'*Candidatus* Liberibacter' in four indigenous Rutaceous species from South Africa

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Declaration

I declare that the thesis/dissertation, which I hereby submit for the degree Masters Scientiae in Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institute.

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SIGNATURE ……

DATE ……

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List of abbreviations

16S Ribosomal RNA	rRNA
˚C	Degrees Celsius
Bp	Basepair
BSA	Bovine serum albumin
CGA	Citrus Growers Association
CTAB	Hexadecyltrimethylammonium bromide
Ct	Cycle Threshold
DNA	Deoxyribonucleic acid
g	gram
HLB	Huanglongbing
ICAN	Isothermal and chimeric primer-initiated amplification of nucleic acids
IGR	Insect Growth Regulator
IOCV	International Organization of Citrus Virologist
Kb	Kilo bases
Laf	'*Candidatus* Liberibacter africanus'
LafC	'*Candidatus* Liberibacter africanus subsp. capensis'
Lam	'*Candidatus* Liberibacter americanus'
LAMP	Loop-mediated isothermal amplification
Las	'*Candidatus* Liberibacter asiaticus'
Leu	'*Candidatus* Liberibacter europaeus'
Lso	'*Candidatus* Liberibacter solanacearum'
MA	Monoclonal Antibodies
Mb	Mega bases

min	Minutes
MLO	Mycoplasma-like organism
omp	Outer membrane protein gene
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electrophoresis
qPCR	Quantitative Polymerase Chain Reaction
rcf	relative centrifugal force
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
sec	Seconds
SNP	Single nucleotide polymorphism
spp.	Species
SSR	Simple sequence repeat
subsp.	Subspecies
USA	United States of America
USD	American dollar
VNTR	Variable tandem repeat numbers

Summary

Greening disease in South Africa is associated with a phloem-limited member of the Alphaproteobacteria known as '*Candidatus* Liberibacter africanus' (Laf). Spread of this bacterium is assisted by the flight and feeding activities of its triozid vector, *Trioza erytreae.* In addition to Laf, '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) has been described from this country. LafC is widely associated with *Calodendrum capense,* an indigenous Rutaceous species. This dissertation aims to determine (i) whether reservoir hosts exist for Laf amongst indigenous rutaceous species that also act as native hosts to *T. erytreae* and (ii) whether LafC can be transmitted vertically through *Ca. capense* seeds. To achieve the first aim, a total of 234 *Clausena anisata*, 289 *Vepris lanceolata* and 231 *Zanthoxylum capense* specimens were assessed for the presence of Liberibacter by making use of a Liberibacter generic real-time PCR assay. Positive samples were further characterized by using a multi-gene phylgenetic approach based on the nucleotide sequences for the *16S*, *rplJ* and *omp* genes, which respectively encode the 16S ribosomal RNA (rRNA) subunit, 50S ribosomal subunit protein L10 and the outer membrane protein. The tree host species from which Liberibacter sequences were obtained were DNA barcoded using standard procedures. Of the trees tested, 33 *Cl. anisata*, 17 *V. lanceolata*, 9 *Z. capense* and 1 *Zanthoxylum davyi*, tested positive for Liberibacter. Phylogenetic analysis of the *rplJ* and *omp* gene regions, revealed unique Liberibacter clusters associated with each tree species. Phylogenetic analysis of *16S* data indicated that the strains detected in *V. lanceolata* and *Cl. anisata* were grouped with LafC, while those from *Zanthoxylum* species grouped separately. The presence of these bacteria in indigenous Rutaceous species should be further investigated to determine whether they are capable of being transmitted to and causing disease on commercial citrus species. To address the second aim of the study, seeds of *Ca. capense* were collected from two mother trees with known LafC infection. Following total DNA extraction from the midribs of leafs grown from these seeds, a LafC directed real-time PCR system was used to detect the bacterium. However, none of the samples had Ct values <35, the positive/negative threshold. From this study, no indication of seed-transmission was obtained within the limited number of samples which could be

tested. The lack of vertical transmission of both Laf and LafC is seeminly a characteristic shared amongst African Liberibacter species.

Chapter 1

Introduction and objectives of the study

1.1 Introduction

South Africa is ranked globally as the third largest citrus exporter having exported 1,376 thousand tons of citrus during 2010/11 (CGA, 2012). This important industry, however, is under constant pressure from various diseases. One such disease is known locally as Greening disease and is caused the phloem-restricted bacterium (Garnier and Bové, 1983) '*Candidatus* Liberibacter africanus' (Laf), which is a member of the class Alphaproteobacteria (Jagouiex et al., 1994). Laf is primarily spread amongst orchards in South Africa by the triozid, *Trioza erytreae* Del Guercio (McClean and Oberholzer, 1965; Burckhard and Ouvrard, 2012). Typically, leaves from infected branches will have a mottled appearance similar to that of a nutrient deficiency and fruits produced from these branches will be small, lopsided and bitter tasting (McClean and Oberholzer, 1965) thus being unfit for exportation.

To limit the spread and impact of the Greening disease stringent control strategies are implemented. These involve the planting of disease free material, removal of infected trees and branches and chemical control of vector populations within orchards (Buitendag and von Broembsen, 1993). The disease, neverteless, remains a persistent problem, especially in cooler production areas. It has been suggested that Laf is continually introduced into orchards from alternative reservoir hosts amongst natural vegetation surrounding orchards. *Clausena anisata, Vepris lanceolata* and *Zanthoxylum capense* are known native hosts of *T. erytreae,* which have been shown to be capable of supporting all development stages of this triozid (Moran, 1968). Also, Laf has previously been identified from *V. lanceolata* through hybridization (Korsten et al., 1996) and *Cl. anisata* have been shown to possibly serve as a host to Laf by graft inoculation studies (van den Berg et al., 1991-1992). However, no formal studies have attempted to determine whether these native hosts of *T. erytreae* naturally act as reservoir sources for the continual introduction of Laf into citrus orchards.

In the Western Cape, South Africa, mottling symptoms characteristic of Greening disease was observed on the leaves of a *Calodendrum capense* (Cape chestnut) tree growing in close proximity to a citrus orchard where Laf was identified (Garnier et al., 1999). Subsequent analyses form the *Ca. capense* tree revealed that it was infected

with a strain of Liberibacter that is related to Laf and is now known as '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) (Garnier et al., 2000). Since its discovery, LafC have been found widely associated with *Ca. capense* trees in South Africa (Phahladira et al., 2012) and have thus far not been identified from commercial citrus species (Pietersen et al., 2010). It is however unknown how LafC is transmitted amongst *Ca. capense* trees and whether this bacterium is capable of being vertically transmitted through seeds. Although Laf has been shown not to be seed-transmissible (van Vuuren et al., 2011), the close relationship between Laf and LafC suggest that this will probably also be true for the latter. However, no formal studies have thus far attempted to determine whether LafC is capable of being vertically transmitted.

1.2 Objectives of study

The first objective (Chapter 3) was to determine whether the native hosts of *T. erytreae* namely, *Cl. anisata, V. lanceolata* and *Z. capense* serve as reservoir hosts for Laf. The identification of Laf from these hosts would lead to the advancement of control strategies to include these indigenous rutaceous hosts that exist in natural vegetation surrounding orchards.

The second objective (Chapter 4) was to assess whether LafC is vertically transmitted through the seeds of infected *Ca. capense* trees. The results of this study will determine whether the lack of vertical transmission is a characteristic shared amongst African Liberibacter species.

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Chapter 2

Literature Review

2.1 Introduction

Citrus production in South Africa has come a long way since the arrival of the first orange trees on South African soil, June 14th 1654, as noted in the journal of Jan van Riebeeck. These first trees were succeeded by a shipment of about 300 orange trees from St. Helena in 1656 (Anonymous, 1926). The citrus industry in South Africa however, only really started to feature as a global competitor in 1930 when citrus exports exceeded 1 900 000 cases (Anonymous, 1930). Today, South Africa is ranked 13th amongst the world's fresh citrus production with 60 355 hectares of land dedicated to the planting of commercial citrus, of which Valencia orange production alone comprises 25 398 hectares. Globally, the country is the third largest exporter of citrus behind Spain and Turkey, exporting 1,376 thousand tons of citrus during 2010/11 (CGA, 2012).

Due to the economic importance of this industry, extensive research is being done in South Africa to ensure that the industry thrives, even under threat from various diseases that may cause collapse of citrus production. One such disease is locally known as Greening disease. This disease has been known in production areas such as Tzaneen, Rustenburg and White River since 1929 (Oberholzer et al., 1963; McClean and Oberholzer, 1965a) where it eliminated most commercial citrus orchards during the 1970s (Pretorius and van Vuuren, 2006). By the end of the 1980s, it was estimated that this disease caused an annual loss of R35 million (3.8 million USD) to the South African citrus industry (van den Berg et al., 1987). The disease has since spread to citrus producing areas across the country with only parts of the Eastern and Southern Cape being Greening free to date.

A disease similar to Greening disease from South Africa is known as Huanglongbing (HLB), and has been described from various citrus growing regions including Southern China, India, Brazil and Florida. HLB is considered to be more aggressive than Greening disease and has caused the destruction of over 100 million commercially grown citrus trees in Asia alone (Zhang et al., 2010). The detrimental effect of HLB is clearly demonstrated when one considers that by 2007 over 1 million trees have been removed from São Paulo State, Brazil (Gottwald et al., 2007), since the discovery of

HLB there in 2004 (Colleta-Filho et al., 2004). The aggressive nature of HLB ensured an honorable mention of the disease amongst the top 10 plant pathogenic bacteria, despite not making the list (Mansfield et al., 2012).

The following review focuses on various aspects of the organism responsible for Greening disease in South Africa as well as the causal agent associated with HLB described from other citrus producing countries.

2.2 History and discovery of the agents associated with citrus Greening and HLB

HLB was first observed in South Western China during the late 1800s by Citrus farmers but it was not until 1956 that the yellowing and leaf mottling symptoms characteristic of the disease in the province Guangdong was formally described (Lin and Lin, 1956). Similar disease symptoms to those described by farmers in Southern China were observed in South Africa, where the disease was named Greening (Oberholzer et al., 1963). In other citrus producing areas across the world similar leaf mottling symptoms became apparent and different names were given to describe the disease. In the Philippines the disease was known as mottle-leaf disease (Lee, 1921; Salibe and Cortez, 1968), Likubin in Taiwan, die-back in India (Fraser and Singh, 1968) and vein-phloem degeneration in Indonesia (Bové, 2006).

During the 1960s, various authors suggested that these diseases may be related (Martinez and Wallace, 1967; Fraser and Singh, 1968; Salibe and Cortez, 1968) and at the 13th Conference of International Organization of Citrus Virologist (IOCV) hosted in China, 1995 it was decided that the official name of the disease will from then on be Huanglongbing in honour of Prof Lin Kongxiang (Moreno et al., 1996). The name was decided based on the international rules of nomenclature, which states that a disease must be named after the name given in the first official description of the disease. However, for the purpose of this study, Greening is considered different to HLB due to differences in etiological agent to be discussed. Therefore, the name Greening will be used when referring to the disease when the causal agent is from Africa and HLB will be used when referring to other affected citrus producing regions where a different etiological agent is present

2.2.1 Causal agent

The cause of the disease was initially attributed to a range of factors such as mineral deficiencies, (Lin and Lin, 1956; Oberholzer et al., 1963; Fraser and Singh, 1968), water logging, infestation by nematodes, or the presence of a fungus (Lin and Lin, 1956). Lin and Lin (1956) conducted graft transmission studies which suggested that HLB in China was caused by a graft transmissible agent. In South Africa, similar graft transmission studies were conducted and based on the results from these experiments it was concluded that Greening disease in South Africa is caused by a graft transmissible virus (McClean and Oberholzer, 1965a). It became widely accepted that Greening disease, HLB, mottle leaf and Likubin were all caused by the "Greening virus" (Fraser and Singh, 1968). This idea however, was soon rejected as it was discovered that certain viral plant diseases such as mulberry dwarf and yellows disease were in fact not caused by viruses but rather by mycoplasma-like organisms (MLOs). Laflèche and Bové (1970) then performed electron microscopy studies on HLB-infected sweet oranges which revealed the presence of similar MLOs in the sieve tubes of these trees. These MLOs associated with HLB infected citrus trees, however, had a thicker envelope than MLOs and resembled the cell walls of bacteria (Garnier and Bové, 1977). The bacterial nature of the causal agents of HLB and Greening disease were further demonstrated by the sensitivity of these agents to penicillin and tetracycline treatments (Bové et al., 1980). Finally, Garnier et al. (1984) proved through a combination of electron microscopy and antibiotic studies on infected citrus and periwinkle that both HLB and Greening disease were caused by a gram-negative bacterium that remained restricted within the sieve tubes of the plants they infect (Garnier and Bové, 1983). It was later shown that these gram negative bacteria belonged to the class alphaproteobacteria based on 16S ribosomal RNA (rRNA) comparative studies (Jagoueix et al., 1994)

Despite the similarities between symptom expression and morphological characteristics of the African and Asian forms of the disease, they differ in various aspects. In South Africa, the causal agent of Greening disease is vectored by *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) (McClean and Oberholzer, 1965b; Burckhard and Ouvrard, 2012) whereas the Asian form is transmitted by *Diaphorina citri* Kuwayama (Hemiptera:

Liviidae) (Capoor et al, 1967; Martinez and Wallace; 1967; Burckhard and Ouvrard, 2012). Trees infected with African Greening typically recovers from disease symptoms at temperatures above 25˚C whereas trees infected with Asian HLB will remain symptomatic at temperatures up to 32˚C (Garnier and Bové, 1983). The heat sensitivity of the organism associated with African Greening is in direct correlation with its triozid vector, which is also heat sensitive and only occurs in cooler areas (Catling, 1969b). The strains responsible for the two forms of the disease also differ in serological properties as monoclonal antibodies directed against the Asian form do not react with the African form (Garnier et al., 1991).

These organisms have to date not been obtained in culture, contributing to the prolonged characterization of these causal agents. In 1994, by sequencing and comparing the 16S rRNA gene region of the African and Asian forms of the bacterium, Jagoueix et al. (1994) could for the first time reveal that, despite the high similarity between the two isolates, they were unique. The Asian form was thus named '*Candidatus* Liberobacter asiaticum' and the African form '*Candidatus* Liberobacter africanum' (Jagoueix et al., 1994). These names were later changed to '*Candidatus* Liberibacter asiaticus' (hereafter referred to as Las) and '*Candidatus* Liberibacter africanus' (hereafter referred to as Laf) to comply with international rules of nomenclature (Garnier et al., 2000).

In 2004, Las was found to be present in South America for the first time where it was found to infect citrus orchards in São Paulo, Brazil (Coletta-Filho et al., 2004). Extensive surveys were conducted across São Paulo, which led to the discovery of a new species that was named '*Candidatus* Liberibacter americanus' (hereafter referred to as Lam). This newly characterized species was responsible for 98% of HLB infections in Brazil at the time (Teixeira et al., 2005). Lam is more closely related to Las than to Laf and is vectored by *D. citri* (Teixeira et al., 2005). As with Laf, Lam is also heat sensitive (Lopes et al., 2009b). However, the disease symptoms caused by Lam are similar to those described for infection by both Las and Laf (Lopes et al., 2008).

2.3 Transmission

2.3.1 Vector transmission

HLB and Greening disease are primarily spread in orchards by feeding activities of their insect vectors. Laf in South Africa is transmitted by *T. erytreae* (McClean and Oberholzer 1965b) and Las and Lam are vectored by *D. citri* (Capoor et al., 1967; Teixeira et al., 2005). However, both insects are capable of efficiently transmitting Las and Laf (Massonie et al., 1976; Aubert, 1987). Once Laf and Las have been acquired by these insects, the bacteria spreads to the salivary glands, haemolymph (Moll and Martin., 1973), filter chamber, midgut, ovaries and muscles where it multiplies (Ammar et al., 2011), establishing a persistent infection within the insect vector (Xu et al., 1988; Hung et al., 2004). This multiplication already occurs at nymphal stages (Hung et al, 2004) and is essential for efficient transmission of the bacteria (Inoue et al., 2009).

Both males and females are capable of transmitting the bacteria after a feeding period of 24 hours (Catling and Atkinson, 1974; Capoor et al, 1974). The pathogen has been shown to be acquired by both *D.citri* and *T. ertytrea* during nymphal stages. Capoor et al. (1974) and Xu et al. (1988) demonstrated that 4th and 5th nymphs of *D. citri* are capable of not just acquiring but also transmitting Las. Hung et al. (2004), however, found that Las can be acquired by all nymphal stages in *D. citri.*

Las have been shown to be transmissible from male to female *D. citri* during courtship where it becomes persistent in the reproductive organs of the female (Mann et al., 2011a). Despite the presence of this bacterium within the reproductive organs of female liviids, various studies have shown that Las is not transmitted transovarially (Capoor et al., 1974, Xu et al., 1988, Hung et al., 2004). In South Africa, one report indicated that Laf is transmitted transovarially by *T. erytreae* (van den Berg et al., 1991-1992), although these results have not yet been repeated.

T. eytreae is capable of completing its life cycle on three indigenous Rutaceae hosts, *Vepris lanceolata* (Lam.) G. Don (previously *V. undulata*), *Clausena anisata* (Willd.) J. Hk. Ex Benth. and *Zanthoxylum capense* (Thunb.) Harv. (previously *Fagara capensis*) (Moran, 1968a). The triozid vector of Laf is attracted to these trees in addition to

Calodendrum capense (L.F.) Thunb., however, if given a choice between indigenous Rutaceae and *Citrus limon* (L.) Burm.f., triozids will be preferentially attracted to *Ci. limon* (Moran, 1968b). This attraction may be explained by the physiology of lemon leaves, which are more suited for oviposition compared to indigenous leaves (Moran and Buchan, 1975). *D. citri* have also been found on *Murraya paniculata* (L.) Jack (Miyakawa, 1980), which is considered to be its natural host, *Limonia acidissima* (L.) (Koizumi et al., 1996) and *Ficus carica* (L.) (Thomas and De León, 2011).

Despite the presence of alternative hosts for *T. erytreae* and *D.citri*, both are preferentially attracted to citrus. Mann et al. (2012) suggested that Las may play a direct role in increasing the attractiveness of infected plants to *D. citri* compared to healthy plants. They postulated that Las causes the release of volatiles from the plant that attracts the liviid vector of this bacterium. The liviid will then feed on an infected plant, acquiring the bacteria, and soon after move to a healthy plant, possibly due to the low nutritional properties of the infected plant, promoting the spread of Las.

Fig 2.1: Vectors of Laf and Las **a)** Adult *T. erytreae* feeding on new flush of *V. lanceolata* (photo courtesy of Pietersen, G.); and **b)** adult *D. citri* feeding on citrus.

In addition to *T. erytreae* and *D.citri,* the liviid *Diaphorina communis* Mathur (Donovan et al., 2011) and the psylla *Cacopsylla citrisuga* Yang & Li (Cen et al., 2012) have been shown to be infected with Las. The role that these alternative vectors play in the epidemiology of Las has not yet been determined.

The interaction between citrus infecting Liberibacters and their vectors are not yet fully understood. Sequencing of the genomes of *D. citri*'s bacterial endosymbionts are currently underway (Saha et al., 2012). Information from these sequencing projects may

help uncover whether the endosymbiotic population of this liviid plays a role in the fitness of *D. citri* thus contributing to Las transmission (Saha et al., 2012).

2.3.2 Seed transmission

Las has not been found to be able to infect seed embryos (Tatineni et al., 2008) and HLB is therefore not considered to be a seed borne disease. This however does not exclude the possibility that this pathogen may be seed transmissible as the pathogen can be detected from the seed coat of seeds from symptomatic material (Tatineni et al., 2008; Hilf, 2011). Seed transmission of citrus Liberibacters is cumbersome as rootstock varieties in the USA are grown from seed (Hilf, 2011). If Las is transmitted vertically through seeds, then seedlings grafted onto infected rootstocks will aquire Las prior to being planted, ultimately leading to the perpetuation of HLB within an orchard. Capoor et al. (1974) performed seed transmission tests where they planted 2400 seeds from symptomatic *Citrus sinensis* (L.) Osbeck (sweet orange) and 850 seeds from symptomatic *Ci. paradisi* MacFad (grapefruit). After germination of a high percentage of these seeds, all seedlings were healthy, showing no typical disease symptoms. Although these results suggested that the bacterium is not transmissible though seed, the possibility that Las may be present in these seedlings could not be exclude as sensitive molecular techniques were not present at the time to verify the absence of Las. Later studies on seed transmission had conflicting results. In the experiments conducted by Graham et al. (2008), Albrecht and Bowman (2009) and Hilf (2011) a low percentage of seedlings grown from the seed of symptomatic fruit tested positive for Las whereas studies done by Shokrollah et al. (2009), Hartung et al. (2010a) and van Vuuren et al. (2011) concluded that neither Las nor Laf are transmitted through seeds. Therefore, no conclusive answers yet exist as to whether citrus infecting Liberibacters are vertically transmitted.

2.3.3 Experimental transmission

Las, Laf and Lam can be transmitted artificially to a recipient host plant by grafting and by making use of dodder (Garnier and Bové, 1983), which have been shown to be more efficient than natural transmission. These two means of transmission are exploited by

researchers to aid in investigating aspects pertaining to the pathogen such as host range, host response and determining how aggressive the pathogen is (Lopes and Frare, 2008). For graft transmission experiments, the best source of inoculum is 4cm long budwood from infected trees (Lopes and Frare, 2008). Las is, however, transmitted more efficiently than Lam by grafting (Lopes et al., 2009a).

Various species of dodder have been shown to transmit citrus associated Liberibacters, including *Cuscuta reflexa* Roxb. (da Graça, 1991), *Cu. pentagona* Engelmann (Zhang et al., 2010), and *Cu. indecora* Choisy (Hartung et al., 2010b). Although all Liberibacter species are efficiently transmitted by dodder, some characteristics of this host plant may interfere in transmission studies. For example, dodder prefers not to colonize plant hosts from different species once it has been established on a source plant. One way to overcome this is by winding the tendrils of the dodder around the recipient plant, although the dodder may return to the donor plant in which case it may be necessary to cut the tendrils away from the host plant (Hartung et al., 2010b).

Catharanthus roseus, (L.) G. Don, commonly known as periwinkle, is an ideal experimental model for Liberibacter species as the plant grows well and Las and Laf are capable of multiplying to higher titres within this host than citrus hosts (Garnier and Bové, 1983). HLB infected periwinkle have been used to characterize the bacterium using electron microscopy (Garnier et al., 1984) and aided in Las serotypes studies through the use of monoclonal antibodies (Garnier et al., 1990).

2.4 Symptom expression and host response to Liberibacter infection

Symptom expression of both HLB and Greening disease are similar, but HLB symptoms are more aggressive than that of Greening disease (Manicom and van Vuuren, 1990). Due to similarities in symptom expression of the two diseases, symptoms will be described based on the first extensive publication explaining disease symptoms of Greening disease in South Africa from McClean and Oberholzer, (1965b).

All parts of a tree are affected by HLB and Greening as the bacteria which causes these diseases spreads systematically in plants and can be found in all floral and fruit parts except the endosperm and embryo of seeds (Tatineni et al., 2008) and can even be

found infecting tree roots (Shokrollah et al., 2009). Symptom expression of infected trees is dependent on a linear relationship that exists between the time required for visible symptoms to be presented and the concentration of bacterial populations within the host plant (Coletta-Filho et al., 2010). The severity of the disease may be influenced by the age of the host tree. If a tree becomes infected at a later stage, the tree will have predominantly normal branches with only single branches showing symptoms; however, if a tree becomes infected at an early stage, the tree will be severely affected by the disease. In the latter case, all branches will show symptoms of the disease, become stunted and will be unproductive (McClean and Oberholzer, 1965a).

2.4.1 Foliar symptoms

One of the most pronounced symptoms of both HLB and Greening is the mottled appearance of leaves from infected branches (Bové, 2006). Leaves have an irregular yellow discoloration along the midrib and veins that spreads laterally across the leaf surface from the vein. The severity of the mottled appearance is influenced by both the age of the leave, with mature leaves showing more pronounced symptoms, and the time of the year (McClean and Oberholzer, 1965a). This mottled appearance of HLB/Greening affected leaves however also resembles the chlorotic leaf patterns of a Zinc deficiency (Schneider, 1968) and alone cannot verify the presence of the organisms associated with HLB and Greening.

Fig 2.2: Blotchy mottled appearance of citrus leaves infected with (a) Laf in Mpumalanga, South Africa and (b) Las in Orlando, Florida, USA.

New flush from an infected branch is commonly upright and narrow. These new leaves are either completely yellow or are yellow with green specks. As these leaves mature, the mottling will become more pronounced. The leaves from severely infected trees are dropped prematurely leaving the tree sparsely foliated (McClean and Oberholzer, 1965a).

Schneider (1968) proposed that the foliar symptoms are attributed to localized phloem necrosis caused by invasion of the Greening causal agent. He also noted an increase in the size of starch granules within the chloroplast of infected cells, causing the outer membrane of chloroplast to expand. Due to the collapse of phloem cells, the cell become filled with starch and may impart a leathery feel and appearance to the leaves (Schneider, 1968). These starch granules consist of more highly polymerized carbohydrates compared to those produced by stressed plants, and is therefore regarded as characteristic of Las infection (Gonzalez et al., 2012).

Through microarray analyses of Las infected *Ci. sinensis* trees, Albrecht and Bowman (2008) demonstrated that significant transcriptional changes occurs within Las infected leaves. In infected cells, the transcription of beta-amylase, an enzyme used in starch degradation, is down regulated whereas the expression of Glucose-6-phosphate/phosphate translocator, which plays a role in starch synthesis, is up regulated. Furthermore, they verified the increased level of starch granules from infected compared to healthy leaves through microscopy studies and iodine staining. They also found that the expression of a phloem-specific lectin PP2-like protein was induced, 13-17 weeks post-inoculation. They speculated that this protein causes necrosis of phloem cells so that the translocation stream is blocked, ultimately halting an increase in bacterial populations. These results were reproduced by Kim et al. (2009) who concluded that symptom development of HLB following Las infection of *Ci. sinensis* trees is due to the alteration of host gene expression by Las and not due to the aggregation of bacterial cells within the phloem of infected leaves.

The concentration of carbohydrates such as sucrose and glucose also increases in Las infected cells. An increase in the concentration of these two carbohydrates can cause a

reduction of photosynthesis. This may contribute to the yellowing of infected leaves (Fan et al., 2010).

Infection of *Ci. sinensis* trees also has an effect on the metabolic signature of that tree. The concentration of essential amino acids such as arginine and proline are greatly reduced in both symptomatic and asymptomatic Las infected leaves. This may be a mechanism used by the bacterium to suppress host defense responses (Slisz et al., 2012).

2.4.2 Fruit and seed symptoms

Fruit produced from infected branches are of a poor quality and are of no economic value. Typically HLB/Greening affected fruits are much smaller than their healthy counterparts and will remain green at the stylar end (McClean and Oberholzer, 1965a; Bassanenzi et al., 2009). The greened colour of these fruits may be linked to lower ethelyne concentrations of the fruit, which causes an increase in photosynthesis (Martinelli et al., 2012).

Symptomatic fruits are also lopsided, bitter tasting with the flesh of the fruit having a greenish brown appearance and are dropped prematurely (McClean and Oberholzer, 1965a). Even in the absence of symptom expression, fruits may already be infected with the causal agents of either HLB or Greening. These fruits have a lower juice percentage and are characterized by low Brix/acidity ratios (Bassanezi et al., 2009). Juice produced from Las infected fruits with no obvious symptoms is bitter tasting, has a metallic quality to it and is described by trained tasters as salty and sour. However, if the juice from such fruits is mixed with healthy fruits, as occurs during production, no obvious differences can be noted (Baldwin et al., 2010).

2.5 Epidemiology

Las has a wider geographic distribution than Laf, being present in many citrus producing countries around the world (Table 2.1). Lam has only been identified from citrus in Brazil (Lopes and Frare, 2008). Commercially grown citrus from South Africa have been shown to be free from Las and only contains infection by Laf (Pietersen et al., 2010).

This was also the case in other citrus producing African countries up until 2010 when Las was reported from a citrus orchard in Ethiopia (Saporani et al., 2010). As *T. erytreae* have been shown to be able to acquire and transmit Las in addition to Laf (Massonie et al., 1976), the spread of Las southward throughout Africa is anticipated unless Las infected trees are eliminated from Ethiopia. This anticipated spread will be detrimental to African citrus production as heat-tolerant Las (Garnier and Bové, 1983) will be able to infect citrus in warmer areas where heat-sensitive Laf has not yet been able to thrive.

Table 2.1: Geographical distribution of the three citrus infecting Liberibacter species

Country	Las	Laf	Lam	Reference
Swaziland	-	X	-	Catling and Atkinson, 1974
Saudi Arabia	X	-	-	Bové and Garnier, 1984
Yemen	X	-	-	Bové and Garnier, 1984
Malawi	-	X	-	Aubert et al., 1988
Burundi	-	X	-	Aubert et al., 1988
Kenya	-	X	-	Aubert et al., 1988
Somalia	-	X	-	Aubert et al., 1988
Ethiopia	X	X	-	Aubert et al., 1988; Saporani et al., 2010
Cameroon	X	-	-	Aubert et al., 1988
India	X	-	-	Garnier and Bové, 1996
Vietnam	X	-	-	Garnier and Bové, 1996
Indonesia	X	-	-	Garnier and Bové, 1996
Taiwan	X	-	-	Garnier and Bové, 1996
Nepal	X	-	-	Garnier and Bové, 1996
Cambodia	X	-	-	Garnier and Bové, 1996
Thailand	X	-	-	Garnier and Bové, 1996
China	X	-	-	Garnier and Bové, 1996
Sri Lanka	X	-	-	Garnier and Bové, 1996
Malaysia	X	-	-	Garnier and Bové, 1996
Phillipines	X	-	-	Garnier and Bové, 1996
Reunion	X	X	-	Garnier et al., 1996
Mauritius	X	X	-	Garnier et al., 1996

South Africa	-	X	-	Garnier and Bové, 1996
Zimbabwe	-	X	-	Garnier and Bové, 1996
Laos	X	-	-	Garnier and Bové, 1999
Myanmar	X	-	-	Garnier and Bové, 1999
East Timor	X	-	-	Weinert et al., 2004
Papua new guinea	X	-	-	Weinert et al., 2004
Brazil	X	-	X	Coletta-Filho et al., 2004; Teixeira et al., 2005
Florida	X	-	-	Halbert, 2005
Pakistan	X	-	-	Bové, 2006
Bhutan	X	-	-	Bové, 2006
Bangladesh	X	-	-	Bové, 2006
Southern Japan	X	-	-	Bové, 2006
Madagascar	X	X	-	Bové, 2006
Cuba	X	-	-	Luis et al., 2009
California	X	-	-	Stokstad, 2012
Texas	X	-	-	Stokstad, 2012
Argentina*	X	-	-	Outi et al., 2013

*Las has only been detected in backyard citrus trees and not yet from commercial citrus groves

2.6 Detection and identification of citrus infecting Liberibacter species

Early detection and identification of citrus associated Liberibacters from infected trees is a vital part of controlling HLB and Greening. By being able to identify infected trees from an orchard before symptoms appear, the inoculum source can be removed and spread of the disease in an orchard can be greatly reduced. However, determining whether a tree is infected with either disease through visual inspection of HLB/Greening symptoms alone cannot verify the presence of Las or Laf from a sample as nutrient deficiencies causes similar mottling patterns on affected leaves (Fraser and Singh, 1968). Additional methods are therefore needed to detect and identify the causal agents of these diseases, although these are associated with a number of technical difficulties. This is primarily because these bacteria are usually present in low concentrations and they are unevenly distributed within the tissue of citrus trees (McClean and Oberholzer, 1965a).

2.6.1 Early Liberibacter diagnostics

Once it had been established that Greening disease and HLB were caused by a graft transmissible agent, it became possible to confirm the presence of the causal organism in affected orchards through biological indexing following visual inspection for mottling on indicator plants. This method is time consuming with indicator seedlings only becoming symptomatic after a period of three months. Another setback of biological indexing is that transmission is not always achieved leading to false-negative results (da Graça, 1991). It was therefore necessary to develop a detection method for Greening disease that was both specific and time efficient.

Schwarz (1965) identified the presence of a fluorescent marker from *Ci. sinensis* trees showing typical Greening symptoms that was absent from healthy trees. The presence of the phenolic fluorescent marker in suspect trees could be tested for by using thin layer chromatography methods (Schwarz, 1968a; Schwarz, 1968b, Schwarz and van Vuuren, 1970). This phenolic substance was identified as gentisoyl glucose, a compound also found in trees with stubborn, die-back, stem-pitting and leaf-mottle (Feldman and Hanks, 1969). Gentisoyl glucose is present in the albedo of *Ci. sinensis* fruit prior to maturation of the fruit making it possible to determine which fruit will be affected with Greening disease before symptoms are observed (Schwarz, 1970). However, this method was not reliable as it was demonstrated that trees under environmental stress also produced gentisoyl glucose, even in the absence of Greening disease (Feldman and Hanks, 1969). Despite being non-specific, the detection of gentisoyl glucose as an indicator for Greening disease from citrus material remained common practice in South Africa till the late 1980's (van Lelyveld et al., 1988)

Other early detection methods for HLB and Greening disease involved the use of electron microscopy (Laflèché and Bové, 1970), fluorescent microscopy (Nariani et al., 1975), serological methods such as monoclonal antibodies (MA) (Garnier et al., 1991), ELISA (Varma et al., 1993) and Southern hybridization (Bové et al., 1993). The use of MA became widely accepted but could only detect specific geographic populations of Liberibacters. The limited use of MA led to the development of DNA probes (i.e., In-0.6, In-2.6 and In-1.9) to be used in hybridization studies (Villechanoux et al., 1992). Probe

In-0.6 was only able to hybridize with and detect the India Poona strain whereas probes In-2.6 and In-1.9 were both able to hybridize with all Asian strains of the organism. Probe In-2.6 was later characterized and shown to hybridize to the *nus*G-*rpl*KAJL-*rpo*B gene cluster on the chromosome of the bacterium associated with HLB (Villechanoux et al., 1993). This probe was also the only probe capable of detecting the African strain of the Greening-associated organism yielding a band of 2.1 Kilo base pairs (kbp) compared to the larger 2.6 kbp band of the Asian strains using less stringent conditions (Villechanoux et al., 1993). Another probe, As 1.7 (Planet et al., 1995), was used to detect various isolates of Laf across South Africa (Korsten et al., 1996).

2.6.2 Sequence-dependant diagnostics

With the advancement of molecular techniques such as PCR and sequencing, Jagouiex et al. (1994) were able to amplify and sequence the 16S rRNA gene for both Las and Laf. This was followed by the amplification and partial sequencing of the *rpl*KAJL-*rpo*BC operon that was previously used as targets for probe design in Southern hybridization assays (Planet et al., 1995). These sequences were obtained for Laf with the intention of designing new probes that would allow its detection in Southern and dot-blot hybridization assays.

The first PCR assay for the detection of both Las and Laf was developed in 1996 by Jagouiex et al. and involved restriction fragment length polymorphism (RFLP) analysis of a specific amplicon. Primer pair OI1/OI2c was specifically designed to detect 16S rRNA of both known Liberibacter species, yielding an amplicon size of 1160 bp. The two Liberibacter species could be distinguished from one another following digestion with the restriction enzyme, *xba*I, which would yield 640 and 520 bp bands for Las and 520 and 506 bp bands for Laf.

Like all PCR-RFLP based diagnostic methods, the need for enzymatic digestion of the amplification product to distinguish Las from Laf meant that the assay was costly and time-consuming. Hocquellet et al. (1999b) developed an alternative PCR assay in which the ribosomal protein gene, *rplJ,* is amplified using primer pair A2/J5. Following

amplification with primers A2/J5, Las can be distinguished from Laf because the resulting amplicon is 703 bp long for Las and 669 bp for Laf.

The advantage of using PCR-based techniques over Southern blotting assays for early detection of citrus infecting Liberibacters lies in the increased sensitivity and time-efficiency of PCR compared to Southern blotting. Liberibacter DNA can be detected from asymptomatic material in less than one day using PCR, making it possible to determine infection prior to symptom expression. PCR based assays for the detection of Liberibacters is still being developed and have replaced the use of serological and Southern hybridization techniques for routine diagnostics. Such assays include the detection of viable Las cells by the addition of ethidium monozide to a PCR reaction using primer pair A2/J5 (Trivedi et al., 2009). Other PCR assays include the development of a competitive assay for the quantification of Liberibacter DNA from a sample (Kawabe et al., 2006), duplex PCR system that amplifies both *omp* and *phopol*2 gene regions of Las (Donnua et al., 2012) and a single closed tube dual primer TaqMan system that incorporates a nested-PCR system within a single tube (Lin et al., 2010).

Li et al. (2006) designed the first real-time or quantitative PCR (qPCR) assay for the specific detection of various Liberibacter species (Las, Laf, Lam and '*Candidatus* Liberibacter solanacearum'). Primers and probes were designed based on a conserved 70 bp region of the 16S rRNA gene of the known '*Candidatus* Liberibacter' spp. This method has been modified by redesigning the forward primer so that it is capable of detecting all known '*Candidatus* Liberibacter' spp in a single reaction (Pietersen, *unpublished data*). Due to the small size of the amplicon, this qPCR assay has been shown to be even more sensitive, reproducible and rapid than conventional PCR strategies (Li et al., 2007). The sensitivity of this qPCR assay can also be attributed to the choice of targeting the 16S rRNA region. This is because the 16S rRNA gene is present in a higher copy number in Las (three copies) than the *rpl*KAJL*rpo*B (single copy) gene cluster, which is also commonly targeted in PCR design (Kim and Wang, 2009). Other qPCR assays have been developed based on the design of primers targeting different genes including *rpo*B (Ananthakrishan et al., 2012), and hypothetical genes located within the prophage gene region, *hyvI* and *hyvII* (Morgan et al., 2012).

The use of PCR-based diagnostics tools for the identification of HLB from citrus orchards requires well-equipped laboratories which is not always available, especially in developing countries where citrus is grown. Such obstacles can be overcome by developing molecular diagnostic tools which requires minimal sample processing and the use of simple equipment such as a water bath. Loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) and Isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (Uemori et al., 2007) are two recently developed molecular diagnostic tools which fulfill such requirements. Okuda et al. (2005) designed a LAMP protocol from the *nus*G-*rpl*KAJL-*rpo*B gene cluster for the detection of most Las strains. They showed that this technique was as sensitive as conventional PCR with the added benefit of being both robust and time-efficient. An ICAN system based on the amplification of a portion of the 16S rRNA gene was designed by Urasaki et al. (2008). The diagnostic system described can be performed in a single tube and the presence of Las is visualized by fluorescence.

Other novel detection methods have been described such as the detection of micro-RNAs and small interfering RNAs from citrus which can be used as markers for early detection of Las infections (Jin, 2013). Researchers have also explored the possibility of relying on visible-near infrared spectroscopy (Sankaran et al., 2011) and the use of high-resolution aerial imaging (Garcia-Ruiz et al., 2013) for the detection of HLB from citrus orchards. Despite these techniques still being in their infancy and requiring more extensive research, they hold the promise of being effective diagnostic tools that can aid in future control of HLB.

2.7 Management of Greening and HLB

Control of HLB and Greening is not an easy task. This is primarily because all commercial citrus species and cultivars are susceptible to these diseases, irrespective of the rootstock used (McClean and Oberholzer, 1965a; Cheema et al., 1982; Shokrollah et al., 2011; Albrecht et al., 2012; Albrecht and Bowman, 2012). In fact, it has been demonstrated in Brazil that if the disease is not controlled in an orchard, the infected grove may become unprofitable within 5-10 years after its initial introduction (Belasque et al., 2010).

2.7.1 Therapeutic control

The use of antibiotics to control HLB in orchards, as well as eliminating the organisms from budwood, was initially attempted. It was demonstrated that tetracycline was most effective in suppressing the organisms associated with the disease form both Asia (Martinez et al., 1970; Nariani et al., 1975) and South Africa (Schwarz and van Vuuren, 1971). Tetracycline could be applied to infected trees either as a foliar spray (Martinez et al., 1975; Nariani et al., 1975) or by trunk injections (Schwarz et al., 1974; Su and Chang, 1976). However, suppression of disease symptoms was only temporary due to the bacteriostatic nature of tetracycline, requiring follow-up applications of antibiotics which are expensive (Buitendag and von Broembsen, 1993; Chung and Zhisheng, 1991). Other side-effects included the presence of high levels of residue in fruits from treated trees, production of small fruits as well as phytotoxic effects at the sight of injection (Buitendag and von Broembsen, 1993; Schwarz et al., 1974). Despite these drawbacks, recent studies have been done that focuses on the use of penicillin G and streptomycin as possible control agents against Las (Zhang et al., 2010; Zhang et al., 2011).

2.7.2 Management through quarantine

Quarantine measures were implemented as part of a HLB control strategy in the Philippines to prevent the spread of the disease to unaffected areas. This strategy included planting of disease-free trees and spraying of insecticides to control vector populations (Altamirano et al., 1976). In South Africa, Greening disease has been successfully managed since the early 1990s by the implementation of a three-pronged approach that involves the use of disease-free material, vector control (to be discussed separately) and the removal of inoculum sources (Buitendag and von Broembsen, 1993). Under the citrus improvement program, farmers receive disease-free citrus trees that were grown in psyllid-proof nurseries. These nurseries, in turn, receive budwood from the Foundation Block, located in the Eastern Cape, one of the last Greening-free areas in the country (von Broembsen and Lee, 1988). In order to reduce inoculum sources in the field, trees younger than 10 years with significant infection (infection>75%) are removed whilst in trees older than 10 years, only branches showing

infection are removed (Buitendag and von Broembsen, 1993). Management of HLB through this integrated approach have also been adopted in Brazil (Belasque et al., 2010), China (Shokrollah et al., 2011) and Taiwan (Hung et al., 2000).

2.7.3 Novel control strategies

Temperature dependent techniques such as cryopreservation (Ding et al., 2008) and thermotherapy (Hoffman et al., 2013) have been evaluated as tools to eliminate Las from germplasm and breeding material. Other novel control strategies that are being explored includes the use of antagonistic bacteria to Las isolated from healthy citrus roots (Trivedi et al., 2011) and the use of antimicrobial compounds that specifically inhibits the ATPase activity of *sec*A (Akula et al., 2012). The use of transgenic trees has also been considered (Miyata et al., 2012) and Dutt et al. (2012) evaluated four phloem specific promoters that could potentially be targeted for transgene expression of an antimicrobial construct specifically designed for the elimination of Las.

2.7.4 Vector control

Control of the vector species of Laf, Las and Lam form a vital part of integrated control strategies of both HLB and Greening. Insecticide application is synchronized with flushing rhythms of trees which coincide with higher vector populations as these vectors breed exclusively on young flush (Catling, 1969d; Aubert, 1987). The application of monocrotophos as foliar sprays (Pyle, 1977; Supriyanto and Whittle, 1991) and petroleum sprays (Rae et al., 1997) to control adult triozid and liviid vector species have been applied. The insect growth regulators (IGRs) buprofezin and diflubenzuran have also been shown to be effective in controlling vector populations. These IGRs act by suppressing the emergence of adults by disrupting mottling stages (Tiwari et al., 2012).

Vector populations are naturally controlled by the presence of parasites (Catling, 1969a) and predators, although predators seem to only play a minor role (Catling, 1969c). These agents have been considered as viable biological control agents. In Reunion, where both *T. erytreae* and *D. citri* occurs, the biological control of vector populations was proven to be successful (Aubert and Quilici, 1988). The ectoparasites *Tetrastichus radiates*, Waterston, which is the main parasite of *T. erytreae* (Catling, 1969a) and

Tetrastichus dryi Waterston, were introduced into Reunion (Ettienne and Aubert, 1980) and within four years significant reductions in populations sizes were noted (Aubert and Quilici, 1984). Within 8 years after the introduction of these parasites, *T. erytreae* was completely eliminated from the island and *D. citri* populations were greatly decreased (Aubert and Quilici, 1988). Similar success was achieved in Mauritius (van den Berg, 1990). Complete biological control have not been achieved elsewhere and may be attributed to the presence of hyperparasites, which are absent in Reunion (Aubert and Quilicci, 1984).

Various other biological agents have been considered that can be used as part of an integrated control strategy. In Mexico, the honey wasp *Brachygastra mellifica* Say, have been identified as a potential control agent of *D. citri* (Reyes-Rosas et al., 2011), although no studies have thus far been conducted to evaluate the effectiveness of this predaceous wasp. Studies have shown that the predatory mite *Amblyseius swirskii* Athias-Henriot 1962 may be an effective control agent for *D. citri* (Juan-Blasco et al., 2012). The entomopathogenic fungi, *Isaria fumosorosea* Wize, have also shown promising results under both laboratory (Stauderman et al., 2012) and field conditions (Lezama-Gutierrez et al., 2012). Novel strategies for the control of vector populations such as the use of volatiles emitted by species of *Allium* L. (Garlic) (Mann et al., 2011b) and more molecular approaches such as the use of RNAi specifically targeted at *D. citri* (Wuriyanghan et al., 2011) are being explored.

2.8 Host Range of Citrus infecting Liberibacter species

Las, Laf and Lam are considered to infect all commercially grown citrus species and cultivars irrespective of the rootstocks used. In South Africa, Laf has been found associated with Greening disease from *Ci. sinesis, Ci. reticulate* Blanco (Tangerine and Mandarin), *Ci. pardisi, Ci. pardisi x Ci. reticulate* (Tangelo), *Ci. limon* (Lemon), *Ci. jambhiri* Lush. (Rough lemon), *Ci. Aurantium* L. (Sour orange), *Poncirus trifoliate* (L.) Raf. (Trifoliate orange), *Ci. aurantifolia* (Christm.) Swingle (Lime) (McClean and Schwarz, 1970) and *Ci. reticulate* (Clementine) (Korsten et al., 1996). The response to infection of these citrus species by Laf can be classified as severe (Sweet orange, mandarin, tangelos), moderate (grapefruit, lemon, sour orange) and tolerant (pummelo,

trifoliate orange) (Manicom and van Vuuren, 1990). Similar disease trends are described for citrus infected with different Las genotypes from Japan (Mayikawa, 1980) and Florida (Folimonova et al., 2009). In Brazil, Lam was inoculated onto various citrus varieties of *Ci. sinesis*, *Ci. Reticulate x Ci. Sinesis* (Tangor), *Ci. reticulate* and *Ci. limon*. From these studies it was shown that all the tested varieties were susceptible to infection by Lam to variable degrees, as seen with Las and Laf (Lopes and Frare, 2008).

2.8.1 Alternative Rutaceous hosts

The presence of alternative hosts to the three known citrus infecting Liberibacter species and their insect vectors makes these hosts potential reservoirs for the continual introduction of Greening in South Africa and HLB in other countries into commercial citrus orchards. Because of this, studies have been conducted to determine whether such hosts exist, either naturally or experimentally.

One such potential host for Las, which have been studied extensively, is *M. paniculata* (Orange Jasmine) (Mayikawa, 1980; Hung et al., 2000; Damsteegt et al., 2010, Zhou et al., 2007; Lopes et al., 2010; Walter et al., 2012). *M. paniculata* is a common ornamental tree in many citrus producing countries and is also host to *D. citri* (Tsai and Liu, 2000). Transmission studies of Las from citrus to *M. paniculata* have been performed using both grafting and vector transmission approaches. Hung et al. (2000) concluded that Las is not capable of being transmitted to and multiplying within *M. paniculata.* In contrast, Damsteegt et al. (2010) demonstrated that *D. citri* successfully transmitted Las to *M. paniculata* in which Las replicated for up to 10 months. Despite the low success rate of artificial transmission of Las to *M. paniculata,* this bacterium have been found to naturally infect *M. paniculata* trees in urban areas around Sáo Paulo (Lopes et al., 2010) and Florida (Walter et al., 2012). In Florida, *M. paniculata* plays a minor role as a reservoir for Las as only 1.8% of the trees tested were positive for Las which was present at low titres (Walter et al., 2012). Lam was also found to naturally infect *M. paniculata* trees in Brazil and it was shown that Lam is more adapted to multiplying in adult *M. paniculata* trees than Las (Lopes et al., 2010). It has also been

demonstrated that *D. citri* is capable of transmitting Lam from *M. paniculata* to *Ci. sinesis* trees (Gasparoto et al., 2010).

L. acidissima (wood apple) and *Severinia buxifolia* (Poir.) Ten. (Chinese box orange) have been identified experimentally as possible reservoir hosts of Las (Hung et al., 2000; Hung et al., 2001). Other rutaceous members which have been identified to be naturally infected with Las includes; *Cl. lansium* (Lour.) Skeels (Wampee) (Ding et al., 2005) in China and *Ci. decumana* (L.) Murr. (Beejapuraka) in India (Adkar-Purushothama et al., 2011). In South Africa, *V. lanceolata* has been identified as an alternative host of a Liberibacter through Southern blotting (Korsten et al., 1996) and *Cl. anisata* inoculated with bark strips from greening trees presented symptoms similar to Greening disease (van den Berg et al., 1991-1992).

2.8.2 Non-Rutaceous hosts

Las has been successfully transmitted to non-rutaceous species including *Ca. roseus* (Garnier and Bové, 1983), *Nicotiana tabacum* (L.) (Garnier and Bové, 1993) as well as *Lycopersicon esculentum* (Mill.) (Tomato) (Duan et al., 2008) through the use of dodder species. Laf has also been transmitted to *Ca. roseus* from infected *Ci. sinesis* through dodder (Garnier and Bové, 1983). Lam has been shown to infect both *N. tabacum* (Francischini et al., 2007) and *Cu. indecora* (Hartung et al., 2010b). During a survey conducted in Jamaica, Las was identified from three weed species namely; *Cleome rutidosperma* DC (Family: Capparaceae), *Pisonia aculeate* (L.) (Family: Nyctaginaceae) and *Trichostigma octandrum* (L.) H.Walter (Family: Phytolaccaceae) (Brown et al., 2011). The ability of these '*Candidatus* Liberibacter' species to multiply within non-rutaceous hosts suggest that members of the Alphaproteobacteria may be less host specific amongst botanical families than previous suspected.

2.9 '*Candidatus* Liberibacter species' related to Laf, Las and Lam

Various other species belonging to the Alphaproteobacteria have been identified that are closely related to Las, Laf and Lam. These novel species have been identified from

different botanical hosts across a range of families. In the section below, a brief overview of these are provided.

2.9.1 '*Candidatus* Liberibacter africanus subsp. capensis'

In 1994, Greening symptoms were observed on *Ci. reticulate* trees in the Western Cape South Africa, for the first time. These samples were confirmed to be positive for Laf leading to the Western Cape losing its Greening-free status (Garnier et al., 1999). Mottling symptoms similar to those associated with the Greening disease were observed on *Ca. capense* tree that bordered the *Ci. reticulate* orchard where Greening was first observed. PCR results indicated that this *Ca. capense* tree was infected with Laf and it was thought that this indigenous ornamental Rutaceae was the origin of the introduction of Greening in the Western Cape (Garnier et al., 1999). Subsequent phylogenetic studies based on the 16S rRNA, 16S-23S rRNA intergenic region and part of the *rpl*KAJL-*rpo*B operon demonstrated that the Liberibacter present in *Ca. capense* was not Laf but a new Liberibacter closely related to Laf. This novel Liberibacter was subsequently named '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) (Garnier et al., 2000) and has been shown to be widely distributed in South Africa in association with *C. capense* (Phahladira et al., 2012).

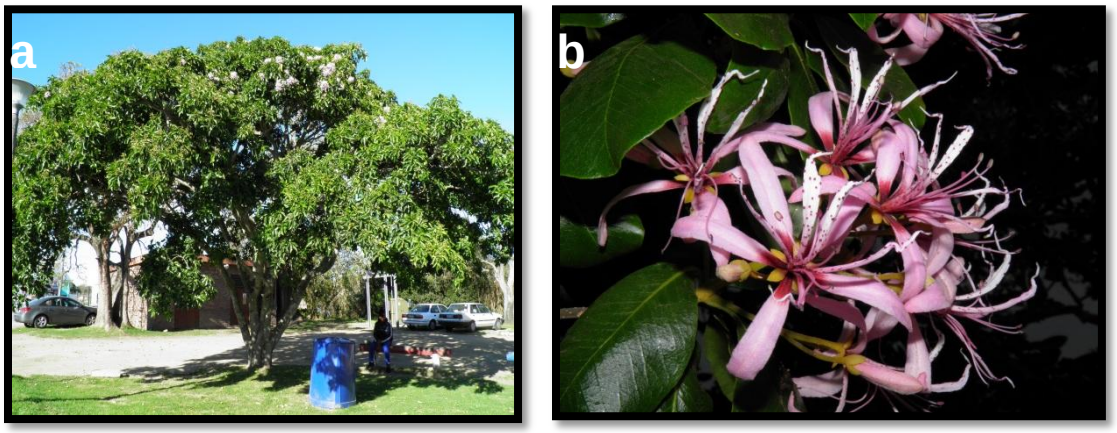

Fig 2.3: *Ca. capense* **a)** in the Western Cape, South Africa; **b)** Flowers of a *Ca. capense* tree.

2.9.2 '*Candidatus* Liberibacter solanacearum'

A Liberibacter was found to be associated with Zebra chip disease of *Solanum tubersum* (L.) (potato) in New Zealand (Liefting et al., 2008a). The same Liberibacter species was also identified from tomato and pepper and is now known as '*Candidatus* Liberibacter solanacearum' (Lso). Subsequently, Lso has been associated with *Solanum betaceum* (Cav.) (Tamarillo or tree tomato), *Physalis peruviana* (L.) (Cape gooseberry) (Liefting et al., 2008b) and *Daucus carota* (L.) (Carrot) (Munyaneza et al., 2010). Lso is vectored by the potato psyllid *Bactericera cockerelli* Sulc (Liefting et al., 2009) and in these host causes mottling symptoms similar those caused by HLB and Greening on citrus.

2.9.3 '*Candidatus* Liberibacter europaeus'

In 2011 another '*Candidatus* Liberibacter species' was identified from *Pyrus communis* L. (pear) in Europe (Raddadi et al., 2011). This species is vectored by *Cacopsylla pyri* L. (Homoptera: Psyllidae) and is known as '*Candidatus* Liberibacter europaeus' (Leu). Leu has been found to be widespread across Europe being present in 9/14 species within the genus *Cacopsylla* and have been found associated with *Prunus spinosa* (L.) (black thorn), *Malus domestica* (Borkh.) (apple) and *Crataegus monogyna* (Jaquin) (hawthorn) trees (Camerota et al., 2012). Unlike other known Liberibacters, Leu causes no disease on *P. communis* despite reaching high titres in host plants and is therefore considered to be an endophyte (Raddadi et al., 2011). This endophyte has also been reported from New Zealand where it was discovered from the invasive West European shrub, *Cytsus scoparius* (L.) (Link) (scotch broom), where it is vectored by the broom psyllid, *Arytainilla spartiophila* Foerster (Thompson et al., 2013).

2.9.4 *Liberibacter crescens*

More recently a Liberibacter has been identified from *Vasconcellea pubescens* (A.) DC (mountain papaya) (Leonard et al., 2012). This is the first member of the genus Liberibacter to have been grown in culture which allowed researches to easily sequence the complete genome of *L. crescens* (CP003789) (Leonard et al., 2012). Because this

is the only species within the genus *Liberibacter* to be obtained in culture, *L. crescens* (Leonard et al., 2013) lacks "*Candidatus*" epithet. This bacterium could ultimately act as a model to better understand other Liberibacter species which cannot be obtained in culture.

2.10 Molecular characterization of Laf, Las and Lam

The unculturable nature of the three known citrus infecting '*Candidatus* Liberibacter' species has slowed their molecular characterization. The first sequence to be cloned and characterized for both Las and Laf was the *nus*G-*rpl*KAJL-*rpo*BC gene cluster, the DNA target of probes ln-2.6 for Las isolates (Villechanoux et al., 1993) and As-1.7 for Laf isolates (Planet et al., 1995). At about the same time, the 16S rRNA gene region for these two bacterial species were also obtained by PCR and cloning (Jagoueix et al., 1994). Later, upon discovery of a novel '*Candidatus* Liberibacter' species being present in citrus from São Paulo, the above mentioned sequences were used to characterize Lam (Teixeira et al., 2005). In addition, by using a random amplified polymorphic DNA assay (RAPD), Hocquellet et al. (1999a) succeeded in characterizing four additional genes (*nus*G, *pgm, omp* and a gene encoding a hypothetical protein) from the chromosomes of Las and Laf.

Molecular characterization of Las occurred at a more rapid rate than that of Laf due the growing importance of the organism in disease epidemiology of HLB in Florida. Through thermal asymmetric interlaced PCR (TAIL-PCR), the *nus*G-*rpl*KAJL-*rpo*B gene cluster could be further characterized to include *tuf*B and *sec*E (Okuda et al, 2005). Lin et al. (2008) extended the known sequence length of the three gene regions (*tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*BC gene cluster, *omp* and 16/23S rDNA) by 14.7 kbp using a modified genomic walking method. They were able to characterize an additional 8,564 bp from these three gene regions using this same method (Doddapaneni et al., 2008). This method was also used to obtain the sequence for the complete β-operon (*rpl*KAJL-*rpo*BC operon) for Laf and Lam as well as *tuf*B, *sec*E and *nus*G genes for Lam (Teixeira et al., 2008).

In 2009, Duan et al. succeeded in obtaining the complete genome of Las ('*Candidatus* Liberibacter asiaticus' strain Psy62; NC_012985.2) through a metagenomics approach. This was achieved from DNA extracted from a single psyllid was subjected to multiple displacement amplification (Dean et al., 2002)) followed by pyrosequencing on the Roche 454 pyrosequencer (Duan et al., 2009).

The available genome information revealed several characteristics of Las which are unique to obligate intracellular bacteria (Duan et al., 2009). For example, Las has a single, circular genome which is greatly reduced (1.23Mb) when compared to other members of the Rhizobiaceae of which it is a member. Las is also characterized by a low GC content of only 36.5%. Of the 1,136 coding regions identified, 74% encode putative products with known function. However, relatively few genes enode products involved in the biosynthesis of compounds and regulatory elements, whereas the products of a large proportion of genes assist in cell motility and active transport of elements. The availability of the Las genome will therefore help scientists understand the interaction of the pathogen with its host and its vector. The availabiltity of this information may also help to determine the specific growth conditions needed for Las to be cultured, should it be possible.

The Las genome sequence information was also exploited for phylogenetic analyses. Based on the concatenated data for 92 protein sequences, Duan et al. (2009) showed that Las is an early-branching member of the Rhizobiales. Later studies supported this phylogenetic placement as Las shares protein orthologs and various other characteristics with other members of the Rhizobiales (Hartung et al., 2011).

Neither Laf nor Lam whole genome sequences are available at present. However, the genome of Lam has been estimated to be a circular genome of 1.29-1.34Mb in size through the use of PFGE and hybridization (Wulff et al., 2009). Attempts are underway to complete whole genomes of both Laf (Li and Pietersen, *per comm*) and Lam (Lin et al., 2013).

2.11 Genetic diversity amongst Las isolates

Determining the genetic diversity amongst geographically isolated Las populations can help give insight into the origin of Las. Such information is valuable for understanding how possible disease introductions occurred and how future introductions can be prevented/limited. For Las a number of intraspecific markers have been developed. Gao et al. (1993) defined seven distinct Asian serogroups being present for Las through the use of monoclonal antibodies. Single nucleotide polymorphism (SNP) analysis of the 16S rRNA and β-operon regions have also been used to identify the genetic diversity of Las in Karnataka state, India (Adkar-Purushothama et al., 2009). These gene regions however are highly conserved and forms part of the core genome making them poor candidates in genetic diversity studies (Tomimura et al., 2009; Chen et al., 2010).

Bastianel et al. (2005) suggested using the outer membrane gene (*omp*) to study genetic variation between different Liberibacter species as well as for determining the genetic variation within species. Despite the fact that this gene is part of the core genome PCR-RFLP analysis of the *omp* gene allows resolution of geographically distinct Las isolates. The use of *omp* based PCR-RFLP has since been used to differentiate Las strains infecting both *Ci. reticulate* and *Ci. grandis* Osbeck trees in Taiwan (Ahmad et al., 2009), as well as for studying the genetic diversity of Las isolates found in China (Hu et al., 2011).

Tomimura et al. (2009) compared the use of 5 molecular markers (16S rRNA, 16S-23S rRNA intergenic spacer region, *omp*, *trn-tuf*B-*nus*G-*rpl*KAJL-*rpo*B and bacteriophage-type DNA polymerase) to differentiate genetically diverse Las isolates from South-East Asia. They found that the bacteriophage-type gene region was the most variable marker used and allowed separation of the Las isolates from South East Asia into three groups. Isolates from Japan, however, did not have a detectable bacteriophage sequence present in their genomes.

Due to their hypervariable nature, phage related gene sequences are becoming increasingly popular for differentiating Las isolates. Liu et al. (2011) distinguished Las isolates from Guangdong and Yannan in China by using a prophage terminase gene as

molecular marker whereas Zhou et al. (2011) cloned and sequenced prophage genes *hyv*$_{I}$ and *hyv*$_{II}$ from Las isolates collected from different hosts and geographic areas. The use of *hyv*$_{I}$ and *hyv*$_{II}$ proved to be excellent for differentiating Las isolates from different countries regardless of the host used. Of these genes *hyv*$_{I}$ showed greater genetic resolution than *hyv*$_{II}$.

The use of variable tandem repeat numbers (VNTR) or microsatellite markers have also been used to distinguish Las populations. Chen et al. (2010) selected locus CLIBASIA_01645 based on the presence of the tandem repeat sequence AGACACA within this locus. Through the use of this VNTR marker they found evidence to suggest that Las populations from Guangdong are distinct from the populations found in Florida. It was also hypothesized that Las was introduced on two separate occasions into Florida based on the presence of two distinct Las populations. Kotah et al. (2011) also used VNTR markers to differentiate 21 Las isolates from Okinawa into 17 different genetic groups. By combining SNP markers that are located 200bp upstream of the VNTR locus, this research group believes that more accurate predictions can be made concerning the dispersal of Las (Katoh et al., 2012). More recently, Islam et al. (2012) through the use of 7 polymorphic microsatellite markers were able to identify 117 Las genotypes from Asia, North America and South America. The lower genetic diversity of Las as in Florida and Brazil compared to the vast diversity as seen in Asia suggest that Las was only recently introduced into the Americas and did that it not originate from an indigenous source. Data from this study therefore supports the findings of Chen et al. (2010) that Las was introduced into Florida by two separate introduction events.

2.12 The origin of '*Candidatus* Liberibacter' species

The origins of the causal agents of HLB and Greening disease have been a point of considerable discussion over the past years. '*Candidatus* Liberibacter species' are considered to have evolved along with members of the orders Rhizobiales and Rhodobacteriales (Doddapaneni et al., 2008). However, based on the phylogenetic analyses of the 16S rRNA region of known citrus infecting Liberibacter species, this group of bacteria emerged prior to the expansion of the order Rhizobiales

(Doddapaneni et al., 2008). Two schools of thought exists concerning the origin citrus infecting Liberibacters, both of which will be discussed here.

2.12.1 Multi-continental Hypothesis

The initial hypothesis on the origin of HLB and Greening is that each citrus infecting Liberibacter evolved independently from one another on separate continents, i.e., Las evolved in Asia, Laf in Africa and Lam in South America (Bové, 2006). The origin of Las from China has been widely accepted, as most commercially grown citrus originates from this country (Beattie et al., 2008), and some of the earliest reports of HLB are from Guangdong province, China (Lin and Lin, 1956). Molecular evidence that supports the Chinese origin of Las was produced by Islam et al. (2012) who showed high levels of microsatellite-based genetic diversity amongst Las isolates from China. This diversity is is consistent with the idea that long-standing populations such as these have accumulated significantly more mutations over time than recently introduced populations. Various so-called “founder haplotypes” were also detected among the isolates from China further supporting an origin of Las in this region. However, similar high levels of genetic diversity and “founder haplotypes” also been found in Las populations in India (Islam et al, 2012), supporting the idea that Las originated in India and spread eastward to China (Beattie et al., 2008; Teixeira et al., 2008).

Upon the discovery of Las in São Paulo, Lam was the dominant Liberibacter species present in citrus orchards compared to Las incidence. This suggested that Lam was present in Brazil prior to the introduction of Las (Teixeira et al., 2005). However, Lopes et al. (2009b) demonstrated that Las reach higher titres in commercial citrus trees than Lam suggesting that the association of citrus with Lam is not as well-established as the relationship between Las and citrus. The higher incidence of Lam within orchards as explained here as well as the relative low fitness of Lam within citrus suggest that Lam may have originated from an indigenous source present in Brazil.

The African origin of Laf is supported by two factors. Firstly, Laf has only ever been identified from commercial citrus orchards in Africa (Garnier and Bové, 1996) and secondly, citrus is not indigenous to the African continent (Beattie et al., 2008). As

mentioned above, Laf and a related Liberibacter, LafC, have been identified from *V. lanceolata* (Korsten et al, 1996) and *Ca. capense* (Garnier et al., 2000) respectively. The presence of Liberibacters on indigenous Rutaceae trees in South Africa and the ability of *T. eytreae* to feed on these trees (Moran, 1968) may suggest that Laf originated from an indigenous source. One such an example is given here.

2.12.1.1 LafC as possible Laf ancestor

Since the discovery of LafC (Garnier et al., 2000), this Liberibacter has been widely associated with *Ca. capense* trees from across South Africa in both Greening and Greening-free areas (Phahladira et al., 2012). It has also been demonstrated that LafC is not found in commercially grown citrus (Garnier et al., 2000; Pietersen et al., 2010) and it does not seem likely that LafC is transmitted to commercial citrus under natural conditions (Phahladira et al., 2012). LafC also causes no clear symptoms on *Ca. capense* indicating the possibility that this bacterium co-evolved with *Ca. capense*. This long-standing association is further supported by the common occurrence of LafC with *Ca. capense* even in isolated, natural regions that are far removed from citrus. The presence of LafC in South Africa prompted the hypothesis that this close relative of Laf may represent a parent lineage of Laf and that the association of Laf with citrus emerged through a host jumping event possibly assisted by an intermediate host such as *V. lanceolata* (Phahladira et al., 2012).

2.12.2 Single Australasian origin

Beattie et al. (2008) has proposed that Las and Laf evolved from a common ancestor in Africa and that speciation of Las occurred in India. Their hypothesis dictates that an ancestral '*Candidatus* Liberibacter species' was present in *V. lanceolata* and that this ancestor was transmitted to *Ci. sinensis* or *Ci. reticulate* trees by *T. eytreae.* Such an event is proposed to have occurred in a European colony on the Southeast coast of Africa and that infected trees/material were shipped to the Indian subcontinent. Here, the disease could have been spread by the acquisition of the bacteria by *D. citri* and assisted through changes by horticultural practices that increased the flushing rhythms of commercially grown citrus.

It has been demonstrated that Lam diverged from Las and Laf 309 million years ago (mya) and that Laf and Las again diverged from one another 147 mya (Teixeira et al., 2008). These findings support the idea that a putative Gondwanan Liberibacter ancestor existed which resulted in the speciation of Las and Laf following the dislocation and fractioning of Gondwana. Africa split from India 160 mya in which African and Indian Liberibacter lineages became isolated and could possibly have given rise to Las and Laf as it is known today (Teixeira et al., 2008).

2.13 Concluding remarks

'*Candidatus* Liberibacter species' represents a group of alphaproteobacteria that is diverse in terms of their host range, vectors, temperature preferences and symptom expression. The presence of both pathogenic (Las, Laf, Lam and Lso) and endophytic (LafC, Leu) species within this group supports the idea proposed by Lopes et al. (2010), that members of this group may be opportunistic pathogens in non-native hosts. By evaluating different potential hosts of these Liberibacters and determining whether these Liberibacters are pathogenic on any of these putative hosts, it may be able to gain insight into the evolution of Liberibacter species as they are known today.

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Chapter 3*

'*Candidatus* Liberibacter' amongst native hosts of *Trioza erytreae* in South Africa

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3.1 Abstract

Greening disease in South Africa is associated with '*Candidatus* Liberibacter africanus' (Laf), a phloem limited bacterium vectored by *Trioza erytreae.* A Liberibacter related to Laf, '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) was found infecting *Calodendrum capense*. The widespread presence of LafC in South Africa suggests that, in addition to *Ca. capense,* other Rutaceae trees from the country may be infected with Liberibacters related to Laf. The current study aimed to investigate the existence of such hosts. Samples from 234 *Clausena anisata,* 289 *Vepris lanceolata* and 231 *Zanthoxylum capense* were collected throughout the natural distribution of these trees in South Africa. Total DNA was extracted and tested for the presence of Liberibacters by real-time PCR. '*Candidatus* Liberibacters' present in positive samples was characterized by amplifying and sequencing the *rplJ*, *16S* and *omp* regions. To confirm the identity of the tree host species from which Liberibacter sequences were obtained, tree hosts were barcoded. Of the trees tested, 33 *Cl. anisata,* 17 *V. lanceolata,* 9 *Z. capense* and 1 *Zanthoxylum davyi*, tested positive for a Liberibacter. Phylogenetic analysis of the *rplJ* and *omp* regions, revealed unique clusters for Liberibacters associated with each tree species. Phylogenetic analysis of 16S rRNA sequence indicated that Liberibacters obtained from *V. lanceolata* and *Cl. anisata* were similar to *16S* sequences for LafC, whereas those from *Zanthoxylum* species grouped separately. The presence of Laf-related Liberibacters from indigenous Rutaceous species should be further investigated to determine whether these Liberibacters are capable of being transmitted to and causing disease within commercial citrus species.

3.2 Introduction

During the late 1920's a disease commonly known as Greening was observed from citrus orchards in Rustenburg, South Africa. The disease soon spread to other production regions within the country and by the late 1970's, Greening virtually eliminated citrus production in Rustenburg, Tzaneen, White River and production regions west of Nelspruit (Pretorius and van Vuuren, 2006). This disease is estimated to have caused annual losses of R35 million (3.8 million USD) by the 1980's (van den Berg et al., 1987). Today, Greening incidence have been lowered to economic acceptable levels through the implementation of stringent control strategies but remains a reoccurring problem in cooler production areas.

Initially it was thought that Greening was caused by a transmissible virus (McClean and Oberholzer, 1965a), but it was later demonstrated that a phloem-limited (Garnier and Bové, 1983) member of the Alphaproteobacteria (Jagoueix et al., 1994) namely, '*Candidatus* Liberibacter africanus' (Laf) was associated with diseased plants (Garnier et al., 2000; Pietersen et al., 2010). Laf is primarily spread through the feeding and flight activities of the triozid, *Trioza erytreae* Del Guercio (McClean and Oberholzer, 1965b). This Liberibacter has thus far only been identified from citrus orchards in Africa and the Mascarene islands (Garnier and Bové, 1996; Garnier et al., 1996) whereas a related bacterium, '*Candidatus* Liberibacter asiaticus' (Las) (Jagoueix et al., 1994, Garnier et al., 2000), has been associated with a disease similar to Greening known as citrus Huanglongbing (HLB) from Asia (Garnier and Bové, 1996), and the Americas (Coletta-Filho et al., 2004; Halbert, 2005). Las has only recently been identified from a citrus orchard in Ethiopia (Saporani et al., 2010), being the first report of this bacterium on the African continent. A third citrus infecting Liberibacter species, '*Candidatus* Liberibacter americanus' (Lam), is described from Brazil (Teixeira et al, 2005a). Both Las and Lam are vectored by the liviid, *Diaphorina citri* Kuwayama (Capoor et al., 1967; Teixeira et al., 2005b). The insect vectors involved in the spread of HLB and Greening respectively, have been shown to efficiently transmit both Las and Laf (Masonie et al., 1967; Aubert, 1987). Laf and Lam are considered to be heat sensitive (Garnier and Bové, 1983; Lopes et al., 2009), whereas Las is heat tolerant (Garnier and Bové, 1983).

Infection of citrus trees with any of the three citrus infecting Liberibacter species are characterized by mottled appearance of leaves from infected branches, the production of small, unripe fruit and tree stunting (McClean and Oberholzer, 1965b; Lopes et al., 2008).

In addition to the three Liberibacter species associated with commercial citrus, a Liberibacter has been described from an ornamental Rutaceae tree, *Calodendrum capense* (L.f.) Thunb (Cape Chestnut) indigenous to South Africa (Garnier et al., 2000). Initially it was speculated that the bacterium identified from a *Ca. capense* in close proximity to a citrus orchard in the Western Cape, was identical to Laf and may be the origin of the introduction of Greening disease in this province (Garnier et al, 1999). Subsequent sequence analyses, however, revealed that this Liberibacter, whilst having some homology with Laf, was unique from both Laf and Las and was subsequently named '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) (Garnier et al, 2000). LafC, despite being widely associated with *Ca. capense* (Phahladira et al., 2012), has not been identified from commercial citrus is South Africa (Pietersen et al., 2010), indicating that this bacterium does not play a direct role in the epidemiology of greening disease.

Both Greening disease and HLB are managed by a three pronged approach which includes the planting of disease free material, the removal of inoculum sources through elimination of infected trees and branches from an orchard, and chemical control of vectors (Buitendag and von Broembsen, 1993; Belasque et al., 2010; Hung et al., 2000; Shokrollah et al., 2011). This strategy is directed solely at the removal of inoculum sources within orchards and may therefore be flawed. The insect vectors of all known citrus infecting Liberibacters have been observed colonizing indigenous vegetation surrounding commercial orchards (Moran 1968a, Miyakawa, 1980) which is excluded from control measures. The existence of indigenous hosts to the vectors of these Liberibacters poses two distinct challenges towards complete elimination of both Greening and HLB. Firstly, indigenous vegetation surrounding orchards are excluded from spraying programs, aiding the perpetuation of vector populations. Van den Berg et al. (1991) found that the flushing period of native hosts of *T. erytreae,* which follows that

of commercial citrus species, prolonged the breeding period of this triozid, increasing vector population sizes dramatically. Secondly, indigenous plant species may act as alternative hosts to citrus infecting Liberibacters resulting in reinfection of commercial citrus species with Liberibacters through the migration of triozid and liviid vectors between indigenous vegetation and orchards (van den Berg et al., 1991). Las has been found to naturally infect *Murraya paniculata* (L.) Jack (Orange jasmine) in São Paulo (Lopes et al., 2010) and Florida (Walter et al., 2012) whereas Lam has been identified from this host in Brazil (Lopes et al., 2010). In addition to *M. paniculata,* other Rutaceous trees such as *Clausena lansium* (Lour.) Skeels. (Ding et al., 2005) and *Citrus decumana* (L.) L. (Adkar-purushothama et al., 2011), have been found naturally infected with Las. This Liberibacter has also been identified from the non-rutaceous weeds *Cleome rutidosperma* DC (Family: Capparaceae), *Pisonia aculeate* L. (Family: Nyctaginaceae) and *Trichostigma octandrum* (L.) H.Walter (Family: Phytolaccaceae) in Jamaica (Brown et al., 2011). No formal studies have thus far dealt with the presence of naturally occurring alternate host to Laf in South Africa. The existence of such host could explain why Greening disease remains an economic important disease of citrus in cooler production areas.

T. erytreae have been observed feeding on and colonizing members within the Rutaceae family, indigenous to South Africa (Moran, 1968a; Moran, 1968b). Moran (1968a) recorded *Cl. anisata* (Willd.) J.Hk. ex Benth. (Horsewood), *Vepris lanceolata* (Lam.) G.Don (White ironwood) (Previously *Vepris undulata*) and *Zanthoxylum capense* (Thunb.) Harv. (Small forest knobwood) (Previously *Fagara capensis*) (Fig. 1) as native hosts of *T. erytreae,* supporting the development of all life stages of this triozid (Moran, 1968a). He found that whilst preferentially feeding on *Ci. Limon* (L.) Burm.f. if given a choice*, T. erytreae* would also feed on these three host species in addition to *Ca. capense* (Moran, 1968b). Previously Laf has been identified from *V. lanceolata* by Southern hybridization (Korsten et al., 1996), whereas *Cl. anisata* was shown to be a potential host of this bacterium through grafting (van den Berg et al., 1991-1992). The association and common occurrence of *T. erytreae* with *Cl. anisata, V. lanceolata* and *Z. capense* and the possibility that *Cl. anisata* and *V. lanceolata* may harbor Laf, makes

these three hosts likely alternate hosts of Laf which acts as native reservoir sources for this bacterium.

Fig. 3.1. Native hosts of *T. erytreae* **a)** A healthy and **b)** triozid infested *Cl. anisata*. **c)** A healthy growing *V. lanceolata* in Knysna, Western Cape **d)** Leaves of a *V. lanceolata* showing characteristic *T. erytreae* infestation. **e)** Bark of *Z. capense* found in KwaZulu-Natal **f)** *Z. capense* leave with evidence of previous triozid infestation

Laf has thus far only been identified from commercial citrus in Africa and the Mascarene islands (Garnier et al., 1996; Garnier and Bové, 1996; Pietersen et al., 2010), being absent from Asia where most commercially grown citrus varieties were developed (Beattie et al., 2008). This suggests that Laf may have originated on the African continent within an indigenous source. Phahladira et al. (2012) proposed that LafC from *Ca. capense* may represent such an ancestral lineage of Laf which underwent a host shift from *Ca. capense* to citrus, possibly assisted by *V. lanceolata* which acted as an intermediate host. The involvement of *V. lanceolata* in the evolution of Laf as proposed by Phahladira et al. (2012) is supported by the ability of *T. erytreae* to colonize this plant as well as the identification of Laf through hybridization from this host species (Korsten

et al., 1996, Beattie et al., 2008). However, no studies have been conducted to ascertain whether the Liberibacter identified from *V. lanceolata* is related to either Laf or LafC or represents a novel Liberibacter species. *T. erytreae* additionally feeds on *Cl. anisata* and *Z. capense* indicating the possibility that these hosts may be naturally infected with either of the African Liberibacter species, having had a more direct role in the evolution of Laf from citrus than LafC from *Ca. capense*.

A survey was conducted in which samples of *Cl. anisata, V. lanceolata* and *Z. capense* were collected within the natural distribution of these species in South Africa. Samples were then analyzed for Liberibacters. The identification of indigenous hosts of Laf may help identify possible reservoirs involved in the spread of greening disease in South Africa. Additionally, the identification of Liberibacters from alternative rutaceous host may give insight into the evolution of Laf from the African continent.

3.3 Materials and Methods

3.3.1 Sample Collection

Leaf and petiole samples of *Cl. anisata, V. lanceolata* and *Z. capense* were collected from across South Africa within the natural distribution of these species (Fig. 3.2). Trees were located and visually identified with help from both amateur and seasoned botanists. GPS coordinates were taken of trees sampled and each tree was tagged with a unique accession number. Trees were sampled from both natural and urban settings, irrespective of the presence of symptoms. Most samples were collected during winter months and early spring, with a small number of samples being collected during summer.

3.3.2 DNA extraction

Total DNA was extracted from leaf petioles and midribs following a CTAB extraction method as described by Doyle and Doyle, (1990), with modifications of Fundecitrus. Pooled petioles and midribs (0.5g per sample) were macerated in filter separated bags containing 5ml CTAB with 0.2% mercaptoethanol using the Homex 6 Homogenizer (Bioreba, AG, Switzerland). Following this, 2ml macerate was transferred to a tube and

incubated at 60˚C for 30 min whilst shaking after which it was centrifuged for 5 min at 3000rcf. 900µl of supernatant was recovered and transferred to a new tube containing an equal volume of 24:1 chloroform:isoamyl alcohol, and mixed. Tubes were then centrifuged at 14000rcf for 5 min at 10˚C. Following centrifugation, 800µl of the upper aqueous phase was transferred to a new tube containing 480µl ice-cold iso-propanol and left overnight at room temperature. Samples were then centrifuged at 14000rpm for 20 min at 4˚C. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellet and tubes were centrifuged at 14000rpm for 10 min at 4˚C. The supernatant was discarded and the washing step was repeated. After the final washing step, samples were left to air dry for 20min. Pellets were re-suspended in 100µl TE buffer and stored at -80˚C.

3.3.3 Identification of Liberibacter positive samples by real-time PCR

To identify Liberibacter-positive samples, extracted DNA was subject to a Liberibacter generic real-time PCR. This assay was a modification of the real-time PCR protocol as described by Li et al. (2006) with the forward primer being redesigned to target a region of 16S rRNA which is conserved amongst known Liberibacter species, enabling the detection of various Liberibacter species within a single assay (Pietersen, *unpublished*). 1µl of DNA template was added to a final reaction volume of 10µl containing 5.0µl 2X Taqman® universal Master Mix II (ABI, Foster City, CA, USA), 500nM forward primer LibUF (5'-GGC AGG CCT AAC ACA TGC-3'), 500nM reverse primer HLBr (GCG TTA TCC CGT AGA AAA AGG TAG), 150nM probe HLBp (5'-AGA CGG GTG AGT AAC GCG-3'), 2ng/ml BSA and 3.4µl dH_2O. Reaction mixture and DNA template were loaded into capillaries and the reaction was performed using a LightCycler® 1.5 (Roche Diagnostics, Manheim, Germany) capillary based thermocycler. Reaction conditions were modified from the protocol described by Li et al. (2006) to allow optimum detection of unknown Liberibacters using Taqman® universal Master Mix II, with initial denaturation of 10 min at 95˚C, 45 cycles 95˚C for 10s, 62˚C for 50s and 72˚C for 5s, followed by final cooling of 30s at 40˚C. Fluorescence was measured and Ct values were determined using LightCycler® 1.4 software (Roche Diagnostics, Mannheim,

Germany). A positive/negative crossing threshold (Ct) of Ct<35 was used as samples with Ct>35 does not yield amplification with conventional PCR reactions.

3.3.4 Identification of Liberibacters from samples by PCR

Samples yielding Ct values within the threshold limit (Ct<35) were subject to the following three conventional PCR reactions, the products of which were sequenced in order to identify the Liberibacters present.

3.3.4.1 PCR amplification of *rplJ*

Liberibacter *rplJ* was amplified as described by Hocquellet et al., (1999). 0.5µl of DNA template was added to a final reaction volume of 50µl consisting of 0.1% of 2% Triton X-100, 5µl 10X NH_4 reaction buffer (Bioline, Boston, USA), 0.2mM deoxynucleotide triphosphate mix (dNTP), 0.5µM primer A2 (5'-TAT AAA GGT TGA CCT TTC GAG TTT-3'), 0.5µM primer J5 (5'-ACA AAA GCA GAA ATA GCA ACA A-3'), 0.05M $MgCl_2$, 2000µg/ml BSA, 5u/µl of 2.5 units (U) Biotaq® (Bioline, Boston, USA) and made up to a final reaction volume with molecular grade H_2O (Sigma-Aldrich, St. Louis, MO, USA). PCR cycling reaction was performed on a T100™ Thermal Cycler (Bio-Rad, CA, USA). Cycling conditions were set up as follow; initial denaturation at 92˚C for 3 min, followed by 35 cycles of denaturation at 92˚C for 20s, annealing at 62˚C for 20s and elongation at 72˚C for 45s, with a final elongation step at 72˚C for 5 min. Following amplification, products were visualized by gel electrophoreses.

3.3.4.2 PCR amplification of outer membrane protein (*omp*)

The outer membrane protein (*omp*) of Liberibacters was amplified from Liberibacter universal real-time PCR positive samples. The protocol of Bastianel et al. (2005) was used with modifications for the detection of unknown Liberibacters.

PCR was set up as for amplification of *rplJ* but with HPIinv (5'-ATG AAT TTG TTG CCT ATT CC-3') and OMP8inv (5'-TCA CGA CGA ATC ACA GAA TC-3') primers. Amplification of template DNA was performed on a T100™ Thermal Cycler (Bio-Rad, California, USA) with cycling conditions as follows; initial denaturation at 92˚C for 3 min, followed by 35 cycles of denaturation at 92˚C for 30s, annealing at 55˚C for 30s and

elongation at 72˚C for 2 min, with a final elongation step at 72˚C for 10 min. Amplification products were visualized on a 1% agarose gel following electrophoreses as previously explained.

3.3.4.3 PCR amplification of 16S ribosomal gene

The protocol described by Jagoueix et al. (1996) was followed for the amplification of Liberibacter 16S rRNA sequences. Minor modifications were made to detect unknown Liberibacters from samples.

PCR was performed by the addition of 0.5µl of DNA template to 50µl reaction mix as previously described using OA1 (5'-GCG CGT ATT TTA TAC GAG CGG CA-3') and OI2c (5'-GCC TCG CGA CTT CGC AAC CCA T-3') primers. Amplification was carried out under the following conditions; initial denaturation at 92˚C for 5 min, followed by 35 cycles of denaturation at 92˚C for 30s, annealing at 62˚C for 30s and elongation at 72˚C for 90s, with a final elongation step at 72˚C for 10 min. Amplification products were visualized following gel electrophoreses as previously described.

3.3.5 Direct sequencing of amplification products (*rplJ, omp, 16S*)

To remove single stranded DNA from PCR products, 0.5µl of 10 U exonuclease (Fermentas, Maryland, USA) and 2µ of 2U FastAP® (Fermentas, Maryland, USA) as per manufacturer's instructions. Purified amplicons were sequenced in both directions using Big Dye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, USA) as per manufacturer's instructions. Sequences of amplicon products were determined using an ABI 3500xL automated sequencer (Applied Biosystems, Foster City, CA) at the core sequencing facility of the University of Pretoria, South Africa.

3.3.6 Phylogenetic analyses

Sequences obtained were compiled into data sets according to each gene sequenced and aligned online with Mafft. Aligned data sets were trimmed using BioEdit and used in Jmodel test analyses. The phylogenetic relationships of aligned Liberibacter data sets were assessed using Mega version 5 (Tamura et al., 2011). In each data set, sequences from known Laf and LafC sources were included (Laf sequences 11-2015,

11-2016, 11-2019, 11-2021, 11-2025; LafC sequences 10-2241, 10-2281, 10-2282). Laf sources were obtained from citrus planted at the experimental farm, University of Pretoria whereas LafC sources were collected from *Ca. capense* in the Sundaysriver valley, Western Cape.

3.3.7 Confirmation of tree host species by DNA barcoding

To verify the nature of the tree host from which Liberibacters were identified, tree host species were identified by amplification and direct sequencing of the large subunit of ribulose 1,5-biphosphate carboxylase-coding gene (*rbcL*), the standard, plant barcoding gene.

Amplification was in 50µl reactions as described for Liberibacter PCRs above, but using primer rbcLa F (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3') (Levin et al., 2003) and primer rbcLa R (5'-GTA AAA TCA AGT CCA CCR CG-3') (Kress and Erickson, 2007). PCR cycling reaction was performed on a T100™ Thermal Cycler (Bio-Rad, California, USA). Cycling conditions were set up as follow; Initial denaturation at 92˚C for 3 min, followed by 35 cycles of denaturation at 92˚C for 20s, annealing at 55˚C for 20s and elongation at 72˚C for 90s, with a final elongation step at 72˚C for 5 min. Amplification products were then gel electrophoresed and visualized under an ultraviolet trans-illuminator. Amplicons were sequenced and subject to phylogenetic analyses as previously discussed.

3.4 Results

A total of 234 *Cl. anisata*, 289 *V. lanceolata* and 231 *Zanthoxylum spp specimens* were sampled from across South Africa, of which 33 *Cl. anisata,* 16 *V. lanceolata,* 9 *Z. capense* and 1 *Z. davyi* (as identified by barcoding, Fig. 3.6) tested positive for the presence of a Liberibacter following real-time PCR (Ct<35) (Table 3.1). These samples were obtained from different areas across South Africa in both greening and greening-free regions. Most of the samples were obtained from natural vegetation.

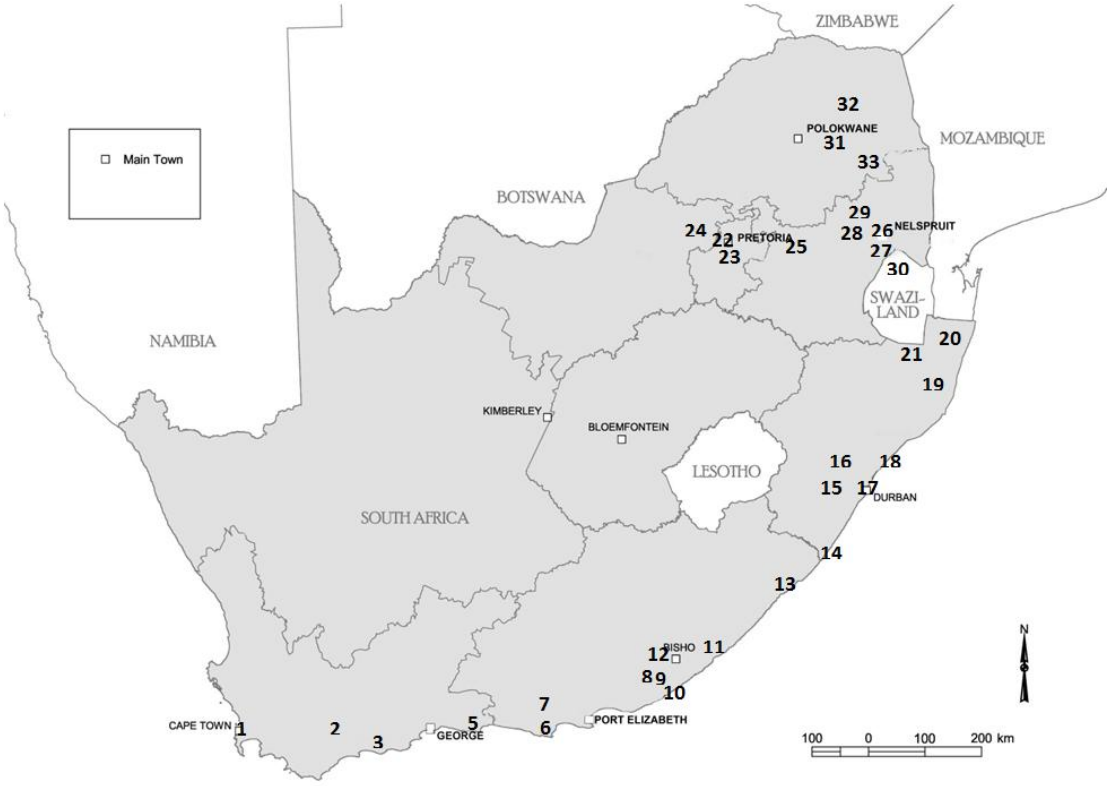

Fig. 3.2. Sampling sites of indigenous Rutaceous trees across South Africa. Site numbers (1-33) correspond to those listed in Table 1.

The Liberibacter specific *rplJ, omp* and *16S* gene sequences were determined for those samples testing positive with Liberibacter generic real-time PCR. None of the "no-template" and healthy control samples included per reaction yielded any amplicons. Amplification of *rplJ* and *16S* genes was successful for all Liberibacter positive samples tested and full length *rplJ* and *16S* sequences could be obtained from different host species. However, for *omp* sequences, Liberibacter sequences form only 29/33 *Cl. anisata,* 14/16 *V. lanceolata*, 8/9 *Z. capense,* 0/1 *Z. davyi* samples were successfully amplified and sequenced. Various attempts were made to lower stringency of the PCR in order to amplify the remaining samples but failed. Samples from which the *omp* gene could not be amplified were 11-4039, 11-4243, 11-4275, 11-4313, 11-4561, 12-0005, 12-0016 and 12-0017 (Appendix A).

Table 3.1: Number of indigenous trees sampled per site and number of Liberibacter positive (Ct<35 with the Liberibacter Universal real-time PCR) samples identified per site

Province	District	Site number (Fig)	#Samples with Ct<35/ #Sampled at site		
			Cl. anisata	*V. lanceolata*	*Z. capense*
Western Cape	Kirstenbosch	1	0	0/1	0
	Swellendam	2	0	0/3	0
	Heuningbos	3	3/13	0	0/9
	George	4	1/1	0/9	0
	Knysna	5	4/11	12/70	5/40
Eastern Cape	St. Francis Bay	6	3/7	0	1/11
	Patensie	7	0/2	0/13	0/10
	Grahamstown	8	0/14	2/23	0/10
	Bathurst	9	0	0/1	0/2
	Port Alfred	10	2/4	0/3	0/7
	East London	11	0	1/19	0/11
	Ngele	12	0/3	0	1/2
	Port St. John	13	0	0/2	0/0
KwaZulu-Natal	Port Edward	14	0/5	1/15	1/12
	Richmond	15	0/5	0/20	0/1
	Pietermaritzburg	16	4/8	0/6	0/1
	Durban	17	0/1	0/6	0/5
	Balito	18	1/1	0	2/7
	Hluhlwe	19	0/4	0/29	0/9
	Kosi Bay	20	0	0/4	0/1
	Pongola	21	0	0/4	0
Gauteng	Pretoria	22	0	0/24	0/21
	Johannesburg	23	0	0	0/2
North West	Rustenburg	24	0	0/17	0/6
Mpumalanga	Schoemanskloof	25	0/46	0/15	0/30
	Nelspruit	26	0	0	0/4
	Baberton	27	4/5	0	0/1
	Sabi	28	0/48	0/2	0/3
	Mount Sheba	29	0/11	0	0/12
Swaziland	Unknown	30	0	0	0/2
Limpopo	Magoebaskloof	31	0/11	0	0/6
	Tzaneen	32	0/16	0/1	0/6
	Lekgalameetse	33	0/18	0/2	0
Totals			33/234	16/289	10/231

Following amplification and sequencing, phylogenetic analyses was performed using the compiled data sets per gene sequence. None of the samples contained any typical

Laf sequences. Unique phylogenetic clusters however were obtained, correlating with the tree host species from which Liberibacter sequences were obtained, for both the *rplJ* (Fig. 3.3) and *omp* (Fig. 3.4) sequences.

For *rplJ*, the aligned sequences of Liberibacter ex *Cl. anisata* samples were 97.0% identical to that of LafC sequences from *Ca. capense* and only 86.4% identical to Laf sequences from citrus. Similarly, for the same region, Liberibacter ex *V. lanceolata* sequences shared greater nucleotide identity with LafC sequences (96.1%) when compared to Laf sequences (85.3%). Liberibacters ex *Z. capense* and the single Liberibacter ex. *Z. davyi,* formed a distinct clade but shared greater nucleotide identity across aligned sequences with Laf (89.0%) than LafC (84.0%). The distinct clades, differing from known Liberibacters, were shown to correlate with the host tree species from which they were obtained.

Aligned *omp* sequences from Liberibacter ex *Cl. anisata* clustered nearer to LafC (95.6% identity) than Laf (77.9% identity). This clustering was also observed for aligned Liberibacter ex *V. lanceolata* sequences which shared 95.7% sequence identity with LafC and only 77.9% identity with Laf sequences. Liberibacter ex *Z. capense* once again clustered nearer to Laf than LafC and shared 87.0% nucleotide identity across aligned *omp* sequences with Laf and only 76.9% sequence identity with LafC. Phylogenetic clustering from both aligned *rplJ* and *omp* sequences indicates distinct Liberibacter phylogenetic clusters that correlate with the host species harboring them.

The clustering of Liberibacter positive samples, as determined for Liberibacter *rplJ* and *omp* gene sequences, were incongruent with those observed for the 16S rRNA sequences. The 16S rRNA sequence of Liberibacter ex *Cl. anisata* and Liberibacter ex *V. lanceolata* shared 100% sequence identity with LafC sequences, and 99.0% sequence homology with Liberibacter ex *Z. capense* and Liberibacter ex *Z. davyi* sequences. Liberibacter ex *Z. capense* 16S rRNA sequences also shared 99.7% sequence homology with Laf sequences and were the only Liberibacter sequences to form a unique phylogenetic cluster separate from Laf and LafC sequences for this gene (Fig. 3.5).

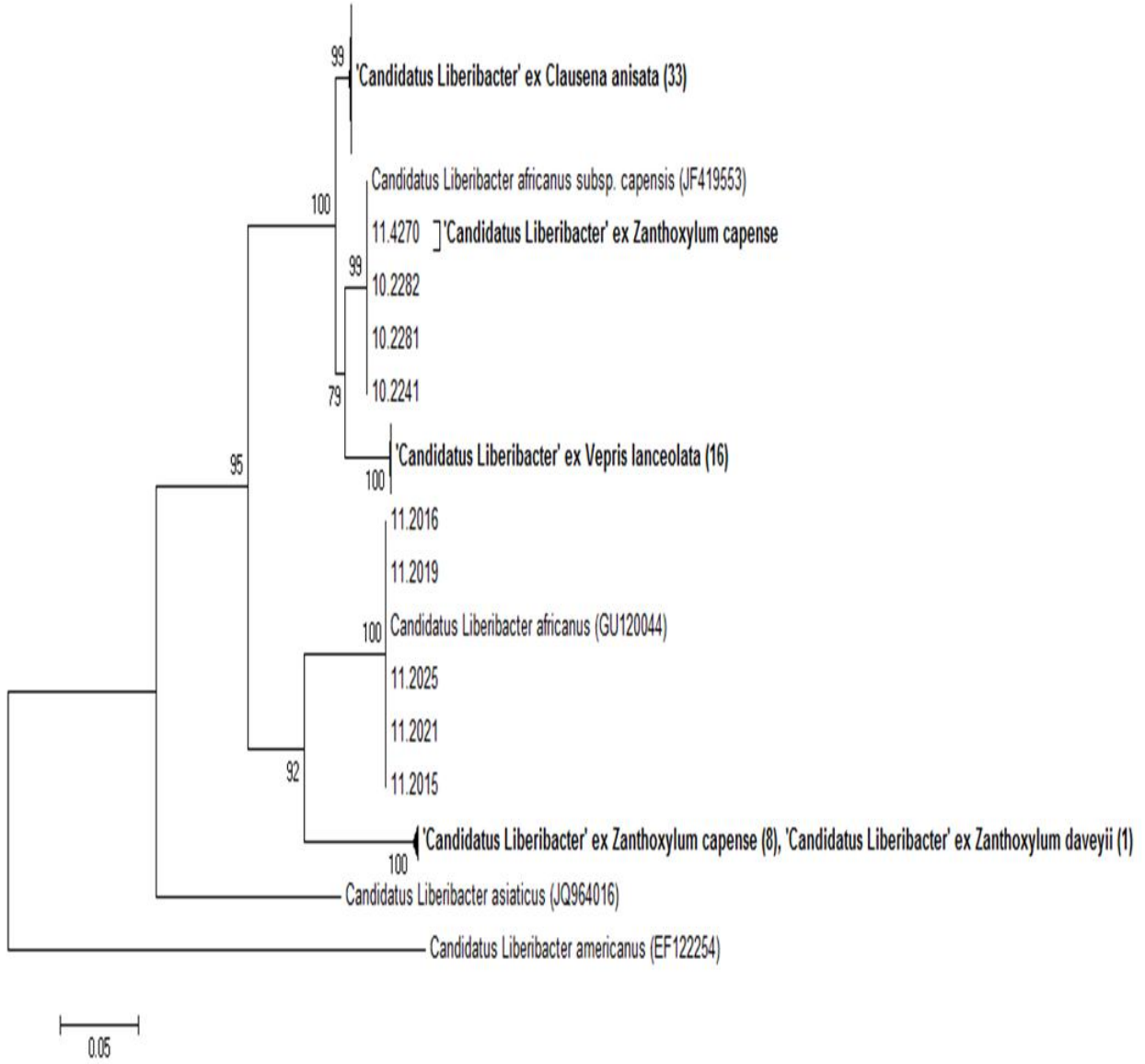

Fig. 3.3. Maximum Likelihood tree generated of aligned *rplJ* sequences obtained from Liberibacter positive *Cl. anisata, V. lanceolata* and *Z. capense* trees in Mega5 with 1000 bootstrap replicates. The number of sequenced Liberibacter positive samples per tree host is indicated in brackets following host name. Branches were collapsed according to tree host species which shared >70 bootstrap values. Las (JQ964016) and Lam (EF122254) were included as outgroups whereas Laf (GU120044) and LafC (JF419553) were included as ingroups. 11-2015, 11-2016, 11-2019, 11-2021 and 11-2025 are Laf sequences obtained from citrus trees collected from the experimental farm at the University of Pretoria. 10-2241, 10-2281 and 10-2282 represents known LafC sequences from *Ca. capense* collected in the Sundaysriver valley, Eastern Cape. Bootstrap values are shown at nodes.

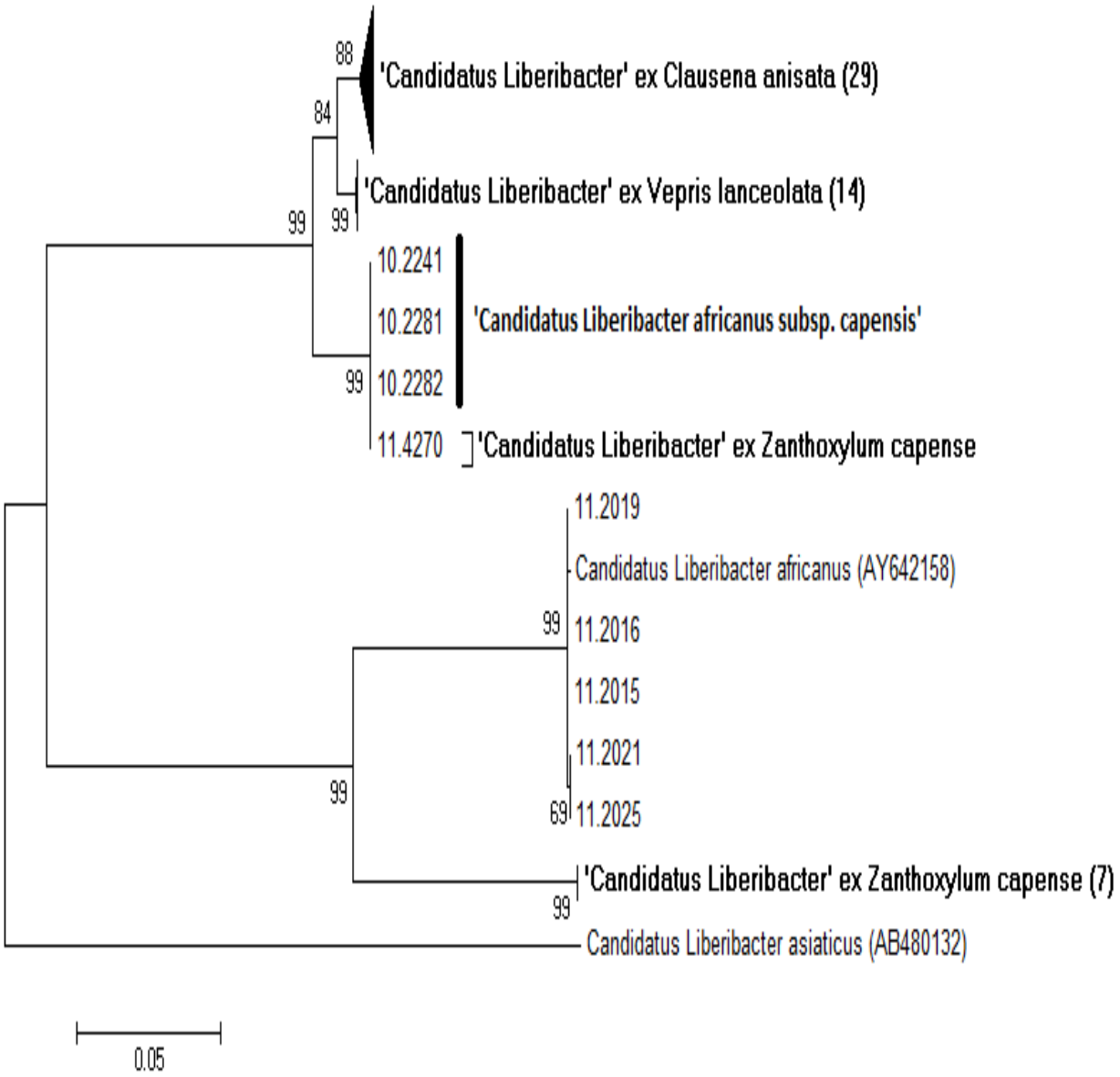

Fig. 3.4. Maximum Likelihood tree generated of aligned *omp* sequences obtained from Liberibacter positive *Cl. anisata, V. lanceolata* and *Z. capense* trees in Mega5 with 1000 bootstrap replicates. The number of successfully sequenced Liberibacter positive samples per tree host is indicated in brackets following host name. Branches were collapsed according to tree host species which shared >70 bootstrap values. Las (AB480132) was included as an outgroup whereas Laf (AY642158) was used as an ingroup. 11-2015, 11-2016, 11-2019, 11-2021 and 11-2025 are known Laf sequences obtained from citrus trees collected from the experimental farm at the University of Pretoria. 10-2241, 10-2281 and 10-2282 represents known LafC sequences from *Ca. capense* collected in the Sundaysriver valley, Eastern Cape. Bootstrap values are shown at nodes.

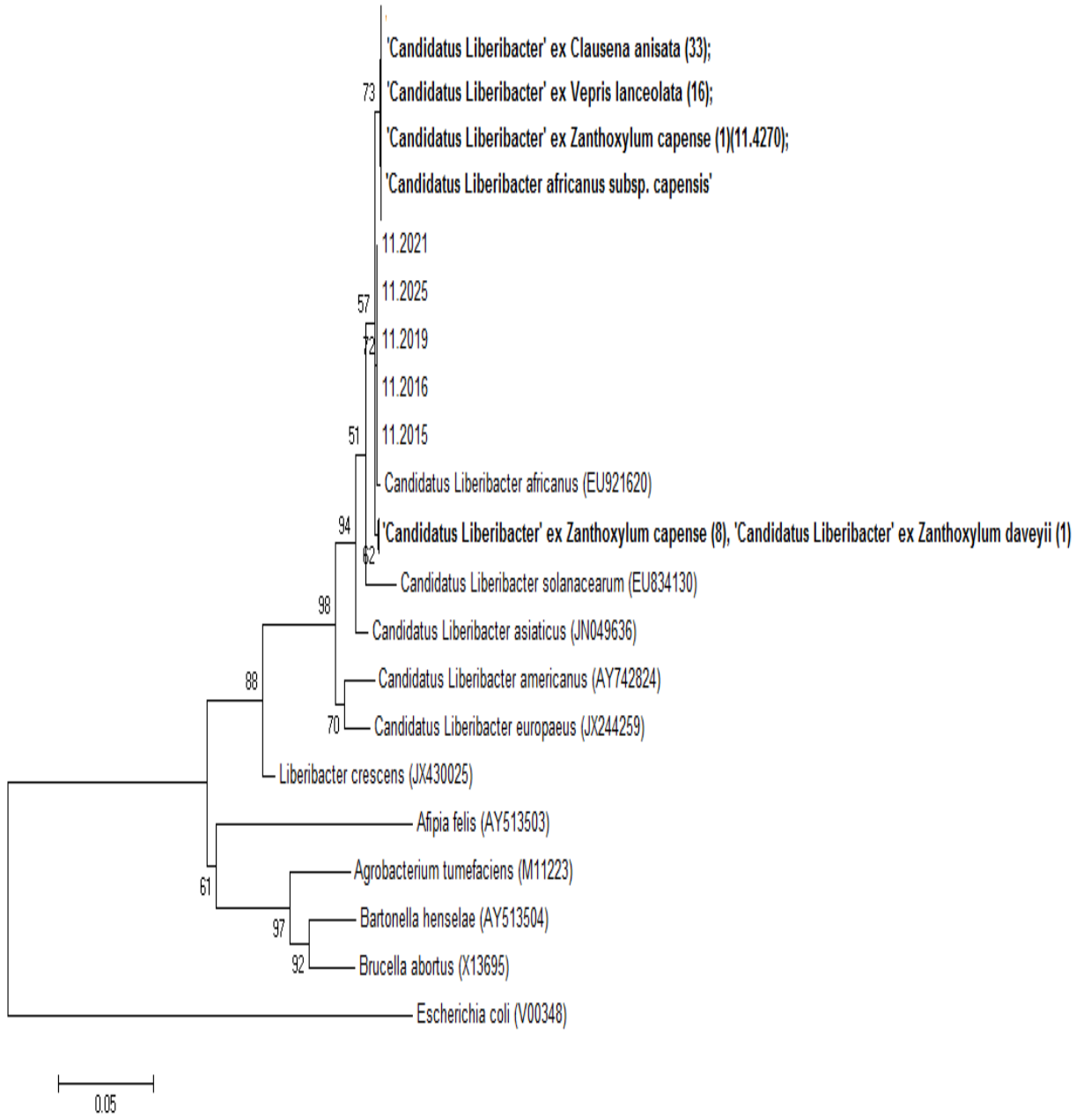

Fig. 3.5. Maximum Likelihood tree generated from aligned *16S* sequences obtained from Universal Liberibacter PCR positive *Cl. anisata, V. lanceolata, Z. capense*, *Z. davyi,* all known Liberibacter species, and other proteobacteria in Mega5 using Jukes-cantor model with 1000 bootstrap replicates. GenBank accession numbers are shown on tree for sequences included in analyses. The number of sequenced Liberibacter positive samples per tree host is indicated in brackets following host name. Branches were collapsed according to tree host species which shared >70 bootstrap values. *E. coli* (V0038) was included as an outgroup. 11-2015, 11-2016, 11-2019, 11-2021 and 11-2025 are known Laf sequences obtained from citrus trees collected from the experimental farm at the University of Pretoria. Bootstrap values are shown on nodes

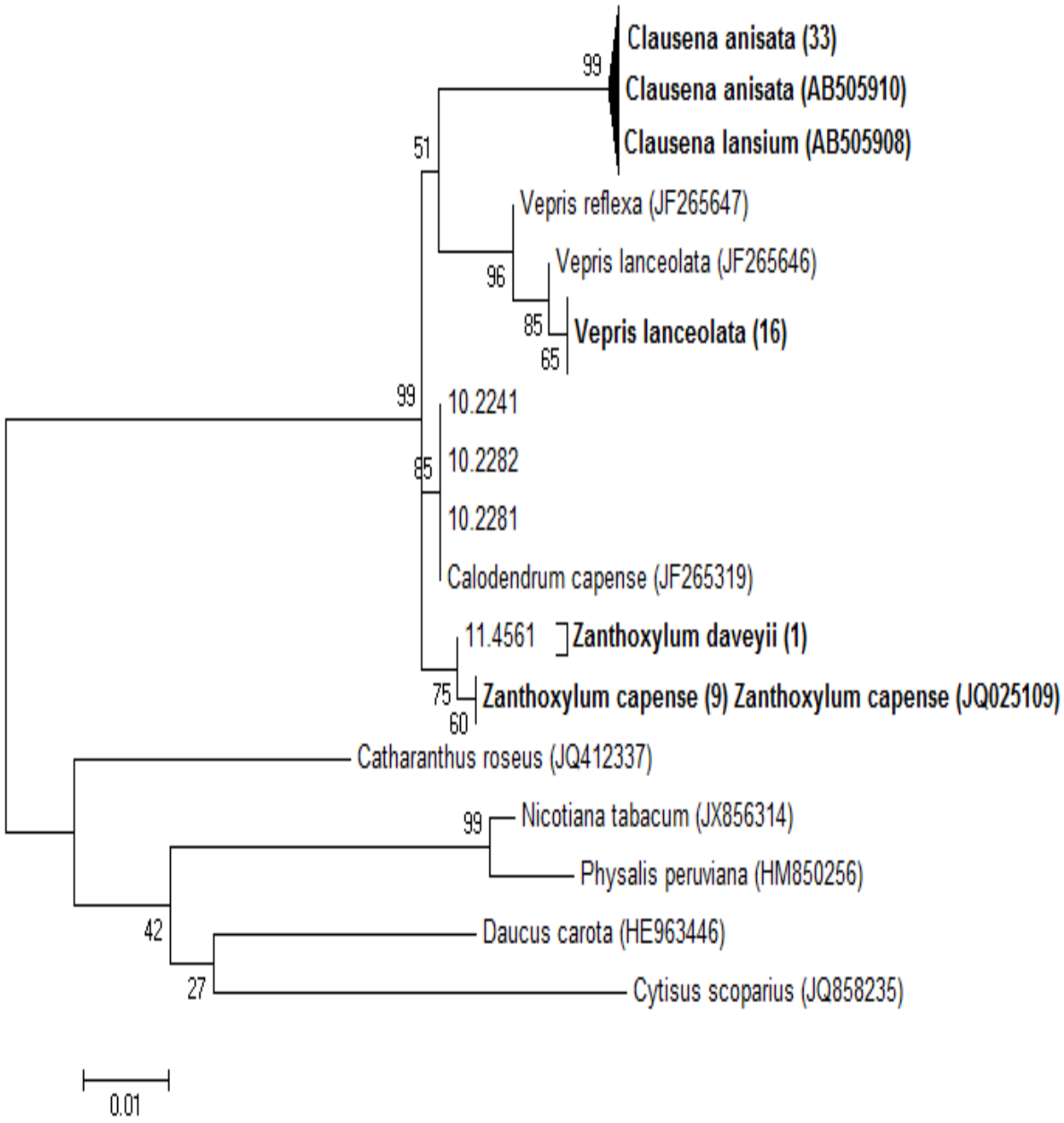

Fig. 3.6. Maximum likelihood tree generated from aligned *rbcL* barcoding gene for tree host species. The number of sequenced tree host specimens testing positive for Liberibacters following a generic Liberibacter real-time PCR assay, are indicated in brackets following host name. Branches sharing >70 bootstrap values were collapsed per host species. Alternative experimental hosts of Las were included as outgroups. GenBank accessions of sequences included are shown on tree. 11-4561 represents a putative *Z. davyi* and is distinct from other *Z. capense* sequences. 10-2241, 10-2281 and 10-2282 represents LafC positive *Ca. capense* samples collected from the Sundaysriver valley, Eastern Cape. Bootstrap values are shown at nodes.

Sequences from sample 11-4270 (Liberibacter ex *Z. capense*) was an exception to the general observed trend for Liberibacters from *Zanthoxylum* sp. All sequences generated (*rplJ, omp, 16S*) from sample 11-4270 shared 100% nucleotide identity to known LafC sequences. This suggests that Liberibacters identified from indigenous trees, despite generally being associated with specific rutaceous species, may on occasion infect another rutaceous host. This is the first report of a Rutaceous host in South Africa with two different Liberibacters.

To assess the correlation of phylogenetic clustering observed for Liberibacter *rplJ* and *omp* gene sequences and specific tree host species, the accepted barcoding gene of plants, ribulose 1,5-biphosphate carboxylase-coding gene (*rbcL*), for the host of Liberibacter positive samples were determined (Fig. 3.6). This confirmed that tree host species and the specific Liberibacter detected within them were clearly associated (Fig. 3.3 and 3.4). It also confirmed that sample 11-4270, clustering with Liberibacters from *Ca. capense* was obtained from a *Z. capense.* Furthermore analysis of the host *rbcL* gene of sample 11-4561 which was obtained from a putative *Z. davyi* specimen rather than *Z. capense* was in fact distinct from other *Z. capense rbcL* sequences.

3.5 Discussion

In the current study, a survey was conducted in which 234 *Cl. anisata,* 289 *Vepris lanceolata,* and 231 *Zanthoxylum capense* specimens were collected from across South Africa within the natural distribution range of these rutaceous trees. These three indigenous rutaceous species are known hosts to the triozid vector of Laf, *T. erytreae* (Moran, 1968b). Camerota et al. (2012) investigated whether '*Candidatus* Liberibacter europaeus' (Leu), an endophytic bacterium of *Pyrus communis* L. (pear) which is transmitted by *Cacopsylla pyri* L. (Raddi et al., 2011) can be acquired and transmitted by alternate species within the *Cacopsylla* genus. Leu was identified from 9 of the 14 *Cacopsylla* species tested. Additionally, Leu was detected from *Prunus spinosa* (L.) (black thorn), *Malus domestica* (Borkh.) (apple) and *Crataegus monogyna* (Jaquin) (hawthorn), the host plants of *C. melanoneura* Förster*, C. pruni* Scopoli and *C. peregrine* Förster suggesting that these psyllids are vectors for this bacterium. By studying native hosts of *T. erytreae* for the presence of Liberibacters, chances are

heighted that these hosts may be naturally infected with Laf. A high proportion of trees sampled during the study had tell-tale nymph-induced depression marks indicating previous infestation by *T. erytreae*. Live adults were also observed on a number of samples tested. However, the lack of visual evidence for the presence of *T. erytreae* does not imply the complete absence of this triozid from a given specimen as leaves containing depression marks from previous infestations could have been dropped. Therefore, samples were collected regardless of the presence of *T. erytreae.*

Liberibacters related to Laf form citrus and LafC from *Ca. capense* (Garnier et al., 2000), were identified from all tree species tested. However none of the Liberibacter sequences obtained from the indigenous sources from this study were typical Laf or LafC sequences. Liberibacter sequences from *V. lanceolata* and *Cl. anisata* shared greater nucleotide identity with LafC sequences whereas Liberibacter sequences from *Z. capense* and the single *Z. davyi* shared greater nucleotide identities with Laf sequences from citrus. Both *Cl. anisata* and *V. lanceolata* have previously been identified as potential hosts of Laf (Korsten et al., 1996; van den Berg et al., 1991-1992) but no studies have been done to ascertain whether these trees were infected with Laf or a related Liberibacter using molecular techniques. Van den Berg et al. (1991-1992) performed graft inoculation studies in which greening material was grafted onto potential indigenous hosts. From this study a single *Cl. anisata* showed mottling symptoms similar to greening disease. At the time this study was performed it was not possible to determine whether Laf could be detected from this plant by PCR and sequencing and the assumption that Laf was transmitted to this indigenous host was purely based visualization of symptoms. Leaf mottling however, can be caused by nutrient deficiencies (Fraser and Singh, 1968) and could possibly explain the observations made by Van den Berg et al. (1991-1992). A number of specimens collected from this study showed mottling symptoms similar to citrus trees infected with Laf. No relationship could be established between specimens with mottling symptoms and the presence of Liberibacter sequences. As samples were often collected within natural settings, it is possible that mottling symptoms observed could be attributed to nutrient deficiencies (Fraser and Singh, 1968) as natural undisturbed soils are not fertilized artificially as is the case in many orchards. Korsten et al. (1996) identified the presence of 'Laf' from *V.*

lanceolata by Southern hybridization. Cross reactivity has been demonstrated on the protein level, where monoclonal antibodies specific to Laf can cross react with LafC due to close relation of these two bacterial species (Garnier et al., 2000). Also, as demonstrated from this study, all Liberibacters identified from indigenous Rutaceae sampled shares great nucleotide homologies amongst all genes tested and probes designed for Southern hybridization analyses may have cross reacted with any of these Liberibacters. As the Liberibacter associated with *V. lanceolata* identified here is more similar to LafC than Laf, it is possible that Korsten et al. (1996) in greater likelihood detected a LafC-like rather than a Laf-like Liberibacter from the *V. lanceolata* sampled.

Liberibacter positive samples were identified in both greening and greening free areas within the natural distribution of the indigenous rutaceous trees sampled. Phahladira et al. (2012) identified LafC positive *Ca. capense* from across South Africa irrespective of the presence of greening disease, suggesting an association between LafC and *Ca. capense* independent of that between Citrus and Laf. Assuming this also holds true for other Liberibacters in indigenous rutaceous hosts, samples were collected from regions in South Africa regardless of the presence of Greening disease. Liberibacters were identified in Rutaceous tree specimens sampled along coastal regions from the Western Cape to Kwazulu-Natal and inland from Mpumalanga. However, none of the specimens collected from Gauteng, North-West and Limpopo provinces yielded Liberibacter positive samples. In both Gauteng and the North-West, only a limited number of indigenous samples could be collected as both *V. lanceolata* and *Cl. anisata* are less prevalent within these two provinces (Van Wyk and van Wyk, 1997) with no *Cl. anisata* specimens being collected. Many of the *V. lanceolata* specimens sampled in these two provinces were located in urban settings either planted by municipalities or home owners. Planted trees are commonly sprayed with insecticides thus reducing vector populations from these trees that carry Liberibacters. Whilst *Z. capense* is more commonly found in both Gauteng and the eastern parts of North-West compared to *Cl. anisata* and *V. lanceolata* (van Wyk and van Wyk, 1997) only a limited number of very young specimens could be collected which showed no signs of triozid infestations, thus attributing to the lack of Liberibacter positive *Z. capense* samples identified from these provinces. *Cl. anisata, V. lanceolata* and *Z. capense* occurs naturally in Limpopo (van

Wyk and van Wyk, 1997). However, a large percentage of land cover within Limpopo is inaccessible either due to agricultural or forestry activities. Therefore, only a limited number of indigenous specimens could be collected in Limpopo. By sampling a larger pool of specimens, chances of identifying a Liberibacter positive specimen is greatly increased.

In all, 33 *Cl. anisata,* 16 *V. lanceolata,* 9 *Z. capense* and 1 *Z. davyi* specimens tested positive for the presence of a Liberibacter following real-time PCR. Of the total trees sampled, the number of Liberibacter positive samples obtained represents 14.1% *Cl. anisata,* 5.5% *V. lanceolata* and 4.3% *Zanthoxylum spp* of the trees tested. This is similar to the percentage of Liberibacter positive samples identified from alternate host surveys conducted for both Las and Lam in São Paulo, Brazil. A total of 786 *M. paniculata* trees were sampled from São Paulo of which only 7.8% and 3.8% tested positive for Lam and Las respectively (Lopes et al, 2010). Similarly, only 1.8% of 1,046 *M. paniculata* trees sampled in Florida tested positive for the presence of Las (Walter et al, 2012). LafC, however, was identified in 38% *Ca. capense* specimens of 278 samples collected across South Africa (Phahladira et al., 2012) suggesting a more established relationship between LafC and *Ca. capense* than that of Las and Lam with *M. paniculata*. In the study conducted by Camerota et al. (2012) the low incidence of Leu positive samples was attributed to the erratic distribution of Liberibacters within their respective hosts as well as the limited number of trees sampled. The erratic distribution of citrus infecting Liberibacters has been demonstrated (Foliminova et al., 2009) and it has previously been suggested that it is more likely to detect Liberibacters from symptomatic material than non-symptomatic plant parts (Bové, 2006; Folimonova et al., 2009). It is unknown whether the Liberibacters as identified from this study causes disease symptoms within their indigenous hosts and samples could not be collected based on mottling symptoms. Therefore, as with Leu, these Liberibacters may have been absent from sampled branches whilst being present in other parts of the same tree due to the erratic distribution characteristic of Liberibacters (Folimonova et al., 2009). The number of specimens collected for each host species was limited as many of these samples were collected from natural undisturbed regions which are not always easy accessible. As with the study conducted by Camerota et al. (2012), the low incidence of

Liberibacter positive samples can be explained by the erratic distribution of Liberibacters in their hosts and the limited number of samples tested.

Phahladira et al. (2012) demonstrated that LafC was not transmitted to citrus under natural conditions within the Sunday's river valley, Eastern Cape. *Ca. capense* is not an ideal host of *T. erytreae* (Moran, 1968a) and does not support the development of this triozid suggesting that LafC is vectored by a vector other than *T. erytreae*. The occurrence of LafC in Greening-free areas and the identification of LafC positive *Ca. capense* grown in close proximity with citrus orchards (Garnier et al., 2000; Phahladira et al., 2012) further demonstrate the probable involvement of an alternative vector assisting in the spread of LafC within *Ca. capense*. This would also help explain why in a recent survey; only Laf was identified from commercial citrus in South Africa (Pietersen et al., 2010) and not LafC. However, in the current study Liberibacters sequences atypical of Laf were identified from *Cl. anisata, V. lanceolata* and *Zanthoxylum* species. As previously mentioned these hosts were selected because of the ability of *T. erytreae* to feed on and colonize (Moran 1968a; Moran 1968b) these rutaceous species heightening chances of identifying Laf. Because this triozid feed on both commercial citrus and the hosts studied here (Moran, 1968b) it is possible that Liberibacters identified from these hosts are transmitted to commercial citrus. In the study conducted by Pietersen et al. (2010) commercial citrus samples showing typical mottling symptoms were collected from across South Africa and the presence of Laf was determined through direct sequencing of β-operon amplicons following PCR using primers A2/J5. It is unknown whether Liberibacters identified from indigenous hosts are capable of causing disease within their respective hosts and whether these Liberibacters causes mottling of commercial citrus varieties should natural transmission occur. Therefore it is possible that asymptomatic trees exist within citrus orchards that are infected with a Liberibacter from an indigenous host that were excluded from the survey conducted by Pietersen et al. (2010). The use of conventional PCR strategies to identify Liberibacters from commercial citrus samples poses an additional bias towards the detection of only Laf. During PCR a dominant Liberibacter genotype will be preferentially amplified and therefore sequenced. Laf may be more adapted to citrus than other Liberibacters from indigenous Rutaceae thus being present in higher titers

explaining why Pietersen et al. (2010) identified Laf as the sole Liberibacter infecting commercial citrus varieties is South Africa. It is therefore important to ascertain whether Liberibacter identified from this study are capable of being transmitted to citrus by *T. erytreae* under controlled conditions. Transmission studies using *T. erytreae* will help determine whether natural transmission of the Liberibacters identified from *Cl. anisata, V. lanceolata, Z. capense* and *Z. davyi* to commercial citrus can occur. Graft inoculation studies should also be performed to determine whether these Liberibacters can multiple and cause disease within commercial citrus varieties. The lack of evidence presented here that the native hosts of *T. erytreae* serves as reservoir hosts for Laf indicates that these hosts should not be included in inoculum reduction strategies until further research is done concerning the transmission of their associated Liberibacters to citrus.

Gene regions amplified in this study (*16S*, *rplJ* and *omp*) all form part of the core genome within Liberibacter species. Both the 16S rRNA and β-operon are commonly used to identify and differentiate Liberibacter species (Jagoueix et al, 1996; Hoqcuellet et al., 1999). Las and Laf were described as two distinct species based on cloning, sequencing and comparison of 16S rRNA and *rplKAJL-rpoB* sequences (Jagoueix et al., 1994). LafC was shown to share greater homology with Laf than Las based on phylogenetic comparison of 16S rRNA, intergenic 16S/23S and partial *rplKAJL-rpoBC* gene sequences with Las and Laf (Garnier et al., 2000) whereas Lam was identified as being unique from both Las and Laf based solely on the 16S rRNA sequence analyses (Teixeira et al., 2005a). Initially Bastianel et al. (2005) proposed that the *omp* region be used to aid in differentiating various genotypes within Liberibacter species following restriction fragment length polymorphism (RFLP) analyses (Bastianel et al., 2005). However, it was found that this gene region is highly conserved between Las isolates obtained from geographically isolated regions. Phylogenetic analyses from the complete *omp* gene resembled similar phylogenetic groupings to 16S rRNA analyses across Liberibacter lineages suggesting that the *omp* gene region forms part of the core genome (Bastianel et al., 2005). The use of sequences within the core genome of Liberibacter species enables the differentiation of Liberibacter species whereas more variable genes resolve genotypes within a given species, which was not the aim of the current study.

Phylogenetic clustering of both *rplJ* and *omp* genes correlates to the tree host species from which the Liberibacters were obtained. This suggests that each tree host species harbors a distinct Liberibacter clade. Amongst these sequences, Liberibacter ex *V. lanceolata* and Liberibacter ex *Cl. anisata* shared greater homology with LafC than Laf, whereas Liberibacter ex *Z. capense* sequences were more homologous to Laf than LafC. This close relationship between Liberibacters identified in this study and either Laf or LafC was even more obvious with 16S rRNA sequences with Liberibacter ex *Cl. anisata* and Liberibacter ex *V. lanceolata* being identical to LafC sequences from *Ca. capense* whereas Liberibacters ex *Zanthoxylum spp* showed greater homology to Laf sequences. Additionally it was found that LafC and Laf 16S rRNA sequences shared greater homology (99.0%) than previously reported (97.4%) (Garnier et al., 2000). This is mainly due to the initial 16S rRNA sequence (GenBank acc. AF137368) containing various unresolved nucleotides resulting in this sequence appearing less homologous to Laf than LafC sequences from this study. This confirms that 16S rRNA sequences are relatively conserved amongst Liberibacter species and that additional sequences within the core genome, such as *rplJ* and *omp,* are better suited to differentiate Liberibacter species and sub-species. Similarly, Ghosh et al. (2013) found that the 16S rRNA gene from Liberibacter spp. provides little or no resolution between closely related species and suggest that more variable ribosomal DNA regions be used to differentiate different Liberibacter isolates. To determine whether novel Liberibacter species are associated with the rutaceous host from this study, further sequence data is required due to conflicting phylogenetic results for all genes studied in which Liberibacter ex *Cl. anisata* and Liberibacter ex *V. lanceolata* grouped differently with LafC in all instances.

Various hypotheses regarding the origin of citrus infecting Liberibacter species are proposed. For the purpose of explanation the main hypothesis will be referred to as the multi-continental and single-origin hypotheses. According to the multi-continental hypothesis, each citrus infecting Liberibacter species originated from the continent where it was initially discovered Las from Asia, Laf from Africa and Lam from South America (Bové, 2006). Las isolates from China and India are genetic diverse indicating that varied populations were formed by mutations that resulted from natural selection and population differentiation (Islam et al., 2012). The presence of founder haplotypes

in both China and India, as shown by eBURTS analyses, further supports the origin of Las from Asia (Islam et al., 2012). Upon the discovery of Las in Brazil it was found that most commercial citrus orchards infected with HLB, Lam incidence exceeded that of Lam suggesting that Lam was present prior to the introduction of Lam (Teixeira et al., 2005b). Las incidence in commercial citrus however soon surpassed that of Lam and it was demonstrated that Las multiplied to higher titres in citrus than Lam, suggesting that Lam's association with citrus is not well established (Lopes et al., 2009). The origin of Laf from Africa is supported by the association of citrus with Laf solely from Africa (Garnier an Bové, 1996), a continent to which citrus is believed to have been introduced (Beattie et al., 2008). The occurrence of LafC (Garnier et al., 2000) and the promiscuous nature of *T. erytreae* (Moran, 1968a; Moran, 1968b) further suggest that Laf originated from an indigenous source in Africa. Contrary to this, the single source origin implies that all known citrus infecting Liberibacter species originated from an indigenous Rutaceous source in Africa (Beattie et al., 2008). Teixeira et al. (2008) presented molecular evidence to suggest that these Liberibacters originated from a common ancestor from Gondwana. According to their findings, Laf and Las diverged from Lam 309 mya after which Las and Laf separated 147 mya into the forms as they are known today (Teixeira et al., 2008). From the current study, Liberibacters closely related to both Laf and LafC were identified. The close relation of these Liberibacter suggest that in Africa, various Liberibacter populations exist which may have arose from a single African ancestor. This is supported by the close homology of Laf, Liberibacter ex *Zanthoxylum spp* and LafC-like 16S rRNA sequences. As previously mentioned, this sequence is highly conserved amongst Liberibacter species and may be reminiscent from a common ancestor which gave rise to all African Liberibacters. As with LafC, Liberibacters positive *Cl. anisata, V. lanceolata* and *Zanthoxylum spp* were often obtained from isolated regions that have remained undisturbed by urban encroachment, suggesting that the association of these Liberibacters with their indigenous rutaceous host is long-standing and may have been due to co-evolution (Phahladira et al., 2012). Phahladira et al. (2012) proposed that Laf in Africa evolved after a host jumping event occurred in which LafC, through the involvement of *V. lanceolata* as an intermediate host, was acquired and transmitted to citrus by *T. erytreae.* Liberibacter sequences

obtained from this study however suggest that Liberibacter ex *Zanthoxylum* spp may have had a more direct role in the evolution of Laf from citrus as it is known today. The close sequence homology shared between Laf and Liberibacter ex *Zanthoxylum spp* for all gene sequences, compared to LafC homologous sequences indicate the likeliness that the Liberibacter associated with *Zanthoxylum spp* represents a predecessor to Laf which could've been acquired and transmitted to citrus by *T. erytreae*. As demonstrated with *Z. capense* where, in addition to Laf-like sequences present, a single sample (11-4270) from this host contained a LafC-like sequence. This supports the possibility that the African Liberibacters may be present in multiple host species, albeit at relative low incidences. The preferential feeding pattern of *T. erytreae* on citrus (Moran, 1968b) and potential multiple host range of African Liberibacters could have assisted in multiple transmission of Liberibacter ex *Z. capense* from this indigenous host to citrus, indirectly placing selective pressure on Liberibacter ex *Z. capense* to evolve and adapt to a new host. To support these claims it would be necessary to perform transmission studies involving both vector and grafting approaches to ascertain whether any of the Liberibacter identified here are capable of being acquired by *T. erytreae* and multiplying within commercial citrus.

3.6 Conclusions

Typical citrus-infecting Laf, was not detected in any of the indigenous rutaceous specimens tested, and does not appear to play a role in the epidemiology of Laf on citrus. A Liberibacter closely related to Laf however, was found present in *Zanthoxylum species* for the first time. LafC-like Liberibacters from *Cl. anisata* and *V. lanceolata* are also reported for the first time. Further studies are needed to determine whether these Liberibacters are transmitted to commercial citrus and whether any of these Liberibacters are capable of causing disease on commercial citrus species. However, the association of various Liberibacter variants with indigenous rutaceous trees presents researchers with a unique opportunity to explore the possible evolution of Laf on citrus from a Liberibacter source indigenous to the African continent.

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Chapter 4*

Lack of Seed Transmission of '*Candidatus* Liberibacter africanus subsp. capensis' in *Calodendrum capense*

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4.1 Abstract

'*Candidatus* Liberibacter africanus subsp. capensis' (LafC) is commonly associated with *Calodendrum capense* trees from across South Africa. This Liberibacter is closely related to '*Candidatus* Liberibacter africanus' (Laf), the phloem limited bacterium considered to be the causal agent of Greening disease of citrus in Africa. Unlike Laf, LafC has not yet been coupled to any disease of *Ca. capense* and it has been suggested to have co-evolved with this Rutaceous host, representing an ancestral lineage of Laf. Laf is primarily spread in citrus orchards by the activity of *Trioza erytreae,* the triozid vector of this bacterium and it has been demonstated that Laf is not vertically transmitted through infected seeds. The aim of this study was to investigate whether the lack of seed transmission is a trait shared amongst Liberibacter lineages from Africa. Seed were collected from two mother trees with known LafC infection, from which respectively 34 and 35 seedlings were obtained. Total DNA was extracted from leaf midribs per seedling and tested for the presence of LafC using a LafC directed real-time PCR system. None of the seedlings tested had a Ct value of Ct<35, the positive/negative threshold. Therefore, from the current study, no indication of seed-transmission was obtained within the limited number of samples tested suggesting that African Liberibacters are not vertically transmitted.

4.2 Introduction

Greening disease of citrus in South Africa is associated with '*Candidatus* Liberibacter africanus' (Laf), a phloem limited bacterium belonging to the class Alphaproteobacteria (Jagoueix et al., 1994). Laf is vectored by the triozid, *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) (McClean and Oberholzer, 1965b). Other citrus producing countries are plagued by a similar disease known as citrus Huanglongbing (HLB) which is associated with a related bacterium, '*Candidatus* Liberibacter asiaticus' (Las). A third citrus infecting Liberibacter has been described from Brazil and is known as '*Candidatus* Liberibacter americanus' (Lam) (Teixeira et al., 2005). Both Las and Lam are vectored by the liviid, *Diaphorina citri* Kuwayama (Hemiptera: Liviideae) (Capoor et al., 1967; Teixeira et al., 2005). Infection with either one of the three Liberibacters causes distinct mottling patterns on the leaves from infected branches (McClean and Oberholzer, 1965a; Lopes and Frare, 2008). Such branches produce fruits that are of no economic value as these fruits are smaller, foul tasting and are dropped prematurely (McClean and Oberholzer, 1965a).

Following an outbreak of Greening disease in the Western Cape, an apparently novel species of Liberibacter was found associated with an indigenous ornamental Rutaceae tree, *Calodendrum capense* (L.F.) Thunb., commonly known as the Cape Chestnut (Garnier et al., 1999). PCR, sequence analysis and Southern hybridization studies revealed that this newly identified Liberibacter is closely related to Laf and was given the subspecies status '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) (Garnier et al., 2000). LafC has been found to be commonly associated with *Ca. capense* trees across South Africa following the natural distribution of this tree species, being present in areas where greening has thus far not yet been found (Phahladira et al., 2012). The insect that vectors this Liberibacter has to date, not yet been identified.

Spread of the three citrus infecting Liberibacter species is primarily assisted by the flight and feeding activities of their insect vectors. These Liberibacters are graft transmissible and can also be transmitted to experimental hosts through dodder (Garnier and Bové, 1983; Hartung et al., 2010b). Once the pathogen has been transmitted to a citrus host, it spreads systemically within the host and can be detected from all parts within the tree,

including seed coats (Tatineni et al., 2008). The presence of Liberibacter within seed coats is, however, a cause for concern as these pathogens may be transmitted to seedlings through wounds during germination. Also, rootstocks used in the USA are grown from seeds and may play a role in the spread of HLB as infecteted rootstocks may transmit the bacterium to the scion, if the pathogen is seed transmissible (Hilf, 2011).

The subject of seed transmission has been dealt with in several previous studies, the results of which were contrasting and inconclusive. Graham et al (2008) and Albrecht and Bowman (2009) detected Las by PCR from a low percentage of seedlings grown from symptomatic fruits. Contrary to this, Shokrollah et al. (2009), Hartung et al. (2010a) and Hilf (2011) found no evidence to suggest that Las is transmitted to seedlings from infected seeds. The latter also appears to be the case for Laf from South Africa as demonstrated by van Vuuren et al., (2011). Thus far, however, very little is known about the transmission of LafC and whether vertical transmission of this bacterium occurs. This study therefore aims to investigate whether LafC is vertically transmitted via *Ca. capense* seeds.

4.3 Materials and Method

4.3.1 Sample preparation

Seeds from two *Ca. capense* trees (accession 09-0749 and 09-0751) with known LafC infection were collected and transplanted into separate pots containing sterile fertilizer. These pots were placed within an insect proof greenhouse and seeds were left to germinate. From mother tree 09-0749, 34 seeds germinated and 35 seedlings were obtained from mother tree 09-0751.

4.3.2 DNA extraction and molecular detection of LafC

Leaves were collected from each seedling and DNA was extracted from 0.5g of midribs using the CTAB extraction method as described by Doyle and Doyle, 1990 with modifications by Fundecitrus (as previously described in Chapter 3).

LafC was detected from samples by real-time PCR according to the protocol of Li et al., 2006 using primer pair HLBafC/HLBr with probe HLBp, specifically targeting LafC 16S rRNA. Real-time PCR reactions were performed in 10µl reaction mixtures containing 2µl 5X Taqman® Universal PCR master mix (Roche Diagnostics, Manheim, Germany), 0.5µl of each 10µM primer (HLBafC, HLBr), 0.3µl of 5µM probe HLBp and 6.7µl dH_2O. Reactions were run in duplicate and always included healthy and infected samples as controls. DNA amplification was performed using the Lightcycler 1.5® (Roche Diagnostics, Manheim, Germany) under the following conditions; initial denaturation at 95˚C for 10 min followed by 45 cycles of denaturation at 95˚C for 10sec, annealing at 58˚C for 40sec and elongation at 72˚C for 1sec (single acquisition) followed by a final cooling step oat 40˚C for 30sec. Samples with a Ct value of Ct<35 were considered positive.

4.4 Results

All controls included acted as expected during real-time PCR reactions. Healthy controls remained negative whereas known positive controls had an average Ct value of Ct=24.

All 34 seedlings grown from the seed of mother tree 09-0749 and 35 seedling from 09-0750 remained negative (Ct<35) with no fluorescence being detected, indicating the absence of LafC. These results suggest that LafC is not seed transmissible at levels which could be detected within the limited number of samples.

4.5 Discussion

The results of this study suggest that LafC is not seed transmissible. This is in agreement previous findings that Laf is not vertically transmitted to seedlings grown from seed taken of symptomatic fruits (van Vuuren et al, 2011). Similar results have also been demonstrated in the case where seedlings obtained from seeds in which seed coats contained Las, the causal agent of HLB in Florida, were negative for the presence of Las (Shokrollah et al., 2009; Hartung et al., 2010a; Hilf, 2011). However, two independent studies have found that Las is transmitted to a low percentage of seedlings from seeds infected with the bacteria (Graham et al., 2008; Albrecht and Bowman, 2009). In the study conducted by Albrecht and Bowman (2009), 1313 sweet

orange seedlings were used of which 918 of these were grown from seeds obtained from Las positive fruits. Seedlings grown from seeds infected with Las were noticeably smaller than those grown from healthy seeds. However, when these seedlings were tested for the presence of Las by PCR, only 3 out of the 431 seedlings analyzed tested positive for Las and when retested after approximately 10 months, none of the seedlings were positive for Las, indicating that the initial results may have been caused by contamination (Hilf, 2011). All seedlings used in the current study appeared healthy for the duration of the experiment.

One drawback of the current study was that only a limited number of samples could be tested. Previous studies regarding seed transmission of citrus infecting Liberibacters relied on screening a large number of seedlings from symptomatic seeds (Albrecht and Bowman, 2009; Hilf et al., 2011). In the present study, seed could only be sampled from two mother trees with known LafC infection based on PCR results. *Ca. capense* trees are indigenous to South Africa and in contrast to commercial citrus, are not mass produced. Therefore seeds could only be obtained from a limited number of trees, and only a relatively small amount of seeds could be harvested.

As LafC does not cause any noticeable disease symptoms on the seed of *Ca. capense* (Phahladira et al., 2012), the seeds used in this study were taken without being associated with symptomatic samples and may have been free of this Liberibacter during the time of testing. Tatineni et al. (2008) demonstrated that in citrus trees infected with Las, the bacterium spreads systemically within the tree and can be detected by PCR from both symptomatic and asymptomatic material. LafC generally multiplies to higher titers in *Ca. capense* when compared to Laf (Pietersen *per comm.*) and in accordance with the results presented by Tatineni et al. (2008), LafC as with Las, may spread systemically within its tree host species, increasing the possibility that this bacterium may be present in the seed coats of seeds from infected *Ca. capense* trees, despite the lack of visual symptoms.

4.6 Conclusion

Not much is thus far known about how LafC is spread amongst *Ca. capense* trees in South Africa. Evidence presented here however suggests that LafC is not vertically transmitted at high levels in *Ca. capense* and that it is more likely that the spread of this Liberibacter is assisted by a yet unknown vector. The lack of demonstrated seed transmission for both Laf and LafC indicates that this may be a trait shared amongst Liberibacter lineages from South Africa.

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Appendix

Appendix A

Table A1: Specimen information

Acc number	Tree host Species	GPS		Ct	Symptoms observed (Mo=Mottling Y=Yellowing; VC=Vein clearing)	Triozids (Em=Emergence marks; T=Live Triozids)
		Latitude (S)	Longitude (E)			
11-4020	*V. lanceolata*	-33.993	22.594	-	-	-
11-4021	*V. lanceolata*	-33.991	22.599	-	-	-
11-4022	*V. lanceolata*	-33.988	22.611	-	-	-
11-4028	*V. lanceolata*	-34.027	22.999	-	-	-
11-4032	*V. lanceolata*	-34.029	23.001	-	-	-
11-4033	*V. lanceolata*	-34.030	23.006	-	-	-
11-4034	*V. lanceolata*	-34.037	23.012	-	-	-
11-4035	*V. lanceolata*	-34.059	23.022	25.77	-	-
11-4036	*V. lanceolata*	-34.059	23.022	24.99	-	-
11-4037	*V. lanceolata*	-34.059	23.022	21.18	-	-
11-4038	*V. lanceolata*	-34.058	23.021	25.15	-	-
11-4039	*V. lanceolata*	-34.058	23.021	29.11	-	-
11-4040	*Z. capense*	-	-	-	-	-
11-4041	*Z. capense*	-34.058	23.021	-	-	-
11-4042	*Z. capense*	-34.058	23.022	19.98	-	-
11-4043	*Z. capense*	-34.058	23.022	-	-	-
11-4044	*V. lanceolata*	-34.058	23.022	26.63	-	-
11-4045	*V. lanceolata*	-34.058	23.022	-	-	-
11-4046	*V. lanceolata*	-34.058	23.022	-	-	-
11-4048	*V. lanceolata*	-34.058	23.022	23.75	-	-
11-4049	*V. lanceolata*	-34.058	23.022	-	-	-
11-4050	*V. lanceolata*	-34.058	23.023	-	-	-
11-4051	*V. lanceolata*	-34.059	23.023	-	-	-
11-4052	*V. lanceolata*	-34.058	23.023	-	-	-
11-4056	*V. lanceolata*	-34.059	23.024	22.02	-	-

11-4059	*V. lanceolata*	-34.059	23.024	-	-	-
11-4060	*Z. capense*	-34.059	23.022	-	-	-
11-4062	*V. lanceolata*	-33.982	22.944	-	-	-
11-4063	*V. lanceolata*	-33.982	22.943	-	-	-
11-4065	*V. lanceolata*	-33.983	22.941	-	-	-
11-4067	*V. lanceolata*	-33.982	22.938	-	-	-
11-4068	*V. lanceolata*	-33.982	22.943	-	-	-
11-4069	*V. lanceolata*	-33.981	22.944	-	-	-
11-4070	*V. lanceolata*	-33.980	22.943	-	-	-
11-4071	*V. lanceolata*	-33.980	22.943	-	-	-
11-4072	*V. lanceolata*	-33.980	22.943	-	-	-
11-4073	*V. lanceolata*	-33.980	22.943	-	-	-
11-4074	*V. lanceolata*	-	-	-	-	-
11-4075	*V. lanceolata*	-34.001	22.990	-	-	-
11-4076	*V. lanceolata*	-34.001	22.992	-	-	-
11-4077	*V. lanceolata*	-34.001	22.992	27.44	-	-
11-4078	*V. lanceolata*	-34.011	23.000	-	-	-
11-4079	*V. lanceolata*	-34.019	22.988	-	-	-
11-4080	*V. lanceolata*	-34.019	22.988	-	-	-
11-4081	*V. lanceolata*	-34.040	23.073	-	-	-
11-4082	*V. lanceolata*	-34.040	23.073	-	-	-
11-4083	*V. lanceolata*	-34.040	23.074	-	-	-
11-4084	*V. lanceolata*	-34.040	23.074	-	-	-
11-4086	*V. lanceolata*	-33.993	23.400	23.56	-	-
11-4087	*V. lanceolata*	-33.993	23.400	25.97	-	-
11-4088	*V. lanceolata*	-33.994	23.400	-	-	-
11-4089	*V. lanceolata*	-	-	-	-	-
11-4090	*V. lanceolata*	-33.995	23.400	-	-	-
11-4095	*V. lanceolata*	-34.000	23.398	-	-	-
11-4096	*V. lanceolata*	-34.034	23.372	-	-	-
11-4097	*V. lanceolata*	-33.967	23.466	-	-	-
11-4098	*V. lanceolata*	-33.967	23.467	-	-	-
11-4099	*V. lanceolata*	-33.965	23.466	23.90	-	-
11-4100	*V. lanceolata*	-33.965	23.455	-	-	-

11-4101	*V. lanceolata*	-34.048	23.235	-	-	-
11-4102	*V. lanceolata*	-34.043	23.105	-	-	-
11-4103	*V. lanceolata*	-34.043	23.105	-	-	-
11-4104	*V. lanceolata*	-34.043	23.105	-	-	-
11-4105	*V. lanceolata*	-34.070	23.068	-	-	-
11-4106	*V. lanceolata*	-34.077	23.063	-	-	-
11-4107	*V. lanceolata*	-34.076	23.063	-	-	-
11-4108	*V. lanceolata*	-34.066	23.064	-	-	-
11-4110	*V. lanceolata*	-34.029	22.943	-	-	-
11-4112	*V. lanceolata*	-	-	-	-	-
11-4113	*V. lanceolata*	-	-	-	-	-
11-4135	*Z. capense*	-	-	-	-	Em
11-4136	*Z. capense*	-	-	-	-	Em
11-4137	*V.lanceolata*	-	-	-	-	Em, T
11-4138	*V.lanceolata*	-	-	-	-	Em, T
11-4139	*V. lanceolata*	-	-	-	-	Em, T
11-4140	*V.lanceolata*	-	-	-	-	-
11-4141	*Z. capense*	-	-	-	-	Em
11-4142	*V. lanceolata*	-	-	-	-	Em
11-4143	*V.lanceolata*	-	-	-	-	Em, T
11-4144	*V.lanceolata*	-	-	-	-	Em
11-4145	*Z. capense*	-25.495	27.305	-	-	Em
11-4156	*V.lanceolata*	-	-	-	-	Em
11-4157	*V. lanceolata*	-	-	-	-	Em
11-4158	*V.lanceolata*	-	-	-	-	Em
11-4159	*V.lanceolata*	-	-	-	-	Em
11-4160	*V. lanceolata*	-	-	-	-	Em
11-4161	*V. lanceolata*	-	-	-	-	Em
11-4162	*V.lanceolata*	-	-	-	-	Em
11-4163	*V. lanceolata*	-	-	-	-	Em
11-4164	*V.lanceolata*	-	-	-	-	Em
11-4165	*V.lanceolata*	-	-	-	-	Em
11-4166	*V.lanceolata*	-	-	-	-	Em, T
11-4167	*V. lanceolata*	-	-	-	-	Em, T

11-4168	*V. lanceolata*	-	-	-	-	Em, T
11-4169	*V. lanceolata*	-	-	-	-	Em, T
11-4170	*V. lanceolata*	-	-	-	-	Em, T
11-4171	*Z. capense*	-	-	-	-	-
11-4172	*Z. capense*	-	-	-	-	-
11-4173	*Z. capense*	-	-	-	-	-
11-4175	*V. lanceolata*	-	-	-	-	Em
11-4176	*Z. capense*	-	-	-	Y	-
11-4177	*Z. capense*	-	-	-	Y	-
11-4178	*Z. capense*	-	-	-	Y	-
11-4179	*Z. capense*	-	-	-	Y	-
11-4180	*V. lanceolata*	-	-	-	Mo	Em
11-4181	*Z. capense*	-	-	-	Y	-
11-4182	*Z. capense*	-	-	-	Y	-
11-4183	*V. lanceolata*	-	-	-	Mo	Em, T
11-4184	*V. lanceolata*	-	-	-	-	Em, T
11-4185	*V. lanceolata*	-	-	-	-	Em, T
11-4186	*V. lanceolata*	-	-	-	-	Em, T
11-4187	*V. lanceolata*	-	-	-	-	Em, T
11-4188	*V. lanceolata*	-	-	-	-	Em, T
11-4189	*V. lanceolata*	-	-	-	-	Em, T
11-4190	*V. lanceolata*	-	-	-	-	Em, T
11-4191	*V. lanceolata*	-	-	-	-	Em, T
11-4192	*V. lanceolata*	-	-	-	-	Em, T
11-4193	*V. lanceolata*	-	-	-	-	Em, T
11-4194	*V. lanceolata*	-	-	-	-	Em, T
11-4195	*V. lanceolata*	-	-	-	Mo	Em
11-4196	*V. lanceolata*	-	-	-	-	Em
11-4197	*V. lanceolata*	-	-	-	Mo	Em, T
11-4198	*V. lanceolata*	-	-	-	-	-
11-4199	*V. lanceolata*	-	-	-	-	-
11-4200	*Z. capense*	-	-	-	-	-
11-4201	*V. lanceolata*	-	-	-	Mo	-
11-4202	*V. lanceolata*	-	-	-	Mo	-

11-4203	*V. lanceolata*	-	-	-	-	Em
11-4204	*V. lanceolata*	-	-	-	-	Em
11-4205	*C. anisata*	-34.369	21.265	26.33	-	-
11-4206	*C. anisata*	-34.369	21.265	-	-	-
11-4207	*Z. capense*	-34.369	21.265	-	-	-
11-4208	*Z. capense*	-34.369	21.265	-	-	-
11-4209	*Z. capense*	-34.369	21.265	-	-	-
11-4210	*Z. capense*	-34.369	21.265	-	-	-
11-4211	*Z. capense*	-34.369	21.265	-	-	-
11-4212	*C. anisata*	-34.369	21.265	-	-	-
11-4213	*Z. capense*	-34.369	21.265	-	-	-
11-4214	*C. anisata*	-34.369	21.265	-	-	-
11-4215	*Z. capense*	-34.368	21.264	-	-	-
11-4216	*C. anisata*	-34.368	21.264	-	-	-
11-4217	*Z. capense*	-34.368	21.264	-	-	-
11-4218	*C. anisata*	-34.368	21.264	-	-	-
11-4219	*C. anisata*	-34.368	21.264	26.99	-	-
11-4220	*C. anisata*	-34.367	21.264	-	-	-
11-4221	*Z. capense*	-34.367	21.264	-	-	-
11-4222	*C. anisata*	-34.367	21.264	-	-	-
11-4223	*C. anisata*	-34.367	21.264	-	-	-
11-4224	*C. anisata*	-34.367	21.264	-	-	-
11-4225	*C. anisata*	-34.367	21.264	26.23	-	-
11-4226	*C. anisata*	-34.367	21.265	-	-	-
11-4227	*C. anisata*	-33.961	22.530	20.43	Mo	Em
11-4228	*V. lanceolata*	-33.959	22.532	-	Mo	Em
11-4229	*V. lanceolata*	-33.959	22.532	-	Mo	-
11-4230	*V. lanceolata*	-33.959	22.532	-	Mo	Em
11-4231	*V. lanceolata*	-33.960	22.533	-	Mo	Em
11-4232	*V. lanceolata*	-33.960	22.532	-	Mo	Em
11-4233	*V. lanceolata*	-33.964	22.568	-	Mo	Em
11-4234	*V. lanceolata*	-33.964	22.568	-	Mo	-
11-4235	*C. anisata*	-34.040	22.836	-	-	-
11-4236	*Z. capense*	-34.037	22.837	-	-	-

11-4237	*Z. capense*	-34.037	22.836	-	-	-
11-4238	*C. anisata*	-34.037	22.836	-	-	-
11-4239	*C. anisata*	-34.037	22.836	-	-	-
11-4240	*C. anisata*	-34.037	22.837	-	-	Em
11-4241	*C. anisata*	-34.036	22.837	-	-	-
11-4242	*C. anisata*	-34.034	22.839	-	-	-
11-4243	*C. anisata*	-34.034	22.847	24.95	-	-
11-4244	*Z. capense*	-34.034	22.847	-	-	-
11-4245	*Z. capense*	-34.033	22.847	-	-	-
11-4246	*C. anisata*	-34.033	22.847	24.87	-	-
11-4247	*Z. capense*	-34.033	22.847	-	-	-
11-4248	*Z. capense*	-34.058	23.021	-	-	Em
11-4249	*Z. capense*	-34.058	23.021	26.13	-	Em
11-4250	*Z. capense*	-34.058	23.022	-	-	-
11-4251	*Z. capense*	-34.058	23.022	-	-	Em
11-4252	*Z. capense*	-34.058	23.021	-	-	-
11-4253	*Z. capense*	-34.058	23.022	-	-	Em
11-4254	*Z. capense*	-34.058	23.022	-	-	-
11-4255	*Z. capense*	-34.058	23.022	-	-	-
11-4256	*Z. capense*	-34.058	23.021	-	-	-
11-4257	*Z. capense*	-34.058	23.022	-	-	-
11-4258	*Z. capense*	-34.058	23.021	-	-	-
11-4259	*Z. capense*	-34.058	23.021	-	-	Em
11-4260	*V. lanceolata*	-33.948	23.628	-	-	-
11-4261	*V. lanceolata*	-33.948	23.628	-	-	-
11-4262	*V. lanceolata*	-33.948	23.628	-	-	-
11-4263	*V. lanceolata*	-33.948	23.628	-	-	-
11-4264	*V. lanceolata*	-33.949	23.630	-	-	-
11-4265	*V. lanceolata*	-33.949	23.633	-	-	-
11-4266	*Z. capense*	-34.161	24.817	-	-	-
11-4267	*Z. capense*	-34.161	24.814	-	-	-
11-4268	*Z. capense*	-34.168	24.809	-	-	-
11-4269	*Z. capense*	-34.162	24.825	-	-	-
11-4270	*Z. capense*	-34.163	24.825	29.96	-	-

11-4271	*Z. capense*	-34.163	24.824	-	-	-
11-4272	*C. anisata*	-34.163	24.824	18.94	-	Em
11-4273	*Z. capense*	-34.163	24.825	-	-	-
11-4274	*Z. capense*	-34.163	24.825	-	-	-
11-4275	*C. anisata*	-34.163	24.825	19.24	-	-
11-4276	*Z. capense*	-34.166	24.829	-	-	-
11-4277	*Z. capense*	-34.166	24.829	-	-	Em
11-4278	*C. anisata*	-34.167	24.829	-	-	-
11-4279	*C. anisata*	-34.167	24.829	14.90	-	Em
11-4280	*C. anisata*	-34.167	24.829	-	-	Em
11-4281	*Z. capense*	-34.167	24.830	-	-	Em
11-4282	*C. anisata*	-34.167	24.830	-	-	Em
11-4283	*C. anisata*	-34.167	24.829	-	-	-
11-4284	*V. lanceolata*	-33.754	24.797	-	-	-
11-4285	*Z. capense*	-33.715	24.742	-	-	-
11-4286	*Z. capense*	-33.715	24.742	-	-	-
11-4287	*Z. capense*	-33.715	24.742	-	-	-
11-4288	*V. lanceolata*	-33.709	24.740	-	-	-
11-4289	*Z. capense*	-33.709	24.740	-	-	-
11-4290	*C. anisata*	-33.709	24.740	-	-	-
11-4291	*Z. capense*	-33.709	24.740	-	-	-
11-4292	*Z. capense*	-33.709	24.740	-	-	-
11-4293	*Z. capense*	-33.709	24.740	-	-	-
11-4294	*V. lanceolata*	-33.709	24.740	-	-	-
11-4295	*Z. capense*	-33.709	24.740	-	-	-
11-4296	*V. lanceolata*	-33.708	24.739	-	-	-
11-4297	*V. lanceolata*	-33.707	24.738	-	-	-
11-4298	*C. anisata*	-33.706	24.738	-	-	-
11-4299	*V. lanceolata*	-33.705	24.736	-	-	-
11-4300	*Z. capense*	-33.705	24.736	-	-	-
11-4301	*Z. capense*	-33.704	24.736	-	-	-
11-4302	*V. lanceolata*	33°56'53"	23°37'41"	-	-	-
11-4303	*V. lanceolata*	33°56'53"	23°37'41"	-	-	-
11-4304	*V. lanceolata*	33°55'15"	23°27'13"	-	-	-

11-4305	*V. lanceolata*	33˚55'15"	23˚27'13"	-	-	-
11-4306	*V. lanceolata*	33˚55'15"	23˚27'13"	-	-	-
11-4307	*V. lanceolata*	33˚53'45"	23˚46'07"	-	-	-
11-4308	*V. lanceolata*	-	-	-	-	-
11-4309	*V. lanceolata*	-33.320	26.520	-	-	-
11-4310	*Z. capense*	-33.320	26.522	-	-	-
11-4311	*V. lanceolata*	-33.320	26.522	-	-	-
11-4312	*V. lanceolata*	-33.320	26.522	-	-	-
11-4313	*V. lanceolata*	-33.320	26.522	27.66	-	-
11-4314	*C. anisata*	-33.328	26.501	-	-	-
11-4315	*C. anisata*	-33.328	26.501	-	-	-
11-4316	*Z. capense*	-33.328	26.501	-	-	-
11-4317	*C. anisata*	-33.328	26.501	-	-	-
11-4318	*C. anisata*	-33.328	26.501	-	-	-
11-4319	*C. anisata*	-33.328	26.502	-	-	-
11-4320	*Z. capense*	-33.328	26.501	-	-	-
11-4321	*Z. capense*	-33.328	26.501	-	-	-
11-4322	*C. anisata*	-33.328	26.501	-	-	-
11-4323	*C. anisata*	-33.328	26.501	-	-	-
11-4324	*Z. capense*	-33.328	26.501	-	-	-
11-4325	*V.lanceolata*	-33.328	26.501	-	-	-
11-4326	*Z. capense*	-33.328	26.500	-	-	-
11-4327	*Z. capense*	-33.328	26.500	-	-	-
11-4328	*C. anisata*	-33.328	26.500	-	-	-
11-4329	*C. anisata*	-33.328	26.500	-	-	-
11-4330	*V. lanceolata*	-33.318	26.518	-	-	-
11-4331	*V. lanceolata*	-33.318	26.518	-	-	-
11-4332	*V. lanceolata*	-33.318	26.518	-	-	-
11-4333	*V. lanceolata*	-33.321	26.517	-	-	-
11-4334	*V. lanceolata*	-33.319	26.522	-	-	-
11-4335	*V. lanceolata*	-33.319	26.521	25.05	-	Em
11-4336	*Z. capense*	-33.319	26.521	-	-	-
11-4337	*V. lanceolata*	-33.319	26.521	-	-‘	Em
11-4338	*V. lanceolata*	-33.319	26.520	-	-	-

11-4339	*V. lanceolata*	-33.319	26.521	-	-	-
11-4340	*V. lanceolata*	-33.319	26.521	-	-	-
11-4341	*V. lanceolata*	-33.318	26.521	-	-	-
11-4342	*V. lanceolata*	-33.314	26.521	-	-	-
11-4343	*V. lanceolata*	-33.314	26.521	-	-	-
11-4344	*V. lanceolata*	-33.312	26.521	-	-	-
11-4345	*V. lanceolata*	-33.312	26.521	-	-	-
11-4346	*V. lanceolata*	-33.386	26.704	-	-	-
11-4347	*Z. capense*	-33.386	26.704	-	-	-
11-4348	*Z. capense*	-33.386	26.704	-	-	-
11-4349	*C. anisata*	-33.389	26.707	-	-	-
11-4350	*C. anisata*	-	-	-	-	-
11-4351	*V. lanceolata*	-33.389	26.707	-	-	-
11-4352	*V. lanceolata*	-33.389	26.707	-	-	-
11-4353	*C. anisata*	-33.389	26.707	-	-	-
11-4354	*C. anisata*	-33.389	26.707	-	-	-
11-4355	*C. anisata*	-33.392	26.707	-	-	-
11-4356	*V. lanceolata*	-33.505	26.830	-	-	-
11-4357	*Z. capense*	-33.505	26.830	-	-	-
11-4358	*Z. capense*	-33.504	26.830	-	-	-
11-4359	*V. lanceolata*	-33.585	26.908	-	Y	-
11-4360	*Z. capense*	-33.597	26.900	-	-	-
11-4361	*C. anisata*	-33.594	26.899	17.53	-	-
11-4362	*C. anisata*	-33.594	26.899	-	-	-
11-4363	*C. anisata*	-33.594	26.899	27.61	-	Em
11-4364	*C. anisata*	-33.594	26.899	-	-	-
11-4365	*Z. capense*	-33.593	26.899	-	-	Em
11-4366	*C. capense*	-33.589	26.902	-	-	-
11-4367	*V. lanceolata*	-33.556	26.980	-	-	Em
11-4368	*Z. capense*	-33.556	26.980	-	-	-
11-4369	*Z. capense*	-33.556	26.980	-	-	-
11-4370	*Z. capense*	-33.556	26.980	-	-	-
11-4371	*Z. capense*	-33.556	26.980	-	-	-
11-4372	*Z. capense*	-33.541	27.038	-	-	-

11-4373	*V. lanceolata*	-32.950	28.015	-	-	-
11-4374	*V. lanceolata*	-32.950	28.015	-	-	-
11-4375	*V. lanceolata*	-	-	-	-	-
11-4376	*V. lanceolata*	-32.939	28.028	-	-	-
11-4377	*V. lanceolata*	-32.935	28.024	-	-	Em
11-4378	*V. lanceolata*	-32.935	28.026	-	Mo	Em, T
11-4379	*V. lanceolata*	-32.934	28.027	-	-	-
11-4380	*V. lanceolata*	-32.934	28.028	24.84	M, VC	Em, T
11-4381	*Z. capense*	-32.934	28.029	-	-	-
11-4382	*V. lanceolata*	-32.934	28.026	-	-	Em
11-4383	*Z. capense*	-32.934	28.026	-	-	Em
11-4384	*Z. capense*	-32.934	28.026	-	-	-
11-4385	*V. lanceolata*	-32.934	28.025	-	-	Em
11-4386	*V. lanceolata*	-32.934	28.025	-	-	Em
11-4387	*Z. capense*	-32.934	28.026	-	-	-
11-4388	*Z. capense*	-32.814	28.023	-	-	-
11-4389	*V. lanceolata*	-32.814	28.023	-	-	-
11-4390	*V. lanceolata*	-32.814	28.017	-	-	-
11-4391	*V. lanceolata*	-32.814	28.017	-	-	-
11-4392	*Z. capense*	-32.814	28.017	-	-	-
11-4393	*V. lanceolata*	-32.807	28.019	-	-	-
11-4394	*V. lanceolata*	-32.806	28.020	-	-	-
11-4395	*Z. capense*	-32.806	28.020	-	-	-
11-4396	*V. lanceolata*	-32.806	28.020	-	-	-
11-4397	*Z. capense*	-32.805	28.020	-	-	-
11-4398	*V. lanceolata*	-32.802	28.020	-	-	-
11-4399	*Z. capense*	-32.802	28.020	-	-	-
11-4400	*Z. capense*	-32.802	28.020	-	-	-
11-4401	*V. lanceolata*	-32.802	28.021	-	-	-
11-4402	*V. lanceolata*	-32.784	28.018	-	-	-
11-4403	*Z. capense*	-32.784	28.018	-	-	-
11-4404	*V. lanceolata*	-	-	-	-	Em
11-4405	*V. lanceolata*	-31.594	29.517	-	-	-
11-4406	*Z. daveyii*	-31.590	29.527	-	-	-

11-4407	*Z. daveyii*	-31.048	30.172	-	-	-
11-4408	*C. capense*	-31.048	30.172	-	-	-
11-4409	*C. capense*	-31.049	30.172	-	-	-
11-4410	*C. anisata*	-31.049	30.172	-	-	Em
11-4411	*C. anisata*	-31.048	30.172	-	-	Em
11-4412	*Z. capense*	-31.048	30.172	-	-	-
11-4413	*C. capense*	-31.046	30.171	-	-	-
11-4414	*V. lanceolata*	-31.034	30.180	-	Mo	Em, T
11-4415	*V. lanceolata*	-31.064	30.176	-	Mo	Em
11-4416	*V. lanceolata*	-31.064	30.176	-	Mo	Em, T
11-4417	*V. lanceolata*	-31.064	30.176	-	-	Em
11-4418	*V. lanceolata*	-31.064	30.176	-	Mo	Em
11-4419	*V. lanceolata*	-31.064	30.176	-	-	Em
11-4420	*V. lanceolata*	-31.064	30.176	-	-	Em
11-4421	*V. lanceolata*	-31.064	30.176	-	-	Em, T
11-4422	*V. lanceolata*	-31.064	30.176	-	-	Em, T
11-4423	*V. lanceolata*	-31.064	30.176	24.26	-	Em, T
11-4424	*V. lanceolata*	-31.064	30.176	-	Mo, VC	Em
11-4425	*V. lanceolata*	-31.064	30.176	-	Mo	Em
11-4426	*Z. capense*	-31.064	30.176	-	-	-
11-4427	*Z. capense*	-30.998	30.259	-	-	-
11-4428	*O. bachmanii*	-30.998	30.257	-	Mo	-
11-4429	*V. lanceolata*	-30.998	30.257	-	Mo	-
11-4430	*T. gerrardia*	-30.998	30.257	-	-	Em
11-4431	*T. natalensis*	-30.998	30.258	-	-	Em
11-4432	*V. lanceolata*	-30.998	30.258	-	-	Em
11-4433	*Z. capense*	-30.998	30.258	-	-	-
11-4434	*Z. capense*	-30.998	30.258	-	-	-
11-4435	*Z. capense*	-30.968	30.284	-	-	-
11-4436	*C. anisata*	-30.968	30.284	-	Mo	Em
11-4437	*T. natalensis*	-30.968	30.285	-	Mo	Em
11-4438	*Z. capense*	-30.958	30.290	-	-	Em
11-4439	*C. anisata*	-30.958	30.290	-	-	-
11-4440	*Z. capense*	-30.958	30.290	-	-	Em, T

11-4441	*C. anisata*	-30.958	30.290	-	-	Em
11-4442	*V. lanceolata*	-30.958	30.290	-	Mo	Em
11-4443	*Z. capense*	-30.956	30.291	24.91	Mo	Em, T
11-4444	*C. anisata*	-29.742	30.416	-	-	-
11-4445	*C. anisata*	-29.742	30.416	-	-	-
11-4446	*C. anisata*	-29.742	30.416	-	-	-
11-4447	*C. anisata*	-29.743	30.417	-	-	-
11-4448	*C. anisata*	-29.743	30.416	-	-	Em
11-4449	*V. lanceolata*	-29.818	30.341	-	-	-
11-4450	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4451	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4452	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4453	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4454	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4455	*V. lanceolata*	-29.818	30.341	-	-	-
11-4456	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4457	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4458	*V. lanceolata*	-29.818	30.341	-	-	EM
11-4459	*Z. capense*	-29.818	30.341	-	-	-
11-4460	*V. lanceolata*	-29.818	30.341	-	-	-
11-4461	*V. lanceolata*	-29.818	30.341	-	Mo	Em
11-4462	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4463	*V. lanceolata*	-29.818	30.341	-	-	Em
11-4464	*V. lanceolata*	-29.818	30.341	-	-	Em, T
11-4465	*V. lanceolata*	-29.818	30.341	-	-	-
11-4466	*V. lanceolata*	-29.818	30.341	-	Mo	Em
11-4467	*V. lanceolata*	-29.818	30.341	-	-	-
11-4468	*V. lanceolata*	-29.817	30.341	-	-	-
11-4469	*V. lanceolata*	-29.817	30.341	-	-	-
11-4470	*V. lanceolata*	-29.606	30.347	-	-	-
11-4471	*V. lanceolata*	-29.606	30.347	-	-	Em
11-4472	*V. lanceolata*	-29.606	30.347	-	-	Em
11-4473	*V. lanceolata*	-29.606	30.347	-	-	Em
11-4474	*C. anisata*	-29.605	30.346	26.95	Mo	Em, T

11-4475	*V. lanceolata*	-29.604	30.346	-	Mo	Em
11-4476	*C. anisata*	-29.604	30.346	15.89	-	-
11-4477	*C. anisata*	-29.604	30.346	19.79	-	Em
11-4478	*V. lanceolata*	-29.604	30.347	-	-	Em, T
11-4479	*C. anisata*	-29.604	30.347	19.89	-	Em, T
11-4480	*C. anisata*	-29.604	30.347	-	-	Em
11-4481	*C. anisata*	-29.603	30.347	-	-	-
11-4482	*C. anisata*	-29.604	30.347	-	-	-
11-4483	*C. anisata*	-29.604	30.347	-	-	Em, T
11-4484	*Z. capense*	-29.605	30.344	-	-	Em
11-4485	*V. lanceolata*	-29.871	30.885	-	-	Em, T
11-4486	*V. lanceolata*	-29.911	30.944	-	-	-
11-4487	*Z. capense*	-29.911	30.944	-	-	-
11-4488	*Z. capense*	-29.911	30.944	-	-	-
11-4489	*V. lanceolata*	-29.910	30.944	-	-	-
11-4490	*V. lanceolata*	-29.909	30.945	-	-	-
11-4491	*Z. capense*	-29.908	30.945	-	-	Em
11-4492	*V. lanceolata*	-29.908	30.945	-	-	Em
11-4493	*Z. capense*	-29.907	30.946	-	-	Em
11-4494	*Z. capense*	-29.907	30.945	-	-	-
11-4495	*C. anisata*	-29.907	30.945	-	-	-
11-4496	*V. lanceolata*	-29.913	30.942	-	-	-
11-4497	*Z. capense*	-29.513	31.228	-	-	Em
11-4498	*Z. capense*	-29.513	31.228	-	-	Em
11-4499	*Z. capense*	-29.513	31.228	-	-	Em
11-4500	*Z. capense*	-29.512	31.227	-	-	Em
11-4501	*Z. capense*	-29.512	31.227	21.17	-	Em
11-4502	*Z. capense*	-29.513	31.226	24.49	-	Em
11-4503	*Z. capense*	-29.526	31.208	-	-	-
11-4504	*Z. capense*	-	-	-	-	-
11-4505	*C. anisata*	-28.688	32.003	20.62	-	-
11-4506	*Z. capense*	-28.688	32.003	-	-	-
11-4507	*V. lanceolata*	-27.978	32.331	-	-	-
11-4508	*V. lanceolata*	-27.978	32.331	-	-	-

11-4509	*V. lanceolata*	-27.978	32.331	-	-	-
11-4510	*Z. capense*	-27.978	32.331	-	-	-
11-4511	*V. lanceolata*	-27.978	32.331	-	-	-
11-4512	*V. lanceolata*	-27.978	32.331	-	-	-
11-4513	*Z. capense*	-27.978	32.331	-	-	-
11-4514	*V. lanceolata*	-27.979	32.338	-	-	-
11-4515	*V. lanceolata*	-27.979	32.338	-	-	-
11-4516	*V. lanceolata*	-27.979	32.338	-	-	-
11-4517	*V. lanceolata*	-27.979	32.338	-	-	-
11-4518	*V. lanceolata*	-27.979	32.338	-	-	-
11-4519	*V. lanceolata*	-27.979	32.338	-	-	-
11-4520	*V. lanceolata*	-27.979	32.342	-	-	-
11-4521	*V. lanceolata*	-27.979	32.342	-	-	-
11-4522	*C. anisata*	-27.979	32.342	-	-	-
11-4523	*V. lanceolata*	-27.980	32.347	-	-	-
11-4524	*Z. capense*	-27.980	32.347	-	-	-
11-4525	*V. lanceolata*	-27.980	32.347	-	-	-
11-4526	*V. lanceolata*	-27.980	32.347	-	-	-
11-4527	*Z. capense*	-27.984	32.348	-	-	-
11-4528	*Z. capense*	-27.984	32.348	-	-	-
11-4529	*V. lanceolata*	-27.801	32.415	-	-	-
11-4530	*V. lanceolata*	-27.801	32.415	-	-	-
11-4531	*V. lanceolata*	-27.801	32.415	-	-	-
11-4532	*V. lanceolata*	-27.801	32.415	-	-	-
11-4533	*Z. capense*	-27.801	32.415	-	-	-
11-4534	*V. lanceolata*	-27.801	32.415	-	-	-
11-4535	*Z. capense*	-27.801	32.415	-	-	-
11-4536	*Z. capense*	-27.801	32.416	-	-	-
11-4537	*V. lanceolata*	-27.453	32.572	-	-	-
11-4538	*C. anisata*	-27.453	32.572	-	-	-
11-4539	*C. anisata*	-27.454	32.572	-	-	-
11-4540	*V. lanceolata*	-27.454	32.572	-	-	-
11-4541	*C. anisata*	-27.453	32.572	-	-	-
11-4542	*V. lanceolata*	-27.238	32.543	-	-	-

11-4543	*V. lanceolata*	-27.083	32.564	-	-	-
11-4544	*V. lanceolata*	-27.083	32.564	-	-	-
11-4545	*V. lanceolata*	-27.083	32.564	-	-	-
11-4546	*V. lanceolata*	-27.083	32.564	-	-	-
11-4547	*Z. capense*	-27.841	32.335	-	-	-
11-4548	*V. lanceolata*	-26.965	32.811	-	-	-
11-4549	*V. lanceolata*	-27.052	32.429	-	-	-
11-4550	*V. lanceolata*	-27.047	32.416	-	-	-
11-4551	*V. lanceolata*	-27.047	32.416	-	-	-
11-4552	*V. lanceolata*	-27.266	31.985	-	-	Em
11-4553	*V. lanceolata*	-27.309	31.977	-	-	-
11-4554	*V. lanceolata*	-27.332	31.990	-	-	-
11-4555	*V. lanceolata*	-	-	-	-	-
11-4556	*Teclea sp*	-	-	-	-	-
11-4557	*V. lanceolata*	-	-	-	-	-
11-4558	*Z. capense*	-	-	-	-	-
11-4559	*C. anisata*	-	-	-	-	-
11-4560	*C. anisata*	-	-	-	-	-
11-4561	*Z. daveyii*	-	-	28.11	-	-
11-4562	*Z. daveyii*	-	-	-	-	-
11-4563	*C. ansiata*	-	-	-	-	-
12-0001	*Z. capense*	-	-	-	-	Em
12-0002	*Z. capense*	-	-	-	-	Em
12-0003	*Z. capense*	-	-	25.14	Y	Em
12-0004	*Z. capense*	-	-	27.63	-	Em
12-0005	*Z. capense*	-	-	29.72	-	Em
12-0006	*Z. capense*	-	-	-	-	Em
12-0007	*Z. capense*	-	-	-	-	Em
12-0008	*Z. capense*	-	-	-	-	Em
12-0009	*Z. capense*	-	-	-	-	Em
12-0010	*Z. capense*	-	-	-	-	Em
12-0011	*Z. capense*	-	-	-	-	Em
12-0012	*Z. capense*	-	-	-	-	Em
12-0013	*Z. capense*	-	-	-	-	Em

12-0014	*Z. capense*	-	-	-	Y	Em
12-0015	*Z. capense*	-	-	-	Y	Em
12-0016	*C. anisata*	-	-	25.73	-	Em
12-0017	*C. anisata*	-	-	23.56	-	Em
12-0018	*Z. capense*	-	-	-	-	Em
12-0019	*C. anisata*	-	-	-	-	Em
12-0020	*Z. capense*	-	-	-	-	Em
12-0021	*C. anisata*	-	-	-	-	Em
12-0022	*V. lanceolata*	-	-	-	-	-
12-0090	*C. anisata*	-25.385	30.566	-	-	Em
12-0091	*C. anisata*	-25.385	30.567	-	Y	-
12-0092	*C. anisata*	-25.385	30.568	-	-	-
12-0093	*C. anisata*	-25.385	30.568	-	-	-
12-0094	*C. anisata*	-25.385	30.568	-	-	Em
12-0095	*C. anisata*	-25.385	30.568	-	-	Em
12-0096	*Z. daveyii*	-25.378	30.543	-	-	-
12-0097	*C. anisata*	-25.376	30.538	-	-	-
12-0098	*C. anisata*	-25.376	30.538	-	Mo	Em
12-0099	*C. anisata*	-25.376	30.538	-	-	-
12-0100	*C. anisata*	-25.365	30.516	-	-	-
12-0101	*Z. daveyii*	-25.364	30.516	-	-	-
12-0102	*Z. daveyii*	-25.364	30.516	-	Y	-
12-0103	*Z. daveyii*	-25.364	30.516	-	-	-
12-0104	*Z. daveyii*	-25.364	30.516	-	-	-
12-0105	*Z. daveyii*	-25.364	30.516	-	-	-
12-0106	*Z. daveyii*	-25.363	30.515	-	-	-
12-0107	*Z. daveyii*	-25.363	30.515	-	Y	Em
12-0108	*Z. daveyii*	-25.365	30.516	-	-	Em
12-0109	*Z. daveyii*	-25.383	30.540	-	-	Em
12-0110	*Z. daveyii*	-25.383	30.540	-	-	-
12-0111	*C. anisata*	-25.414	30.603	-	-	-
12-0112	*Z. daveyii*	-25.414	30.603	-	-	-
12-0113	*C. anisata*	-25.414	30.603	-	-	-
12-0114	*Z. daveyii*	-25.416	30.602	-	-	-

12-0115	*C. anisata*	-25.417	30.601	-	-	-
12-0116	*C. anisata*	-25.417	30.601	-	-	-
12-0117	*C. anisata*	-25.417	30.601	-	-	-
12-0118	*C. anisata*	-25.417	30.601	-	-	-
12-0119	*C. anisata*	-25.417	30.601	-	-	-
12-0120	*C. anisata*	-25.417	30.601	-	-	-
12-0121	*C. anisata*	-25.4177	30.601	-	Mo	-
12-0122	*C. anisata*	-25.422	30.596	-	-	Em
12-0123	*C. anisata*	-25.422	30.596	-	-	-
12-0124	*C. anisata*	-25.422	30.596	-	-	-
12-0125	*Z. daveyii*	-25.422	30.596	-	-	-
12-0126	*C. anisata*	-25.422	30.596	-	-	-
12-0127	*C. anisata*	-25.422	30.596	-	-	-
12-0128	*C. anisata*	-25.422	30.596	-	-	-
12-0129	*C. anisata*	-25.422	30.596	-	-	Em
12-0130	*C. anisata*	-25.422	30.596	-	-	Em
12-0131	*Z. daveyii*	-25.422	30.596	-	-	Em
12-0132	*C. anisata*	-25.421	30.598	-	-	-
12-0133	*C. anisata*	-25.421	30.598	-	-	Em
12-0134	*C. anisata*	-25.421	30.598	-	-	-
12-0135	*C. anisata*	-25.421	30.598	-	-	Em
12-0136	*C. anisata*	-	-	-	-	Em
12-0137	*C. anisata*	-	-	-	-	Em
12-0138	*C. anisata*	-25.417	30.606	-	-	Em
12-0139	*C. anisata*	-25.417	30.606	-	-	Em
12-0140	*Z. daveyii*	-25.396	30.610	-	-	-
12-0141	*V. lanceolata*	-25.396	30.610	-	-	-
12-0142	*V. lanceolata*	-25.396	30.610	-	-	-
12-0143	*V. lanceolata*	-25.396	30.610	-	-	-
12-0144	*V. lanceolata*	-25.396	30.610	-	-	-
12-0145	*V. lanceolata*	-25.396	30.610	-	-	-
12-0146	*V. lanceolata*	-25.396	30.610	-	-	-
12-0147	*V. lanceolata*	-25.396	30.610	-	-	-
12-0148	*V. lanceolata*	-25.396	30.610	-	-	-

12-0149	*V. lanceolata*	-25.396	30.610	-	-	-
12-0150	*V. lanceolata*	-25.396	30.610	-	-	-
12-0152	*V. lanceolata*	-25.396	30.610	-	-	-
12-0153	*V. lanceolata*	-25.396	30.610	-	-	-
12-0154	*V. lanceolata*	-25.396	30.610	-	-	-
12-0155	*V. lanceolata*	-25.396	30.610	-	-	-
12-0156	*Z. daveyii*	-25.435	30.435	-	-	-
12-0157	*Z. daveyii*	-	-	-	-	-
12-0158	*Z. daveyii*	-	-	-	-	-
12-0159	*Z. daveyii*	-	-	-	-	-
12-0160	*C. anisata*	-	-	-	-	-
12-0161	*Z. daveyii*	-	-	-	-	-
12-0162	*C. anisata*	-25.430	30.434	-	-	-
12-0163	*Z. daveyii*	-25.429	30.434	-	-	-
12-0164	*Z. daveyii*	-25.429	30.434	-	-	-
12-0165	*Z. daveyii*	-25.429	30.434	-	-	-
12-0166	*Z. daveyii*	-25.429	30.434	-	-	-
12-0167	*C. anisata*	-25.429	30.434	-	-	-
12-0168	*C. anisata*	-25.429	30.434	-	-	-
12-0169	*C. anisata*	-25.429	30.434	-	-	-
12-0170	*Z. daveyii*	-25.430	30.435	-	-	-
12-0171	*Z. daveyii*	-25.430	30.435	-	-	-
12-0172	*C. anisata*	-25.430	30.435	-	-	-
12-0173	*C. anisata*	-25.430	30.435	-	-	-
12-0174	*C. anisata*	-25.430	30.435	-	-	-
12-0175	*c. anisata*	-25.430	30.436	-	-	-
12-0176	*Z. daveyii*	-25.430	30.438	-	-	-
12-0177	*C. anisata*	-25.430	30.438	-	-	-
12-0178	*Z. daveyii*	-25.436	30.436	-	-	-
12-0179	*Z. daveyii*	-25.436	30.436	-	-	-
12-0180	*C. anisata*	-25.436	30.437	-	-	-
12-0191	*Z. capense*	-	-	-	-	-
12-0192	*Z. capense*	-	-	-	-	-
12-0193	*Z. capense*	-	-	-	-	-

12-0194	*Z. capense*	-	-	-	-	-
12-0195	*Z. capense*	-	-	-	-	-
12-0196	*Z. capense*	-	-	-	-	-
12-0197	*Z. capense*	-	-	-	-	-
12-0198	*Z. capense*	-	-	-	-	-
12-0199	*Z. capense*	-	-	-	-	-
12-0200	*Z. capense*	-	-	-	-	-
12-0201	*Z. capense*	-	-	-	-	-
12-0202	*Z. capense*	-	-	-	-	-
12-0203	*Z. capense*	-	-	-	-	Em
12-0204	*Z. capense*	-	-	-	-	-
12-0205	*Z. capense*	-	-	-	-	-
12-0206	*Z. capense*	-	-	-	-	Em
12-0247	*Z. capense*	-	-	-	-	-
12-0248	*Z. capense*	-	-	-	-	-
12-0249	*Z. capense*	-	-	-	-	-
12-0250	*Z. capense*	-	-	-	-	-
12-0251	*Z. capense*	-25.607	30.977	-	Mo	Em
12-0252	*C. anisata*	-25.945	31.113	32.32	-	-
12-0253	*C. anisata*	-25.945	31.113	25.25	-	Em
12-0254	*C. anisata*	-25.945	31.113	32.93	-	-
12-0255	*C. anisata*	-25.945	31.114	-	-	-
12-0256	*C. anisata*	-25.945	31.114	34.52	-	-
12-0257	*Z. daveyii*	-26.041	31.404	-	-	-
12-0258	*Z. daveyii*	-26.041	31.404	-	-	-
12-0259	*C. anisata*	-25.417	30.734	-	-	-
12-0260	*C. anisata*	-25.417	30.734	-	-	-
12-0261	*C. anisata*	-25.417	30.734	-	-	-
12-0262	*C. anisata*	-25.417	30.734	-	-	-
12-0263	*C. anisata*	-25.417	30.734	-	-	-
12-0264	*V. lanceolata*	-25.417	30.734	-	-	Em
12-0265	*C. anisata*	-25.417	30.734	-	-	-
12-0266	*C. anisata*	-25.417	30.734	-	-	-
12-0267	*C. anisata*	-25.416	30.734	-	-	-

12-0268	*C. anisata*	-25.418	30.735	-	-	-
12-0269	*C. anisata*	-25.376	30.692	-	-	-
12-0270	*C. anisata*	-25.376	30.692	-	-	-
12-0271	*C. anisata*	-25.376	30.692	-	-	0
12-0272	*C. anisata*	-25.376	30.692	-	-	Em
12-0273	*C. anisata*	-25.376	30.692	27.11	-	-
12-0274	*C. anisata*	-25.170	30.764	22.71	-	-
12-0275	*C. anisata*	-25.170	30.764	28.69	-	-
12-0276	*C. anisata*	-25.170	30.764	28.77	-	-
12-0277	*C. anisata*	-25.083	30.726	-	-	-
12-0278	*C. anisata*	-25.083	30.726	-	-	-
12-0279	*C. anisata*	-25.083	30.726	27.34	-	-
12-0280	*C. anisata*	-25.083	30.726	-	-	-
12-0281	*C. anisata*	-25.083	30.726	-	-	Em
12-0282	*C. anisata*	-25.083	30.726	24.54	-	Em
12-0283	*C. anisata*	-25.083	30.726	20.38	-	Em
12-0284	*C. anisata*	-25.083	30.726	-	-	-
12-0285	*V. lanceolata*	-25.082	30.726	-	-	-
12-0286	*C. anisata*	-25.082	30.724	-	-	-
12-0287	*C. anisata*	-25.082	30.724	-	-	-
12-0288	*C. anisata*	-25.082	30.724	-	-	-
12-0289	*C. anisata*	-25.103	30.709	-	-	-
12-0290	*C. anisata*	-25.103	30.710	-	-	-
12-0291	*C. anisata*	-25.103	30.710	-	-	Em
12-0292	*C. anisata*	-25.103	30.709	-	-	Em
12-0293	*C. anisata*	-25.103	30.710	-	-	Em
12-0294	*C. anisata*	-25.103	30.710	-	-	Em
12-0295	*C. anisata*	-25.103	30.710	-	-	Em
12-0296	*C. anisata*	-25.103	30.710	27.30	-	Em
12-0297	*C. anisata*	-25.103	30.710	-	-	Em
12-0298	*C. anisata*	-	-	-	-	Em
12-0299	*C. anisata*	-	-	-	-	-
12-0300	*C. anisata*	-	-	-	-	-
12-0301	*C. anisata*	-24.944	30.810	-	-	Em

12-0302	*C. anisata*	-24.944	30.810	-	-	Em
12-0303	*Z. daveyii*	-24.944	30.810	-	Mo	Em
12-0304	*Z. daveyii*	-24.944	30.810	-	-	-
12-0305	*Z. daveyii*	-24.944	30.810	-	-	-
12-0306	*C. anisata*	-24.944	30.810	-	-	Em
12-0307	*C. anisata*	-24.944	30.811	-	-	Em
12-0308	*C. anisata*	-24.944	30.811	-	-	Em
12-0309	*C. anisata*	-24.944	30.811	-	-	-
12-0310	*C. anisata*	-24.944	30.811	-	-	Em
12-0311	*C. anisata*	-24.944	30.811	-	-	Em
12-0312	*C. anisata*	-24.932	30.808	-	Mo	Em
12-0313	*C. anisata*	-24.932	30.808	-	Mo	Em
12-0314	*C. anisata*	-24.932	30.808	-	Mo	Em
12-0315	*C. anisata*	-24.932	30.808	-	-	Em
12-0316	*C. anisata*	-24.932	30.808	-	-	Em
12-0317	*C. capense*	-24.868	30.695	-	Mo	-
12-0318	*C. anisata*	-24.938	30.713	-	-	Em
12-0319	*Z. capense*	-24.938	30.714	-	-	-
12-0320	*Z. caoense*	-24.938	30.714	-	-	-
12-0321	*Z. daveyii*	-24.938	30.715	-	-	Em
12-0322	*Z. daveyii*	-24.938	30.716	-	-	-
12-0323	*Z. daveyii*	-	-	-	-	Em
12-0324	*Z.daveyii*	-	-	33.53	-	-
12-0325	*C. anisata*	-	-	26.37	-	-
12-0326	*C. anisata*	-	-	33.03	-	Em
12-0327	*C. anisata*	-	-	-	-	Em
12-0328	*Z. daveyii*	-24.939	30.718	-	-	-
12-0329	*Z. daveyii*	-	-	-	-	-
12-0330	*Z. daveyii*	-	-	-	-	-
12-0331	*Z. daveyii*	-	-	-	-	-
12-0332	*C. anisata*	-	-	-	-	-
12-0333	*Z. daveyii*	-	-	-	Mo	Em
12-0334	*Z. daveyii*	-	-	-	-	Em
12-0335	*C. capense*	-	-	-	-	-

12-0336	*Z. capense*	-24.463	30.609	-	-	-
12-0337	*C. capense*	-	-	-	-	-
12-0338	*C. anisata*	-23.858	29.984	-	Mo	Em
12-0339	*C. anisata*	-23.858	29.984	-	-	Em
12-0340	*C. anisata*	-23.858	29.984	-	Mo	Em
12-0341	*C. anisata*	-23.858	29.984	-	Mo	Em
12-0342	*C. anisata*	-23.858	29.984	-	Mo	Em
12-0343	*Z. daveyii*	-23.858	29.984	-	-	-
12-0344	*C. anisata*	-23.857	29.984	-	Mo	Em
12-0345	*C. anisata*	-23.876	29.996	-	Mo	-
12-0346	*C. anisata*	-23.876	29.996	-	Mo	Em
12-0347	*C. anisata*	-23.876	29.996	-	-	-
12-0348	*Z. daveyii*	-23.876	29.997	-	-	-
12-0349	*Z. daveyii*	-23.876	29.996	-	-	-
12-0350	*Z. daveyii*	-23.876	29.996	-	-	-
12-0351	*C. anisata*	-23.876	29.996	-	-	-
12-0352	*C. anisata*	-23.876	29.996	-	-	-
12-0353	*Z. capense*	-23.884	29.996	-	-	-
12-0354	*C. anisata*	-23.884	29.996	-	-	-
12-0355	*C. anisata*	-23.884	29.996	-	Mo, Vc	Em
12-0356	*C. anisata*	-23.884	29.996	-	-	Em
12-0357	*C. anisata*	-23.884	29.996	-	-	Em
12-0358	*C. anisata*	-23.884	29.996	-	Mo	Em
12-0359	*C. anisata*	-23.884	29.996	-	Mo	Em
12-0360	*C. anisata*	-23.884	29.996	-	Mo	Em
12-0361	*C. anisata*	-23.884	29.996	-	-	-
12-0362	*C. anisata*	-23.884	29.996	-	Mo	Em
12-0363	*C. anisata*	-23.884	29.996	-	Mo	Em
12-0364	*Z. daveyii*	-23.884	29.996	-	-	Em
12-0365	*Z. daveyii*	-23.884	29.996	-	-	-
12-0366	*Z. daveyii*	-23.884	29.996	-	-	-
12-0367	*Z. daveyii*	-23.884	29.996	-	-	Em
12-0368	*C. anisata*	-23.884	29.996	-	-	-
12-0369	*C. anisata*	-23.884	29.996	-	-	-

12-0370	*C. anisata*	-23.885	29.996	-	Y	-
12-0371	*C. anisata*	-23.885	29.996	-	Mo	Em
12-0372	*C. anisata*	-23.885	29.996	-	-	-
12-0373	Lemon	-23.885	29.996	-	-	-
12-0374	*C. anisata*	-24.142	30.313	-	Mo	Em
12-0375	*Citrus spp*	-24.142	30.313	-	-	-
12-0376	*C. anisata*	-24.160	30.224	-	Y	-
12-0377	*C. anisata*	-24.160	30.224	-	Mo	Em
12-0378	*C. anisata*	-24.160	30.224	-	Mo	-
12-0379	*C. anisata*	-24.160	30.224	-	-	-
12-0380	*C. anisata*	-24.166	30.248	-	Mo	Em
12-0381	*V. lanceolata*	-24.166	30.248	-	-	Em
12-0382	*C. anisata*	-24.167	30.247	-	Mo	Em
12-0383	*C. anisata*	-24.162	30.255	-	-	Em
12-0384	*C. anisata*	-	-	-	-	Em
12-0385	*C. anisata*	-	-	-	Mo	-
12-0386	*C. anisata*	-24.162	30.255	-	-	Em
12-0387	*C. anisata*	-24.155	30.261	-	Mo	Em
12-0388	*C. anisata*	-	-	-	Mo	Em
12-0389	*C. anisata*	-	-	-	Mo	Em
12-0390	*V. lanceolata*	-	-	-	-	-
12-0391	*C. anisata*	-	-	-	-	Em
12-0392	*C. anisata*	-	-	-	-	Em
12-0393	*C. anisata*	-	-	-	Mo	Em
12-0394	*C. anisata*	-	-	-	Mo	Em
12-0395	*C. anisata*	-	-	-	Mo	Em