Instar	2.69 ± 0.24	2.75 ± 0.22	P=0.7
3rd Instar	3.06 ± 0.39	3.63 ± 0.25	P=0.02
4th Instar	4.38 ± 0.36	4.56 ± 0.31	P=0.4
5th Instar	5.25 ± 0.47	6.06 ± 0.39	P=0.01
Egg to Adult	26.19 ± 0.76	28.94 ±1.30	P=0.001

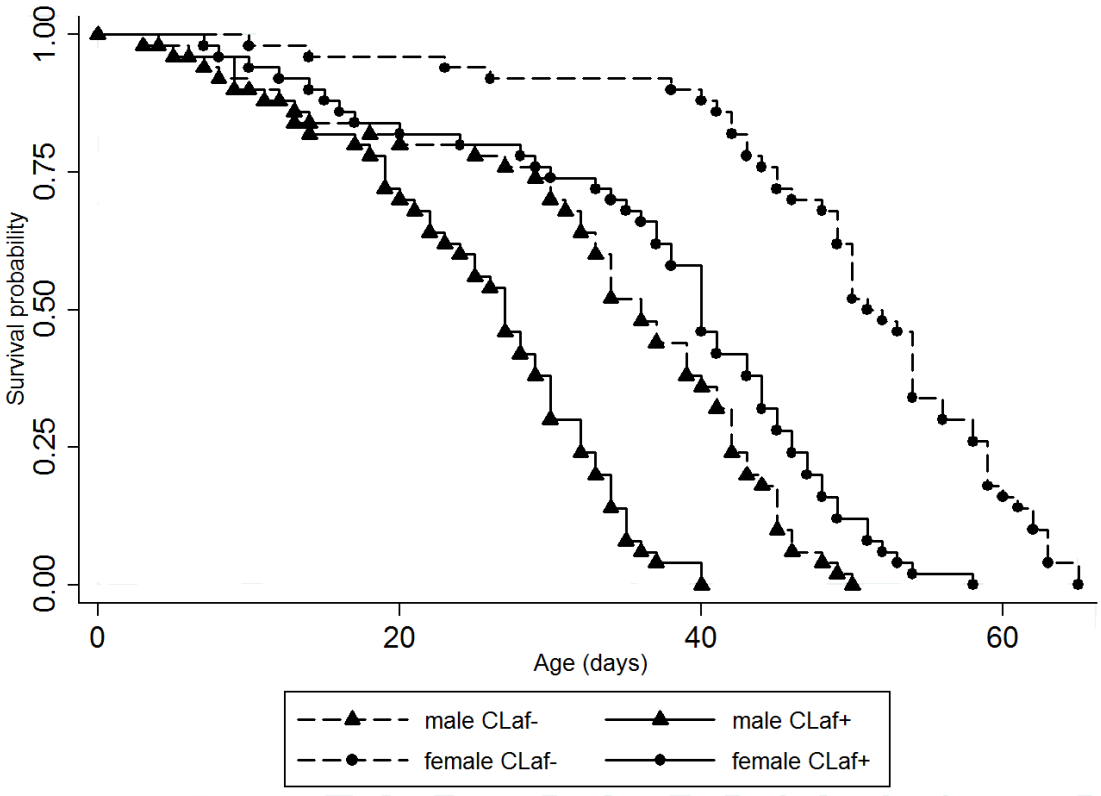
Similarly, CLaf infection had a considerable effect (χ^2=59.76, df=1; P<0.0001) on the survival of all ACT immature stages, with only 23% of CLaf+ and 50% of CLaf- ACT eggs reaching adult stage (Figure 3.1).

Figure 3.1 Effect of CLaf infection on mean survival rate (95% CI) of ACT nymphal stages.



On the other hand, there was no significant difference (t=0.71, df =27, P=0.485) in the mean total number of emerged progeny between the two isofemale lines. However, the proportion of female progeny varied significantly (t=-10.75, df =27, P<0.0001) between CLaf+ and CLaf- ACTs, even though it was female biased for both, i.e. 0.66 and 0.52 for the former and the latter respectively. The sex (χ^2 = 79.78, df =1; P<0.0001) of the psyllid and CLaf infection status (χ^2 = 37.55, df = 1; P<0.0001) as well as their interaction (χ^2 =138.55, df =3; P<0.0001) had a significant effect of ACT survivorship effects (Log rank P<0.0001). Adult survivorship was significantly lower for CLaf+ ACTs relative to their uninfected counterparts, and consequently the longest adult lifespan was recorded for CLaf- adults (Figure 3.2).

Figure 3.2 Kaplan-Meier survival estimates on CLaf+ and CLaf- ACTs.

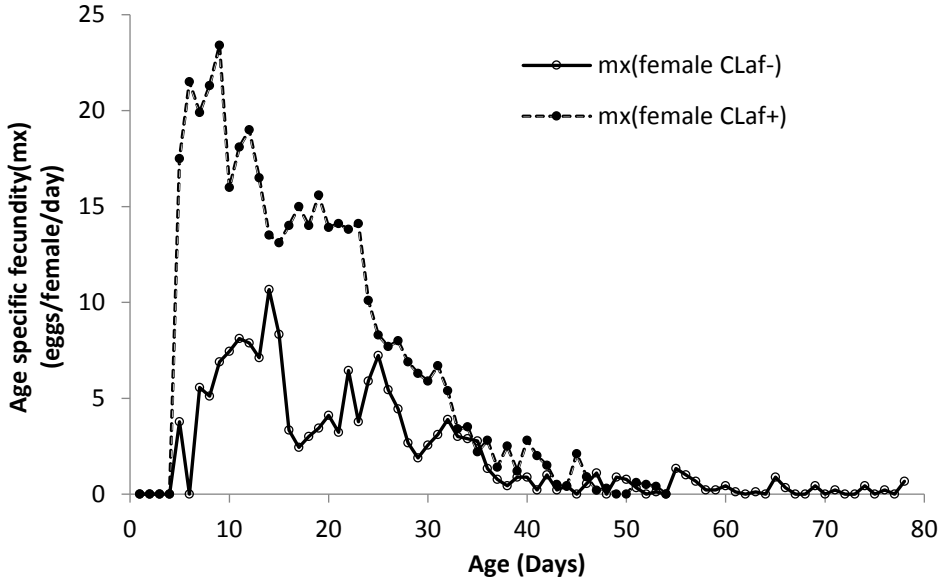


Log rank test for main effects (sex and infection status) and interaction effects was significant P=0.0000

3.4.2 Reproductive capacity and population parameters

For the reproductive lifespan of females, the mean total number of eggs for CLaf+ was 409 eggs vs. 147 eggs for CLaf- ($t = 11.69$, $df = 78$, $P < 0.0001$. The age-specific fecundity (*m*x) curve peaked soon after the onset of reproduction and was considerably affected by a CLaf infection. Daily egg production was also significantly higher in CLaf+ females; however, major peaks of oviposition was observed between day 5 and 25 in both groups, after which the reproductive capacity decreased gradually with age (Figure 3.3).

Figure 3.3 Age-specific fecundity of CLaf+ and CLaf- ACT isofemales reared at 23±1˚C



Fitness parameters of ACT were significantly improved by a CLaf infection (Table 2). CLaf+ isofemales had significantly higher growth rates (intrinsic rate of increase, finite rate of population increase and net reproductive rates) as compared to CLaf- ACTs. Conversely, the doubling time was significantly shorter for CLaf+ individuals as compared to their uninfected counterparts. However, no significant difference was recorded for the generation time of both groups of ACTs (Table 3.2).

Table 3.2: Jackknife life table estimates (means) and associated 95% CL for CLaf+ and CLaf- ACT isofemales reared at 23±1˚C

	CLaf+		CLaf-	
Parameter	Mean	95% CL	Mean	95% CL
Intrinsic rate of Increase - r_m	0.1101	0.1085 - 0.1118	0.0937	0.0906 - 0.0968
Finite rate of increase - λ	1.1164	1.1146 - 1.1183	1.0982	1.0948 - 1.1016
Net Reproduction Rate – R_0	61.6571	55.9494 - 67.3649	37.8573	31.3823 - 44.3323
Mean Generation Time - T (days)	37.4289	36.6246 - 38.2332	38.8170	37.5591 - 40.0748
Doubling Time – dt (days)	6.2928	6.1989 - 6.3867	7.3949	7.1485 - 7.6414

3.4.3 Relative quantification of bacterial 16sRNA CLaf gene

qPCR was used to estimate relative quantities of 16sRNA CLaf gene measured as relative fold change (RFC), and to determine whether increased level of expression in CLaf+ isofemales correlated positively with fitness traits related to each isofemale line and F1 progeny. Except for nymphal survival percentage, a positive correlation was detected between all the other fitness traits (Table 3.3). Thus, the strength of infection in an infected ACT may affect its fitness traits, whereby increased level of infection results in greater numbers of eggs, nymphs and adults, though hatchability was not significantly influenced. Moreover, nymphal survival was greatly reduced by an increased level of infection and vice versa.

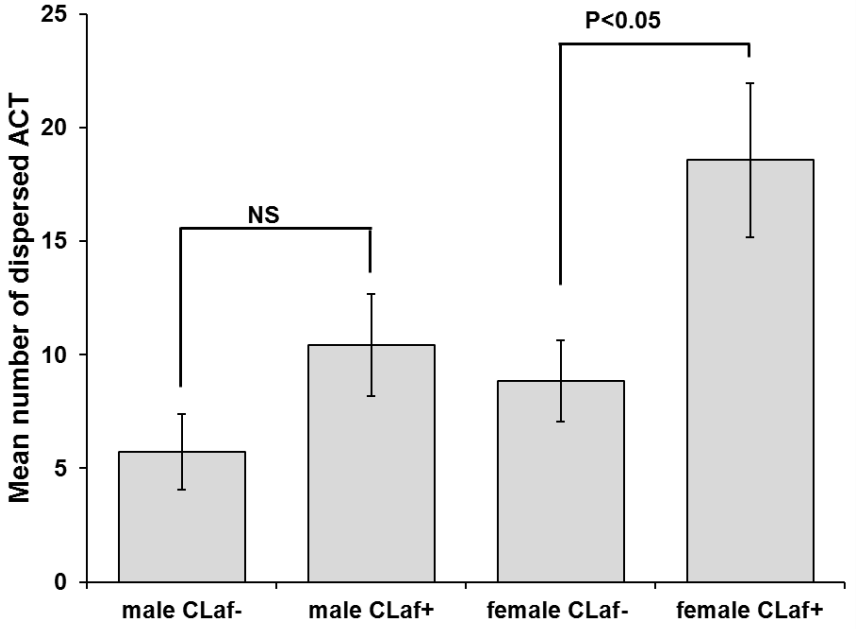
Table 3.3: Correlation between ACT fitness parameters and CLaf relative expression level per insect

	RFC	Fecundity	Total nymph	Hatchability	Total adults
Fecundity	0.66 **0.006**	-	-	-	-
Total nymphs	0.60 **0.01**	0.99 **<0.0001**	-	-	-
Hatchability percentage	0.22 0.4	0.57 **<0.0001**	0.67 **<0.0001**	-	-
Total adults	0.68 **0.004**	0.73 **<0.0001**	0.72 **<0.0001**	-.59 **0.02**	-
Nymph survival percentage	-0.45 0.08	-0.77 **<0.0001**	-0.77 **<0.0001**	-0.39 0.1	-0.20 0.4

3.4.4 Effects of CLaf on ACT dispersal

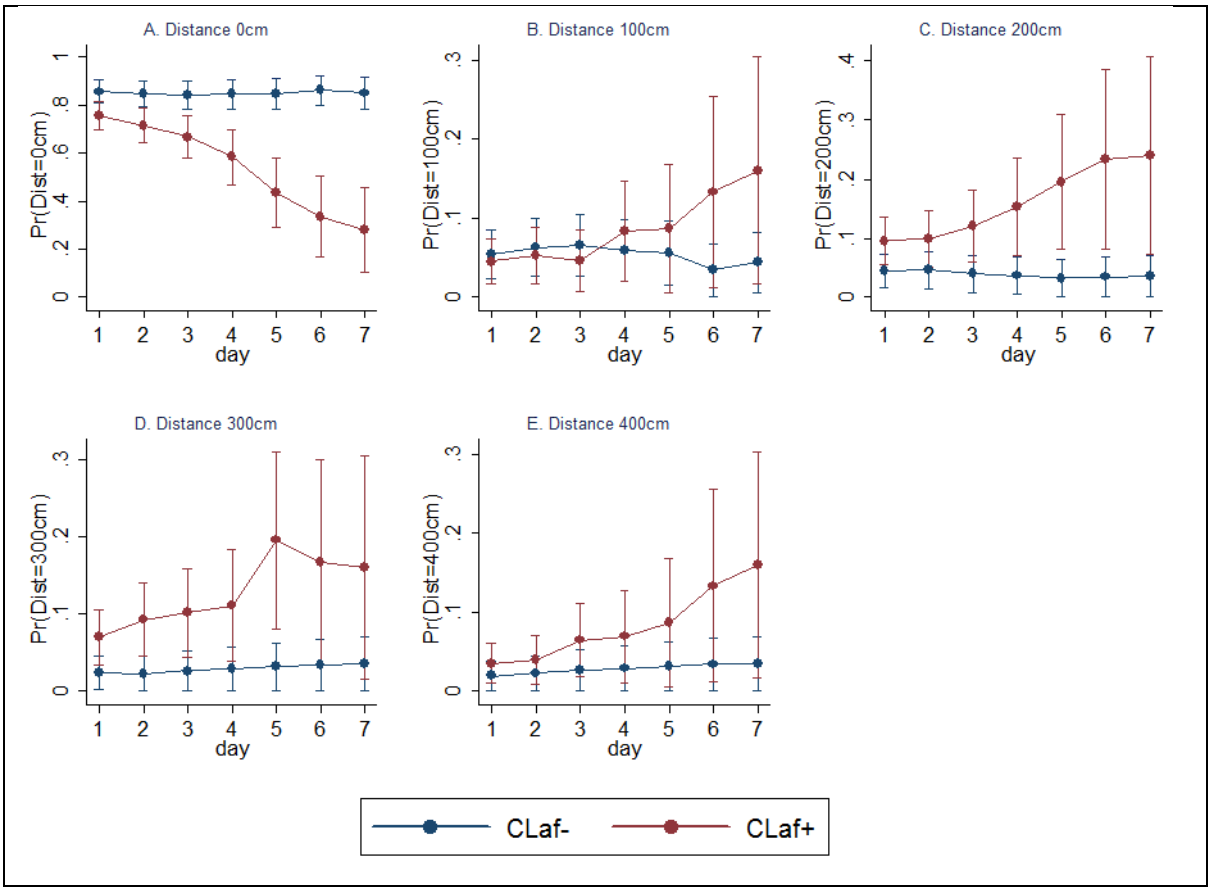
CLaf infection ($F_{1, 24}$ = 9.2, P=0.005) and sex ($F_{2, 24}$ = 3.4, P=0.05) significantly affected dispersal pattern of ACTs, with CLaf+ females having the highest mean numbers of dispersed adults (Figure 3.4).

Figure 3.4: Sex dependent dispersal pattern of CLaf+ and CLaf- ACT males and females expressed in mean (± SE) number of ACT adults (NS = not significant).



We also observed a clear effect of CLaf infection and time on dispersal pattern of ACT to the different distances. Overall, CLaf infection resulted in a higher probability of dispersion by ACT adults (Figure 3.5). Irrespective of CLaf status, the probability of dispersal to all varying distances was < 50%. The probability of both CLaf+ and CLaf- ACTs remaining on the dispersal plant (distance=0cm) gradually declined over time, with CLaf– ACTs exhibiting a very low and almost constant rate of dispersal (Figure 3.5a). CLaf- ACTs frequently dispersed to the closer distance of 100cm with the highest probability of dispersal observed between day 1 and day 3 (Figure 3.5b). However, CLaf+ ACT individuals showed a higher tendency of dispersing to plants placed at the greater distances of 200cm and 300cm, with significant differences in dispersal recorded within and across the observed days (Figures 3.5c and 3.5d). There was no significant difference in the dispersal pattern of both CLaf+ and CLaf- ACTs over time for the distances 100cm and 400cm (Figures 3.5b and 3.5e).

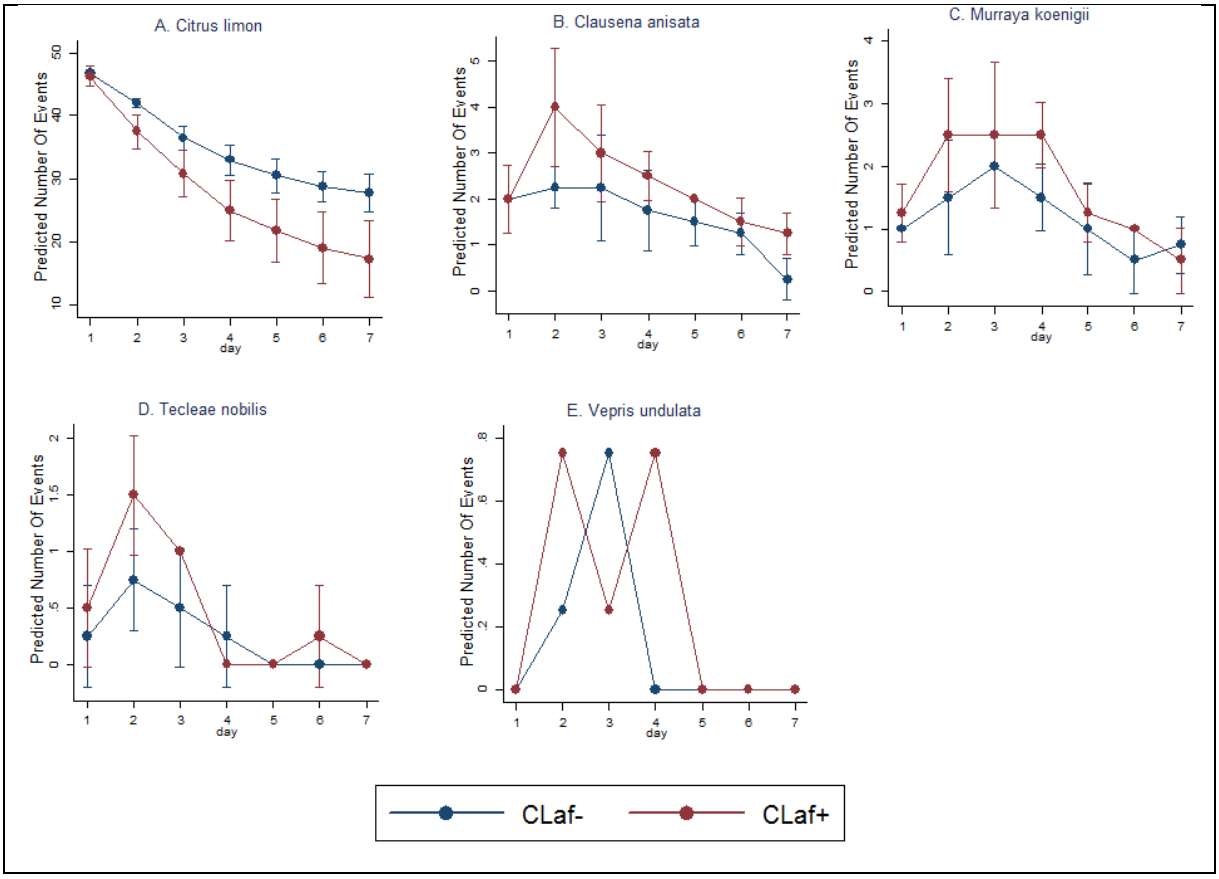
Figure 3.5 Effects of a CLaf infection, distance and time on dispersal behaviour of adult ACTs (mean number of adults and associated 95% CI).



Pairwise comparison of true movements of ACT across the given distances

There was also an effect of time and a CLaf infection on dispersal of ACTs to the different host plants. We observed movement of ACTs over time, with significant interactions between time (measured in days) and CLaf infection status (Figure 3.6). With the exception of *V. undulata*, there was variation in the dispersal pattern of ACTs to other host plants over the days with *M. koenigii* and *C. anisata* recording the highest counts (Figures 3.6b and 3.6c).

Figure 3.6 Host plant dependent dispersal behaviour of CLaf+ and CLaf- ACTs (mean number of adults and associated 95% CI).



3.5 Discussion

In arthropods that are agricultural pests and/ or plant, human or animal disease vectors, a pathogen infection may affect their fitness and increase the speed at which they affect their hosts. An understanding of the role of infection on vector fitness and dispersal capabilities provides important information on how it affects host biology and transmission abilities. Fitness studies are also an essential step in understanding the ecological and evolutionary effects of vector-pathogen interactions and the magnitude of the presumed effects. This knowledge is critical for risk assessments and may offer insights useful for effective management and control of epidemic diseases. Fitness components such as survival, reproduction and development rate are the main parameters used to assess the effects of pathogens on their vectors (McGraw and Caswell 1997; Weintraub and Beanland 2006). The novelty of this study on the epidemiology of ACGD is that it is the first to provide a

comprehensive assessment on effects of CLaf pathogen infection on the ACT vector. Contrary to studies on the closely related Asian citrus psyllid *D. citri*, our research captures an extensive evaluation of fitness traits, ranging from survival, reproductive and population parameters, pathogen infection density and dispersal capabilities as also influenced by citrus and alternate host plants; hence we provide a comprehensive picture of the complex and subtle nature of pathogen–vector interactions.

In this study, a CLaf infection increased fecundity and fertility and resulted in higher growth parameters in ACTs. Generally, oviposition peaked between days 5-25 after the onset of reproduction, but oviposition of CLaf+ females peaked much faster compared to the non-infected ones. Parasite carrying vectors have been shown to compensate fitness costs owing to an infection by increasing their rate of egg production (Adamo 1999; Lefèvre et al. 2008), an effect termed as 'fecundity compensation' (Forbes 1993). Fitness rewards in the form of increased fecundity due to a pathogen infection are, however, rare and there are not many cases where it has been demonstrated. In our study host fecundity increased as a consequence of a CLaf infection which can be viewed as both a pathogen and a host strategy, where both partners gain from the association. The increased fecundity could result in a win-win situation for both the CLaf pathogen and the ACT vector since the higher numbers of eggs produced by CLaf+ females may help increase vector population which in turn will facilitate the spread of the disease. Yet this positive effect was somewhat curtailed by the reduced survival of the nymphs of CLaf+ females, with only 23% of the eggs eventually emerging as adults.

Moreover, CLaf+ adult psyllids had a shorter lifespan compared to their uninfected counterparts, with males generally being more affected than the females. The shorter lifespan in a CLaf+ ACT female could be linked to its high rate of fecundity, suggesting that trade-off may have occurred between survival and fecundity. Physiological trade-offs between reproduction and lifespan has been reported for several organisms, where individuals that reproduce more exhibit shorter lifespans (Glazier 1999; Roff 1992; Williams 1966). Trade-offs in pathogens are of particular interest because they may constrain the evolution and epidemiology of a disease (Susi and Laine 2013).

Furthermore, the survival rate can determine the stability of vector populations and is a critical factor governing their potential in disease transmission (Miller et al. 1973) as low mortality rates increases the likelihood that an infected vector will survive long enough to transmit the pathogen to one or more susceptible hosts and vice versa. Thus, the observed

high mortality in the CLaf+ nymphs, coupled with the shorter overall lifespan, indicate considerable fitness costs for both the ACT vector and the CLaf pathogen, and will result in both reduced population growth of the vector and disease transmission. Therefore, the combined net effects of the increased fecundity, and fertility of CLaf+ females and their higher juvenile mortality and shorter lifespan levelled each other out. Such complex interactions of costs and benefits in vector-pathogen systems renders assessing fitness outcomes very difficult because often individual fitness parameters are intimately related and if looked at in isolation can distort the overall picture.

A previous study on *D. citri* focused on density dependent and dispersal behaviour as affected by CLas exposure and infection (Martini et al. 2015). However, to our knowledge, our study for the first time explores the effects of a citrus greening pathogen on dispersal patterns of a vector as influenced by distance and host plant species variation. We observed greater dispersal behaviour in CLaf+ ACTs, with females exhibiting a significantly increased rate of dispersal. Moreover, higher numbers of CLaf+ ACTs moved to *M. koenigii* and *C. anisata* indicative that the dispersal behaviour of ACT in the field may be influenced by the presence of other host plants in the vicinity and distance from one plant to the next. This enhanced dispersal behaviour, coupled with the observed significantly higher female sex ratio in the CLaf+ isofemales, might affect the epidemiology of the disease and could be one factor for the fast spread of ACGD in Africa.

Greater dispersal of CLaf+ females would additionally increase their chances of finding a mate, leading to more offspring. Therefore, as long as infected females produce on average more daughters than uninfected ones, the pathogen can persist and spread in the population, even if total progeny production is not affected by a pathogen infection as in our study, or even when it is reduced (Caspari and Watson 1959; Turelli 1994; Werren 1997). Feminization has also been described in some hematophagous insects such as mosquitoes (Werren et al. 2008) and this trait tends to confer a reproductive fitness advantage to infected females, allowing infections to spread in the population (Jiggins 2017). Furthermore, since pathogen infection sometimes results in decreased vector lifespan, as also observed in our study, some pathogens rapidly spread/disperse their genes in the population through promoting compensatory sexual activities in vectors, e.g. infected hosts compensate shortened lifespan by developing a greater sexual drive (Abbot and Dill 2001). Other pathogens influence their vectors to increase the probing and feeding rate as observed in

hematophagous insects like tsetse flies, sand flies and mosquitoes (Hogg and Hurd 1995; Lefèvre et al. 2008). Such behavioral modifications benefit the pathogen because enhanced sexual contacts or increased probing activities can result in higher vector fecundity or increase the opportunities for short-term transmission and disease spread during feeding (Drummond et al. 1989; Lefèvre et al. 2008).

We also recorded a significantly shorter egg to adult development time in CLaf+ than in CLaf– individuals. Duration of development is potentially a significant component of overall fitness in the field, as it may determine how long the different developmental stages are exposed to natural enemies like predators and parasitoids (Awmack and Leather 2002). Effectiveness of natural enemies often depends on host/prey growth rates, and especially parasitism can increase when host growth is slowed (Benrey and Denno 1997; Devine et al. 2000). A reduction in development time allows for a faster passage of infected individuals through the nymphal stages, therefore providing a direct beneficial effect to CLaf infected ACTs by reducing their vulnerability to parasitism and predation. From the pathogen's perspective, a reduction in nymphal developmental time is beneficial because infected nymphs will faster reach adulthood and then contribute to pathogen transmission.

In conclusion, most studies of pathogen–vector interactions touch on evidence that support the hypothesis that pathogen infection leads to either a cost or benefit to the host or the pathogen. Very often, the line of argument is in black and white, where the vector is either punished or rewarded. Yet what we observed is that fitness outcomes are not easy to decipher and are often more of a grey area. Hence, pathogen–vector interactions may produce different scenarios and require careful examination, for each host-pathogen system before any firm conclusions can be made. There was no clear-cut distinction between the benefits and costs of a CLaf infection in ACT. The pathogen affected several fitness parameters of the vector in different directions, resulting in fitness gains and costs for CLaf and ACT, and distinguishing the two outcomes can be rather complicated. These complex and subtle interactions between fitness traits and the effects of pathogen infection all contribute to strong ecological impacts and evolutionary pressures on the arthropod host and the pathogen, thus ultimately on the epidemiology of disease. Neglecting any of these outcomes might present a warped picture, not only of the epidemiological patterns, but also for the success of future control strategies. An improved understanding of some of these mutualistic effects might help us to unravel some of the unresolved questions in the study of the pathogen–host interactions

4 Characterization of African citrus triozid, their parasitoids and endosymbionts: intergral resources for biological control success

4.1 Abstract

The African citrus triozid (ACT), *Trioza erytreae* (Del Guercio), is the primary vector of *Candidatus* Liberibacter africanus (CLaf), the causative agent of Africa citrus greening disease. Besides the use of synthetic insecticides, alternative biological control measures are urgently needed for successful pest and disease control. This study evaluates the diversity of ACT parasitoids and further characterizes endosymbionts associated with both *T. erytreae* and its parasitoids. Mitochondrial cytochrome oxidase I gene was used to reconstruct *T. erytreae* and its parasitoids phylogeny, while the 16S rRNA gene was used for the bacterial phylogeny. One well supported clade of ACT was detected within the *Triozidae* phylogeny, while five clades of different ACT parasitoid species were detected within the eulophid and encyrtid phylogeny. The phylogenetic result of parasitoids was supported by morphological identification where five different hymenopteran parasitoid species, the eulophids *Tamarixia dryi* (Waterston) and *Tetrastichus* sp., the encyrtids *Psyllaephagus pulvinatus* (Waterston) and *Aphidencyrtus cassatus* (Annecke) and the figitid *Charipinae* could be identified.

Moreover, four eubacterial symbionts (*Wolbachia, Rickettsia, Arsenophonus* and *Candidatus* Liberibacter sp.) were detected in *T. erytreae* and three symbionts (*Wolbachia, Rickettsia* and *Cardinuim*) in the parasitoids. Maximum likelihood phylogenetic inferences clustered the identified eubacterial symbionts within the α and γ proteobacteria subdivisions. Phylogenetic inferences of 16S rRNA gene sequences indicated that *Wolbachia* strains from ACT and the parasitoids did not form a single monophyletic clade; however, both clustered within Supergroup B. The impacts of these parasitoid species and endosymbionts on ACT are still unknown, but their occurrence and broad distribution indicates the possibility of their future use for rational control of *T. erytreae*.

4.2 Introduction

Bacterial endosymbionts play a crucial role in the biology, ecology and evolution of their arthropod hosts (Dhami et al. 2013; Moran et al. 2008; Werren et al. 2008). Moreover, current emerging research show that insect endosymbionts hold key to novel approaches for managing arthropod-transmitted diseases. The crucial importance of these bacterial

symbionts for key biological processes of their hosts like nutrition (Nikoh et al. 2014), reproduction (Baumann 2005), and disease transmission (Bové 2006) has heightened interest to develop strategies to manipulate and/ or disrupt them as a key element of novel pest and disease vector control strategies (Douglas 2007; Hoffmann et al. 2011).

Endosymbionts are classified either as primary (P-) or secondary (S-) endosymbionts (Baumann 2005; Dale 2006). Primary endosymbionts are obligatory and mutualistic to the host since they are essential for host survival and reproduction (Baumann 2005). They are postulated to have ancient associations with their insect's hosts (Baumann 2005; Rosell et al. 2010). Furthermore they are vertically transovarially transmitted to the host progeny (Balmand et al. 2013; Baumann 2005; Koga et al. 2012; Sloan and Moran 2012), and are located within specialized cells known as mycetomes (bacteriome) (Baumann 2005; Buchner 1965; Rosell et al. 2010). Insects belonging to the hemipteran suborder Sternorrhycha, i.e. psyllids, aphids, mealybugs and whiteflies live on a nutritionally unbalanced diet of phloem that lacks essential amino acids; hence, they form obligate associations with P-endosymbionts that help supplement for the nutritional deficiency (Buchner 1965; Douglas 1998; Moran and Telang 1998). Amongst the most studied primary endosymbionts are *Buchnera aphidicola* in aphids (Munson et al. 1991) and *Candidatus* Carsonella ruddii in psyllids (Thao et al. 2000). S-endosymbionts on the contrary are facultative and either vertically or horizontally transmitted (Chiel et al. 2009; Sintupachee et al. 2006). They tend to have a more recent, less stable association with arthropods and are not essential to host survival but are nonetheless widely distributed among arthropods (Dossi et al. 2014; Douglas 2007; Fukatsu et al. 2000; Sandström et al. 2001;Thao et al. 2000a).

S-symbionts function both as facultative mutualist and reproductive manipulators and can either contribute to host fitness, or manipulate the host's reproduction in ways that enhances its own transmission (Bull 1985; Chiel et al. 2009). Facultative mutualists have diverse functions on their host and can significantly impact on the success of biological control efforts (Hedges et al. 2008; Oliver et al. 2010). For example, S-endosymbionts such as *Rickettsia*, *Hamiltonella*, *Wolbachia*, *Regiella*, and *Serratia* can defend their hosts against parasitoids and fungal or viral pathogens (Ferrari et al. 2004; Hedges et al. 2008; Kambris et al. 2009; Oliver et al. 2003; Scarborough et al. 2005; Vorburger et al. 2010), improve tolerance to heat stress (Feldhaar 2011; Montllor et al. 2002), modify hosts' body color which influences their attractiveness to natural enemies (Tsuchida et al. 2010) and impact changes

in parasitoid oviposition behavior (Oliver et al. 2012). Reproductive manipulators like *Wolbachia*, *Arsenophonus*, *Cardinium* and *Rickettsia* can spread their infections into populations by biasing the sex ratio of their hosts towards production of females (Moran et al. 2008). They manipulate host insects through induction of different reproductive alterations such as thelytokous parthenogenesis (Hagimori et al. 2006; Stouthamer 1993; Stouthamer et al. 1990), feminization of genetic males (Fluger et al. 2012; Rigaud and Juchault 1995; Rousset et al. 1992), male killing (Engelstädter and Hurst 2009; Jiggins et al. 2000; von der Schulenburg et al. 2001; Werren et al. 2008) and oogenesis (Takano et al. 2017). *Wolbachia* and *Cardinium* can also induce cytoplasmic incompatibility (CI), a phenomenon where mating between same-species individuals with different strains or infection status fail to produce viable offspring (Engelstädter and Telschow 2009; Yen and Barr 1973). Such CI-inducing *Wolbachia* and *Cardinium* strains have been exploited to manipulate vector populations by interfering in the transmission of insect-borne diseases (Brelsfoard et al. 2009; Hancock et al. 2011; Iturbe-Ormaetxe et al. 2011; Zabalou et al. 2009).

The African citrus triozid (ACT), *Trioza erytreae* (Del Guercio), is the primary vector of *Candidatus* Liberibacter africanus (CLaf), the causative agent of Africa citrus greening disease (ACGD), the most serious citrus diseases on the African continent (Bové 2006; Graca 1991). The frequent use of synthetic insecticides to control vector populations and manage ACGD is often both economically and environmentally unsustainable; moreover, the use of insecticides has not been sufficient to stop the spread of the disease (Kruse et al. 2017). Many factors contribute to the failure of insecticides to manage citrus greening, including the unique way in which pathogen acquisition is linked to the reproductive cycle of the psyllid vector, the migratory ability of the vector, the ability of the vector to survive on alternate host plants, and the inability to apply insecticides on all citrus groves simultaneously (Kruse et al. 2017). Moreover, citrus greening research is made difficult because the pathogen is an obligate biotroph and cannot be grown in culture, thereby seriously affecting the development of resistant citrus varieties, which is additionally hampered by the lack of strong pathogen resistance in commercial citrus varieties and the lengthy time needed to conduct complicated breeding trials (Dutt et al. 2015). With these factors in mind, alternative methods for vector and disease management are urgently needed and thus more efforts are nowadays directed towards biological control. Parasitoids play an important role in the control of insect pests as they can reduce pest populations in natural ecosystems when they develop in or on their

hosts, extracting nourishment from them, thereby directly or indirectly killing them (Eggleton and Gaston 1990). Endosymbionts have also been shown to influence behavioral traits of parasitoid wasps (Oliver et al. 2012) and such traits could be exploited for control strategies. In ACT's closely related Asian citrus psyllid *Diaphorina citri* (*D. citri*) Kuwayama there are also major attempts to shut down genes that regulate pathogen acquisition, replication, or transmission using endosymbionts (Lu et al. 2012; Slatko et al. 2010; Zélé et al. 2012). Yet, little is known about the diversity and role of parasitoids and secondary symbionts of both *T. erytreae* and its indigenous parasitoids in Africa. To address this, we used diagnostic PCR and DNA sequence-based methods to assess the occurrence of ACT parasitoids and facultative endosymbionts in both *T. erytreae* and its parasitoids. Our aim was to characterize both the hosts and symbiont genes in order to (i) examine the diversity and phylogenetic relationships between *T. erytreae* and its parasitoid species, (ii) detect and identify the presence of ACT and parasitoid endosymbionts, and (iii) examine the phylogenetic relationships of the 16S rRNA gene sequences detected in *T. erytreae* and its parasitoids.

4.3 Materials and methods

4.3.1 Insect collection and identification

ACT specimens and parasitoids were collected between 2015 and 2017 from citrus and alternative *T. erytreae* host plants, from various field sites, principally in eastern, central and western Kenya. Parasitized *T. erytreae* nymphs were collected from a few citrus orchards in the Kericho and Kisumu regions of western Kenya. Developing nymphs on flush shoots of sweet orange (*Citrus sinensis* (L.) Osbeck) and lemon (*C. limon* Osbeck) were excised, placed in aerated plastic containers having moist filter papers and transported to the laboratories of the Nairobi campus of the International Centre of Insect Physiology and Ecology (*icipe*). These flush shoots were placed in separate Perspex cages (30cm × 30cm × 30 cm), kept in a controlled room temperature (24 ± 1^0C) and examined daily for emergence of adult parasitoids (Figure 4.1). A colony was further established by providing the emerged parasitoids with diluted honey and a fresh batch of ACT nymphs comprising of 4^{th} and 5^{th} instars feeding on citrus leaves. The newly emerged parasitoids were first identified based on morphologic traits and later sent for confirmation to *icipe*'s Biosystematics laboratory. Other adult parasitoids were stored in 95% ethanol at -20^0C for subsequent molecular identification.

Figure 4.1 *T.erytreae* and their parasitized nymphs: (a) Adult *T.erytreae* and nymph instars (b) mummified body of *T.erytreae* nymph (c) pre pupa of parasitoid wasp (d) mature pupa (e) parasitized nymphs with thoracic exit hole (f) emerged mature pupa of wasp

4.3.2 Genomic DNA extraction

Prior to extraction, DNA from external microbes that could contaminate the PCR reactions were removed by placing individual insects in sterile 2-mL centrifuge tubes containing 1.5 mL of 70 % alcohol (EtOH), vortexed vigorously for 1 min and the solution discarded, leaving the insects in the tube. The insects were sequentially washed with bleach (6% sodium hypochlorite) and rinsed three times with 2 mL of sterile water, and finally transferred individually to sterile 1.5 mL centrifuge tubes using a camel brush (Jeyaprakash and Hoy 2000; Meyer 2007).

DNA extraction was performed on the entire body of the insects to reduce the risk of missing infection. The insects were individually homogenized with sterile plastic pestles in 30µl of an extraction buffer (Bioline, UK) with 2 µl proteinase K (20mg/ml), and the mixture was incubated overnight at 56^0C followed by a 5-min heating period at 70^0C. Genomic DNA was extracted with the Bioline DNeasy Blood and Tissue kit (Bioline, London UK), following the manufacturer's instructions. The resultant DNA from each sample was eluted in AE buffer (Bioline, UK) to a final volume of 100 µL and quantified using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). The DNA was stored at -20°C for subsequent diagnostic polymerase chain reaction (cPCR) assays.

4.3.3 Screening and sequencing

The standard LepF1/LepR1 primer (Hebert et al. 2004) was used for amplification of the barcoding region of cytochrome oxidase subunit I gene (COI). The 16S ribosomal DNA gene (16S rDNA) was amplified using general 27F/1492R primer (Munson et al. 1991) which amplify this gene across all known eubacteria. Additionally, samples were screened using sequence-specific primers SpixoF/SpixoR (Duron et al. 2008) and WspecF/WspecR (Werren and Windsor 2000) for amplification of *Spiroplasma* and *Wolbachia*, respectively (Table 4.1).

We conducted an initial PCR screening (10 µl PCR reaction) for all the samples and those that scored as positive in the first PCR were then subjected to a second PCR reaction (30 µl total volume) for confirmation and subsequent sequencing. PCR reaction was conducted with 1 unit of MyTaq DNA polymerase (Bioline, UK), MyTaq PCR Buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers) (Bioline, UK), 10 µM of each primer, 25 mM $MgCl_2$ (Thermo Fischer, UK), and 15 ng/µL of DNA template. Amplification was performed in a Mastercycler Nexus Gradient thermocycler (Thermo Scientific, USA), under the following PCR conditions: 2 min at 95°C followed by 40 cycles 95°C for 30 secs, annealing temperature for each primer (Table 4.1) for 45secs, and 72°C for 1 min and a final elongation at 72°C for 10 min. All PCR runs included a negative control (sterile water instead of DNA) to spot any DNA contamination.

Table 4.1: Genes and primers used in polymerase chain reaction (PCR) assays

Primer pair (5'-3')	Target gene (fragment size)	Target group	Annealing temperature	Reference
LepF- ATT CAA CCA ATC ATA AAG ATA T LepR-TAA ACT TCT GGA TGT CCA AAA A	Mitochondrial COI (650 bp)	Most invertebrates	50°C	Hebert et al. 2004
27F- AGAGTTTGATCMTGGCTCAG 1492R- TACCTTGTTACGACTTCAC	16SrRNA (1470bp)	Most bacteria	58.7°C	Munson et al. 1991
WspecF- CAT ACC TAT TCG AAG GGA TAG WspecR- AGC TTC GAG TGA AAC CAA TTC	16SrRNA (438bp)	Wolbachia	56.9°C	Werren and Windsor 2000
SpixoF – TTAGGGGCTCAACCCCTAACC SpixoR – TCTGGCATTGCCAACTCTC	16SrRNA (810 bp)	Spiroplasma	52°C	Duron et al. 2008

The PCR products were then electrophoresed on a 1.5% agarose gel stained with ethidium bromide, and observed under an UV transilluminator (KETA GL imaging system, Wealtec Corp., USA). Extremely weak amplifications that did not yield enough templates for sequencing were regarded as negative, while strongly amplified positive samples with

expected band size were excised and purified using Isolate II PCR and Gel Kit (Bioline, UK) following the manufacturer's instructions. The purified products were then sent for bi-directional sequencing at Macrogen Inc., Europe Laboratories.

4.3.4 Sequence Analysis

The chromatograms were combined into contigs, then manually inspected and cleaned using BioEdit v.7.0.5.2 (Hall 1999), and submitted to NCBI for Blastn (Altschul et al. 1990) to determine the closest sequence identities. If a sequence was ≥95% identical to a sequence found in GenBank, it was assigned to that insect or bacterial species; however, if the sequence in the database was not identical to our sequence (< 95% identity), then our sequence was regarded as a different species or strain.

All sequences of COI and 16S rDNA genes of various hosts and bacteria used for phylogenetic analysis were obtained from GenBank. Multiple alignments of nucleotide sequences were generated by the program CLUSTALW (Thompson et al. 2002) within the MEGA version 7 software package (Kumar et al. 2016). Phylogenetic trees were constructed in MEGA 7 with the Maximum Likelihood method applied as the tree-building algorithm to visualize the patterns of divergence. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms (Gascuel 1997; Saitou and Nei 1987) to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with the superior log likelihood value (Tamura et al. 2013). The reliability of the clustering pattern in the trees was evaluated using a bootstrap analysis with 1,000 replicates.

4.4 Results

4.4.1 Identification of *T. erytreae* and its parasitoid species

Molecular characterization using Lep primer achieved positive DNA amplification with the required DNA concentration in 98 of the 150 *T. erytreae* adult specimens examined. The obtained 650bp COI sequences were subjected to a BLAST search against sequences available in the GenBank database and showed 98-100% level of similarity to *T. erytreae* given GenBank accessions numbers KY754656.1, KU517195.1 and KY754586.1 [*Trioza erytreae* (Hemiptera: Triozidae) Hodgetts et al. 2016; Khamis et al. 2017]. Maximum likelihood phylogeny of our sequence data with those other *Trioza* spp. (Figure 4.2) further

provided strong support for close affiliation to *T. erytreae* identified from Kenyan and South African populations

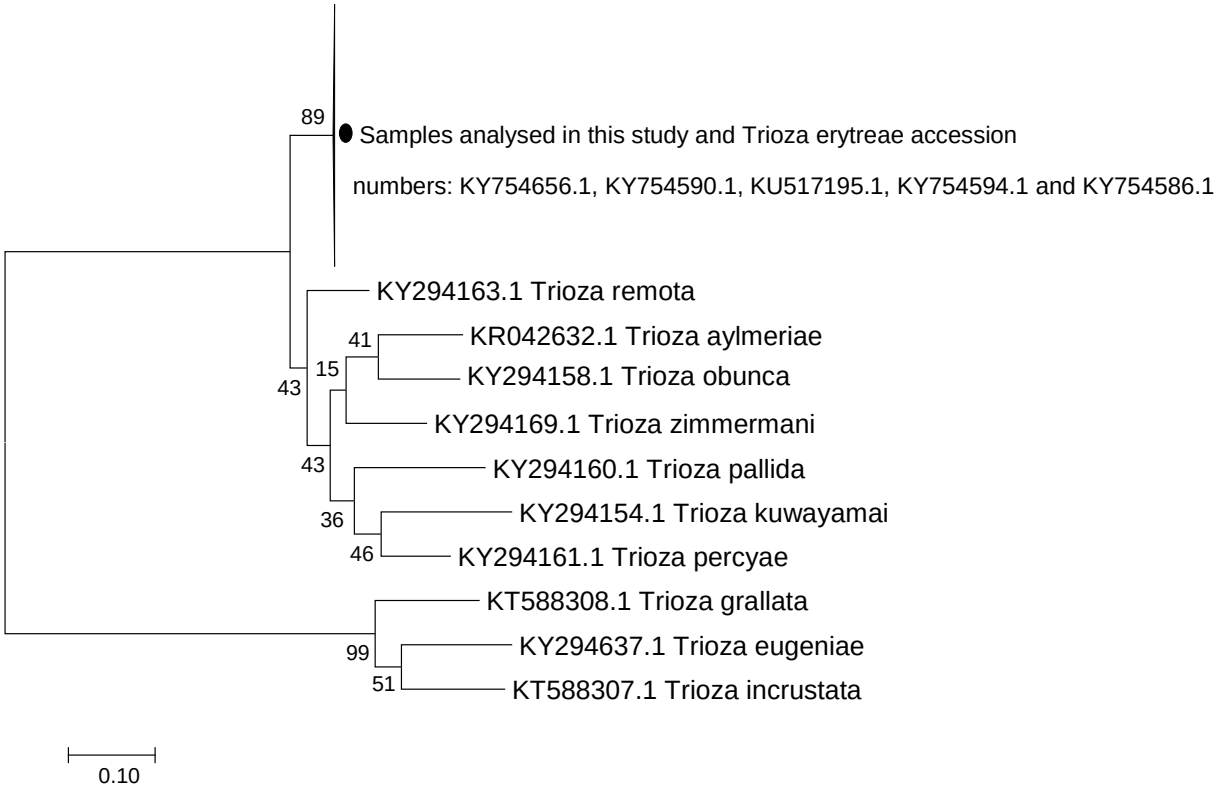


Figure 4.2 Condensed Phylogenetic tree showing evolutionary relationship of mtCOI gene sequences between *T. erytreae* samples from the study with previously submitted mtCOI sequences of other *Trioza* species from NCBI database. Tree was constructed by Maximum Likelihood analysis under Tamura-Nei model model inferred by MEGA 7.

Using the keys and descriptions of Prinsloo (1984) and Van Noort et al. (2015) five hymenopteran parasitoid species were identified based on their unique morphological characteristics; the eulophids *Tamarixia dryi* (Waterston) (as *Tetrastichus dryi* Waterston) and *Tetrastichus* sp., the encyrtids *Psyllaephagus pulvinatus* (Waterston) and *Aphidencyrtus cassatus* (Annecke) and the figitid, *Charipinae* sp. (Figure 4.3).

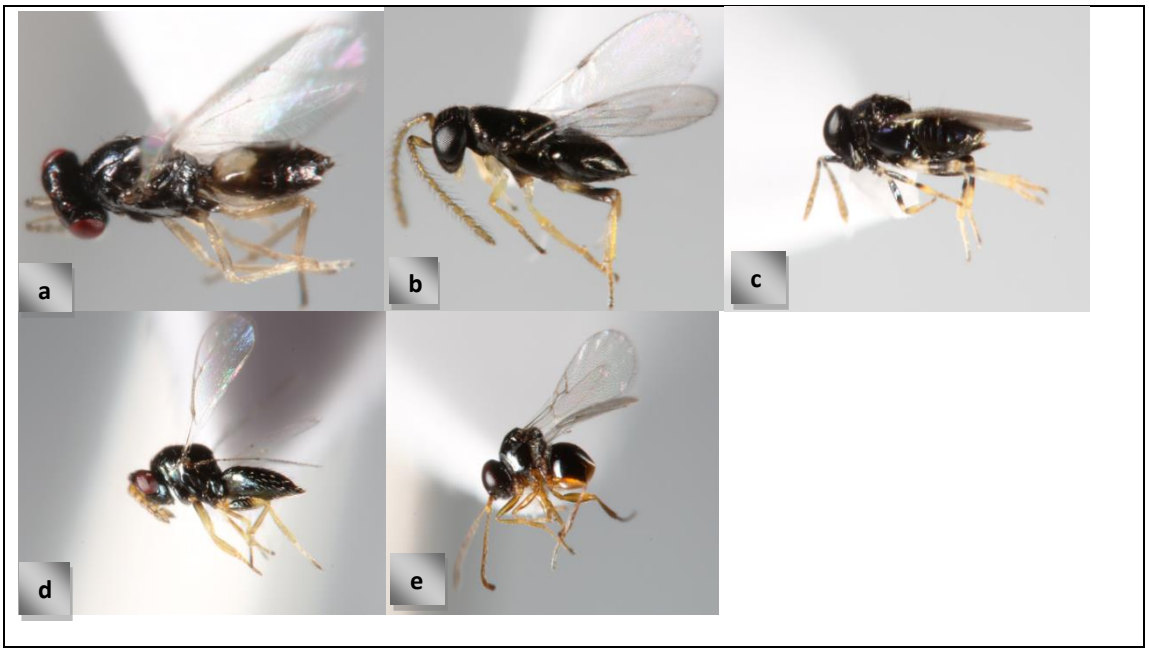


Figure 4.3 Morphologically identified *T.erytreae* parasitoid species from Kenya population: (a) cf. *Tamarixia dryi* (b) cf. *Psyllaephagus pulvinatus* (c) cf. *Aphidencytrus cassatus* (d) cf. *Tetrastichus sp*. (e) *Charipinae*

Additionally, positive DNA amplification was achieved in 76 of the 100 parasitoid specimens examined. Our COI sequences showed 94-95% level of similarity to *T. radiata* (Waterston) and other eulophid and encyrtid sequences in the GenBank, indicating the possibility of close but not identical relationships. Phylogenetic relationships clustered our sequences into five distinct clades (Figure 4.4), further supporting the results of the morphological identification. The first and second clade clustered separately from other identified parasitoid sequences in the GenBank, with the third clade clustering with the eulophid *Aprostocetus aethopis*, the fourth clade closely linking to *Tetrastichinae* spp. and the eulophid *Baryscapus* sp., while the fifth clade was closest to the encyrtid *Diaphorencytrus aligarhensis* (Shafee, Alam and Argarwal) species.

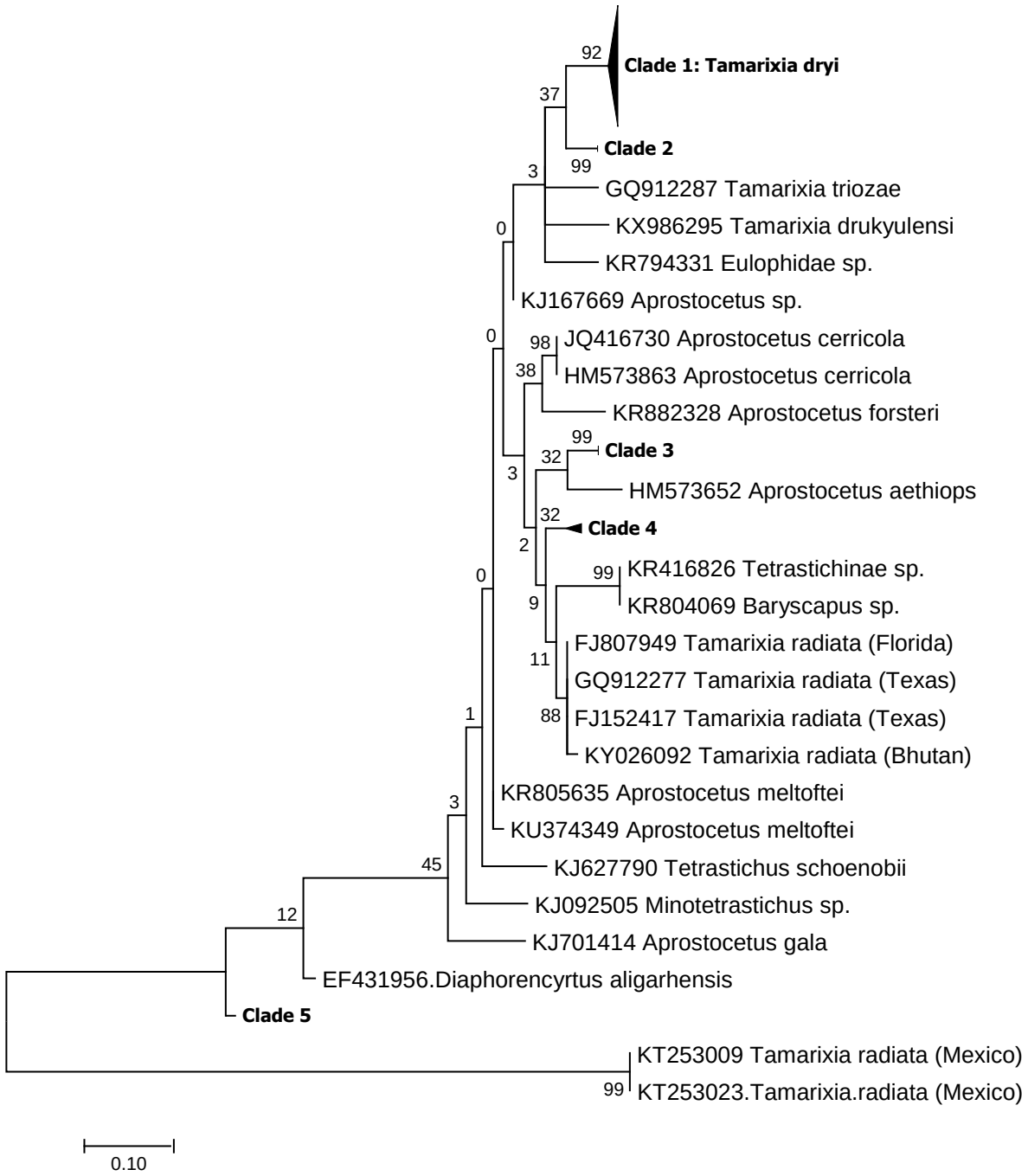


Figure 4.4 Condensed Phylogenetic tree showing evolutionary relationship of mtCOI gene sequences between parasitoid species from this study with the previously submitted mtCOI sequences of other eulophid and encytrid parasitoids from NCBI database. Tree was constructed by Maximum Likelihood analysis under Tamura-Nei model model inferred by MEGA 7.

4.4.2 Detection of bacterial symbionts

Polymerase chain reaction amplification with the general 16S rDNA eubacterial primer and *Wolbachia* specific primer showed products of expected 1,470 bp and 438 bp sizes, respectively. We did not detect *Spiroplasma* in our preliminary screening experiments using

SpixoF/SpixoR primer. CLaf found in *T. erytreae* showed 96-99% identity with sequences given GenBank accession numbers KY000560.1 (CLaf subsp. *capensis*), KX990288.1 (CLaf subsp. *tecleae*), KX990287.1 (CLaf subsp. *zanthoxylii*) and KY000562.1 (CLaf subsp. *clausena*) identified in other infected rutaceous plants and *T. erytreae* specimens (Roberts and Pietersen 2016). *Arsenophonus* found in *T. erytreae* showed 95-98% sequence identity with sequences given GenBank accession numbers AB038366.1 (*Arsenophonus* endosymbiont of *D. citri*), AY264673 (*Arsenophonus* endosymbiont of *Austroliococcus greville*) and MG257478 (*Arsenophonus* endosymbiont of *Hyalopterus pruni*), associated with *D. citri*, the whitefly *Bemisia tabaci* (Gennadius) and aphids, respectively (Subandiyah et al. 2000b; Thao and Baumann 2004; Zouari et al. 2018). We did not, however, detect the presence of *Arsenophonus* in the tested parasitoid specimens.

Furthermore, *Rickettsia* found in *T. erytreae* showed 98% identity to sequences given GenBank accession numbers JQ726774.1 (*Rickettsia* endosymbiont of *Nysius expressus*), KY799068.1 (*Rickettsia* sp.) and NR_074484.2 (*Rickettsia bellii* sp.) associated with stink bugs and other insects (Maina et al. 2017; Matsuura et al. 2012; Ogata et al. 2006). However, *Rickettsia* sequences found in parasitoid wasps showed 99% sequence identity to *Rickettsia* species sequences given GenBank accession numbers FM955311.1 (*Rickettsia* endosymbiont of *Deronectes semirufus*), KU586121.1 (*Rickettsia* symbiont of *Nephotettix cincticeps*) and JQ726774.1 (*Rickettsia Nysius expressus*) described in water beetles (Coleoptera: Dytiscidae), mosquitoes (Diptera: Culicidae) and sting bugs (Hemiptera: Pentatomidae), respectively (Guo et al. 2016; Küchler et al. 2009; Matsuura et al. 2012). Two other parasitoid sequences were 99% identical to *Cardinium hertigii* (DQ854699.1) identified in parasitic wasps (Giorgini et al. 2009) but we found no presence of *Cardinium* in our *T.erytreae* specimens.

The *Wolbachia* sequences of both *T. erytreae* and its parasitoids had at least 98-100% nucleotide identity to known *Wolbachia* 16S rDNA sequences in Supergroup B. Despite the fact that the universal 27F/1492R primer could not amplify *Wolbachia* in *T. erytreae*, it positively detected *Wolbachia* presence in most of the parasitoid specimens and gave 98-100% sequence identities to various *Wolbachia* strains. Nonetheless, targeted screening using *Wspec* specific primer confirmed that all our ACT samples were infected with different strains of *Wolbachia*.

A few other sequences gave 90-95% identities to sequences with GenBank accession numbers AB772201 (Sodalis-like endosymbiont of *Mesoptyelus fascialis*) and AB915782.1 (Sodalis endosymbiont of *Poecilocoris lewisi*) identified in heteropteran bugs (Hosokawa et al. 2015; Koga et al. 2013) AB559929.1 (secondary endosymbiont of *Curculio sikkimensis*) identified in weevils (Coleoptera: Curculionoidea) (Toju and Fukatsu 2011) and JQ063425.1 (endosymbiont of *Columbicola mjoebergi*) identified in lice (Smith et al. 2013). However, the specific genus of these symbionts could not be identified, but they could also represent other symbionts in *T. erytreae* populations. Additionally, the 27F/1492R universal eubacterial primer revealed the presence of *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Shigella*, *Streptococcus* and *Klebsiella*, though, due to the possibility of contamination by free-living bacteria not known to be symbionts of arthropods, these results were disregarded.

4.4.3 Frequency of secondary endosymbionts

The 27F/1492R primer revealed that 63 of the 98 (64%) *T. erytreae* adults and 50 of the 76 (66%) ACT parasitoids harbored S-endosymbiont, indicating that symbionts were common in both the vector and its natural enemies. Moreover, 12 (37%) 16S rDNA eubacterial sequences exhibited a high degree of similarity to sequences from *Arsenophonus*. Members of *Rickettsia* were detected in 10 (16%) of the *T. erytreae* sequences and 5 (10%) of its parasitoid sequences, while *Cardinium* was detected in only 2 (4%) of the parasitoid sequences. We also found low frequencies of a CLaf infection in 10 (16%) of the tested *T. erytreae* specimens. *Wolbachia* was the most frequent endosymbiont observed in both the psyllid and its parasitoids, with high infection rates of 100% (98/98) and 93% (71/76) in *T. erytreae* and its parasitoids, respectively.

4.4.4 Phylogenetic analysis of 16S rRNA gene sequences

Phylogenetic relationships were estimated using the sequences obtained in this study, as well as the 16SrRNA eubacterial sequences from other hosts available in GenBank (supplementary material Table S2). Maximum likelihood phylogenetic inferences clustered the identified eubacterial symbionts within α and γ proteobacteria subdivisions, with the separation strongly supported by the bootstrap method (Figure 4.5).

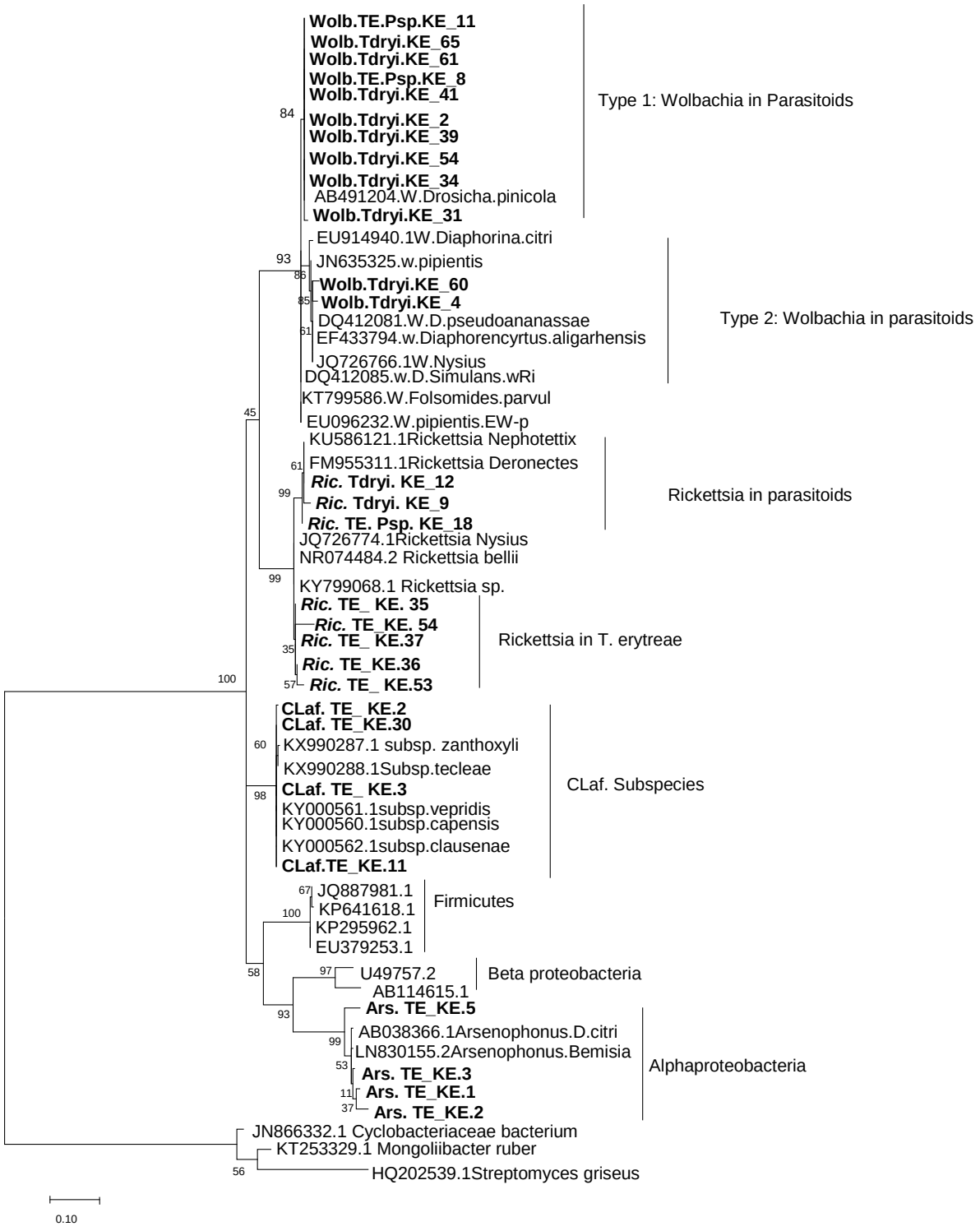


Figure 4.5 Placement of four endosymbiotic bacteria identified from *T.erytreae* and its parasitoids in 16S rDNA phylogeny of the Proteobacteria. Tree was constructed by Maximum Likelihood analysis under Tamura-Nei model model using MEGA 7. The bootstrap values obtained with 1,000 resamplings are shown at the nodes. The numbers in brackets are GenBank accession numbers. The sequences determined in this study are shown in bold.

Arsenophonus endosymbionts of the tested *T. erytreae* clustered in the α-proteobacteria clade together with the *Arsenophonus* symbiont from *D. citri* (AB038366.1) and the *Arsenophonus* endosymbiont of *B. tabaci* (LN830155.2) and the branching was highly supported by the bootstrap scores. The *Rickettsia* infecting *T. erytreae* and those infecting the parasitoids clustered separately, indicating the possibility that the psyllid and its parasitoids harbored different strains of the same bacterium. Phylogenetic analysis showed that the *Rickettsia* infecting *T. erytreae* was nearest to *Rickettsia bellii* and *Rickettsia* sp., while the *Rickettsia* infecting the parasitoid wasps was closest to the *Rickettsia* symbiont of *Deronectes semirufus* and the *Rickettsia* symbiont of *Nephotettix cincticeps*. On the other hand, the *Cardinium* infecting the parasitoids was closely related to the *Cardinium* endosymbiont of *B. tabaci* and *Candidatus* Cardinium hertigii (Figure 4.6).

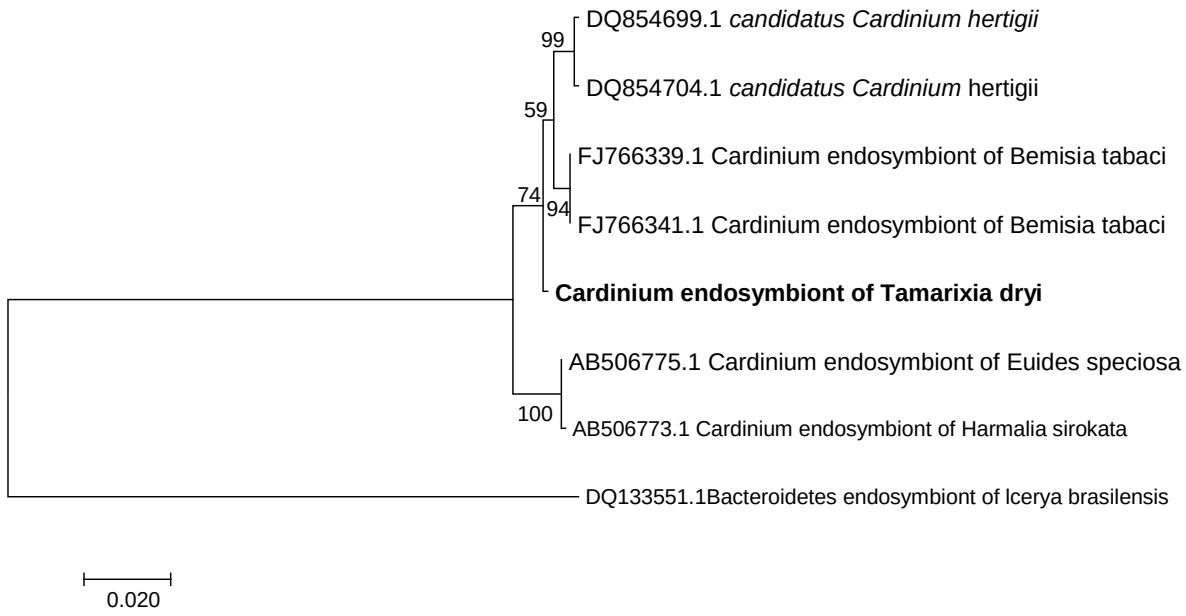


Figure 4.6 Phylogenetic tree showing the relationship of 16S rDNA Cardinium sequences obtained from Tamarixia dryi parasitoid with the previously submitted 16S rDNA sequences of Cardinium from NCBI database. Tree was constructed by Maximum Likelihood analysis under Tamura-Nei model model using MEGA 7. Bacteroidetes endosymbiont of Icerya brasilensis sequence is theoutgroup and numbers on the nodes indicate bootstrap values.

Additionally, phylogenetic analysis was performed using the *Wolbachia* 16S rDNA gene sequences obtained in this study, together with *Wolbachia* sequences from different hymenopteran and hemipteran species available in the GenBank (see Table S2). *Wolbachia* strains infecting both *T. erytreae* and its parasitoids did not cluster together, but all fell within *Wolbachia* Supergroup B (Figure 4.7). The *Wolbachia* infecting only the parasitoids clustered into three distinct clades, while those infecting only *T. erytreae* clustered into two separate clades indicating the likelihood of infections by multiple *Wolbachia* strains. We also noted that *Wolbachia* found in *T. erytreae* were closely related to *Wolbachia* strains previously reported from *D. citri*, *Bryobia praetiosa* (Trombidiformes: Tetranychidae), *Clostera anachoreta* (Lepidoptera: Notodontidae) and *Kleidocerys resedae* (Hemiptera: Lygaeidae), while the *Wolbachia* infecting the parasitoids were closely related to those reported in *Drosichia pinicola* (Hemiptera: Margarodidae), *Hofmannophila pseudospretella* (Lepidoptera: Oecophoridae), *Diaphorencytrus aligarhensis*, and *B. tabaci* (Figure 4.7). Interestingly, one *Wolbachia* sequence obtained from an ACT parasitoid fell into the same clade with a *Wolbachia* endosymbiont of *T. erytreae*, raising the possibility of a horizontal transfer between the two species.

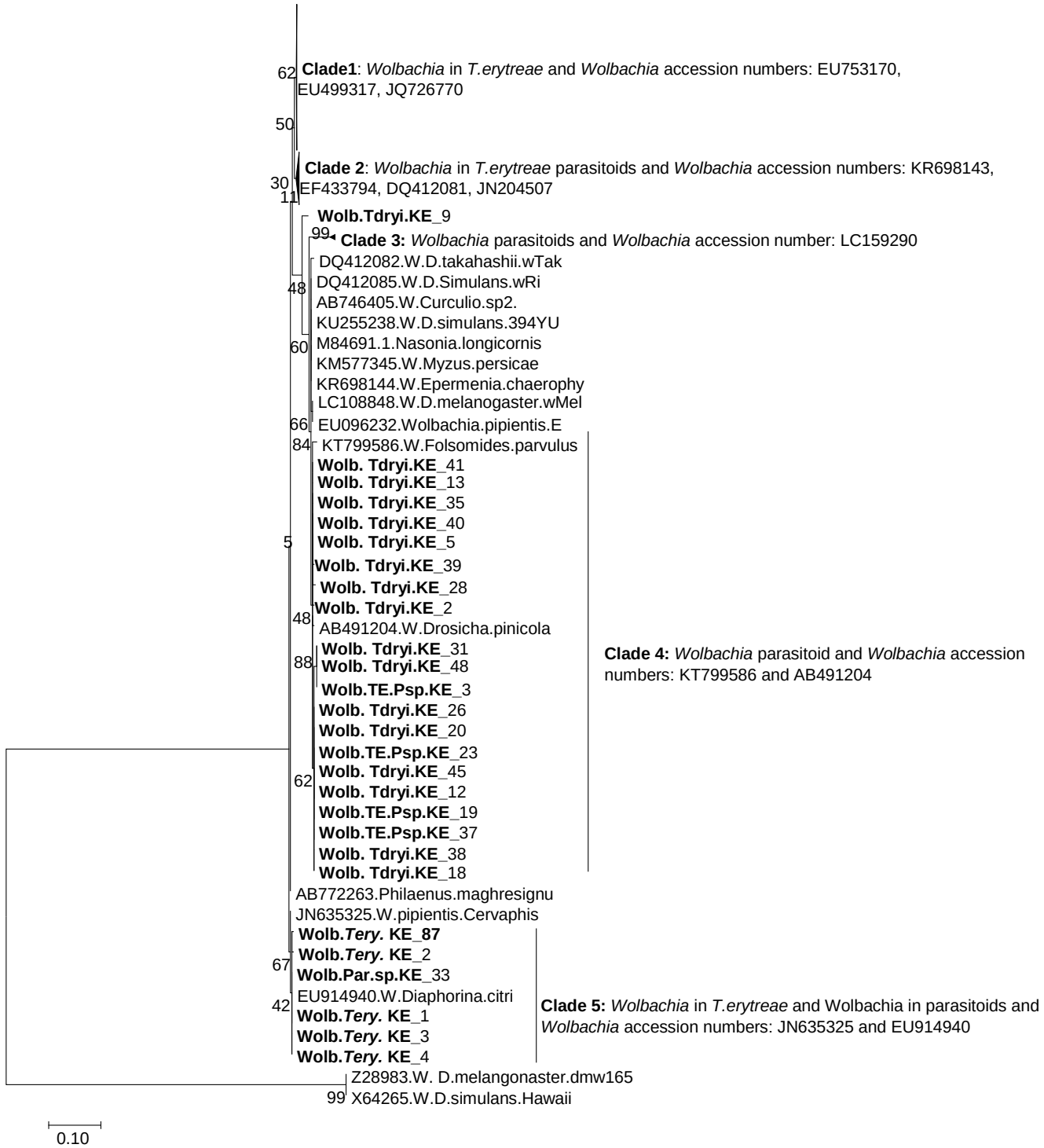


Figure 4.7 Phylogenetic tree showing the relationship of 16S rDNA Wolbachia sequences obtained from *T.erytreae* and parasitoid populations and previously submitted 16S rDNA sequences of *Wolbachia* from NCBI database. Tree was constructed by Maximum Likelihood analysis under Tamura-Nei model model using MEGA 7. Wolbachia dmw 165 strain and simulans Hawaii sequence are the out group and numbers on the nodes indicate bootstrap values.

4.5 Discussion

Current advances in taxonomy are based on the use of both morphological and molecular methodologies to facilitate species characterization and delineation. Moreover, molecular characterization of endosymbionts is a necessary step for understanding their influence on their host and further provides avenues through which these symbionts could be exploited in new approaches for pest and disease management. To our best knowledge, we here report for the first time results from a molecular survey of *T. erytreae* and its parasitoid wasps using DNA barcoding primers and further provide evidence for the presence of symbiotic bacteria in both the psyllid and its natural enemies.

Our study expands on the previous work of Waterston (1922) where conventional taxonomy was used to identify parasitoid species associated with *T. erytreae* in Kenya. We initially carried out a meticulous morphological identification of the found ACT parasitoid specimens. A number of parasitoid species were identified, five of which satisfactorily fit the original description of Waterston for *T. dryi, P. pulvinatus, A. cassatus, Tetrastichus* sp., and *Charipinae* (Annecke and Cilliers 1963; Gahan 1932; Graham 1987; Paretas-Martínez et al. 2007; Robinson 1960; Waterston 1922). Likewise, our molecular and phylogenetic analysis of the mtCOI gene clustered the sequenced parasitoid specimens into five distinct clades. The first two clusters could not successfully link to any barcode accession reference found in the GenBank. Thus, we hereafter refer to the sequences in the first clade as those of *T. dryi*. The third, fourth and fifth clade were closely linked to *Aprostocetus aethiops*, *Tetrastichinae* sp. and *Diaphorencytrus aligarhensis*, respectively. None of our specimens clustered with *T. radiata*, indicating the distant relationship of this species from the parasitoids found in Kenya.

Parasitoids can vary significantly in their effectiveness depending on geographical location and seasonal fluctuations in their populations (Daniel and Moran 1972). For instance, *T. dryi* and *P. pulvinatus* in sync can considerably check ACT populations (Van den Berg and Greenland 2000). For instance, Van der Merwe (1923) observed that >75% of *T. erytreae* nymphs were parasitized by these two species, whereas Catling (1969) found that at least 40-50% of ACT nymphs were parasitized during periods of favorable synchrony between the two parasitoid species and their host, while <10% were parasitized under conditions of poor synchrony. Furthermore, *T. dryi* was introduced from South Africa to Réunion Island and Mauritius for ACT biological control (Aubert 1975; Etienne and Aubert

1980) and successfully reduced the *T. erytreae* populations on both islands (Aubert and Quilici 1983, 1988). Future efforts for effective biological control of ACT and management of ACGD in Kenya should therefore focus on these highly promising two parasitoid species.

Our results indicate substantial diversity and infection frequency of S-endosymbionts among *T. erytreae* and its parasitoid wasps. *Wolbachia* was the most common observed endosymbiont, with fixed infection rates of 100% in ACT and 93% in the parasitoids. Studies on the closely related Asian citrus psyllid *D. citri* also showed that *Wolbachia* is extremely common with 100% infection rates in Floridian and Brazilian populations (Guidolin and Cônsoli 2013; Hoffmann et al. 2014). The fixed infection suggests the possibility that *Wolbachia* may be harbored and integrated in the mycetomal or syncitium endosymbiotic system of ACT (Subandiyah et al. 2000) and may play important biological roles in both the psyllid and its parasitoids i.e. some *Wolbachia* strains harbored by parasitoid insects have been shown to induce thelytokous parthenogenesis in their hosts (Arakaki et al 2000). Since only female parasitoids kill their hosts, a parthenogenetic phenotype in a parasitoid would have several advantages on insect pest control such as (i) a drop in the cost of mass-producing parasitoids for release due to the fact that no males are produced, (ii) rapid population growth due to the higher number of females, and (iii) easier establishment because no mating is required (Bourtzis 2008; Stouthamer 1993). The lack of amplification of *Wolbachia* in *T. erytreae* using the universal 27F/1492R primer in this study could be linked to low titer of *Wolbachia*, an over expression of the other endosymbionts in *T. erytreae* or a lack of an optimized identification (Guidolin and Cônsoli 2013).

Phylogenetic analysis on the basis of 16S rRNA gene sequences placed *Wolbachia* in both ACT and its parasitoid into Supergroup B. Moreover, we observed five distinct *Wolbachia* clades, indicating the possibility of multiple infections with different *Wolbachia* strains within the pest and its parasitoids. *Wolbachia* is sometimes associated with reproductive alterations in their insect hosts, and infection of a species with multiple and/or different strains of *Wolbachia* can result in CI (Engelstädter and Telschow 2009; Yen and Barr 1973). Such *Wolbachia*-induced CI can potentially be employed either for the control of agricultural pests and disease vectors through the Incompatible Insect Technique (IIT), or by spreading a desirable genotype through populations, such as the inability of a vector species to transmit a pathogen (Apostolaki et al. 2011; Kambris et al. 2009; Laven 1967; Turley et al. 2009; Zabalou et al. 2004). Taken together, our results suggest that *Wolbachia* is abundantly

occurring in *T. erytreae* and its parasitoid and further studies should investigate the suitability of these different *Wolbachia* strains for use in insect pest management.

Most of the *Wolbachia* we found in *T. erytreae* clustered separately from those of their parasitoids. Yet in one case a *Wolbachia* sequence from *T. erytreae* occurred in the same clade as that of one of the parasitoid species. This could be an indication of ongoing horizontal transfers of *Wolbachia* between the *T. erytreae* host and its parasitoids. Parasitism attempts could be another potential mechanistic hypothesis for horizontal bacterial transfer between nonrelated hosts. For example, the ovipositor of a parasitoid wasp can become contaminated with *Wolbachia* when it probes non-lethally a *Wolbachia*-infected host and a subsequent probing of a *Wolbachia*-uninfected *T. erytreae* nymph could result in newly and stably infected ACT. Some studies reported on infection of both hosts and parasitoids with *Wolbachia*. For example, West et al. (1998) detected *Wolbachia* in 38% of leaf-mining Gracillarid moths and 28% of their associated wasp parasitoids, but no close relationships were detected using phylogenetic analysis between the *Wolbachia* strains in the host and its parasitoids. In contrast, Vavre et al. (1999) determined a close relationships between several *Wolbachia* strains infecting *Drosophila* spp. and their parasitoid wasps. Yet our data should be interpreted with certain caution since we only found one individual sequence from a parasitoid species clustered with ACT.

We recorded low *Rickettsia* infection frequencies in both *T. erytreae* and its parasitoids depicting its lack of fixed infection. Therefore *Rickettsia* are probably not important for the survival or reproduction of the host insects. On the basis of 16S rRNA gene data, the ACT *Rickettsia* symbionts clustered separately from those of the parasitoid wasps, suggesting that the psyllid and its parasitoids harbor different strains of *Rickettsia*. *Rickettsia* strains show diverse effects in a wide range of insects (Lawson et al. 2001; Sakurai et al. 2005; von der Schulenburg et al. 2001; Weinert et al. 2009; Werren et al. 1994) and further research should try to elucidate in detail the potential impact it can have on ACT and its parasitic wasps.

The *Arsenophonus* symbiont was specifically harbored in *T.erytreae*; however, its imperfect infection rate could suggest that they may be facultative guest microbes of a commensal or parasitic nature (Subandiyah et al. 2000b). Based on the 16S rRNA gene phylogeny, ACT's *Arsenophonus* formed a distinct clade in the gamma subdivision of *Proteobacteria* and was close to those found in *B. tabaci* and *D. citri*. The *Arsenophonus*

genus has been reported in numerous insect species with a diversity of phenotypic effects associated with its infection (Werren et al. 1986; Ferree et al. 2008), though its effects are, however, not fully understood. Some have been reported to behave as obligate mutualists in haematophagous insects, exhibiting nutritional role (Dale et al. 2006), or as facultative mutualists protecting against parasitoid attacks in psyllids (Hansen et al. 2007) while some manipulate the reproduction of various parasitoid wasps by inducing a female-biased sex ratio distortion (Duron et al. 2010; Ferree et al. 2008; Werren et al. 1986).

A *Cardinium* infection was found only in ACT's parasitoid. It has been reported to widely occur in other arthropods like the parasitoid wasp *Encarsia pergandiella* (Hymenoptera: Aphelinidae) (Hunter et al. 2003), the spider mites *Eotetranychus suginamensis* (Prostigmata:Tetranychidae) (Gotoh et al. 2007) and *Bryobia sarothamni* (Prostigmata:Tetranychidae) (Ros and Breeuwer 2009), and the whitebacked planthopper *Sogatella furcifera* (Hemiptera: Delphacidae) (Zhang et al. 2012). It can alter reproductive ability via feminization (Weeks et al. 2001; Giorgini et al. 2009), parthenogenesis (Provencher et al. 2005), and CI (Hunter et al. 2003) of its infected hosts, which in turn, is helpful for its spread within the different host populations (Blackman and Cahill 1998; Giorgini et al. 2009).

In conclusion, our results demonstrates that a large proportion of ACT and parasitoid species are infected by diverse S-endosymbionts and further provide a useful background for investigating their roles in hosts' biology. The high infection frequency of *Wolbachia* warrants to further examine in detail the identified strains to better understand and potentially explore *Wolbachia* as an alternative control strategy for *T. erytreae* in Africa. While most the ACT and its parasitoids tested in our study proved to be positive for an endosymbiont infection, further extensive geographical sampling of ACT and its parasitoids in Africa are needed to precisely estimate the infection frequency and diversity of endosymbionts in their host population. Moreover with the identification of several ACT parasitoid species, mass-rearing programs for augmentative biological control should be explored as part of future biological and integrated pest and disease management strategies against ACT and ACGD, respectively.

5 Synthesis

Agriculture has continued to be a fundamental instrument for sustainable development in many sub-Saharan African (SSA) countries, offering new opportunities and pathways out of poverty through smallholder farming, employment in high-value crop production, entrepreneurship and jobs in the rural and nonfarm economies. Despite enormous potential of agriculture in contributing to the economy of many SSA countries, food and nutritional insecurity remain high and poverty predominates in many rural areas. Improving the productivity, profitability, and sustainability of high value crop production like fruticulture for smallholders is recognized as one of the major pathways for using agriculture for development, improving livelihoods and alleviating poverty in SSA (World Development Report 2008). Among the diversity of fruits cultivated in SSA, citrus ranks high as both food and cash crop, as a large proportion of the rural population, mainly smallholders (Seif 1996), are involved in the cultivation and marketing of the crop.

However, over the last decade, citrus production has been on the decline in Kenya and Tanzania, with minimal efforts to reverse this trend. One of the most economically important pests of citrus is the African citrus triozid, *T. erytreae* which transmits the devastating phloem-limited bacterium, *Candidatus* Liberibacter africanus responsible for causing African citrus greening disease, a disease with no known cure, that has crippled most affected citrus industries and poses a serious threat to the survival of citrus production in the two countries and beyond (Kilalo et al. 2009; Nyambo 2009). Moreover, the recent detection of the Asian citrus psyllid *D. citri*, vector of the Asiatic citrus greening strain *Candidatus* Liberibacter asiaticus (Saponari et al. 2010; Shimwela et al. 2016), in Africa further constitutes an even greater threat to the survival of the citrus industry in Africa.

In this regard, a countrywide survey was conducted to come up with comprehensive data on the extent of incidence and severity of ACGD, with the main goal of providing valuable information on the current status of ACGD in Kenya. This information could be used for rehabilitating and sustaining the local citrus industry in Kenya as well as in Tanzania. An additional objective was to identify the circulating pathogen strains in citrus plant tissues and insect vectors to ascertain the reported cases of CLas and *D. citri* and monitor the spread of the disease. Furthermore, the survey aimed to detect areas where ACGD impact was absent or relatively low for the re-establishment and expansion of insect-proof nurseries for production of clean nursery stock free of the disease.

A widespread occurrence of the CLaf subsp. clausena (CLafcl) on citrus trees was

observed throughout the surveyed regions of Kenya. In contrast, only in a very low proportion of the ACT vectors a CLaf infection could be detected, and none of the analyzed *D. citri* showed any signs of a CLaf or CLas infection. The level of disease incidence and severity varied across the different regions, with chronic greening situation observed in the Upper midland (1,300-1,800 m.a.s.l.) and Lower midland (800-1,300 m.a.s.l.) regions. Moreover, both *T. erytreae* and *D. citri* vectors coexisted at high elevations in the UM and LM regions, illustrating that *D. citri* was spreading fast in these regions, quickly adapting to new geographical areas and ecologies, thus potentially having a high chance of acquiring the CLaf pathogen in the future. The detected absence of the CLaf pathogen and its ACT vector in the major citrus production regions of Makueni and Kitui of Kenya and Muheza in Tanzania could be used to restrict the movement of contaminated planting materials from high disease impact areas to areas of low disease incidence, hence reducing the spread of the disease. The association of CLafcl with citrus also stresses the potential importance of *Clausena anisata* trees as reservoirs for CLaf and shed more light on existence of unrecognized alternative host plants as reservoirs of both CLaf and CLas. Farmers should, therefore, be advised to eliminate *C. anisata* trees or any other alternate host plants in the proximity of their citrus orchards to limit the population build-up of potentially infected psyllid vectors. Yet, more surveys and specimen screening data are needed to rule out the possibility of *D. citri* transmitting CLaf.

On a local level, citrus farming practices were not well developed and there were several constraining factors that impeded citrus growth and development. Citrus production was practiced by small-scale farmers, who committed part of their land to grow citrus as a cash crop or for subsidiary use. Additionally, citrus trees were intercropped with food crops for efficient land use. Unfortunately, these crops may modify the microclimate to favor the rapid multiplication of insect pests, thereby creating more problems in the citrus orchards. Over 90% of the farmers could not recognize ACGD symptoms or the psyllid vector. The farmers principally relied on one another for information on citrus pest management practices due to lack of reliable technical information. Moreover, they often used citrus seedlings from uncertified sources like individual farmers locally producing them in their backyard nurseries. All these factors may have contributed towards the spread of ACGD which calls for urgent control measures by the National Agricultural Research Systems in both countries together with other government agencies and citrus industries in Kenya and Tanzania. These agencies should enable the establishment and improvement of a preventive and corrective plant

quarantine and health system which can restrain the entrance and spread of ACGD and its vector(s). Further, these agencies need to work to integrate different levels of participants such as citrus farmers, extension officers, and pest and disease scouts to collaborate in identifying diseased plants, eradicating them and controlling vector populations with the ultimate aim of increasing the growth and development of citrus production.

The current understanding of vector-pathogen interactions and their implications for the establishment and spread of ACGD remains limited. No studies have explored the direct effects of the CLaf pathogen on fitness-related variables of its ACT vector. Moreover, the link between dispersal of infected and non-infected ACTs in the presence and absence of citrus and alternative host plants is not yet well established to determine safe isolation distances for quarantine and eradication purposes in Africa. Studies on host-pathogen interactions are, therefore, a key to better understand the transmission and epidemiology of important vector-transmitted plant diseases and could lead to the development of successful pest and disease management strategies. In this study it could be established that CLaf infected (CLaf+) ACT conferred some fitness benefits which included increased fecundity, fertility, faster egg-to-adult development time, higher female ratio and dispersal capability, all of which potentially help to promote the spread of infection throughout a host population, leading to more severe damage to citrus crops. However, the CLaf+ ACT vectors also incurred some fitness costs such as reduced nymphal and adult survival. The shorter lifespan in CLaf+ ACT females could be linked to their high rate of fecundity, suggesting trade-offs between survival and fecundity. Such trade-offs in pathogen vectors are of particular interest because they may constrain the evolution and epidemiology of a disease (Susi and Laine 2013). Overall, results of this study suggest that CLaf infection had fitness effects on ACT, but there was no clear-cut distinction between benefits and costs of infection, since the pathogen affected several fitness parameters of the vector in different directions, resulting in simultaneous fitness gains and costs for CLaf and ACT, and distinguishing the two outcomes became rather complicated. These complex and subtle interactions between fitness traits and the effects of pathogen infection can all contribute to strong ecological impacts and evolutionary pressures on the host and the pathogen, and thus ultimately on the epidemiology of disease.

CLaf+ ACTs exhibited increased dispersal capabilities and a subsequent net directed movement from *Citrus limon* to *C. anisata* and *Murraya koenigii*, the latter two ACT alternate host plants. However, it remains to be elucidated what factors caused the

preferential movement of ACT to these alternate host plants. Other studies have shown that plant volatiles can play an important role in the selection of host plants by herbivorous arthropods, thus creating avenues for such host plants to be exploited as trap crops. It would be, therefore, interesting to evaluate some of the volatile profiles from the young leaves of these alternate host plants which could be effectively used as attractants or repellents for greening disease vectors. The potential identification of volatile compounds that elicit behavioral responses in ACT could also be the first step for the development of a push-pull strategy for ACGD vector control.

Biological pest control often employs the use of their predators and parasitoids (van den Berg et al. 1987) and lately as well their endosymbionts (Brelsfoard et al. 2009; Hancock et al. 2011; Iturbe-Ormaetxe et al. 2011) to reduce pest populations. In this context, knowledge on ACT and its parasitoid endosymbionts could be of applied value, as the latter could provide novel avenues for pest control. However, little is known about ACT parasitoids in Kenya (Africa) and no studies on the parasitoid diversity have been undertaken. In addition, no studies have been done on both ACT and parasitoids endosymbionts, and extensive molecular characterization could lead to the discovery of new undescribed species. Thus, the diversity and phylogenetic relationships among ACT parasitoids in Kenya were explored. We compared phylogenies of the 16S rRNA gene sequences detected in the hosts and parasitoids, in the light of association and transmission mode of the endosymbionts. It was possible to describe the main endosymbionts harbored by ACT and its parasitoids. Several ACT parasitoid species could be identified like *Tamarixia dryi*, *Psyllaephagus pulvinatus*, *Tetrastichus* sp, *Aphidencyrtus cassatus* and *Charipinae* sp. In addition, four eubacterial symbionts (*Wolbachia*, *Rickettsia*, *Arsenophonus* and *Candidatus* Liberibacter sp.) were detected in *T. erytreae*, while the parasitoids harbored three symbionts (*Wolbachia*, *Rickettsia* and *Cardinuim*). These results provide a useful starting point for further investigating the roles of these endosymbionts in their hosts' biology. Additionally, the high infection frequency of *Wolbachia* warrants more in detail research of the identified strains to better understand their potential as an alternative control strategy for *T. erytreae* in Africa. Moreover, with the identification of several ACT parasitoid species, there parasitism efficiency should be assessed, and in case of high impact on their host populations mass-rearing programs for augmentative biological control should be explored as part of future biological and integrated pest and disease management strategies against ACT and ACGD, respectively.

6 Outlook

This thesis has examined the greening situation in East Africa and explored interactions between the CLaf pathogen and its ACT vector. It has also laid down the foundation for further citrus integrated pest management research.

Additional experiments and possible future research directions are described in the following sections:

Increased intensive and systematic surveillance for the alien *D. citri*, invasive *T. erytreae* and Liberibacter pathogens

The presence of both two vectors (*D. citri* and *T. erytreae*) and greening pathogens (CLas and CLaf) are now reported in East Africa and the probability of these vectors carrying and transmitting either or both pathogens poses further significant threat to the citrus industry in the region and beyond. Furthermore, the prevalence of the *Candidatus* Liberibacter pathogens on either of the vectors may likely have implications for CGD and psyllid management strategies. This underscores the need for more intensive surveillance activities for detection of the invasive and alien vector and pathogens even at low incidence levels. This is crucial for early warning and may enable the development of successful and cost-effective control and eradication of the greening epidemic. Continuous surveillance for *D. citri* and *T. erytreae* may also lead to the discovery of additional ecological ranges of the vectors. More detailed information on the seasonal phenology of the disease and its vectors will also enable better timed spraying regimes of citrus farmers.

Field monitoring of alternative host plants

The host range of *T. erytreae* and *D. citri* is limited to citrus and some rutaceous relatives and such alternate host plants may accelerate the spread of CGD in citrus. This study established a widespread occurrence of CLaf subsp. clausena (Lafcl) on citrus trees in Kenya. The association of Lafcl with citrus stresses the potential importance of *C. anisata* trees as reservoirs for CLaf and highlights the possible existence of additional so far unrecognized alternative CGD host plants that could act as reservoirs of both CLaf and CLas. Thus, there is need to explore in greater detail the potential reservoir hosts for CLaf within the Rutaceae family and determine whether CGD pathogens can be transmitted to and/or acquired from these other potential rutaceous hosts.

Identification of plant volatiles in non-host plants associated with ACT

The screen house studies indicated that most ACT adults dispersed to *M. koenigii* and *C. anisata* but the factors that caused the preferential movement of ACT to these alternate host plants remain to be elucidated. Thus, the chemical volatile profiles from young leaves of these alternate host plants should be identified and subsequently evaluated as they could be used as effective vector attractants or repellents as a first step in the development of a push-pull strategy for ACGD vector control.

Epidemiological modelling to predict and trace the dynamics of ACGD

Epidemiological models such as temporal stochastic models provide a means to integrate the current status of ACGD knowledge and provide a set of tools to compare the effectiveness of different control scenarios. Such models will help monitor and predict ACGD epidemic incidence and calculate the probability of the disease spread over time using likelihood estimates of the primary and secondary infection foci. The results from these models may be essential for early detection of CGD and thus help control the spread of the pathogens. It may be also useful in formulation of future regulatory policies regarding trade of citrus.

Diagnosis of CLaf using root samples

Early and accurate diagnosis of CGD is essential for effective prevention, control and management of the disease, and roots have been found to serve as a more reliable diagnostic sample unit compared to leaves (Kunta et al. 2014). The preliminary screening of root samples in this study, using the classical OI1/OI2c and A2J5 cPCR primers, produced several non-specific band products while other samples could not produce any amplification product. Therefore, more reliable methods for root sample screening should be explored for accurate detection of the bacterium.

Dispersal studies at a large scale

The dispersal behavior of CLaf+ and CLaf- ACT was studied here at a small scale in a screen house. It would also be interesting to focus on large scale dispersal in open citrus field to bring an additional angle on ACT dispersal abilities.

Establish roles of endosymbionts present in ACT and its parasitoids

The development of environmentally friendly alternative pest management strategies that do not rely on the use of insecticides is critical for the control and elimination of vector-borne diseases in Africa and beyond. One of the most promising alternative vector management strategies involves endosymbiotic bacteria that could decrease the vectoral capacity of their hosts. This study identified diverse endosymbionts in both ACT and its parasitoids. Thus, it would, therefore, be interesting to investigate their roles in both *T. erytreae* and its parasitoids. The abundant occurrence of *Wolbachia* in ACT also warrants further studies to investigate the suitability of the different strains as useful tools for vector or pathogen management strategies.

Mass rearing and release of identified primary parasitoids species

In this study two ACT primary parasitoids, namely *T. dryi* and *P. pulvinatus* were identified. *Tamarixia dryi* has widely been used as a biological control agent of ACT, e.g. on Réunion Island and Mauritius, and significantly helped to reduce ACT populations. Hence, efficient mass rearing techniques for the parasitoids in greenhouses or insectary should be developed for subsequent augmentative releases for control of psyllid populations in Kenya and other ACGD affected countries in Africa.

Breeding for resistance

No CGD resistant citrus seedling trees or scion-rootstock combinations have as yet been identified (da Graça et al. 2015), though some studies have shown variations in sensitivity in different rootstocks (Boava et al. 2015). Thus, production of clean planting material through tissue culture systems, breeding for CLaf resistance (Boava et al. 2015) and utilization of embryo rescued seed from healthy chimera sections of diseased fruits (Vuuren and Manicom 2009) should also be the foci of future research. These could halt or slow the spread of the CGD-associated bacteria by their vectors.

The outstanding future research directions show the need for both researchers and practitioners to work together in addressing the need to develop integrated pest and disease management to combat citrus greening in Africa.

7 References

Abbot P, Dill LM (2001) Sexually transmitted parasites and sexual selection in the milkweed leaf beetle, Labidomera clivicollis. *Oikos 92*(1): 91-100

Adamo SA (1999) Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. *Animal Behaviour 57*(1): 117-124

Akhtar MA, Ahmad I (1999) Incidence of citrus greening disease in Pakistan. *Pakistan Journal of Phytopathology 11*(1): 1-5

Altschul SF, Gish W, Miller W et al (1990) Basic local alignment search tool. *Journal of molecular biology 215*(3): 403-410

Ammar ED, Walter AJ, Hall DG (2013) New excised-leaf assay method to test inoculativity of Asian citrus psyllid (Hemiptera: Psyllidae) with Candidatus Liberibacter asiaticus associated with citrus huanglongbing disease. *Journal of economic entomology 106*(1): 25-35

Annecke CJ (1963) The citrus psylla, Trioza erytreae (Del Guercio), and its parasites. *South African Journal of Agricultural Science 6*(2): 187-191

Anon (1982) Citrus Greening Disease in Kenya and Recommendation for Its Control. Ministry of Agriculture Report Presented to the Director of Agriculture by the Committee on Greening in Citrus and Fruit Tree Nursery Control

Apostolaki A, Livadaras I, Saridaki A et al (2011) Transinfection of the olive fruit fly Bactrocera oleae with Wolbachia: towards a symbiont-based population control strategy. *Journal of Applied Entomology 135*(7): 546-553

Arakaki N, Noda H, Yamagishi K (2000) Wolbachia-induced parthenogenesis in the egg parasitoid Telenomus nawai. *Entomologia Experimentalis et Applicata 96*(2): 177-184

Aubert B (1987) Trioza erytreae Del Guercio and Diaphorina citri Kuwayama (Homoptera: Psylloidea), the two vectors of citrus greening disease: Biological aspects and possible control strategies. *Fruits 42*(3): 149-162

Aubert B, Grisoni M, Villemin M et al (1996, January) A case study of huanglongbing

(greening) control in Reunion. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 13, No. 13)

Aubert B (1975) The integrated control of citrus pests in South Africa and its possible applications in the Mascarene Islands. *Fruits (France)*

Aubert B (1987) Trioza erytreae Del Guercio and Diaphorina citri Kuwayama (Homoptera: Psylloidea), the two vectors of citrus greening disease: Biological aspects and possible control strategies. *Fruits 42*(3): 149-162

Aubert B (1984) The Asian and African citrus psyllid Diaphorina citri Kuwayama, Trioza erytreae (Del Guercio),(Homoptera: Psyllidae) in the south west of Saudi Arabia. *Proposals for an integrated control programme. Report to the Food and Agriculture Organization, Rome, Italy*

Aubert B, Quilici S (1983) Biological control of psyllids in Reunion Island with newly established chalcid hymenopterous [citrus; Trioza erytreae, Diaphorina citri, Trioza eastopi; Tetrastichus dryi, T. radiatus, Diaphorencyrtus aligarhensis]. *Fruits (France)*

Aubert B, Quilici S (1988, January) Monitoring adult psyllas on yellow traps in Reunion Island. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 10, No. 10)

Avery PB, Hunter WB, Hall DG et al (2009) Diaphorina citri (Hemiptera: Psyllidae) infection and dissemination of the entomopathogenic fungus Isaria fumosorosea (Hypocreales: Cordycipitaceae) under laboratory conditions. *Florida Entomologist 92*(4): 608-618

Avery PB, Wekesa VW, Hunter WB et al (2011) Effects of the fungus Isaria fumosorosea (Hypocreales: Cordycipitaceae) on reduced feeding and mortality of the Asian citrus psyllid, Diaphorina citri (Hemiptera: Psyllidae). *Biocontrol Science and Technology 21*(9): 1065-1078

Awmack CS, Leather SR (2002) Host plant quality and fecundity in herbivorous insects. *Annual review of entomology 47*(1): 817-844

Balmand S, Lohs C, Aksoy S et al (2013) Tissue distribution and transmission routes for the tsetse fly endosymbionts. *Journal of invertebrate pathology 112*: S116-S122

Baranwal VK, Majumder S, Ahlawat YS et al (2005) A novel approach for simultaneous detection of Citrus yellow mosaic virus and Citrus greening bacterium by multiplex polymerase chain reaction

Barr NB, Hall DG, Weathersbee III AA et al (2009) Comparison of laboratory colonies and field populations of Tamarixia radiata, an ectoparasitoid of the Asian citrus psyllid, using internal transcribed spacer and cytochrome oxidase subunit I DNA sequences. *Journal of Economic Entomology, 102*(6): 2325-2332

Bassanezi RB, Gottwald TR (2009) Epidemiology of HLB and potential pathways for introduction. *Taller internacional de plagas cuarentenarias de los citricos. Villahermosa, Tabasco, México* 27-31

Bassanezi RB, Montesino LH, Gasparoto MCG et al (2011) Yield loss caused by huanglongbing in different sweet orange cultivars in São Paulo, Brazil. *European journal of plant pathology 130*(4): 577-586

Bassanezi RB, Montesino LH, Stuchi ES (2009) Effects of huanglongbing on fruit quality of sweet orange cultivars in Brazil. *European Journal of Plant Pathology 125*(4): 565

Batool A, Iftikhar Y, Mughal SM et al (2007) Citrus Greening Disease–A major cause of citrus decline in the world–A Review. *Hort. Sci.(Prague) 34*(4): 159-166

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol. 59*: 155-189

Belliure B, Janssen A, Maris PC et al (2005) Herbivore arthropods benefit from vectoring plant viruses. *Ecology Letters 8*(1): 70-79

Benrey B, Denno RF (1997) The slow-growth–high-mortality hypothesis: a test using the cabbage butterfly. *Ecology 78*(4): 987-999

Best A, White A, Boots M (2008) Maintenance of host variation in tolerance to pathogens and parasites. *Proceedings of the National Academy of Sciences 105*(52): 20786-20791

Best A, White A, Boots M (2014) The coevolutionary implications of host tolerance. *Evolution 68*(5): 1426-1435

Birch L (1948) The intrinsic rate of natural increase of an insect population. *The Journal of*

Animal Ecology 15-26

Blackman RL, Cahill M (1998) The karyotype of Bemisia tabaci (Hemiptera: Aleyrodidae). *Bulletin of Entomological Research 88*(2): 213-215

Blowers VC (1967) Notes on the female reproductive system of the South African citrus psylla, Trioza erytreae (Del Guercio) (Homoptera: Psyllidae). *Journal of the Entomological Society of Southern Africa 30*(1): 75-81

Boava LP, Sagawa CHD, Cristofani-Yaly M et al (2015) Incidence of 'Candidatus Liberibacter asiaticus'-infected plants among citrandarins as rootstock and scion under field conditions. *Phytopathology 105*:518-524

Bourtzis K (2008) Wolbachia-based technologies for insect pest population control. In *Transgenesis and the management of vector-borne disease* (pp. 104-113). Springer, New York, NY

Bové JM (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of plant pathology* 7-37

Brelsfoard CL, St Clair W, Dobson SL (2009) Integration of irradiation with cytoplasmic incompatibility to facilitate a lymphatic filariasis vector elimination approach. *Parasites & vectors 2*(1): 38

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

Bull JJ (1985) Sex determining mechanisms: an evolutionary perspective. *Experientia 41*(10): 1285-1296

EPPO/CABI (1997) *Trioza erytreae* and Citrus greening bacterium. Quarantine Pests for Europe, 2nd edn, pp. 547–550 and pp. 971–976. CAB International, Wallingford (GB)

Capoor SP, Rao DG, Viswanath SM (1974, January) Greening disease of citrus in the Deccan Trap Country and its relationship with the vector, Diaphorina citri Kuwayama. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 6, No. 6)

Carey JR (1993) Applied Demography for Biologists with Special Emphasis on Insects:

Oxford University Press. *New York NY*

Caspari E, Watson GS (1959) On the evolutionary importance of cytoplasmic sterility in mosquitoes. *Evolution 13*(4): 568-570

Caspi-Fluger A, Inbar M, Mozes-Daube N et al (2012) Horizontal transmission of the insect symbiont Rickettsia is plant-mediated. *Proc. R. Soc. B 279*(1734): 1791-1796

Catara A, Azzaro A, Mughal SM et al (1988, March) Virus, viroid and prokaryotic diseases of citrus in Pakistan. In *Proceedings of 6th Conference of the International Organization of Citrus Virologist. Tel Aviv* (pp. 957-962)

Catling HD, Annecke DP (1968) Ecology of citrus psylla in the Letaba district of Northern Transvaal. *S. Afr. Citrus J 410*: 8-11

Catling HD (1969) The bionomics of the South African citrus psylla, Trioza erytreae (Del Guercio)(Homoptera: Psyllidae) 3. The influence of extremes of weather on survival. *Journal of the Entomological Society of Southern Africa 32*(2): 273-290

Catling HD (1970) Distribution of the psyllid vectors of citrus greening disease, with notes on the biology and bionomics of Diaphorina citri. *FAO Plant Protection Bulletin 18*(1): 8-15

Cheema SS, Kapur SP, Chohan JS (1982) Evaluation of rough lemon strains and other rootstocks against greening-disease of citrus. *Sci. Hortic 18*:71-75

Cheema SS, Kapur SP, Bansal RD (1985) Efficacy of various therapeutic agents against greening disease of citrus. *J. Res. Punjab Agric. Univ.* 22: 479–482

Cheema SS, Kapur SP, Sharma OP (1986) Chemo-therapeutic controls of greening disease of citrus through bud dip treatment. *Indian J Virol*. *2*: 104-107

Chen X, Stansly PA (2014) Biology of Tamarixia radiata (Hymenoptera: Eulophidae), parasitoid of the citrus greening disease vector Diaphorina citri (Hemiptera: Psylloidea): a mini review. *Florida entomologist 97*(4): 1404-1413

Chiel E, Zchori-Fein E, Inbar M et al (2009) Almost there: transmission routes of bacterial symbionts between trophic levels. *PLoS One* 4(3): 1–11

Chiel E, Inbar M, Mozes-Daube N et al (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): 413-418

Chien CC (1995) The role of parasitoids in the pest management of citrus psyllid. In *proceedings of the symposium on research and development of citrus in Taiwan, Taichung, Taiwan* (pp. 245-261)

Chien CC, Chiu SC, Ku SC (1989) Biological control of Diaphorina citri in Taiwan. *Fruits 44*: 401-407

Chien CC, Chu YI, Ku SC (1991) Parasitic strategy, morphology and life history of Tamarixia radiata (Hymenoptera; Eulophidae). *Chinese Journal of Entomology 11*(4): 264-281

Chiu RJ, Tsai MY, Huang CH (1979) Distribution and retention of tetracyclines in healthy and likubin-affected citrus trees following trunk transfusion. *Plant Protection Bulletin* 1 (1979): 143-152

Chiu SC, Aubert B, Chien CC (1988, January) Attempts to establish Tetrastichus radiatus Waterson (Hymenoptera, Chalcidoidea), a primary parasite of Diaphorina citri Kuwayama in Taiwan. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 10, No. 10)

Chow A, Dunlap CA, Jackson MA et al (2016) Oviposition behavior and survival of Tamarixia radiata (Hymenoptera: Eulophidae), an ectoparasitoid of the Asian citrus psyllid, Diaphorina citri (Hemiptera: Liviidae), on hosts exposed to an entomopathogenic fungus, Isaria fumosorosea (Hypocreales: Cordycipitaceae), under laboratory conditions. *Journal of economic entomology*. *109*(5): 1995-2005

da Graça JV, Kunta M, Sétamou M et al (2015) Huanglongbing in Texas: Report on the first detections in commercial citrus. *Journal of Citrus Pathology 2*(1)

da Graça JV, Douhan GW, Halbert SE et al (2016) Huanglongbing: An overview of a complex pathosystem ravaging the world's citrus. *Journal of Integrative Plant Biology 58*(4): 373-387

Dale C, Beeton M, Harbison C et al (2006) Isolation, pure culture, and characterization of "Candidatus Arsenophonus arthropodicus," an intracellular secondary endosymbiont

from the hippoboscid louse fly Pseudolynchia canariensis. *Applied and Environmental Microbiology 72*(4): 2997-3004

Dale C, Moran NA (2006) Molecular interactions between bacterial symbionts and their hosts. *Cell 126*(3): 453-465

Das AK (2004) Rapid detection of Candidatus Liberibacter asiaticus, the bacterium associated with citrus Huanglongbing (Greening) disease using PCR. *Current Science 87*(9): 1183-1185

DeAngelis JD, Sether DM, Rossignol PA (1993) Survival, development, and reproduction in western flower thrips (Thysanoptera: Thripidae) exposed to impatiens necrotic spot virus. *Environmental Entomology 22*(6): 1308-1312

De Bary A (1879) *Die erscheinung der symbiose*. Verlag von Karl J. Trübner

Devine GJ, Wright DJ, Denholm I (2000) A parasitic wasp (Eretmocerus mundus Mercet) can exploit chemically induced delays in the development rates of its whitefly host (Bemisia tabaci Genn.). *Biological Control 19*(1): 64-75

Dhami MK, Buckley TR, Beggs JR et al (2013) Primary symbiont of the ancient scale insect family Coelostomidiidae exhibits strict cophylogenetic patterns. *Symbiosis 61*(2): 77-91

Dossi FCA, da Silva EP, Cônsoli FL (2014) Population dynamics and growth rates of endosymbionts during Diaphorina citri (Hemiptera, Liviidae) ontogeny. *Microbial ecology 68*(4): 881-889

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annual review of entomology 43*(1): 17-37

Douglas AE (2007) Symbiotic microorganisms: untapped resources for insect pest control. *Trends in Biotechnology 25*(8): 338-342

Drummond FA, Casagrande RA, Logan PA (1989) Population dynamics of Chrysomelobia labidomerae Eickwort, a parasite of the Colorado potato beetle. *International journal of acarology 15*(1): 31-45

Duron O, Bouchon D, Boutin S et al (2008) The diversity of reproductive parasites among

arthropods: Wolbachia do not walk alone. *BMC biology 6*(1): 27

Duron O, Wilkes TE, Hurst GD (2010). Interspecific transmission of a male-killing bacterium on an ecological timescale. *Ecology Letters 13*(9): 1139-1148

Dutt M, Barthe G, Irey M et al (2015) Transgenic citrus expressing an Arabidopsis NPR1 gene exhibit enhanced resistance against Huanglongbing (HLB; Citrus Greening). *PloS one 10*(9): 1–17

Eggleton P, Gaston KJ (1990) " Parasitoid" species and assemblages: convenient definitions or misleading compromises?. *Oikos* 417-421

Ekesi S (2015) Arthropod pest composition and abundance on citrus sinensis in the lowland and highland production locales of Kenya. *Acta Hortic*. *1065*: 1019-1026

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

Engelstädter J, Hurst GD (2009) The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology, Evolution, and Systematics 40*: 127-149

Etienne J (1978) Contrôle biologique à la Réunion de Trioza erytreae (Homopt. Psyllidae) au moyen de Tetrastichus dryi (Hym. Eulophidae). *Fruits 33*(12): 877-882

Etienne J, Aubert B (1980, January) Biological control of psyllid vectors of greening disease on Reunion Island. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 8, No. 8)

Étienne J, Quilici S, Marival D et al (2001) Biological control of Diaphorina citri (Hemiptera: Psyllidae) in Guadeloupe by imported Tamarixia radiata (hymenoptera: Eulophidae). *Fruits 56*(5): 307-315

Evers G, Grisoni M (1991) Present situation of the citrus greening disease in Tanzania and proposal for control strategies. *FRUITS-PARIS 46*: 171-171

FAO (2006) Citrus Fruit Fresh and Processed. *CCP.CI/ST/2006, FAO*: p 47

FAOSTAT. 2012 *http://faostat.fao.org*.

Farnsworth D, Grogan KA, van Bruggen AH et al (2014) The potential economic cost and response to greening in Florida citrus. *Choices 29*(3): 1-6

Feldhaar H (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecological Entomology 36*(5): 533-543

Ferrari J, Darby AC, Daniell TJ et al (2004) Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology 29*(1): 60-65

Ferree PM, Avery A, Azpurua J et al (2008) A bacterium targets maternally inherited centrosomes to kill males in Nasonia. *Current Biology 18*(18): 1409-1414

Flores D, Ciomperlik M (2017) Biological control using the ectoparasitoid, Tamarixia radiata, against the Asian citrus psyllid, Diaphorina citri, in the lower Rio Grande Valley of Texas. *Southwestern Entomologist*. *42*(1): 49-59

Forbes MR (1993) Parasitism and host reproductive effort. *Oikos* 444-450

Friedli J, Bacher S (2001) Mutualistic interaction between a weevil and a rust fungus, two parasites of the weed Cirsium arvense. *Oecologia 129*(4): 571-576

Fukatsu T, Nikoh N, Kawai R et al (2000) The secondary endosymbiotic bacterium of the pea aphid Acyrthosiphon pisum (Insecta: Homoptera). *Applied and environmental microbiology 66*(7): 2748-2758

Gahan AB (1932, October) Two new species of Encyrtidae (Hym.) from Kenya Colony. In *Proceedings of the Royal Entomological Society of London. Series B, Taxonomy* (Vol. 1, No. 10, pp. 220-222). Oxford, UK: Blackwell Publishing Ltd

Garnier M, Bové JM (1996, January) Distribution of the huanglongbing (greening) Liberobacter species in fifteen African and Asian countries. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 13, No. 13)

Garnier M, Bové JM (1983) Transmission of the organism associated with citrus greening disease from sweet orange to periwinkle by dodder. *Phytopathology 73*(10): 1358-1363

Garnier M, Jagoueix S, Toorawa P et al (1996, January) Both huanglongbing (greening) liberobacter species are present in Mauritius and Reunion. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 13, No. 13)

Garnier M, Jagoueix-Eveillard S, Cronje PR et al (2000) Genomic characterization of a

liberibacter present in an ornamental rutaceous tree, Calodendrum capense, in the Western Cape Province of South Africa. Proposal of'Candidatus Liberibacter africanus subsp. capensis'. *International Journal of Systematic and Evolutionary Microbiology 50*(6): 2119-2125

Gascuel O (1997) BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Molecular biology and evolution 14*(7): 685-695

Gavarra MR, Mercado BG (1988, November) Progress report on studies dealing with the psyllid vector (Diaphorina citri Kuwayama) of greening disease in the Philippines. In *Proc. 2nd FAO-UNDP Reg. Workshop Asian/Pacific Citrus Greening, Lipa, Philippines*

Gavarra MR, Mercado BG, Gonzales CI et al (1990, February) Progress report: D. citri trapping, identification of parasite and possible field establishment of the imported parasite, Tamarixia radiata in the Philippines. In *Proceedings of the Fourth International Asia Pacific Conference on Citrus Rehabilitation, Chiang Mai, Thailand* (pp. 4-10)

Giordano R, Jackson JJ, Robertson HM (1997) The role of Wolbachia bacteria in reproductive incompatibilities and hybrid zones of Diabrotica beetles and Gryllus crickets. *Proceedings of the National Academy of Sciences 94*(21): 11439-11444

Giorgini M, Monti MM, Caprio E et al (2009) Feminization and the collapse of haplodiploidy in an asexual parasitoid wasp harboring the bacterial symbiont Cardinium. *Heredity 102*(4): 365–371

Glazier DS (1999) Trade-offs between reproductive and somatic (storage) investments in animals: a comparative test of the Van Noordwijk and De Jong model. *Evolutionary Ecology 13*(6): 539-555

Gotoh T, Noda H, Ito S (2007) Cardinium symbionts cause cytoplasmic incompatibility in spider mites. *Heredity 98*(1): 13

Gottwald TR (2010) Current epidemiological understanding of citrus huanglongbing. *Annual review of phytopathology 48*: 119-139

Gottwald TR, da Graça JV, Bassanezi RB (2007) Citrus huanglongbing: the pathogen and its impact. *Plant Health Progress 6*(1): 1-18

Graca JD (1991) Citrus greening disease. *Annual review of phytopathology 29*(1):109-136

Grafton-Cardwell EE, Stelinski LL, Stansly PA (2013) Biology and management of Asian citrus psyllid, vector of the huanglongbing pathogens. *Annual Review of Entomology 58*: 413-432

Graham AL, Shuker DM, Pollitt LC et al (2011) Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. *Functional Ecology 25*(1): 5-17

Graham MDV (1987) A reclassification of the European Tetrastichinae (Hymenoptera: Eulophidae), with a revision of certain genera. *A reclassification of the European Tetrastichinae (Hymenoptera: Eulophidae), with a revision of certain genera. 55*(1)

Guidolin AS, Cônsoli FL (2013) Molecular characterization of Wolbachia strains associated with the invasive Asian citrus psyllid Diaphorina citri in Brazil. *Microbial ecology 65*(2): 475-486

Guo WP, Tian JH, Lin XD et al (2016) Extensive genetic diversity of Rickettsiales bacteria in multiple mosquito species. *Scientific reports 6*: 38770

Hagimori T, Abe Y, Date S et al (2006) The first finding of a Rickettsia bacterium associated with parthenogenesis induction among insects. *Current microbiology 52*(2): 97-101

Halbert SE, Manjunath KL (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. *Florida entomologist 87*(3): 330-353

Hall DG, Hentz MG (2010) Sticky trap and stem–tap sampling protocols for the Asian citrus psyllid (Hemiptera: Psyllidae). *Journal of Economic Entomology 103*(2): 541-549

Hall TA (1999, January) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic acids symposium series* (Vol. 41, No. 41, pp. 95-98). [London]: Information Retrieval Ltd., c1979-c2000

Hancock PA, Sinkins SP, Godfray HCJ (2011) Strategies for introducing Wolbachia to reduce transmission of mosquito-borne diseases. *PLoS neglected tropical diseases 5*(4): e1024

Hansen AK, Jeong G, Paine TD et al (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): 7531-7535

Hatcher MJ, Dick JT, Dunn AM (2012) Diverse effects of parasites in ecosystems: linking interdependent processes. *Frontiers in Ecology and the Environment 10*(4): 186-194

Hebert PD, Stoeckle MY, Zemlak TS et al (2004) Identification of birds through DNA barcodes. *PLoS biology 2*(10): e312

Hedges LM, Brownlie JC, O'neill SL et al (2008) Wolbachia and virus protection in insects. *Science 322*(5902): 702

Hernández AG (2003) Trioza erytreae (Del Guercio 1918): nueva plaga de los citricos en Canarias. *Phytoma España: La revista profesional de sanidad vegetal* (153): 112-118

Hocquellet A, Toorawa P, Bove JM et al (1999) Detection and identification of the two Candidatus liberobacter species associated with citrus huanglongbing by pcr amplification of ribosomal protein genes of theβ operon. *Molecular and Cellular Probes 13*(5): 373-379

Hoddle MS, Pandey R (2014) Host range testing of Tamarixia radiata (Hymenoptera: Eulophidae) sourced from the Punjab of Pakistan for classical biological control of Diaphorina citri (Hemiptera: Liviidae: Euphyllurinae: Diaphorinini) in California. *Journal of economic entomology 107*(1): 125-136

Hoddle MS (2012) Foreign exploration for natural enemies of Asian citrus psyllid, Diaphorina citri (Hemiptera: Psyllidae), in the Punjab of Pakistan for use in a classical biological control program in California USA. *Pakistan entomol*. *34*(1): 1-5

Hodgetts J, Ostojá-Starzewski JC, Prior T et al (2016) DNA barcoding for biosecurity: case studies from the UK plant protection program. *Genome 59*(11): 1033-1048

Hoffmann AA, Montgomery BL, Popovici J et al (2011) Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature 476*(7361): 454

Hoffmann AA, Clancy D, Duncan J (1996) Naturally-occurring Wolbachia infection in Drosophila simulans that does not cause cytoplasmic incompatibility. *Heredity 76*(1): 1

Hoffmann M, Coy MR, Gibbard HK et al (2014) Wolbachia infection density in populations of the Asian citrus psyllid (Hemiptera: Liviidae). *Environmental entomology 43*(5): 1215-1222

Hogg JC, Hurd H (1995) Plasmodium yoelii nigeriensis: the effect of high and low intensity of infection upon the egg production and bloodmeal size of Anopheles stephensi during three gonotrophic cycles. *Parasitology 111*(5): 555-562

Hosokawa T, Kaiwa N, Matsuura Y et al (2015) Infection prevalence of Sodalis symbionts among stinkbugs. *Zoological letters 1*(1): 5

Hunter MS, Perlman SJ, Kelly SE (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): 2185-2190

Hurst GD, Jiggins FM, von der Schulenburg JHG et al (1999) Male–killing Wolbachia in two species of insect. *Proceedings of the Royal Society of London B: Biological Sciences 266*(1420): 735-740

Husain MA, Nath D 1923 The Life-history of Tetrastichus radiatus parasitic on Euphalerm citri, Euw.; and its Hyperparasite." *In Rep. Proc. 5th Entomol. Mtg. Pusa*: 122–128

Ingwell LL, Eigenbrode SD, Bosque-Pérez NA (2012) Plant viruses alter insect behavior to enhance their spread. *Scientific reports 2*: 578

Iturbe-Ormaetxe I, Walker T, O'Neill SL (2011) Wolbachia and the biological control of mosquito-borne disease. *EMBO reports 12*(6): 508-518

Jackson MA, Dunlap CA, Jaronski ST (2010) Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. *BioControl*. *55*(1): 129-145

Jackson MA, Mcguire MR, Lacey LA et al (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus Paecilomyces fumosoroseus. *Mycological Research*. *101*(1): 35-41

Jackson MA, Payne AR (2007) Evaluation of the desiccation tolerance of blastospores of Paecilomyces fumosoroseus (Deuteromycotina: Hyphomycetes) using a lab-scale, air-drying chamber with controlled relative humidity. *Biocontrol Science and Technology*

17(7): 709-719

Jagoueix S, Bové JM, Garnier M (1994) The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. *International Journal of Systematic and Evolutionary Microbiology 44*(3): 379-386

Jagoueix S, Bové JM, Garnier M (1996) PCR detection of the two «Candidatus» liberobacter species associated with greening disease of citrus. *Molecular and cellular probes 10*(1): 43-50

Jensen DD (1959) A plant virus lethal to its insect vector. *Virology 8*(2): 164-175

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

Jiggins FM, Hurst GDD, Jiggins CD et al (2000) The butterfly Danaus chrysippus is infected by a male-killing Spiroplasma bacterium. *Parasitology 120*(5): 439-446

Jiggins FM (2017) The spread of Wolbachia through mosquito populations. *PLoS biology 15*(6) : e2002780

Kalyebi A, Aisu G, Ramathani I et al (2015) Detection and identification of etiological agents (Liberibacter spp.) associated with citrus greening disease in Uganda. *Uganda Journal of Agricultural Sciences 16*(1): 43-54

Kambris Z, Cook PE, Phuc HK et al (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. *Science 326*(5949): 134-136

Kapur SP, Cheema SS, Bansal RD et al (1986) Chemotherapeutic Control of Citrus Greening through Foliar Spray Effect on Chlorophyll and Greening Marker Substances (GMS). *Indian J. Virol.* 2: 68–72

Kapur SP, Cheema SS, Dhillon RS (1984) Reaction of certain citrus stionic combinations to viral/mycoplasmal diseases. *Indian Journal of Horticulture 41*(1and2): 142-143

Khamis FM, Rwomushana I, Ombura LO et al (2017) DNA Barcode Reference Library for the African Citrus Triozid, Trioza erytreae (Hemiptera: Triozidae): Vector of African Citrus Greening. *Journal of economic entomology 110*(6): 2637-2646

Kilalo D, Olubayo F, Obukosia S et al (2009) Farmer management practices of citrus insect pests in Kenya. *African Journal of Horticultural Science 2*

Kluth S, Kruess A, Tscharntke T (2002) Insects as vectors of plant pathogens: mutualistic and antagonistic interactions. *Oecologia 133*(2): 193-199

Kobori Y, Nakata T, Ohto Y et al (2011) Dispersal of adult Asian citrus psyllid, Diaphorina citri Kuwayama (Homoptera: Psyllidae), the vector of citrus greening disease, in artificial release experiments. *Applied Entomology and Zoology 46*(1): 27-30

Koella JC, SÖrensen FL, Anderson RA (1998) The malaria parasite, Plasmodium falciparum, increases the frequency of multiple feeding of its mosquito vector, Anopheles gambiae. *Proceedings of the Royal Society of London B: Biological Sciences*, *265*(1398): 763-768

Koga R, Meng XY, Tsuchida T et al (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. *Proceedings of the National Academy of Sciences 109*(20): E1230-E1237.

Koga R, Bennett GM, Cryan JR et al (2013) Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. *Environmental Microbiology 15*(7): 2073-2081

Korsten L, Jagoueix S, Bové JM et al (1996, January) Huanglongbing (greening) detection in South Africa. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 13, No. 13)

Kruse A, Fattah-Hosseini S, Saha S et al 2017 Combining'omics and microscopy to visualize interactions between the Asian citrus psyllid vector and the Huanglongbing pathogen Candidatus Liberibacter asiaticus in the insect gut. *PloS one 12*(6): p.e0179531

Küchler SM, Kehl S, Dettner K (2009) Characterization and localization of Rickettsia sp. in water beetles of genus Deronectes (Coleoptera: Dytiscidae). *FEMS microbiology ecology 68*(2): 201-211

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution 33*(7): 1870-1874

Kunta M, Viloria Z, Hilda S et al (2014) Diverse DNA extraction methods and PCR

primers for detection of Huanglongbing-associated bacteria from roots of ‘Valencia’sweet orange on sour orange rootstock. *Scientia Horticulturae 178*: 23-30

Kutzer MA, Armitage SA (2016) The effect of diet and time after bacterial infection on fecundity, resistance, and tolerance in Drosophila melanogaster. *Ecology and evolution 6*(13): 4229-4242

Lallemand J, Fos A, Bové JM (1986) Transmission de la bactérie associée à la forme africaine de la maladie du" greening" par le psylle asiatique Diaphorina citri Kuwayama. *Fruits 41*(5): 341-343

Laven H (1967) Eradication of Culex pipiens fatigans through cytoplasmic incompatibility. *Nature 216*(5113): 383-384

Lawson ET, Mousseau TA, Klaper R et al (2001) Rickettsia associated with male-killing in a buprestid beetle. *Heredity 86*(4)497–505

Lefèvre T, Roche B, Poulin R et al (2008) Exploiting host compensatory responses: the ‘must’of manipulation?. *Trends in parasitology 24*(10): 435-439

Lefèvre T, Lebarbenchon C, Gauthier-Clerc M et al (2009) The ecological significance of manipulative parasites. *Trends in Ecology & Evolution 24*(1): 41-48

Li W, Hartung JS, Levy L (2006) Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. *Journal of microbiological methods 66*(1): 104-115

Li W, Levy L (2009) Citrus Huanglongbing Diagnosis based on molecular detection of associated Liberibacter species. *Taller internacional de plagas cuarentenarias de los cítricos. Villahermosa, Tabasco, México*, 27-31

Lin KH (1964) A preliminary study on the resistance of yellow shoot virus and citrus budwood tissue to heat. *Acta Phytopathologica Sinica 7*:61-63

Lin SJ, Tao CC (1979) A New Psyllaephagus Ashmead Parasitising Diaphorina Citri Kuwayama in Taiwan. *App. Entomol.* 32: 117–21

List GM (1939) The effect of temperature upon egg deposition, egg hatch and nymphal development of Paratrioza cockerelli (Sulc). *Journal of Economic Entomology 32*(1):

30-36

Liu R, Zhang P, Pu X et al (2011) Analysis of a prophage gene frequency revealed population variation of ‘Candidatus Liberibacter asiaticus’ from two citrus-growing provinces in China. *Plant disease 95*(4): 431-435

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods 25*(4): 402-408

Lu P, Bian G, Pan X et al (2012) Wolbachia induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS neglected tropical diseases 6*(7): e1754

Magomere TO, Obukosia SD, Mutitu E et al (2009) Molecular characterization of' Candidatus Liberibacter'species/strains causing huanglongbing disease of citrus in Kenya. *Electronic Journal of Biotechnology 12*(2):5-6

Maina AN, Klein TA, Kim HC et al (2017) Molecular characterization of novel mosquito-borne Rickettsia spp. from mosquitoes collected at the Demilitarized Zone of the Republic of Korea. *PloS one 12*(11): e0188327

Manjunath KL, Halbert SE, Ramadugu C et al (2008) Detection of ‘Candidatus Liberibacter asiaticus’ in Diaphorina citri and its importance in the management of citrus huanglongbing in Florida. *Phytopathology 98*(4): 387-396

Maris PC, Joosten NN, Goldbach RW et al (2004) Tomato spotted wilt virus infection improves host suitability for its vector Frankliniella occidentalis. *Phytopathology 94*(7): 706-711

Martinez AL, Nora DM, Armedilla AL (1970) Suppression of symptoms of citrus greening disease in the Philippines by treatment with tetracycline antibiotics. *Plant Disease Reporter 54*: 1007-1009

Martini X, Hoffmann M, Coy MR et al (2015) Infection of an insect vector with a bacterial plant pathogen increases its propensity for dispersal. *PLoS One 10*(6): e0129373

Match Maker Associations Limited (MMA) 2008. *Citrus for Local and Regional Markets SubSector Quick Scan Tanzania*. Tanzania: Match Maker Associates Limited

Matsuura Y, Kikuchi Y, Meng XY et al (2012) A novel clade of alphaproteobacterial endosymbionts associated with stinkbugs and other arthropods. *Applied and environmental microbiology* AEM-00673

Mc Daniel JR, Moran VC (1972) The parasitoid complex of the citrus psyllaTrioza erytreae (Del Guercio)[Homoptera: Psyllidae]. *Entomophaga 17*(3): 297-317

McClean RE (1970) Greening or blotchy-mottle disease of citrus. *Phytophylactica 2*(3): 177-194

McClean APD (1974) The efficiency of citrus psylla, Trioza erylreae (Del. G.) as a vector of greening disease of citrus. *Phytophylactica 6*(1): 45-54

McDaniel JR (1970) The parasite complex of the citrus psylla, Trioza erytreae (Del Guercio)(Homoptera)

McGraw JB, Caswell H (1996) Estimation of individual fitness from life-history data. *The American Naturalist 147*(1): 47-64

Medeiros RB, Resende RDO, de Ávila AC (2004) The plant virus Tomato Spotted Wilt Tospovirus activates the immune system of its main insect vector, Frankliniella occidentalis. *Journal of virology 78*(10): 4976-4982

Medzhitov R, Schneider DS, Soares MP (2012) Disease tolerance as a defense strategy. *Science 335*(6071): 936-941

Meyer JM (2007) *Microbial associates of the Asian citrus psyllid and its two parasitoids: symbionts and pathogens* (Doctoral dissertation, University of Florida)

Michaud JP (2001) Numerical response of Olla v-nigrum (Coleoptera: Coccinellidae) to infestations of Asian citrus psyllid,(Hemiptera: Psyllidae) in Florida. *Florida Entomologist* 608-612

Michaud JP (2004) Natural mortality of Asian citrus psyllid (Homoptera: Psyllidae) in central Florida. *Biological control 29*(2): 260-269

Michaud JP, Olsen LE (2004) Suitability of Asian citrus psyllid, Diaphorina citri, as prey for ladybeetles. *BioControl 49*(4): 417-431

Michaud JP 2002 Biological Control of Asian Citrus Psyllid (Homoptera: Psyllidae) in Florida. A Preliminary Report. *Entomological News* 113: 216–222

Milinski M (1985) Risk of predation of parasitized sticklebacks (Gasterosteus aculeatus L.) under competition for food. *Behaviour 93*(1): 203-216

Miller JW, Coon BF (1964) The effect of barley yellow dwarf virus on the biology of its vector the English grain aphid, Macrosiphum granarium. *Journal of Economic Entomology 57*(6): 970-974

Miller DR, Weidhaas DE, Hall RC (1973) Parameter Sensitivity in Insect Population Modeling. *Journal of Theoretical Biology* 42(2): 263–74

Milne DL (1977) Control of citrus nursery pests by soil applications of dimethoate. *Citrus and sub-tropical fruit journal* 525: 5–7

Milne DL, De Villiers EA (1977) Dimethoate applied by microjet and drip irrigation for control of citrus psylla, Trioza erytreae, the vector of greening disease. *Citrus and sub-tropical fruit journal* (525): 15–17

Ministry of Agriculture (1982). Citrus Greening Disease in Kenya and Recommendations for Its Control. A Report to the Director of Agriculture by the Committee on Greening Disease in Citrus and Fruit Nursery Management : 80pp

Moll JN, Van Vuuren SP, Milne DL (1980) Greening disease, the South African situation. In *Proc. 8th Conf. Int. Org. Citrus Virol., Univ. Calif* (pp. 109-117)

Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. *Ecological Entomology 27*(2): 189-195

Monzó C, Urbaneja A, Tena A (2015) Los psílidos Diaphorina citri y Trioza erytreae como vectores de la enfermedad de cítricos Huanglongbing (HLB): reciente detección de T. erytreae en la Península Ibérica. *Boletín SEEA 1*: 29-37

Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annual review of genetics 42*: 165-190

Moran NA, Telang A (1998) Bacteriocyte-associated symbionts of insects. *Bioscience 48*(4): 295-304

Moran PJ, Patt JM, Cabanillas HE et al (2011) Localized autoinoculation and dissemination of Isaria fumosorosea for control of the Asian citrus psyllid in South Texas. *Subtropical plant science 63*: 23-35

Moran PJ (2001) The effects of wilt symptom development and peroxidase induction on interactions between vascular wilt bacteria and cucumber beetles. *Entomologia experimentalis et applicata 98*(2): 149-156

Moran VC, Buchan PR (1975) Oviposition by the citrus psylla, Trioza erytreae (Homoptera: Psyllidae), in relation to leaf hardness. *Entomologia experimentalis et applicata 18*(1): 96-104

Moran JR (1967) On the biology of the South African citrus psylla, Trioza erytreae (Del Guercio) (Homoptera: Psyllidae). *Journal of the Entomological Society of Southern Africa 30*(1): 96-106

Munson MA, Baumann P, Clark MA et al (1991) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *Journal of bacteriology 173*(20): 6321-6324

Nachappa P, Levy J, Pierson E et al (2014) Correlation between "Candidatus Liberibacter solanacearum" infection levels and fecundity in its psyllid vector. *Journal of invertebrate pathology 115*: 55-61

Nachappa P, Shapiro AA, Tamborindeguy C (2012) Effect of 'Candidatus Liberibacter solanacearum'on fitness of its insect vector, Bactericera cockerelli (Hemiptera: Triozidae), on tomato. *Phytopathology 102*(1): 41-46

Nadarasah G, Stavrinides J (2011) Insects as alternative hosts for phytopathogenic bacteria. *FEMS microbiology reviews 35*(3): 555-575

Nakata T (2008) Effectiveness of micronized fluorescent powder for marking citrus psyllid, Diaphorina citri. *Applied entomology and zoology 43*(1): 33-36

Nariani TK, Bhagabati KN (1980, January) Studies on the therapeutic and insect vector control of the greening disease of citrus in India. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 8, No. 8)

Nelson WR, Munyaneza JE, McCue KF et al (2013) The Pangaean origin of" Candidatus Liberibacter" species. *Journal of Plant Pathology* 455-461

Nurhadi F (1987, December) Records of important parasites attacking Diaphorina citri in East Java, Indonesia. In *Reg. Workshop Citrus Greening Disease, Huanglungbin, FAO-*

UNDP Project, Fuzhou, China

Nwugo CC, Lin H, Duan Y et al (2013) The effect of 'Candidatus Liberibacter asiaticus' infection on the proteomic profiles and nutritional status of pre-symptomatic and symptomatic grapefruit (Citrus paradisi) plants. *BMC plant biology 13*(1): 59

Nyambo B (2009) Integrated pest management plan (IPMP): the agricultural sector development program-Republic of Tanzania. *Online at: http://www-wds. worldbank. org/.../E21940P11429101IPMP 1P1142911P115873. doc (accessed 6 December 2009)*

Obergolzer PCJ, Von Standen DFA, Basson WJ (1965, January) Greening disease of sweet orange in South Africa. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 3, No. 3)

Obukosia SD, Waithaka K (2000) Nucellar embryo culture of Citrus sinensis L. and Citrus limon L. *African Crop Science Journal: The journal of tropical crop science and production 8*(2): 109-116

Ogata H, La Scola B, Audic S et al (2006) Genome sequence of Rickettsia bellii illuminates the role of amoebae in gene exchanges between intracellular pathogens. *PLoS genetics2*(5): p.e76.

Oliver KM, Russell JA, Moran NA et al (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences 100*(4): 1803-1807

Oliver KM, Noge K, Huang EM et al (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC biology 10*(1): 11

Oliver KM, Degnan PH, Burke GR et al (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual review of entomology 55*: 247-266

Paretas-Martínez J, Melika G, Pujade-Villar J (2007) Description of Lobopterocharips arreplegata gen. n. & sp. n.(Hymenoptera: Figitidae: Charipinae) from Nepal, with notes on its phylogenetic position. *Insect systematics & evolution 38*(4): 473-479

Pelz-Stelinski KS, Killiny N (2016) Better together: association with 'Candidatus Liberibacter asiaticus' increases the reproductive fitness of its insect vector, Diaphorina

citri (Hemiptera: Liviidae). *Annals of the Entomological Society of America 109*(3): 371-376

Phahladira MNB, Viljoen R, Pietersen G (2012) Widespread occurrence of "Candidatus Liberibacter africanus subspecies capensis" in Calodendrum capense in South Africa. *European journal of plant pathology 134*(1): 39-47

Pietersen G, Viljoen R, Nkgobe MB (2010) Background, current situation and managemet of HLB and its vector in south. 2 o Taller internacional sobre el Huanglongbing y el Psilido asiatico de los cítricos. *Mérida, Yucatán Senasica, SAGARPA*

Pietersen G, Arrebola E, Breytenbach JHJ et al (2010) A survey for 'Candidatus Liberibacter'species in South Africa confirms the presence of only 'Ca. L. africanus' in commercial citrus. *Plant Disease 94*(2): pp.244-249

Pluke RW, Qureshi JA, Stansly PA (2008) Citrus flushing patterns, Diaphorina citri (Hemiptera: Psyllidae) populations and parasitism by Tamarixia radiata (Hymenoptera: Eulophidae) in Puerto Rico. *Florida Entomologist*, *91*(1), 36-42

Pole FN, Ndung'u JM, Kimani JM et al (2010) Citrus Farming in Kwale District: A Case Study of Lukore Location." In *Proceedings of the 12th KARI Biennial Conference: Transforming Agriculture for Improved Livelihoods through Agricultural Product Value Chains.* 629–35

Poulin R (1994) The evolution of parasite manipulation of host behaviour: a theoretical analysis. *Parasitology 109*(S1): S109-S118

Poulin R, Thomas F (1999) Phenotypic Variability Induced by Parasites: Extent and Evolutionary Implications. *Parasitology Today 15*(1): 28-32

Prinsloo GL (1981) On the encyrtid parasites (Hymenoptera: Chalcidoidea) associated with psyllids (Hemiptera: Psylloidea) in southern Africa. *Journal of the Entomological Society of southern Africa 44*(2): 199-244

Provencher LM, Morse GE, Weeks AR et al (2005) Parthenogenesis in the Aspidiotus nerii complex (Hemiptera: Diaspididae): a single origin of a worldwide, polyphagous lineage associated with Cardinium bacteria. *Annals of the Entomological Society of America 98*(5): 629-635

Purcell AH (1982) Insect vector relationships with procaryotic plant pathogens. *Annual Review of Phytopathology 20*(1): 397-417

Pyle KR (1977) Control of citrus psylla Trioza erytrease (Del. G.) with sprays of endosulfan on Mazoe Citrus Estates. *Citrus and sub-tropical fruit journal* 520: 7–11

Quilici S (1986) Rapport de visite a` Maurice du 2 au 7 Juin 1986. Doc. IRAT-Re´union, Inst. Rech. Agron. Trop., Réunion, France

Qureshi JA, Stansly PA (2008) Rate, placement and timing of aldicarb applications to control Asian citrus psyllid, Diaphorina citri Kuwayama (Hemiptera: Psyllidae), in oranges. *Pest Management Science 64*(11): 1159-1169

Qureshi JA, Rogers ME, Hall DG et al (2009) Incidence of invasive Diaphorina citri (Hemiptera: Psyllidae) and its introduced parasitoid Tamarixia radiata (Hymenoptera: Eulophidae) in Florida citrus. *Journal of Economic Entomology 102*(1): 247-256

Råberg L, Graham AL, Read AF (2009) Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society of London B: Biological Sciences 364*(1513): 37-49

Rigaud T, Juchault P (1995) Success and failure of horizontal transfers of feminizing Wolbachia endosymbionts in woodlice. *Journal of Evolutionary Biology 8*(2): 249-255

Rivero A, Grillo R (2000) Natural enemies of Diaphorina citri Kuwayama (Homoptera: Psyllidae) in the central region of Cuba. *Centro Agricola 27*(3) : 87-88

Roberts R, Cook G, Grout TG et al (2017) Resolution of the identity of ‘Candidatus Liberibacter’species from Huanglongbing-affected citrus in East Africa. *Plant disease 101*(8): pp.1481-1488

Roberts R, Pietersen G (2017) A novel subspecies of ‘Candidatus Liberibacter africanus’ found on native Teclea gerrardii (Family: Rutaceae) from South Africa. *Antonie van Leeuwenhoek 110*(3): 437-444

Roberts R, Steenkamp ET, Pietersen G (2015) Three novel lineages of ‘Candidatus Liberibacter africanus’ associated with native rutaceous hosts of Trioza erytreae in South Africa. *International journal of systematic and evolutionary microbiology 65*(2): 723-731

Robinson DM (1960) Synonymic notes on Aphidencyrtus pulvinatus (Waterston) (Hymenoptera: Encyrtinae). *Journal of Natural History 3*(32): 491-492

Roca E, Aramburu J, Moriones E (1997) Comparative host reactions and Frankliniella occidentalis transmission of different isolates of tomato spotted wilt tospovirus from Spain. *Plant pathology 46*(3): 407-415

Roff DA (1992) The Evolution of Life Histories: Theory and Analysis Chapman and Hall New York Google Scholar

Rohrig E, Shirk PD, Hall DG et al (2011) Larval development of Diaphorencyrtus aligarhensis (Hymenoptera: Encyrtidae), an endoparasitoid of Diaphorina citri (Hemiptera: Psyllidae). *Annals of the Entomological Society of America*, *104*(1): 50-58

Roistacher CN (1996, January) The economics of living with citrus diseases: huanglongbing (greening) in Thailand. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 13, No. 13)

Ros VID, Breeuwer JAJ (2009) The effects of, and interactions between, Cardinium and Wolbachia in the doubly infected spider mite Bryobia sarothamni. *Heredity 102*(4): 413

Rosell RC, Blackmer JL, Czosnek H et al (2009) Mutualistic and dependent relationships with other organisms. In *Bemisia: Bionomics and management of a global pest* (pp. 161-183) Springer, Dordrecht

Rousset F, Bouchon D, Pintureau B et al (1992) Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B 250*(1328): 91-98

Roy BA, Kirchner JW (2000) Evolutionary dynamics of pathogen resistance and tolerance. *Evolution 54*(1): 51-63

Rwomushana I, Khamis FM, Grout TG et al (2017) Detection of Diaphorina citri Kuwayama (Hemiptera: Liviidae) in Kenya and potential implication for the spread of Huanglongbing disease in East Africa. *Biological Invasions 19*(10): 2777-2787

Gopal K, Pradeepthi E, Gopi V et al (2007) Occurrence, molecular diagnosis and suitable time of detection of citrus greening disease in sweet orange. *Acta Phytopathologica et Entomologica Hungarica 42*(1): 49-58

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution 4*(4): 406-425

Sakurai M, Koga R, Tsuchida T et al (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): 4069-4075

Salibe AA, Cortez RE (1966) Studies on the Leaf Mottling Disease of Citrus in the Philippines. *FAO Plant Prot. Bull* 14: 141–44

Samson RA (1974) Paecilomyces and some allied hyphomycetes. *Studies in mycology 6*1-119

Samways MJ (1984) Biology and economic value of the scale predator, Chilocorus nigritus (F.)(Coccinellidae). *Biocontrol News and Information 5*:91–105

Samways MJ, Grech NM (1986) Assessment of the fungus Cladosporium oxysporum (Berk. and Curt.) as a potential biocontrol agent against certain Homoptera. *Agriculture, ecosystems & environment 15*(4): 231-239

Sandström JP, Russell JA, White JP et al (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. *Molecular ecology 10*(1): 217-228

Sanofer AA (2014) Role of citrus fruits in health. *Journal of Pharmaceutical Sciences and Research 6*(2): 121

Saponari M, De Bac G, Breithaupt J et al (2010) First report of 'Candidatus Liberibacter asiaticus' associated with huanglongbing in sweet orange in Ethiopia. *Plant disease 94*(4): 482-482

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. *Science 310*(5755): 1781-1781

von der Schulenburg JHG, Habig M, Sloggett JJ et al (2001) Incidence of Male-Killing Rickettsia spp.(α-Proteobacteria) in the Ten-Spot Ladybird BeetleAdalia decempunctata L.(Coleoptera: Coccinellidae). *Applied and Environmental Microbiology 67*(1): 270-277

Schwarz RE, Green GC (1972, January) Heat requirements for symptom suppression and

inactivation of the greening pathogen. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 5, No. 5)

Schwarz RE, Van Vuuren SP (1971) Decrease in fruit greening of sweet orange by trunk injection of tetracyclines. *Plant Disease Reporter* 55: 747–50

Seif AA, Whittle AM (1984) Diseases of citrus in Kenya. *FAO Plant Protection Bulletin 32*(4): 122-127

Seif AA (1996) Integrated pest management for citrus: An option for Kenya. In *Crop Protection for Resource-poor Farmers in Kenya. Proceedings of the First Kenya Agricultural Research Institute Biennial Crop Protection Conference, Nairobi, Kenya*

Sétamou M, da Graça JV, Sandoval JL (2016) Suitability of native North American Rutaceae to serve as host plants for the Asian citrus psyllid (Hemiptera: Liviidae). *Journal of Applied Entomology 140*(9): 645-654

Shimwela MM, Narouei-Khandan HA, Halbert SE et al (2016) First occurrence of Diaphorina citri in East Africa, characterization of the Ca. Liberibacter species causing huanglongbing (HLB) in Tanzania, and potential further spread of D. citri and HLB in Africa and Europe. *European journal of plant pathology 146*(2): pp.349-368

Skelley LH, Hoy MA (2004) A synchronous rearing method for the Asian citrus psyllid and its parasitoids in quarantine. *Biological Control 29*(1): 14-23

Slatko BE, Taylor MJ, Foster JM (2010) The Wolbachia endosymbiont as an anti-filarial nematode target. *Symbiosis 51*(1): 55-65

Sloan DB, Moran NA (2012) Genome reduction and co-evolution between the primary and secondary bacterial symbionts of psyllids. *Molecular biology and evolution 29*(12): 3781-3792

Smith RS, Kramer DL (1987) Effects of a cestode (Schistocephalus sp.) on the response of ninespine sticklebacks (Pungitius pungitius) to aquatic hypoxia. *Canadian journal of zoology 65*(7): 1862-1865

Smith WA, Oakeson KF, Johnson KP et al (2013) Phylogenetic analysis of symbionts in feather-feeding lice of the genus Columbicola: evidence for repeated symbiont replacements. *BMC evolutionary biology 13*(1): 109

Stauderman K, Avery P, Aristizábal L et al (2012) Evaluation of Isaria fumosorosea (Hypocreales: Cordycipitaceae) for control of the Asian citrus psyllid, Diaphorina citri (Hemiptera: Psyllidae). *Biocontrol science and technology 22*(7): 747-761

Stouthamer R (1993) The use of sexual versus asexual wasps in biological control. *Entomophaga 38*(1): 3-6

Stouthamer R, Luck RF, Hamilton WD (1990) Antibiotics cause parthenogenetic Trichogramma (Hymenoptera/Trichogrammatidae) to revert to sex. *Proceedings of the National Academy of Sciences 87*(7): 2424-2427

Stumpf CF, Kennedy GG (2007) Effects of tomato spotted wilt virus isolates, host plants, and temperature on survival, size, and development time of Frankliniella occidentalis. *Entomologia experimentalis et applicata 123*(2): 139-147

Subandiyah S, Nikoh N, Sato H et al (2000a) Isolation and characterization of two entomopathogenic fungi attackingDiaphorina citri (Homoptera, Psylloidea) in Indonesia. *Mycoscience*, *41*(5), 509-513

Subandiyah S, Nikoh N, Tsuyumu S et al (2000b) Complex endosymbiotic microbiota of the citrus psyllid Diaphorina citri (Homoptera: Psylloidea). *Zoological Science 17*(7): 983-989

Sule H, Muhamad R, Omar D et al (2014) Parasitism rate, host stage preference and functional response of Tamarixia radiata on Diaphorina citri. *International Journal of Agriculture & Biology 16*(4): 783–88

Susi H, Laine AL (2013) Pathogen life-history trade-offs revealed in allopatry. *Evolution, 67*(11): 3362-3370

Svensson EI, Råberg L (2010) Resistance and tolerance in animal enemy–victim coevolution. *Trends in Ecology & Evolution 25*(5): 267-274

Swai IS, Evers G, Gumpf DJ et al (1992) Occurrence of citrus greening disease in Tanzania. *Plant disease 76*(11)

Tabachnick WJ (2015) Diaphorina citri (Hemiptera: Liviidae) vector competence for the citrus greening pathogen 'Candidatus Liberibacter asiaticus'. *Journal of economic entomology 108*(3): 839-848

Takano SI, Tuda M, Takasu K et al (2017) Unique clade of alphaproteobacterial endosymbionts induces complete cytoplasmic incompatibility in the coconut beetle. *Proceedings of the National Academy of Sciences 114*(23):6110-6115

Tamesse JL, Messi J (2002) Incidence de Trioza erytreae (del Guercio)(Homoptera: Triozidae), Psylle Vecteur du Greening sur la Sensibilité des Plantules d'Agrumes dans une Pépinière au Cameroun. *International Journal of Tropical Insect Science 22*(2): 97-103

Tamura K, Stecher G, Peterson D et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution 30*(12): 2725-2729

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular biology and evolution 10*(3): 512-526

Tang YQ (1989) A preliminary survey on the parasite complex of Diaphorina citri Kuwayama (Homoptera: Psyllidae) in Fujian. In *Proc. 2nd FAOUNDP Regional Workshop on the Asian-Pacific Citrus Greening Disease* (pp. 10-16)

Tang YQ (1990, February) On the parasite complex of Diaphorina citri Kuwayama (Homoptera: Psyllidae) in Asian-Pacific and other areas. In *Proc. 4th International Conference on Citrus Rehabilitation, Chiang Mai, Thailand* (Vol. 4, No. 10, pp. 240-245).

Texeira DDC, Ayres J, Kitajima EW et al (2005) First report of a huanglongbing-like disease of citrus in São Paulo State, Brazil and association of a new Liberibacter species,"Candidatus Liberibacter americanus", with the disease. *Plant Disease 89*(1): 107-107

Thao ML, Baumann P (2004) Evidence for multiple acquisition of Arsenophonus by whitefly species (Sternorrhyncha: Aleyrodidae). *Current microbiology 48*(2): 140-144

Thao ML, Clark MA, Baumann L et al (2000a) Secondary endosymbionts of psyllids have been acquired multiple times. *Current Microbiology 41*(4): 300-304

Thao ML, Moran NA, Abbot P et al (2000b) Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Applied and environmental microbiology, 66*(7): 2898-2905

Thomas F, Cezilly F, De Meeüs T et al (1997) Parasitism and ecology of wetlands: a review. *Estuaries 20*(3): 646-654

Thompson JD, Gibson TJ, Plewniak F et al (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic acids research, 25*(24): 4876-4882

Thompson JD, Gibson TJ, Higgins DG (2003) Multiple sequence alignment using ClustalW and ClustalX. *Current protocols in bioinformatics* 1 : 2-3

Toju H, Fukatsu T (2011) Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Molecular Ecology 20*(4): 853-868

Torres MLG, Nava DE, Gravena S et al (2006) First report of Tamarixia radiata (Waterston) (Hymenoptera: Eulophidae) on Diaphorina citri Kuwayama (Hemiptera: Psyllidae) in Brazil. *Revista de Agricultura (Piracicaba) 81*(1) : 112-117

Tsai JH, Wang JJ, Liu YH (2002) Seasonal abundance of the Asian citrus psyllid, Diaphorina citri (Homoptera: Psyllidae) in southern Florida. *Florida Entomologist 85*(3): 446-451

Tschirley DL, Muendo KM, Weber MT (2004) *Improving Kenya's domestic horticultural production and marketing system: current competitiveness, forces of change, and challenges for the future (volume II: horticultural marketing)* (No. 55156). Michigan State University, Department of Agricultural, Food, and Resource Economics

Tsuchida T, Koga R, Horikawa M et al (2010) Symbiotic bacterium modifies aphid body color. *Science 330*(6007): 1102-1104

Turelli M (1994) Evolution of incompatibility-inducing microbes and their hosts. *Evolution 48*(5): 1500-1513

Turley AP, Moreira LA, O'Neill SL et al (2009) Wolbachia infection reduces blood-feeding success in the dengue fever mosquito, Aedes aegypti. *PLoS Neglected Tropical Diseases 3*(9): e516

Turner T, Burri BJ (2013) Potential nutritional benefits of current citrus consumption. *Agriculture 3*(1): 170-187

Vale PF, Little TJ (2012) Fecundity compensation and tolerance to a sterilizing pathogen in Daphnia. *Journal of evolutionary biology 25*(9): 1888-1896

Van den Berg MA, Van Vuuren SP, Deacon VE (1992) Studies on greening disease transmission by the citrus psylla, Trioza erytreae(Hemiptera: Triozidae). *Israel Journal of Entomology 25*:51-56

Van den Berg MA (1984) Introduction of natural enemies against citrus psylla, Trioza erytreae (Del Guercio)(Hemiptera: Triozidae). *Proc. Greening Syrup. Citrus and Subtropical Fruit Research Institute, Nelspruit, South Africa, CSFRI Publication*, 120-127

Van den Berg MA, Deacon VE, Fourie CJ et al (1987) Predators of the citrus psylla, Trioza erytreae (Hemiptera: Triozidae), in the Lowveld and Rustenburg areas of Transvaal. *Phytophylactica 19*(3): 285-289

Van den Berg MA (1990) The citrus pyslla, Trioza erytreae (Del Guercio)(hemiptera: Triozidae): A review. *Agriculture, ecosystems & environment 30*(3-4) : 171-194

Van den Berg MA, Greenland J (2000) Tamarixia dryi, parasitoid of the citrus psylla, Trioza erytreae: a review. *African Plant Protection 6*(1): 25-28

Van der Merwe CP (1923) The citrus psylla (Trioza merwei, Pettey). *J. Dept. Agric., Union S. Afr., Pretoria. Government Printing and Stationery Office, Reprint*

Van Vuuren SP, da Graca JV (1978) Effects of exposure time and monocrotophos on citrus greening transmission by psylla (Trioza erytreae Del G.). *Citrus and sub-tropical fruit journal*. 536: 13–14

Vavre F, Fleury F, Lepetit D et al (1999) Phylogenetic evidence for horizontal transmission of Wolbachia in host-parasitoid associations. *Molecular Biology and Evolution*. *16*(12): 1711-1723

Vorburger C, Gehrer L, Rodriguez P (2010) A strain of the bacterial symbiont Regiella insecticola protects aphids against parasitoids. *Biology letters 6*(1): 109-111

Vuuren SV, Manicom BQ (2009) Attempts to obtain Huanglongbing resistant or tolerant sweet orange by embryo rescue from healthy chimeras of diseased citrus fruit. *South African Journal of Plant and Soil 26*(4): 220-224

Wade MJ, Stevens L (1985) Microorganism mediated reproductive isolation in flour beetles (genus Tribolium). *Science 227*(4686): 527-528

Wang N, Trivedi P (2013) Citrus huanglongbing: a newly relevant disease presents unprecedented challenges. *Phytopathology 103*(7): 652-665

Waterston J (1922) On the chalcidoid parasites of psyllids (Hemiptera, Homoptera). *Bulletin of Entomological Research 13*(1): 41-58

Weeks AR, Marec F, Breeuwer JA (2001) A mite species that consists entirely of haploid females. *Science 292*(5526): 2479-2482

Weinert LA, Werren JH, Aebi A et al (2009) Evolution and diversity of Rickettsia bacteria. *Bmc Biology 7*(1): 6

Weinert MP, Jacobson SC, Grimshaw JF et al (2004) Detection of huanglongbing (citrus greening disease) in Timor-Leste (East Timor) and in Papua New Guinea. *Australasian Plant Pathology 33*(1): 135-136

Weintraub PG, Beanland L (2006) Insect vectors of phytoplasmas. *Annu. Rev. Entomol. 51*: 91-111

Werren JH, Hurst GD, Zhang W et al (1994) Rickettsial relative associated with male killing in the ladybird beetle (Adalia bipunctata). *Journal of Bacteriology 176*(2): 388-394

Werren JH, Skinner SW, Huger AM (1986) Male-killing bacteria in a parasitic wasp. *Science 231*(4741): 990-992

Werren JH, O'Neill SL (1997) The Evolution of Heritable Symbionts. In Influential Passengers: Inherited Microorganisms and Arthropod Reproduction. Eds. O'Neill SL, Hoffmann AA, Werren JH *Oxford University Press, New York, NY*: 1–41

Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology 6*(10): 741

Werren JH, Windsor DM (2000) Wolbachia infection frequencies in insects: evidence of a global equilibrium?. *Proceedings of the Royal Society of London B: Biological Sciences 267*(1450): 1277-1285

Werren JH (1997) Biology of wolbachia. *Annual review of entomology 42*(1): 587-609

West SA, Cook JM, Werren JH et al (1998) Wolbachia in two insect host–parasitoid communities. *Molecular ecology 7*(11): 1457-1465

Wijkamp I, Goldbach R, Peters D (1996) Propagation of tomato spotted wilt virus in Frankliniella occidentalis does neither result in pathological effects nor in transovarial passage of the virus. *Entomologia Experimentalis et Applicata 81*(3): 285-292

Williams GC (1966) Natural selection, the costs of reproduction, and a refinement of Lack's principle. *The American Naturalist 100*(916): 687-690

World Bank (2007) *World Development Report 2008: Agriculture for Development*. New York: Oxford University Press

Wortmann GB, Schafer E (1977) Control of sucking insects with particular reference to citrus psylla (Trioza erytreae Del G.) by means of a soil applied systemic insecticide. *Citrus and sub-tropical fruit journal* 520: 14–16

Xie PH, Su C, Lin ZG (1988) A preliminary study on an entomogenous fungus [Verticillium lecanii] of Diaphorina citri Kuwayama (Hom.: Psyllidae). *Chin. J. Biol. Control,4*(2): 92

Yang XB, Zhang YM, Henne DC et al (2013) Life tables of Bactericera cockerelli (Hemiptera: Triozidae) on tomato under laboratory and field conditions in southern Texas. *Florida Entomologist* 904-913

Yang Y, Huang MC, Beattie GA et al (2006). Distribution, biology, ecology and control of the psyllid Diaphorina citri Kuwayama, a major pest of citrus: a status report for China. *International Journal of Pest Management, 52*(4), 343-352

Yen JH, Barr AR (1973) The etiological agent of cytoplasmic incompatibility in Culex pipiens. *Journal of invertebrate pathology 22*(2): 242-250

Yesuf M (2013) Pseudocercospora leaf and fruit spot disease of citrus: Achievements and challenges in the citrus industry: A review. *Agricultural Sciences* 4(7): 324–28

Zabalou S, Riegler M, Theodorakopoulou M et al (2004) Wolbachia-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the*

National Academy of Sciences 101(42): 15042-15045

Zabalou S, Apostolaki A, Livadaras I et al (2009) Incompatible insect technique: incompatible males from a Ceratitis capitata genetic sexing strain. *Entomologia Experimentalis et Applicata 132*(3): 232-240

Zchori-Fein EINAT, Perlman SJ (2004) Distribution of the bacterial symbiont Cardinium in arthropods. *Molecular Ecology13*(7): 2009-2016

Zélé F, Nicot A, Duron O et al (2012) Infection with Wolbachia protects mosquitoes against Plasmodium-induced mortality in a natural system. *Journal of evolutionary biology 25*(7): 1243-1252

Zhang XS, Holt J, Colvin J (2000) A general model of plant-virus disease infection incorporating vector aggregation. *Plant Pathology 49*(4): 435-444

Zhang XF, Zhao DX, Hong XY (2012) Cardinium—the leading factor of cytoplasmic incompatibility in the planthopper Sogatella furcifera doubly infected with Wolbachia and Cardinium. *Environmental entomology 41*(4): 833-840

Zhao XY (1981) Citrus yellow shoot disease (Huanglongbing)-a review. In *Proc. Int. Soc. Citricult.* (Vol. 1, pp. 466-469)

Zouari S, Ben Halima MK, Reyes-Prieto M et al (2018). Natural Occurrence of Secondary Bacterial Symbionts in Aphids from Tunisia, with a Focus on Genus Hyalopterus. *Environmental entomology 47*(2): 325-333

8 Appendices

Table S1. Farmers (%) reporting on resources and pest control measures during surveys in Kenya and Tanzania (2015-2017)

Variable	Characteristics	Overall response (n=105)	Frequency (%)
Farm category	Backyard	95	90
	Orchard	10	9
Farm size	<5ha	58	55
	1-5ha	37	35
	>5ha	10	9
Cultivated citrus varieties	Valencia	52	50
	Washington navel	56	53
	Lemon	65	62
	Tangerines	29	28
	Pixie mandrin	15	11
	Lime	4	3
Age of citrus trees	<5 yrs	11	10
	5-10 yrs	71	68
	>10yrs	23	22
Farming practices	Mono cropping	11	10
	Mixed cropping	94	90
Purpose of planting citrus	Cash crop	53	50
	Subsistence	52	50
Nature of planted trees	Grafted	90	86
	Ungrafted	15	14
Status of citrus trees	Symptomatic	59	56
	Healthy	46	44
Presence of Alternate host plants	Yes	29	28
	No	76	72
Knowledge of CGD	Yes	5	5
	No	100	95
Knowledge of vector	Yes	10	10
	No	95	90
Pesticides application	Yes	55	52
	No	50	48
Fertilizer application	Yes	19	18
	No	86	82
Pruning of trees	Yes	42	40
	No	63	60
Advise on management of pest and diseases	Own advise	47	45
	Agricultural commercial retailers	45	43
	Government extension officers	13	12

Table S2: Species used in this study and their GenBank accession numbers

Gene	Species	GenBank no.	Reference
COI	***Trioza* species**		
	Trioza erytreae (TeSA1)	KY754594	Khamis et al. 2017
	Trioza erytreae (TeSA7)	KY754590	Khamis et al. 2017
	Trioza erytreae (TeCA5)	KY754586	Khamis et al. 2017
	Trioza erytreae (TeKE1A)	KY754656	Khamis et al. 2017
	Trioza erytreae	KU517195	Hodgetts et al. 2016
	Trioza aylmeriae	KR042632	Gwiazdowski et al 2015
	Trioza eugeniae	KY294637	Percy 2017
	Trioza zimmermani	KY294169	Percy 2017
	Trioza remota	KY294163	Foerster, 1848; Percy 2017
	Trioza percyae	KY294161	Taylor, 2013; Percy 2017
	Trioza pallida	KY294160	Uichanco, 1919; Percy 2017
	Trioza obunca	KY294158	Fang and Yang, 1986; Percy 2017
	Trioza kuwayamai	KY294154	Enderlein, 1914; Percy 2017
	Trioza grallata	KT588308	Percy et al. 2015
	Trioza incrustata	KT588307	Percy et al. 2015
COI	***Tamarixia* species**		
	Tamarixia radiata (Mexico)	KT253009.1	Guzman-Larralde 2015 (unpublished)
	Tamarixia radiata (Texas)	FJ152417.1	Barr et al 2009
	Tamarixia radiata (Florida)	FJ807949.1	de León and Sétamou 2010
	Tamarixia radiata (Texas)	GQ912277.1	de León and Sétamou 2010
	Tamarixia radiata (Mexico)	KT253023.1	Guzman-Larralde et al. (unpublished)
	Tamarixia radiata (Bhutan)	KY026092.1	Om,N., Yefremova 2017
	Tamarixia triozae (Texas)	GQ912287.1	de León and Sétamou 2010
	Tamarixia drukyulensis	KX986295.1	Om,N., Yefremova 2017
	***Tetrastichus* species**		
	Tetrastichus schoenobii	KJ627790.1	Reetha et al. 2014 (unpublished)
	Tetrastichinae sp.	KR416826.1	Dewaard 2013 (Unpublished)
	Minotetrastichus sp.	KJ092505.1	Dewaard 2014 (Unpublished)
	***Aprostocetus* species**		
	Aprostocetus meltoftei	KU374349.1	Wirta et al. 2016
	Aprostocetus meltoftei	KR805635.1	Hebert et al. 2016
	Aprostocetus gala	KJ701414.1	Shylesha and Abraham (unpublished)
	Aprostocetus forsteri	KR882328.1	Hebert et al. 2016
	Aprostocetus cerricola	HM573863.1	Kaartinen et al. 2010
	Aprostocetus cerricola	JQ416730.1	Stone et al. 2012
	Aprostocetus aethiops	HM573652.1	Kaartinen et al. 2010
	Aprostocetus .sp	KJ167669.1	Dewaard 2014
	Baryscapus .sp.	KR804069.1	Hebert et al 2016
	Eulophidae.sp	KR794331.	Hebert et al. 2016
	Diaphorencyrtus aligarhensis	EF431956.	Meyer and Hoy 2007
16S rRNA	**Rickettsia species**		
	Rickettsia sp.	KY799068.1	Maina et al. 2017
	Rickettsia Nysius	JQ726774.1	Matsuura et al. 2012
	Rickettsia bellii	NR_074484.2	Ogata et al. 2006
	Rickettsia *Nephotettix cincticeps*	KU586121.1	Guo et al. 2016
	Rickettsia *Deronectes semirufus*	FM955311.1	Kuchler et al. 2009
	Arsenophonus species		

	Arsenophonus *Bemisia tabaci*	LN830155.2	Bibi et al.(unpublished)
	Arsenophonus *Diaphorina citri*	AB038366.1	Subandiyah et al. 2000
	Arsenophonus *Hyalopterus*	MG257478.1	Zouari et al. 2018
	Candidatus Liberibacter species		
	Ca. L. subspecies capensis	KY000560.1	Roberts 2016
	Ca. L. subspecies tecleae	KX990288.1	Roberts 2016
	Ca. L. subspecies zanthoxyli	KX990287.1	Roberts 2016
	Ca. L. subspecies clausena	KJ152131.1	Roberts 2014
	Wolbachia species		
	Wolb. Diaphorencyrtus aligarhensis	EF433794.1	Meyer and Hoy,.2008
	Wolb. Diaphorina citri	EU914940.1	Salvador & Consoli,2008
	Wolb. Nysius	JQ726766.1	Matsuura et al. 2012
	Wolb. pipientis Cervaphis quercus	JN635325	Wang and Qiao, 2011
	Wolb. D.Simulans (wRi)	DQ412085	Mateos et al. 2006
	Wolb. D.melangonaster (dmw165)	Z28983	Bourtzis 1994
	Wolb.Folsomides.parvulus. (wFpar)	KT799586.	Ma et al. 2015
	Wolb. Drosicha pinicola	AB491204.	Matsuura et al. 2009
	Wolb. D.pseudoananassae (wPana)	DQ412081.	Mateos et al. 2006
	Wolb. pipientis (EW-p)	EU096232.	Ryu et al. 2007
	Wolb. D.takahashii (wTak)	DQ412082.	Mateos et al. 2006
	Wolb. Philaenus maghresignus	AB772263.	Koga et al. 2013
	Wolb. Kleidocerys.Resedae(clone.KrWlbOkn2)	JQ726770.	Matsuura et al. 2012
	Wolb.Clostera anachoreta (strain)	EU753170.	Song and Shen 2008
	Wolb. Otiorhynchus.coecus.Strain.WOco	GQ167629.	Kajtoch et al. 2009
	Wolb. D.melanogaster.wMel	LC108848.	Morioka et al. 2016
	Wolb. Ornithomya avicularia	MF461480.	Sochova et al. 2017
	Wolb. Icosta sp.	MF461477.	Sochova et al. 2017
	Wolb. Linyphia triangularis	EU333939.	Duron et al. 2008
	Wolb. Bemisia tabaci.Sirsa	JN204507.	Singh et al. 2012
	Wolb. Hofmannophila pseudospretella	KR698143.	Gutzwiller et al. 2015
	Wolb. D.simulans.394YU	KU255238.	Lefoulon et al 2016
	Wolb. Epermenia.chaerophyllella	KR698144.	Gutzwiller et al. 2015
	Wolb. Myzus persicae	KM577345.	Liu and Liu 2014
	Wolb. Nasonia longicornis	M84691.1	Breeuwer et al. 1992
	Wolb. sp.Jabalpur2014	LC159290.	Noda et al. 1992
	Wolb. Curculio.sp2.	AB746405.	Toju et al. 2013
	Wolb. D.simulans Hawaii	X64265.	Solignac 1992
	Wolb. Bryobia praetiosa	EU499317.	Ros et al. 2008
	Wolb. Pipientis culex pipiens	X61768.	O'Neill et al. 1992
	Wolb. Diaphorina citri	EF433793.	Meyer and Hoy 2007
	Cardinium species		
	Cardinium hertigii	DQ854699.1	Giorgini et al. 2006
	candidatus Cardinium hertigii	DQ854704.1	Giorgini et al. 2006
	Cardinium endosymbiont of Bemisia tabaci	FJ766339.1	Gueguen et al. 2009
	Cardinium endosymbiont of Bemisia tabaci	FJ766341.1	Gueguen et al. 2009
	Cardinium endosymbiont of Euides speciosa	AB506775.1	Nakamura et al. 2009
	Cardinium endosymbiont of Harmalia sirokata	AB506773.1	Nakamura et al. 2009
	Bacteroidetes endosymbiont of *Icerya brasilensis*	DQ133551.1	Gruwell et al. 2005

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