'*Candidatus* Liberibacter africanus' in non-rutaceous alternate host species from South Africa

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Declaration of Originality

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

Rochelle Janse van Rensburg

SIGNATURE ……

DATE ……

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"Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment." ~ Rosalind Franklin

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List of Abbreviations

16S rRNA	16S ribosomal RNA subunit
˚C	Degrees Celsius
α	Alpha
β	Beta
γ	Gamma
µl	Microlitres
bp	Base pair
CA	California
CFR	Cape Floristic Region
CGA	Citrus Growers Association
Ct	Cycle Threshold
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
ed(s).	Editor(s)
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence *in situ* hybridization
FL	Florida
g	Grams
HCl	Hydrogen chloride
HLB	Huanglongbing
IOCV	International Organization of Citrus Virologist
Laf	'*Candidatus* Liberibacter africanus'
LafC	'*Candidatus* Liberibacter africanus subsp. capensis'

LafCl	‘*Candidatus* Liberibacter africanus subsp. clausenae’
LafV	‘*Candidatus* Liberibacter africanus subsp. vepridis’
LafT	‘*Candidatus* Liberibacter africanus subsp. teclea’
LafZ	‘*Candidatus* Liberibacter africanus subsp. zanthoxyli’
Lam	‘*Candidatus* Liberibacter americanus’
Las	‘*Candidatus* Liberibacter asiaticus’
Lbr	‘*Candidatus* Liberibacter brunswickensis’
Lcar	‘*Candidatus* Liberibacter caribbeanus’
Lcr	*Liberibacter crescens*
Leu	‘*Candidatus* Liberibacter europaeus’
Lso	‘*Candidatus* Liberibacter solanacearum’
Mb	Mega bases
min	Minutes
ml	Millilitres
MLO	Mycoplasma-like organism
NaCl	Sodium chloride
ng/µl	Nanograms per microlitre
NGS	Next-generation Sequencing
omp	Outer membrane protein gene
PCR	Polymerase Chain Reaction
Pp.	Pages
PVP	Polyvinylpyrrolidone
qPCR	Quantitative Polymerase Chain Reaction
rbcL	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit

RNA	Ribonucleic acid
rplJ	50S ribosomal subunit protein L10 gene
rpm	Rotations per minute
rRNA	Ribosomal RNA
s	Seconds
SDS	Sodium Dodecyl Sulfate
spp.	Species
subsp.	Subspecies
TEM	Transmission electron microscopy
Tris	Trisaminomethane
USA	United States of America

Summary

Non-rutaceous plant species, potentially hosts to '*Candidatus* Liberibacter africanus' (Laf) *sensu lato*, were sampled throughout the Cape Floristic Region from the Fynbos and Succulent Karoo biomes in South Africa, and tested for the presence of the insect-transmitted bacterial pathogen associated with 'Citrus Greening disease' (CG) in *Citrus* species (Rutaceae). Laf is considered a persistent problem to the production of citrus in South Africa as fruits produced from CG infected citrus are smaller in size, lopsided or misshaped and have a characteristic bitter taste. The information on the potential host range in indigenous and other plant species in South Africa is limited. In the current study three surveys were carried out during September 2017 (spring), January 2018 (summer), and August 2018 (winter) in the natural vegetation in Robertson, Worcester, Slanghoek, Vredendal, Lutzville and Klawer. Potential psyllid vectors were collected with vacuum sampling from approximately 20 randomly selected plant samples per plant species at each site. Branches and flowers, when available, of the same plants were collected for morphological identification. Leaf and petiole samples of 989 plant specimens, representing 19 plant families and 42 species, were collected. No typical galls induced on leaves by psyllid nymphs were observed on the plants. Psyllids were only collected from two *Roepera foetida (*Zygophyllaceae) plants*.* Of the 989 plant specimens of alternate host species tested for the presence of Liberibacters by real-time polymerase chain reaction (real-time PCR) assays 142 yielded a Ct value below the selected positive/negative threshold of 31 following the Liberibacter 'Universal' real-time PCR assay. Conventional PCR tests, including the amplification of the 16S rRNA, *omp* and *rplJ* genes of Laf, were conducted on these 142 plant specimens. Seven of these yielded very faint bands after gel electrophoresis analysis of the 16S rRNA conventional PCR test. Sanger sequencing of these suggested they were non-target amplicons. Therefore, none of the 42 plant species tested positive for '*Ca.* Liberibacter africanus', the causal agent for CG in South African citrus. As a number of *Atriplex semibaccata* plants had yielded real-time PCR values <31 and yielded some amplicons with the 16S rRNA PCR, a sample with a DNA concentration above 250 ng/µl was selected for next-generation sequence (NGS) analysis using an Illumina HiSeq 2000 platform at Life Sequencing (Spain) to

attempt to identify the presence of a potentially divergent Liberibacter. NGS data indicated that the bacterial entity amplified by the Liberibacter 'Universal' real-time PCR was not Liberibacter spp., but an, as yet, unidentified member of the Gammaproteobacteria. This bacterium may also be present in a number of other *Atriplex* samples tested during this study, including *A. lindleyi* and *A. nummularia*, which had also yielded amplicons in the real-time PCR assays. It may also be present in some of the other plant species testing positive in the real-time PCR test. The data generated from this study, and from the studies done in conjunction with this one, will be used for biological and epidemiological studies and the development of management strategies.

Chapter 1

Introduction, aims and objectives of the study

1.1. Introduction

Huanglongbing (HLB) (common name: yellow dragon disease) is one of the world's most devastating diseases of citrus trees. HLB is associated with the gram-negative, phloem-limited bacterium which is a member of the class Alphaproteobacteria, known as '*Candidatus* Liberibacter asiaticus' (Las) that spreads through citrus trees, causing decline and then death of the trees (Lin & Lin, 1956). HLB has previously been described from various citrus-growing regions around the world (Bové, 2016). HLB does not occur in South African citrus orchards (Oberholzer *et al*., 1963). In 1963 the term 'Citrus greening' was used to describe a disease in citrus orchards from South Africa when HLB-like symptoms were observed (Oberholzer *et al*., 1963; Jagoueix *et al.,* 1994).

'Citrus Greening' (CG) disease is associated with a related bacterium known as '*Candidatus* Liberibacter africanus' (Laf) (Jagoueix *et al.,* 1994). Transmission of this bacterium is primarily mediated through its triozid insect vector, *Trioza erytreae* Del Guercio (Hemiptera: Triozidae), which transmits Laf amongst South African orchards by feeding on infected hosts and spreading the disease to susceptible host plants (McClean & Oberholzer, 1965; Burckhardt & Ouvrard, 2012). Infected citrus trees can be identified based on observable symptoms which include the mottled appearance of infected leaves, but these symptoms are similar to that of a nutrient deficiency (McClean & Oberholzer, 1965). Other observable symptoms include fruit with reduced size, as well as fruits that are lopsided and bitter tasting (McClean & Oberholzer, 1965). The infected fruits are unfit for exportation from South Africa and hence the disease can cause vast economic losses for the South African industry.

In South Africa stringent control strategies are implemented to manage and limit the spread of CG within and between citrus orchards. Some of the control strategies typically include (1) planting of disease-free plant material, (2) elimination of infected branches and citrus trees, or (3) using chemical strategies for the control of the vector populations within the targeted citrus orchards (Buitendag & von Broembsen, 1993). Even after these management systems have been implemented in the citrus orchards, the

disease remains a persistent and continuous problem, specifically in production areas where the temperatures are below 25˚C (Garnier & Bové, 1983). It has previously been proposed that Laf can be continuously reintroduced into orchards, even after control strategies have been implemented, via the insect vector feeding on infected reservoir hosts amongst the natural vegetation surrounding the orchards and re-spreading the disease to the orchards (Phahladira *et al.,* 2012).

Several studies on both Las and Laf have attempted to determine whether natural vegetation, such as the indigenous plant species surrounding the orchards, can serve as alternate or reservoir sources for the continual introduction of Liberibacters into citrus. For example, Brown *et al.* (2011) identified Las in weeds in Jamaica. Four Laf-subspecies have also been identified from indigenous Rutaceous hosts in South Africa, namely '*Candidatus* Liberibacter africanus subsp. clausenae' (LafCl), '*Candidatus* Liberibacter africanus subsp. vepridis' (LafV), '*Candidatus* Liberibacter africanus subsp. teclea' (LafT) and '*Candidatus* Liberibacter africanus subsp. zanthoxyli' (LafZ) (Roberts *et al.,* 2015; Roberts & Pietersen*,* 2017). All of these plants are native hosts of *T. erytreae*, the triozid vector of Laf (McClean & Oberholzer, 1965; Moran, 1968; Roberts *et al.,* 2015). Fynbos is the dominant natural shrubland and heathland vegetation type in the Cape floristic region of the Western Cape, South Africa (Brown, 1993), and this area is exceptionally species rich (Bond & Goldblatt, 1984). It is also exceptionally rich in Rutaceous shrubs. Research is currently being pursued on the presence of Liberibacters in Fynbos represented Rutaceous species, to attempt to identify reservoir hosts for Laf (Roberts, unpublished).

1.2. Aims of the study

During this investigation we aimed to determine whether non-rutaceous indigenous and non-indigenous weed plant species from the Cape floristic region of the Western Cape, South Africa, serve as natural alternate hosts for Laf. With subsequent analysis we might be able to gain some insight into the evolution and survival of Liberibacter species and the effect they have on their plant hosts when identifying and evaluating potential host plant species of Laf.

1.3. Objectives of the study

The first objective of the study was to determine whether the various non-rutaceous indigenous and non-indigenous weed plant species found in the Cape floristic region of the Western Cape, South Africa, can serve as natural alternate host species for Laf. The identification of alternate host species of Laf would lead to the advancement of management strategies by including the non-rutaceous, indigenous and non-indigenous plant species that occur in natural vegetation in the areas surrounding orchards.

The second objective of the study was to analyse data from next-generation Sequencing (Illumina Sequencing) of a single sample selected as it yielded a positive reaction in the Liberibacter 'Universal' real-time PCR assay used to screen for the presence of Liberibacters in the selected plant species. The aim being to generate further sequence data to identify possible Liberibacter related bacterial infections present within the host plant species found in the Cape floristic region of the Western Cape, South Africa.

1.4. References

Bond P & Goldblatt P, 1984. Plants of the Cape flora. *Journal of South African Botany, Supply* **13**: 1-455.

Bové JM, 2016. Huanglongbing or yellow shoot, a disease of Gondwanan origin: Will it destroy citrus worldwide? *Phytoparasitica* **42**: 579-583.

Brown, NAC, 1993. Promotion of germination of fynbos seeds by plant-derived smoke. *New Phytologist* **123**: 575-583.

Brown SE, Oberheim AP, Barrett A & McLaughlin WA, 2011. First report of '*Candidatus* Liberibacter asiaticus' associated with Huanglongbing in the weeds *Cleome rutidosperma*, *Pisonia aculeate* and *Trichostigma octandrum* in Jamaica. *New Disease Reports* **24**: 25.

Buitendag CH & von Broembsen LA, 1993. Living with citrus greening in South Africa. Pp. 269-273 *In* P Moreno, JV da Graça and LW Timmer (eds.), In Proceedings of the 12th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Burckhardt D & Ouvrard D, 2012. A revised classification of the jumping plant-lice (Hemiptera: Psylloidea). *Zootaxa* **3509**: 1-34.

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

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

Lin KH & Lin KH, 1956. The citrus huang lung bin (Greening) disease in China. *Acta Phytopathologica Sinica* **2**: 14-38.

McClean APD & Oberholzer PCJ, 1965. Greening disease of the sweet orange: Evidence that it is caused by a transmissible virus. *South African Journal of Agricultural Science* **8**: 253-276.

Moran VC, 1968. The development of the citrus psylla, *Trioza erytreae* (Del Guercio) (Homoptera: Psyllidae), on *Citrus limon* and four indigenous hosts plants. *Journal of the Entomological Society of South Africa* **31**: 391-402.

Oberholzer PCJ, von Staden DFA & Basson WJ, 1963. Greening disease of sweet orange in South Africa. Pp. 213-219 *In* WC Price (ed.), In Proceedings of the 3rd Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Phahladira MNB, Viljoen R & Pietersen G, 2012. Widespread occurrence of '*Candidatus* Liberibacter africanus subspecies capensis' in *Calodendrum capense* in South Africa. *European Journal of Plant Pathology* **134**: 39-47.

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

Roberts R, Steenkamp ET & Pietersen G, 2015. Three novel lineages of '*Candidatus* Liberibacter africanus' associated with native rutaceous hosts of *Trioza erytreae* in South Africa. *International Journal of Systematic and Evolutionary Microbiology* **65**: 723-731.

Chapter 2

Literature Review

2.1. Introduction

2.1.1. Huanglongbing and Citrus Greening disease of citrus

Citrus Huanglongbing (HLB), also known as 'yellow shoot' disease of citrus, is associated with the phloem-limited bacterium '*Candidatus* Liberibacter asiaticus' (Las) (Jagoueix *et al.,* 1994). HLB is a devastating, insect-transmissible citrus disease that is spreading around the world (Bové, 2006; da Graça, 2008) and no established efficient management or control measures exist for it yet (Canales *et al.,* 2016). HLB causes decline in citrus trees and has led to the death of millions of trees (Lin & Lin, 1956; Zhang *et al.,* 2010). It can take up to two years after the initial infection of Las before any noticeable symptoms become apparent, therefore making detection of infected trees very difficult (Slisz *et al.,* 2012).

The original record of HLB-like symptoms observed in citrus orchards were from the 18th century in India (Capoor, 1963; da Graça, 2008). It is suspected that the pathogen responsible for HLB was probably present in native rutaceous plants from which the disease causing pathogen may have been transmitted to new citrus trees planted in nearby areas (da Graça, 2008). HLB was also identified in China in the late 19th century by a Chinese farmer (Zhao, 1981), and it is therefore suggested that infected citrus may have been transported from India to China (da Graça, 2008). In recent years, HLB has been described from various citrus growing areas around the world, including Argentina, Brazil, China, Ethiopia, India, Madagascar, Philippines, Saudi Arabia, and USA (California, Florida and Texas), just to name but a few (Bové & Garnier, 1984; Aubert *et al.,* 1988; Garnier & Bové, 1996; Coletta-Filho *et al*., 2004; Halbert, 2005; Teixeira *et al*., 2005; Bové, 2006; Saponari *et al*., 2010; Stokstad, 2012; Outi *et al.,* 2013).

During the late 1960s the disease had different names in other parts of the world. In the Philippines the disease was termed 'mottle leaf' disease (Lee, 1921; Salibe & Cortez, 1968), and in India the term 'dieback' is used to describe similar symptoms in citrus (Fraser & Singh, 1968; Bové, 2006), in Taiwan it was named 'Likubin', and in Indonesia it was called 'citrus vein-phloem degeneration' (CVPD) (Bové, 2006; Wirawan *et al.,* 2017). In all these Asian countries the disease was later found to be associated with Las.

HLB does not occur in South Africa, but symptoms similar to HLB symptoms described by the citrus farmers in Southern China were observed in South Africa in 1963 (Oberholzer *et al*., 1963). The disease, which proved later not to be associated with Las but rather with another species of Liberibacter, was named citrus 'Greening' disease (Oberholzer *et al*., 1963). The heat sensitive disease with similar but less severe symptoms than HLB in citrus appeared in the Western Transvaal, South African in 1928 under the name 'yellow shoot' (Schwarz & van Vuuren, 1971; Wirawan *et al.,* 2017). The disease was named 'Greening' in Eastern Transvaal and 'yellow branch' in Western Transvaal (McClean & Oberholzer, 1965a). The true nature of the disease in South Africa was not immediately understood. In 1937 'Greening' was described to be being due to mineral toxicity (van der Merwe & Andersen, 1937). McClean and Oberholzer (1965) however demonstrated that the South African citrus greening (CG) disease can be transmitted through grafting (McLean & Oberholzer, 1965a). Thereafter the graft transmissible agent referred to as the 'Greening virus' became widely accepted as the causal agent of CG, HLB, mottle leaf and Likubin (Fraser & Singh, 1968).

The 'greening virus' idea was rejected soon after because it was discovered that the causal agent of certain "viral" plant diseases, including yellows disease and mulberry dwarf disease, were not viruses, but rather mycoplasma-like organisms (MLOs) (Laflèché & Bové, 1970). Laflèche and Bové (1970) discovered MLOs that were present within the sieve tubes of orange trees infected with HLB through electron microscopy studies. It was discovered that the MLOs associated with HLB infections of citrus trees had a thicker envelope compared to other MLOs, and that these organisms' appearance resembled bacterial cell walls (Garnier & Bové, 1977). By exposing the causal agents of HLB and CG to tetracycline treatments and testing the sensitivity of these agents to penicillin, the bacterial nature of the agents was demonstrated by Bové *et al*. (1980). It was later shown that gram-negative bacterial agents were associated with both HLB and CG by using a combination of antibiotic studies and electron microscopy on infected citrus and periwinkle samples (Garnier *et al.,* 1984). Garnier & Bové (1983) observed that the gram-negative bacteria associated with these citrus

diseases remained restricted within the sieve tubes of the infected citrus plants. It was only later, in 1994, that 16S ribosomal RNA (rRNA) comparative studies were used to identify that the gram-negative bacteria that caused HLB and CG respectively belonged to the class Alphaproteobacteria and that they were in fact unique, despite their high similarity (Jagoueix *et al.,* 1994). From there on the African form was termed '*Candidatus* Liberobacter africanum', and the Asian form was termed '*Ca.* Liberobacter asiaticum' (Jagoueix *et al.,* 1994). Based on the international rules of nomenclature, both of these names were later changed respectively to '*Ca.* Liberibacter africanus' (referred to as Laf from here on) and '*Ca.* Liberibacter asiaticus' (referred to as Las) (Garnier *et al*., 2000).

At the 13th Conference of International Organization of Citrus Virologist (IOCV), which was hosted in China in 1995, it was decided that the correct name to use for the global citrus disease, in honour of Prof Lin Kongxiang, will be 'Huanglongbing' from then on (Moreno *et al*., 1996). The disease name was decided based on the international rules of nomenclature, which states that the first official description of the pathogen must be used to give the disease a name.

Our group (Pietersen, personal communication) contend that HLB and the Citrus Greening disease found in Africa should be treated as two diseases. The vector of Laf was identified as *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) (McClean & Oberholzer, 1965b; Burckhardt & Ouvrard, 2012), whereas the vector of Las was identified as *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Capoor *et al.,* 1967; Martinez & Wallace; 1967; Burckhardt & Ouvrard, 2012). The agent associated with HLB, Las, was transmitted by graft-inoculation from infected citrus to susceptible citrus for the first time in the 1950s in China (Lin & Lin, 1956). While HLB was the disease name used to describe the first graft-transmission of the causal agent, and therefore has priority over other names (Moreno *et al.,* 1996), plants affected by Laf appear to recover from the symptoms at 32°C but not at 27°C (Bové *et al.,* 1984), and the trees rarely die from the disease. Therefore, HLB and CG should be considered two separate diseases with Laf associated with the milder CG vectored naturally by *T. erytreae* and Las

associated with the more serious HLB, vectored naturally by *D. citri*, and therefore in this dissertation we refer to the two diseases separately based on which species of Liberibacter is associated with them. The literature reviewed is considered in this context.

Research done in South Africa, where citrus was infected with CG, indicated that the presence of Laf is associated with CG in citrus (Garnier *et al*., 1999). The unculturable characteristic of both Laf and Las has contributed to the prolonged and continued characterisation of these bacteria. In 2009 the cultivation of all three citrus infecting Liberibacter species – Laf, Las and '*Candidatus* Liberibacter americanus' (discussed below) – has been made possible by designing a new medium, now known as Liber A (Sechler *et al*., 2009). The Liber A medium was designed to contain citrus vein extract and a growth factor that sustained the growth of the '*Ca.* Liberibacter' species (Sechler *et al*., 2009).

In recent years HLB was detected in infected citrus orchards in Säo Paulo, Brazil, South America for the first time (Coletta-Filho *et al.,* 2004). After conducting widespread surveys across São Paulo, an additional newly characterised Liberibacter species was identified and named '*Ca.* Liberibacter americanus' (referred to as Lam from here on). It was discovered that this new species of Liberibacter was initially responsible for almost all (nearly 98%) of the HLB infections at that time in Brazil (Teixeira *et al.,* 2005) but has subsequently been relegated to being rarely found due to the increase in Laf infections. After sequencing and analysis of the 16S rRNA gene, it was discovered that Lam is more closely related to Las compared to Laf. The vector that transmits Lam between citrus trees was also identified as *D. citri*. (Teixeira *et al*., 2005).

2.1.2. Host response to Liberibacter infection and the symptom expression in host plants

The expression of symptoms of both CG and HLB infections of citrus are similar, but CG does tend to cause less aggressive symptoms in infected trees (Manicom & van Vuuren, 1990). The bacterial entities associated with both these diseases affect all parts of a tree as the diseases spread

systematically through the plant (Tatineni *et al.,* 2008). The bacteria are present in the fruit and floral parts after systematic spread throughout the infected host, except the endosperm and embryo of the seeds (Tatineni *et al.,* 2008). Even the tree roots can be infected by the bacterial agents associated with the two diseases (Shokrollah *et al.,* 2009).

The linear relationship that exists between the concentration of the bacterial populations within the host and the time required for the appearance of visible symptoms determines the symptom expression of infected trees (Coletta-Filho *et al.,* 2010). The age of the host plant also influences the severity of the disease (McClean & Oberholzer, 1965b). For instance, during the early stages of the tree's life, the disease will severely affect the tree when infected, as compared to a tree that becomes infected during a later stage having predominately healthy branches with the youngest branches typically showing disease symptoms (McClean & Oberholzer, 1965b). In the case where young trees become infected, all the branches will be symptomatic, and the branches will be underdeveloped and unproductive (McClean & Oberholzer, 1965b).

For the purpose of this study only CG will be discussed in detail with some references to HLB, because this study is based on the probability of finding Laf-like infections in alternative host plants from South Africa as only Laf-like infections have previously been identified from South Africa.

2.1.2.1. Foliar symptoms

CG infected trees can be identified within orchards as the disease cause a blotchy appearance and mottling of the leaves of infected branches (Bové, 2006). The infected branches can be identified by looking at the leaves that have an uneven yellowing in colour along the midrib and veins that typically spreads in a lateral direction across the surface of the leaf from the vein, and the yellow discolouration may appear on a single shoot or branch of infected trees (Chung & Brlansky, 2005; Batool *et al.,* 2007). The age of the leaves and the time of the year influences the severity of the mottled appearance, where more pronounced symptoms are visible on mature leaves (McClean & Oberholzer, 1965a). However, the characteristic mottled appearance CG

infected leaves also resemble the effects of Zinc deficiency that causes the chlorotic leaf pattern (Schneider, 1968) (see Figure 1). Therefore, the presence of mottled leaves cannot confirm the presence of the causal agent associated with CG. New flushes from infected branches are typically narrow and in an upright position, with the infected leaves either completely yellow in colour or the leaves are yellow with flecks of green (see Figure 2). The leaves will become more distinct and noticeable as the leaves mature (McClean & Oberholzer, 1965a). Severely infected trees are typically sparsely foliated as the leaves from these trees are prematurely dropped (McClean & Oberholzer, 1965a).

Figure 1: Difference in leaf colouration of citrus trees in California, USA. (**A**) Healthy leaf, (**B**) infected with HLB (similar to CG symptoms) (**C**) Zinc deficiency, and (**D**) HLB infected and Zinc deficient leaf (Pourreza, 2016).

Figure 2: Mottled, yellowing appearance of citrus tree leaves infected with (**A**) '*Candidatus* Liberibacter asiaticus' in Florida, USA, and (**B**) '*Candidatus* Liberibacter africanus' in Mpumalanga, South Africa. (photos courtesy of Ronel Roberts).

Schneider (1968) proposed that localised phloem necrosis caused by the invasion and infection of the causal agent of CG leads to the foliar symptoms. It was also noted that the infected cells' starch granules within the chloroplast have an increased size, which causes the outer membrane of the chloroplast

to enlarge (Schneider, 1968). The collapse of the phloem cells then causes the cells to fill with starch, which may cause the leathery appearance and feel of the leaves (Schneider, 1968).

2.1.2.2. Fruit and seed symptoms

Symptomatic citrus fruit from CG infected trees are typically smaller in size, they also have poor colouration, they remain green at the stylar end, and they often contain aborted or partially developed seeds compared to fruit from healthy trees (McClean & Oberholzer, 1965a). Therefore, the poor quality of the fruit produced from infected branches makes them unsuitable for exportation. As with HLB, the greened colour of fruit from infected branches may be linked to reduced ethylene concentrations, which cause photosynthesis to increase (Martinelli *et al.,* 2012). The symptomatic fruit are typically misshapen or lopsided, they have a bitter taste (McClean & Oberholzer, 1965a). These fruits also drop prematurely with a greenish-brown discolouration of the flesh of the fruit (McClean & Oberholzer, 1965a). The causal agent of CG also produces infected fruit in asymptomatic trees as for HLB, where the fruits have reduced juice percentage and characteristic Brix/acidity ratios (Bassanezi *et al.,* 2009).

2.1.3. Transmission of Citrus Greening disease

2.1.3.1. Vector transmission

The primary spread of CG in orchards is facilitated by the feeding and flight activity of the triozid vector, *T. erytreae,* in South Africa (McClean & Oberholzer, 1965b). The insect vector is able to efficiently transmit Laf by feeding on infected citrus and flying to and feeding on susceptible citrus (Aubert, 1987; van den Berg, 1990; Cocuzza *et al.*, 2016; Aidoo *et al*., 2019). Three indigenous rutaceous host plant species from South Africa have been identified on which *T. erytreae* insects can complete their life cycle, including (1) *Clausena anisata* (Willd.) Hook. f. ex Benth. (Horsewood), (2) *Vepris lanceolata* (Lam.) G.Don (white ironwood) (previously *V. undulata*), and (3) *Zanthoxylum capense* (Thunb.) Harv. (small forest ironwood) (previously *Fagara capensis*) (Moran, 1968a*)*. *T. erytreae* is also attracted to *Calodendrum capense* (L.f.) Thunb (Cape chestnut) (Garnier *et al.,* 1999). However, if given the choice, *T. erytreae* is preferably attracted to *Citrus limon*

(L.) Burm.f. when compared to the indigenous rutaceous species (Moran, 1968b). The preferred attraction of the insect vector to *Ci. limon* may be explained based on physiological aspects of the lemon leaves, as the leaves are more suited for oviposition compared to the leaves of the other three indigenous trees (Moran & Buchan, 1975).

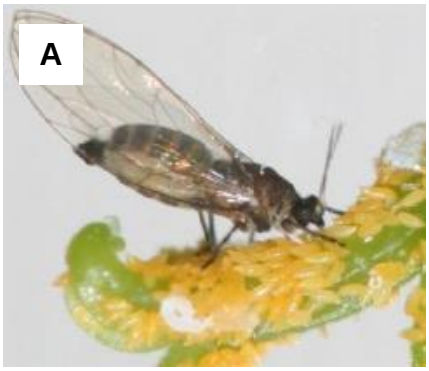


Figure 3: (**A**) *Trioza erytreae* adult feeding on *Vepris lanceolata* flush (photo courtesy of Gerhard Pietersen).

Acquisition of Laf and Las by the respective insect vectors only occurs when the psyllid insects feed on currently infected citrus plants. In HLB, after Las is acquired by the vector the bacterium spreads to the insect salivary glands, then to the haemolymph, the filter chamber, the midgut, the ovaries and the muscles of the insect where accumulation and multiplication ultimately occurs (Moll & Martin, 1973; Ammar *et al.*, 2011). This then allows the establishment of a tenacious Las infection within the insect ready to be transmitted to susceptible trees (Xu *et al.,* 1988; Hung *et al*., 2004). For Las, the multiplication of the bacteria within the insect vector is essential for efficient bacterial transmission to susceptible hosts (Inoue *et al.,* 2009). This has not been tested for Laf. After a feeding period of about 24 hours both male and female psyllids (*T. erytreae* and *D. citri*) are capable of transmitting the bacterial agents (Laf and Las respectively) (Catling & Atkinson, 1974; Capoor *et al.*, 1974). The pathogen can be acquired by the nymphal stages of *T. erytreae* (Hung *et al.,* 2004). Therefore, bacterial multiplication can already occur during the nymphal stages (Hung *et al.,* 2004). A South African study has indicated previously that Laf can be transmitted transovarially by *T. erytreae*, i.e. Laf can be transmitted from parent to offspring, although the results have not been repeated yet (van den Berg *et al*., 1991-1992). Gottwald *et al.* (2007) demonstrated the experimental transmission of Laf by *T. erytreae*

and Las by *D. citri*. They indicated that under experimental conditions, both species are able to transmit the respective causal agents (Gottwald *et al.,* 2007).

2.1.3.2. Graft- and dodder transmission

The artificial transmission of Laf to a susceptible host via grafting and using dodder has been done (Garnier & Bové, 1983). It has been demonstrated that these forms of experimental transmission of Laf are more efficient than natural transmission (Garnier & Bové, 1983). The graft transmission experiments involved inoculum from Laf infected citrus trees, with the best source of inoculum being a small piece of infected budwood (Garnier & Bové, 1983).

Various species of dodder have been identified that may aid in the transmission of citrus associated Liberibacter species, including *Cuscuta reflexa* Roxb. (da Graça, 1991), *Cu. indecora* Choisy (Hartung *et al*., 2010), and *Cu. pentagona* Engelmann (Zhang *et al*., 2010) (Cuscutaceae). Although efficient transmission by dodder of all Liberibacter species has been shown, some characteristics of the host may restrict transmission studies, for example once dodder has been established on a source plant it does not prefer to colonize plant hosts from different species (Hartung *et al.,* 2010). The use of dodder and graft inoculation as a means of transmission have been exploited by researchers to assist in investigating aspects relating to the pathogen, for instance host responses to infections, bacterial host ranges and determining the aggressiveness of the pathogen (Lopes & Frare, 2008).

An ideal experimental model for Laf is periwinkle, *Catharanthus roseus* (L.) G. Don (Apocynaceae), as Laf can multiplying to higher titres within this species, and this host plant grows very fast and well (Garnier & Bové, 1983). Periwinkle plants infected with HLB have also been used to characterise Las using electron microscopy techniques (Garnier *et al*., 1984), and similar studies also assisted in Las serotype studies through the use of monoclonal antibodies (Garnier *et al.,* 1991; Gao *et al.,* 1993). These studies can be used to further study the Laf bacterium to better understand and characterise the bacterium in the future.

2.1.3.3. Seed transmission

Determining the transmission of citrus Liberibacters through plant seeds is cumbersome and very unlikely, but very important as rootstocks are typically grown from seedlings (Hilf, 2011). Recently van Vuuren *et al.* (2011) concluded that vertical transmission of Laf through seeds does not occur. They examined seedlings for symptoms and attempted to detect bacterial DNA in the seedlings germinated from symptomatic trees by using conventional PCR tests as well as qPCR and found no evidence of vertical seed transmission (Hilf *et al.,* 2013). Hilf *et al*. (2013) conducted a study to attempt to visualise Las cells in the vascular bundle of citrus seed coats using fluorescence *in situ* hybridization (FISH) and transmission electron microscopy (TEM) techniques and concluded that Las colonises seed coats and that the bacteria were exclusively found in the seed coat. Therefore, removal of the seed coat is recommended as a standard practice during germination of citrus seeds (Hilf *et al*., 2013). No studies involving the aforementioned techniques have been used to determine if Laf is present in seeds from CG infected citrus. Based on the research done on the possibility of vertical seed transmission of citrus infecting Liberibacters, no conclusive answer exists yet regarding the possibility of Laf seed transmission. It appears to be unlikely, because there is no direct vascular connection between the parent plant and the embryo within the seed.

2.1.4. Host range of Laf

The three Liberibacter species, Laf, Las and Lam, can infect all commercially grown citrus cultivars and species, regardless of the rootstocks used. In South Africa, all the citrus host species for Laf have been identified as: *Citrus sinensis* (L.) Osbeck (sweet orange)*, Ci. paradisi* MacFad (grapefruit)*, Ci. reticulata* Blanco (tangerine and mandarin), *Ci. paradisi x Ci. reticulata* (tangelo), *Ci. limon* (lemon), *Ci. jambhiri* Lush. (rough lemon), *Ci. aurantium* L. (sour orange), *Ci. aurantifolia* (Christm.) Swingle (Mexican lime), *Ci. reticulata* var. clementine (clementine), and *Poncirus trifoliate* (L.) Raf. (trifoliate orange) (McClean & Schwarz, 1970; Korsten *et al*., 1996). Manicom and van Vuuren (1990) classified the host responses to Laf infections as

tolerant (pomelo and trifoliate orange), mild/moderate (lemon, grapefruit and sour orange), and severe (mandarin, sweet orange and tangelos).

2.1.4.1. Alternative rutaceous host species

The continual introduction of CG into commercially grown South African citrus orchards in spite of implementing control strategies has led to the suggestion that non-agricultural host plants may serve as possible reservoirs for Laf and its insect vector, *T. erytreae*. Previous studies done on HLB have attempted to identify reservoir plant host species of Las and Lam, both naturally and experimentally. It has been demonstrated that both bacteria can naturally infect the rutaceous *Murraya paniculata* (L.) Jack (orange jasmine), which in many citrus producing countries is a common ornamental tree and natural host of *D. citri* (Mayikawa, 1980; Hung *et al.,* 2000; Zhou *et al.,* 2007; Damsteegt *et al*., 2010; Lopes *et al.,* 2010; Jantasorn *et al.,* 2012; Walter *et al*., 2012). Alternative rutaceous host species of Laf has also been identified. These include three indigenous rutaceous host plant species, namely *Cl. anisate*, *V. lanceolata* and *Z. capense* (Moran, 1968a; Roberts *et al.,* 2015*)*, as mentioned before. The triozid vector of Laf can complete its life cycle on all three these plant species. *T. erytreae* is also attracted to *Ca. capense* in addition to the three tree species mentioned previously (Garnier *et al.,* 1999; Phahladira *et al.,* 2012). Though, if given the choice, *T. erytreae* prefers to inhabit *Ci. limon* when compared to the indigenous rutaceous species (Moran, 1968b). No other rutacous host species have been identified from South Africa.

2.1.4.2. Non-rutaceous host species

No alternative non-rutaceous plant species have been identified as possible hosts for Laf yet. The successful transmission of Las to non-rutaceous species have been done through the use of dodder species (Garnier & Bové, 1983; Garnier & Bové, 1993; Duan *et al.,* 2008), these include *Ca. roseus* (Garnier & Bové, 1983), *Nicotiana tabacum* L. (Solanaceae: tobacco) (Garnier & Bové, 1993), and *Solanum lycopersicum* L. (Solanaceae: tomato) (synonyms: *Lycopersicon lycopersicum* (L.) H. Karst., *Lycopersicon esculentum* Mill.) (Duan *et al*., 2008). Las was also identified from three weed plant species present in Jamaica, including *Cleome*

rutidosperma DC (Capparaceae), *Pisonia aculeata* L. (Nyctaginaceae), and *Trichostigma octandrum* (L.) H.Walter (Phytolaccaceae) (Brown *et al.,* 2011). Both *N. tabacum* (Francishini *et al*., 2007) and *Cu. indecora* (Hartung *et al*., 2010) have been shown to be infected with Lam. Another Liberibacter species named '*Candidatus* Liberibacter solanacearum' (referred to as Lso) has been identified and detected in various countries around the world, including in tomato plants in Mexico (Munyaneza *et al*., 2009), in carrots from Africa and Norway (Munyaneza *et al.*, 2014; Tahzima *et al.,* 2014), and in potato infected with zebra chip disease in New Zealand (Secor *et al.,* 2009).

The fact that these '*Ca.* Liberibacter' species can multiply within non-rutaceous host plants suggests that the members of Alphaproteobacteria, including Laf, may have a wider host range amongst botanical families than previously suspected. This may be due to the presence of different psyllid species capable of transmitting the same bacteria to a wide variety of host plants, due to the psyllid vector feeding preferences. For example, the psyllid species responsible for transmitting Lso in carrots is *T. apicalis* Förster (Hemiptera: Triozidae), which differs from the psyllid species responsible for transmitting Lso in tomato and potato, known as *Bactericera cockerelli* (Hemiptera: Triozidae). It is therefore possible that non-rutaceous host plants exists in South Africa.

2.1.5. Molecular characterisation Laf.

Due to the unculturable nature of the citrus infecting Liberibacters, the process of molecular characterization of the citrus infecting '*Ca*. Liberibacter' species has been very slow. The *nusG-rplKAJL-rpoBC* gene cluster was the first sequence that was cloned and characterised for Laf, which is the target of the DNA probe As-1.7 for Laf isolates (Villechanoux *et al.,* 1993; Planet *et al*., 1995). The 16S rRNA gene region of Laf was also obtained during that time by PCR and cloning techniques (Jagoueix *et al.,* 1994). Four additional genes, namely *nusG, omp, pgm* and a gene encoding a hypothetical protein, were later characterised from the Laf chromosome (Hocquellet *et al*., 1999).

2.1.5.1. Complete genome of Laf

The complete genome of Laf was sequenced and produced by Lin *et al.* (2015) from the '*Candidatus* Liberibacter africanus' strain PTSAPSY (GenBank accession: CP004021.1) by analysing DNA extracted from *T. erytreae* from Pretoria, South Africa. Lin *et al.* (2015) indicated that the Laf genome is circular in shape and 1.92 Mb in size. They tested extracted DNA during real-time PCR assays, and the DNA samples that had high Laf titres were used for whole genome amplification (Lin *et al.,* 2015). The Laf amplicons were used to construct a sequencing library and ultimately used to obtain the complete genome sequence of the PTSAPSY strain by using an Illumina HiSeq 2000 sequencing approach (Lin *et al.,* 2015). Lin *et al.* (2015) concluded that the Laf genome consisted of a 34.5% GC content, 1,017 predicted protein coding sequences or open reading frames (ORF), 44 transfer RNAs (tRNAs), and the complete sequence copies of the16S, 23S and 5S rRNAs (Lin *et al.,* 2015).

2.1.6. Detection and identification of Laf infections.

Identification of Laf infections can be done accurately by using real-time PCR detection methods. The original real-time PCR, also referred to as quantitative PCR (qPCR), system for the specific identification and detection of various Liberibacters, including Laf, Las, Lam and Lso, was developed by Li *et al*. (2006). Li *et al.* (2006) designed primers and probes for the detection of a conserved region of the 16S rRNA gene of the known '*Ca*. Liberibacter' species, that was only 70 bp in size. A modified version of the method was used for the accurate detection of all known '*Ca*. Liberibacter' species in a single reaction by redesigning the forward primer (Roberts *et al*., 2015). The qPCR assay developed by Roberts *et al.* (2015) is more sensitive, rapid and reproducible than the conventional PCR strategies used before (Li *et al.,* 2007), due to the small size of the amplicon. The 16S rRNA gene is the target region of this qPCR assay, which attributed to the increased sensitivity of the assay, because the 16S rRNA gene has three copies in the Liberibacter genome. Well-equipped laboratories are required for the accurate identification of CG from citrus orchards by using PCR-based diagnostics and

techniques, which is not always easily accessible, particularly in citrus growing developing countries.

2.1.7. Origin hypotheses of Laf

The origins of Liberibacter species is a highly discussed subject. It has been considered that '*Ca.* Liberibacter' species have evolved alongside other members of the Rhizobiales (Rhizobiaceae) and the Rhodobacteriales, but a phylogenetic analysis of the 16S rRNA gene region of the known citrus infecting Liberibacters indicated that this bacterial group emerged before the members of the Rhizobiales (Doddapaneni *et al.,* 2008). Two hypotheses that might explain the origin of the citrus infecting Liberibacter species are discussed below.

2.1.7.1. Multi-continental Hypothesis

The original hypothesis regarding the origin of the bacterial agents of CG and HLB is that the Liberibacters infecting citrus evolved independently on separate continents, i.e. Laf in Africa, Las in Asia, and Lam in South America (Bové, 2006). For the purpose of this study, only the hypothesis concerning Laf will be discussed.

2.1.7.1.1. Hypothesis that Laf originated in Africa.

Two factors supports the hypothesis that Laf originated in Africa, (1) the presence of Laf infections has only ever been identified in African commercially grown citrus orchards (Garnier & Bové, 1996), and (2) citrus is not naturally found and therefore not indigenous to Africa (Beattie *et al.,* 2008). As mentioned previously, Laf has been identified and isolated from *V. lanceolata* trees (Korsten *et al.,* 1996), which are native to Africa. Furthermore, a related Liberibacter, '*Candidatus* Liberibacter africanus subsp. capensis' (LafC), has been identified and isolated from *Ca. capense* trees (Garnier *et al.,* 2000). It is also possible that Laf originated from an indigenous source as the presence of Liberibacter infections has been identified in indigenous rutaceous tree species from South Africa, and the ability of *T. erytreae* to inhabit and feed on these trees also supports this hypothesis (Moran *et al*., 1968b; Phahladira *et al.,* 2012; Roberts *et al.,* 2015; Roberts *et al.,* 2017).

2.1.7.1.2. LafC as the possibly ancestor of Laf

Since its discovery, LafC has been widely associated with *Ca. capense* trees in both greening-affected and greening-free regions across South Africa (Garnier *et al.*, 2000; Phahladira *et al.,* 2012). LafC has not been identified in citrus from South Africa that are grown commercially (Garnier *et al.,* 2000; Pietersen *et al.,* 2010) and the natural transmission of LafC to commercially grown citrus seems very unlikely (Phahladira *et al.*, 2012). LafC infections of *Ca. capense* seems to be asymptomatic with no visible symptoms on the trees (Phahladira *et al.,* 2012), which may possibly indicate that LafC co-evolved with this plant host. The common association of LafC with *Ca. capense* in natural, isolated regions, far removed from citrus, also supports this long-standing association between the bacterium and the plant species. In South Africa, the presence of LafC also promotes the hypothesis that LafC, which is closely related to Laf, may represent a parent lineage of Laf (Phahladira *et al.,* 2012). It has also been suggested that a host jumping event by an intermediate host plant, such as *V. lanceolata,* may have possibly assisted in the association of Laf with citrus (Phahladira *et al.,* 2012).

2.1.7.2. Single Australian origin

In 2008 it has been proposed that Laf and Las evolved from a common African ancestor and the speciation of Las subsequently occurred in India (Beattie *et al*., 2008). Beattie *et al.* (2008) hypothesised that within *V. lanceolata* an ancestral '*Ca.* Liberibacter' species was present and that *T. erytreae* transmitted the ancestor from *V. lanceolata* to *Ci. sinensis* or *Ci. reticulata* trees. It was proposed that such an event occurred on the Southeast coast of Africa in a European colony from which infected plant material was shipped to the Indian subcontinent (Beattie *et al*., 2008). In India the Las bacterium could have been acquired by *D. citri*, which may have allowed the spread of the disease through India (Beattie *et al*., 2008).

Teixeira *et al.* (2008) demonstrated that Lam diverged from Laf and Las approximately 309 million years ago, and the Laf and Las, the respective bacterial causal agents of CG and HLB, diverged from one another approximately 147 million years ago. This supports the hypothesis of the existence of a common Liberibacter ancestor, which ultimately resulted in the

speciation of Laf and Las following the dislocation and fractioning of Gondwana. African and Indian Liberibacter lineages became isolated from one another when Africa and India split 160 million years ago, and this occurrence may have possibly led to the presence of the bacteria known respectively today as Laf and Las (Teixeira *et al*., 2008).

2.1.8. *'Candidatus* Liberibacter' species related to Laf

A variety of novel subspecies of the citrus infecting Laf and other species closely related to Laf have been identified from various host plant species across a range of plant families. The Laf subspecies previously identified and the other members of the Liberibacter group are discussed below.

2.1.8.1. '*Candidatus Liberibacter africanus subsp. capensis'*

Symptoms of CG were observed in the Western Cape, South Africa, on *Ci. reticulata* trees for the first time in 1994 (Garnier *et al.,* 1999). Laf infections were confirmed from these plants by Garnier *et al.* (1999) which lead to the Western Cape province losing its greening-free status. Mottling of the tree leaves, similar to the symptoms associated with CG, was first observed on *Ca. capense* trees that bordered CG infected *Ci. reticulata* orchards (Garnier *et al.,* 1999). Garnier *et al.* (1999) also suspected that this indigenous ornamental rutaceous plant species may have been the source of the initial introduction of CG in the Western Cape. Following the identification of CG in South Africa, phylogenetic studies based on the *rplKAJL-rpoB* operon indicated that the species present in the *Ca. capense* trees was a novel Liberibacter subspecies of Laf (Garnier *et al*., 2000). The Liberibacter subspecies was subsequently named '*Ca.* Liberibacter africanus subsp. capensis' (LafC) (Garnier *et al.,* 2000), and since its identification LafC has been recorded across South Africa, predominantly in association with *Ca. capense* trees (Phahladira *et al.,* 2012).

2.1.8.2. Four additional Laf-subspecies (LafCl, LafT, LafV and LafZ)

Four additional Laf-subspecies have been identified from indigenous rutaceous host plants in South Africa. Three of these, namely '*Ca.* Liberibacter africanus subsp. clausenae' (LafCl) present in *Clausena anisate* (Willd.) Hook. f. ex Benth., '*Ca.* Liberibacter africanus subsp. vepridis' (LafV)

present in *Vepris lanceolata* (Lam.) G.Don (previously known as *V. undulata*), and '*Ca.* Liberibacter africanus subsp. zanthoxyli' (LafZ) present in *Zanthoxylum capense* (Thunb.) Harv. (previously known as *Fagara capensis*), were described from known native hosts of *T. erytreae* (McClean & Oberholzer, 1965b; Moran, 1968a; Roberts *et al.,* 2015). The fourth Laf-subspecies, named '*Ca.* Liberibacter africanus subsp. teclea' (LafT), was recorded from *Teclea gerrardii* I.Verd. (Roberts & Pietersen, 2017). Trees testing positive for the respective Laf-subspecies did not display the typical leaf mottling symptoms associated with Laf infections. This may suggest that Laf-subspecies may also act as endophytes within their hosts (McClean & Oberholzer, 1965a; Moran, 1968a; Roberts *et al.,* 2015; Roberts & Pietersen, 2017). Nontarget amplification with real-time PCR suggest that a Liberibacter species from Uganda and Tanzania (Kalyebi *et al.,* 2015; Shimwela *et al.,* 2016) identified as HLB is most likely LafCl (Roberts *et al*., 2017).

*2.1.8.3. Other '**Candidatus Liberibacter' species***

The other '*Ca.* Liberibacter' species previously identified in different citrus growing areas around the world include:

(1) '*Ca.* Liberibacter solanacearum' (Lso) (GenBank accession: CP002371.1), which has been found in association with the zebra chip disease of *Solanum tuberosum* L. (Solanaceae: potato) in New Zealand and the psyllid yellows disease of *Solanum lycopersicum* L. (Solanaceae: tomato) and *Piper nigrum* L. (Solanaceae: pepper). The psyllid associated with Lso in these diseases is *B. cockerelli* (Hemiptera: Triozidae).

(2) '*Ca.* Liberibacter europaeus' (Leu) (GenBank accession: PSQJ01000001.1), identified from *Pyrus communis* L. (Rosaceae: pear) trees in Europe (Raddadi *et al.,* 2011) is transmitted by the insect vector, *Cacopsylla pyri* L. (Hemiptera: Psyllidae).

(3) *Liberibacter crescens*, which was detected in *Vasconcellea pubescens* A.DC (Caricaceae: mountain papaya) with Papaya bunchy top disease symptoms (Leonard *et al*., 2012; Fagen *et al.,* 2014). *L. crescens* is the only other member of the genus Liberibacter, excluding the three citrus infecting Liberibacter species, that can be grown in culture which enabled

researchers to sequence the complete genome of *L. crescens* (Leonard *et al.* 2012) (GenBank accession: CP003789.1).

(4) '*Ca.* Liberibacter caribbeanus' (Lcar), which has been recorded from both the Asian citrus psyllid*, D. citri*, and *Ci. sinensis* from Cordoba in the north-eastern region of Columbia, South America through real-time PCR assays (Keremane *et al*., 2015) (GenBank accession: KP012550.1 and KP012551.1).

(5) '*Ca.* Liberibacter brunswickensis' (Lbr), the most recently discovered Liberibacter, which is associated with the eggplant psyllid, *Acizzia solanicola* Kent & Taylor (Hemiptera: Psyllidae), in Australia (Morris *et al.,* 2017). The whole genome sequence for Lbr is currently not available.

2.2. Concluding remarks

'*Ca.* Liberibacter' species from the Rhizobiaceae clade includes gram-negative, phloem-limited bacteria that represents a unique group of Alphaproteobacteria. Liberibacters have diverse symptom expressions, host ranges, vectors, and temperature preferences. These bacteria are predominantly transmitted via insect vectors belonging to the Psylloidea (McClean & Oberholzer, 1965b; Capoor *et al.,* 1967; Secor *et al.,* 2009; Raddadi *et al.,* 2011, Teixeira *et al*., 2005). Liberibacters have reduced genomes of an average size of 1.2 Mb (Duan *et al*., 2009; Wulff *et al*., 2014; Lin *et al.,* 2015), with the complete circular genome of Laf being 1.92 Mb in size (Lin *et al.,* 2015).

Currently there are eight known Liberibacter species which are found around the world, namely Laf, Las, Lam, *L. crescens*, Lso, Leu, Lcar and Lbr (Jagoueix *et al.,* 1994; Duan *et al.,* 2009; Liefting *et al.,* 2009; Munyaneza *et al.,* 2010; Raddadi *et al.,* 2011; Leonard e*t al.,* 2012; Phahladira *et al.,* 2012; Munyaneza *et al.*, 2014; Tahzima *et al.,* 2014; Keremane *et al*., 2015; Morris *et al.,* 2017). There are also five known Laf-subspecies, namely LafC, LafCl, LafT, LafV and LafZ (McClean & Oberholzer, 1965a; Moran, 1968a; Garnier *et al.,* 2000; Roberts *et al.,* 2015; Roberts & Pietersen, 2017). In 2009 a newly designed medium, designated Liber A, has allowed the cultivation of the three citrus infecting Liberibacter species, which includes Laf, Las and Lam (Sechler *et al*., 2009).

Laf infections has previously been described from Burundi (Aubert *et al.,* 1988), Ethiopia (Aubert *et al.,* 1988; Saponari *et al*., 2010), Kenya (Aubert *et al.,* 1988), Madagascar (Bové, 2006), Malawi (Aubert *et al.,* 1988), Mauritius (Garnier *et al.,* 1996), Reunion island (Garnier *et al.,* 1996), Somalia (Aubert *et al.,* 1988), South Africa (Garnier & Bové, 1996), Swaziland (Catling & Atkinson, 1974) and Zimbabwe (Garnier & Bové, 1996). Laf is the only Liberibacter species identified in South Africa. So far, no alternative non-rutaceous host species of Laf have been identified from South Africa. Further studies regarding alternative host plants, including plants that may act as reservoir hosts for Liberibacters, will lead to better control strategies of all citrus infecting Liberibacters around the world.

2.3. References

Aidoo OF, Tanga CM, Khamis FM, Rasowo BA, Mohamed SA, Badii BK, Salifu D, Sétamou M, Ekesi S, Borgemeister C, 2019. Host suitability and feeding preference of the African citrus triozid *Trioza erytreae* Del Guercio (Hemiptera: Triozidae), natural vector of “Candidatus Liberibacter africanus”. *Journal of Applied Entomology* **143**: 262-270.

Ammar ED, Shatters RG Jr & Hall DG, 2011. Localization of *Candidatus* Liberibacter asiaticus, associated with citrus Huanglongbing disease, in its psyllid vector using fluorescence *in situ* hybridization. *Journal of Pathology* **159**: 726-734.

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

Aubert B, Garnier M, Cassin JC & Bertin Y, 1988. Citrus greening disease survey in East and West African countries south of Sahara. Pp. 231-237 *In* LW Timmer, SM Garnsey and L Navarro (eds.), In Proceedings of the 10th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Bassanezi RB, Montesino LH & Stuchi ES, 2009. Effects of Huanglongbing on fruit quality of sweet orange cultivars in Brazil. *European Journal of Plant Pathology* **125**: 565-572.

Batool A, Iftikhar Y, Mughal SM, Khan MM, Jaskani MJ, Abbas M & Khan IA, 2007. Citrus Greening Disease - A major cause of citrus decline in the world - A Review. *Horticultural Science (Prague)* **34**: 159-166.

Beattie GAC, Holford P, Mabberley DJ, Haigh AM & Broadbent P, 2008. On the origins of *Citrus,* Huanglongbing, *Diaphorina citri* and *Trioza erytreae.* Pp. 23-56 *In* TR Gottwald and JH Graham (eds.), In Proceedings of the International Research Conference of HLB. Plant Management Network, Orlando, FL, USA.

Bové JM, 2006. Invited Review: Huanglongbing: A destructive, newly emerging, century-old disease of citrus. *Journal of Plant Pathology* **88**: 7-37.

Bové JM, Bonnet P, Garnier M & Aubert B, 1980. Penicillin and Tetracycline treatments of greening disease-affected citrus plants in the glasshouse, and the bacterial nature of the procaryote associated with greening. Pp. 91-10 *In* EC Calavan, SM Garnsey and LW Timmer (eds.), In Proceedings of the 8th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Bové JM & Garnier M, 1984. Citrus greening and psylla vectors of the disease in the Arabian Peninsula. Pp. 258-263 *In* P Moreno, JV da Graça and LW Timmer (eds.), In Proceedings of the 13th Conference of the International Organization of citrus Virologists. University of California, Riverside, CA, USA.

Brown SE, Oberheim AP, Barrett A & McLaughlin WA, 2011. First report of '*Candidatus* Liberibacter asiaticus' associated with Huanglongbing in the weeds *Cleome rutidosperma, Pisonia aculeate* and *Trichostigma octandrum* in Jamaica. *New Disease Reports* **24**: 25.

Burckhardt D & Ouvrard D, 2012. A revised classification of the jumping plant-lice (Hemiptera: Psylloidea). *Zootaxa* **3509**: 1-34.

Canales E, Coll Y, Hernández I, Portieles R, Rodríguez García M, López Y, Aranguren M, Alonso E, Delgado R, Luis M, Batista L, Paredes C, Rodríguez M, Pujol M, Ochagavia ME, Falcón V, Terauchi R, Matsumura H, Ayra-Pardo C, Llauger R, del Carmen Pérez M, Núñez M, Borrusch MS, Walton JD, Silva Y, Pimentel E, Borroto C & Borrás-Hidalgo O, 2016. '*Candidatus* Liberibacter asiaticus', Causal Agent of Citrus Huanglongbing, Is Reduced by Treatment with Brassinosteroids. *PLoS One* **11**: e0146223.

Capoor SP, 1963. Decline of citrus trees in India. *Bulletin National Institute of Science India* **24**: 48-64.

Capoor SP, Rao DG & Viswanath SM, 1967. *Diaphorina citri* Kuway., A vector of the greening disease of citrus in India. *Indian Journal of Agricultural Science* **37**: 572-575.

Capoor SP, Rao DG & Viswanath SM, 1974. Greening disease of citrus in the Deccan trap country and its relationship with the vector, *Diaphorina citri* Kuwayama. Pp. 43-49 *In* LG Weathers and M Cohen (eds.), In Proceedings of the 6th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Catling HD & Atkinson PR, 1974. Spread of Greening by *Trioza erytreae* (Del Guercio) in Swaziland. Pp. 33-39 *In* LG Weathers and M Cohen (eds.), In Proceedings of the 6th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Chung KR & Brlansky RH, 2005. Citrus Diseases exotic to Florida; Huanglongbing (citrus greening). Fact Sheet PP-210. Plant Pathology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences. University of Florida, Gainesville, FL, USA.

Cocuzza G, Urbaneja A, Hernandez-Suarez E, Siverio F, Di Silvestro S, Tena Alejandro & Rapisarda C, 2016. A review on *Trioza erytreae* (African citrus psyllid), now in mainland Europe, and its potential risk as vector of huanglongbing (HLB) in citrus. *Journal of Pesticide Science* **90**: 1-17.

Coletta-Filho HD, Carlos EF, Alves KCS, Pereira MAR, Bosxariol-Camargo RL, de Souza AA & Machado MA, 2010. *In planta* multiplication and graft transmission of '*Candidatus* Liberibacter asiaticus' revealed by real-time PCR. *European Journal of Plant Pathology* **126**: 53-60.

Colleta-Filho HD, Tagon MLPN, Takita MA, de Negri JD, Pompeu Jr J & Machado MA, 2004. First report of the causal agent of Huanglongbing ("*Candidatus* Liberibacter asiaticus") in Brazil. *Plant Disease* **88**: 1382.

Da Graça JV, 1991. Citrus greening disease. *Annual review of Phytopathology* **29**: 109-136.

Da Graça JV, 2008. Biology, history and world status of Huanglongbing, *In* I Taller Internacional sobre Huanglongbing de los cítricos (Candidatus Liberibacter spp.) y el psílido asiático de los cítricos (Diaphorina citri) (Hermosillo), 1-7.

Damsteegt VD, Postnikova EN, Stone AL, Kuhlmann M, Wilson C, Schaad NW, Brlansky RH & Schneider WL, 2010. *Murraya paniculata* and related species as potential host and inoculums reservoirs of '*Candidatus* Liberibacter asiaticus', causal agent of Huanglongbing. *Plant Disease* **94**: 528-533.

Doddapaneni H, Liao H, Lin H, Bai X, Zhao X, Civerolo EL, Irey M, Coletta-Filho H & Pietersen G, 2008. Comparative phylogenomics and multi-gene cluster analyses of the citrus Huanglongbing (HLB)-associated bacterium *Candidatus* Liberibacter. *BMC Research Notes* **1**: 72.

Duan Y, Gottwald T, Zhou LJ & Gabriel DW, 2008. First report of dodder transmission of '*Candidatus* Liberibacter asiaticus' to tomato (*Lycopersicon esculentum*). *Plant Disease* **92**: 831.

Duan Y, Zhou L, Hall DG, Li W, Doddapaneni H, Lin H, Liu L, Vahling CM, Gabriel DW, Williams KP, Dickerman A, Sun Y & Gottwald T, 2009. Complete genome sequence of citrus Huanglongbing bacterium, '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. *Molecular Plant-Microbe Interactions* **22**: 1011-1020.

Fagen JR, Leonard MT, McCullough CM, Edirisinghe JN, Henry CS, Davis MJ & Triplett EW, 2014. Comparative genomics of cultured and uncultured strains suggests genes essential for free-living growth of Liberibacter. *PLoS One* **9**: 1-11.

Francischini FJB, Oliveira KDS, Astúa-Monge G, Novelli A, Lorenzino R, Matiollo C, Kemper E, da Silva ACR, James R, Maxwell C & Kitajima EW, 2007. First report on the transmission of '*Candidatus* Liberibacter americanus' from citrus to *Nicotiana tabacum* cv. Xanthi. *Plant Disease* **91**: 631.

Fraser LR & Singh D, 1968. Citrus dieback in India - the contribution of Greening Virus. Pp. 141-144 *In* JFL Childs (ed.), In Proceedings of the 4th Conference of the International Organization on Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M & Bové JM, 1977. Structure trilamellaire des deux membranes qui entourent les organismes procaryotes associés à la maladie du “greening” des agrumes. *Fruits* **32**: 749-752.

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

Garnier M & Bové JM, 1993. Citrus greening disease and the Greening bacterium. pp. 212-219 *In* P Moreno, JV da Graça and LW Timmer (eds.), In Proceedings of the 12th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M & Bové JM, 1996. Distribution of the Huanglongbing (Greening) Liberobacter species in fifteen African and Asian countries. Pp. 388-391 *In* J V da Graça, RF Lee and RK Yokomi (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M, Bové JM, Cronje PR, Sanders GM, Korsten L & Le Roux H, 1999. Presence of ‘*Candidatus* Liberibacter africanum’ in the Western Cape province of South Africa. Pp. 369-372 *In* JV da Graça, RF Lee and RK Yokomi (eds.), In Proceedings of the 14th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M, Danel N & Bové JM, 1984. The greening organism is a gram-negative bacterium. Pp. 115-124 *In* SM Garnsey, LW Timmer and JA Dodds (eds.), In Proceedings of the 9th International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M, Gao SJ, He YL, Villechanoux S, Gandar J & Bové JM, 1991. Study of the greening organism (GO) with monoclonal antibodies: Serological identification, morphology, serotypes and purification of the GO. Pp. 428-435 *In* RH Brlansky, RF Lee and LW Timmer (eds.), In Proceedings of the 11th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M, Jagoueix-Eveillard S, Cronje PR, Le Roux HF & Bové JM, 2000. Genomic characterization of a Liberibacter present in an ornamental rutaceous tree, *Calodendrum capense,* in the Western Cape province of South Africa. Proposal of '*Candidatus* Liberibacter africanus subsp. capensis'. *Journal of Systematic and Evolutionary Microbiology* **50**: 2119-2125.

Garnier M, Jagoueix S, Toorawa P, Grisoni M, Mallessard R, Dookun A, Saumtally S, Autrey JC & Bové JM, 1996. Both Huanglongbing (Greening) Liberobacter species are present in Mauritius and Reunion. Pp. 271-275 *In* JV da Graça, P Moreno and RK Yokomi (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Gao S, Garnier M & Bové JM, 1993. Production of monoclonal antibodies recognizing most strains of greening BLO by *in vitro* immunization with an antigenic protein purified from BLO. Pp. 224-249 *In* P Moreno, JV da Graça, and LW Timmer (eds.), In Proceedings of the 12th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Gottwald TR, da Graça JV & Bassanezi RB, 2007. Citrus Huanglongbing: The pathogen and its impact. *Plant Health Progress* doi: 10.1094/PHP-2007-0906-01-RV.

Halbert SE, 2005. The discovery of Huanglongbing in Florida. Proceedings of the 2nd international citrus canker and Huanglongbing research workshop. Florida Citrus Mutual, Orlando, 2005, H-3.

Hartung JS, Paul C, Achor D & Brlansky RH, 2010. Colonization of dodder, *Cuscuta indocera,* by '*Candidatus* Liberibacter *asiaticus'* and '*Ca.* Liberibacter *americanus'. Phytopathology* **100**: 756-762.

Hilf ME, 2011. Colonization of citrus seed coats by '*Candidatus* Liberibacter asiaticus': Implications for seed transmission of the bacterium. *Phytopathology* **101**: 1242-1250.

Hilf ME, Sims KR, Folimonova SY & Achor DS, 2013. Visualization of '*Candidatus* Liberibacter asiaticus' cells in the vascular bundle of citrus seed coats with fluorescence in situ hybridization and transmission electron microscopy. *Phytopathology* **103**: 545-554.

Hocquellet A, Bové JM & Garnier M, 1999. Isolation of DNA from the uncultured '*Candidatus* Liberobacter' species associated with citrus Huanglongbing by RAPD. *Current Microbiology* **38**: 176-182.

Hung TH, Hung SC, Chen CN, Hsu MH & Su HJ, 2004. Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus Huanglonbing in vector psyllids: application to the study of vector-pathogen relationships. *Plant Pathology* **53**: 96-102.

Hung TH, Wu ML & Su HJ, 2000. Identification of alternative hosts of the fastidious bacterium causing citrus greening disease. *Journal of Phytopathology* **148**: 321-326.

Inoue H, Ohnishi J, Ito T, Tommimura K, Miyata S, Iwanami T & Ashihara W, 2009. Enhanced proliferation and efficient transmission of *Candidatus* Liberibacter asiaticus by adult *Diaphorina citri* after acquisition feeding in the nymphal stage. *Annals of Applied Biology* **155**: 29-36.

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

Jantasorn A, Duan Y, Puttamuk T, Zhang S, Thaveechai N, 2012. Association of '*Candidatus* Liberibacter asiaticus', the causal agent of citrus huanglongbing in *Murraya paniculata* and *Diaphorina citri* in Thailand. *Thai Journal of Agricultural Science* **45**: 161-170.

Kalyebi A, Aisu G, Ramathani J, Ogwang J, McOwen N & Russel P, 2015. Detection and identification of etiological agents (Liberibacter spp.) associated with citrus greening disease in Uganda. *Journal of Agricultural Science* **16**: 43-54.

Keremane ML, Ramadugu C, Castaneda A, Diaz JEP, Chen EA, Duan YP, Halbert SE & Lee RF, 2015. Report of *Candidatus* Liberibacter caribbeanus, a new citrus- and psyllid-associated Liberibacter from Colombia, South America. *In* American Phytopathological Society Annual meeting.

Korsten L, Jagoueix S, Bové JM & Garnier M, 1996. Huanglongbing (Greening) detection in South Africa. Pp. 395-398 *In* JV da Graça, P Moreno and RK Yokomi (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Laflèche D & Bové JM, 1970. Mycoplasmes dans les agrumes atteints de "greening", de stubborn, ou de maladies similaries. *Fruits* **25**: 455-465.

Lee HA, 1921. The relation of stocks to mottle leaf of citrus trees. *The Philippine Journal of Science* **18**: 85-95.

Leonard MT, Fagen JR, Davis-Richardson AG, Davis MJ & Triplett EW, 2012. Complete genome sequence of *Liberibacter crescens* BT-1. *Standards in Genomic Sciences* **7**: 271-283.

Li W, Hartung JS & Levy L, 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus Huanglongbing. *Journal of Microbiological Methods* **66**: 104-115.

Li W, Hartung JS & Levy L, 2007. Evaluation of DNA amplification methods for improved detection of '*Candidatus* Liberibacter species' associated with citrus Huanglongbing. *Plant Disease* **91**: 51-58.

Liefting LW, Sutherland PW, Ward LI, Paice KL, Weir BS & Clover GRG, 2009. A new '*Candidatus* Liberibacter' species associated with diseases of solanaceous crops. *Plant Disease* **93**: 208-214.

Lin KH & Lin KH, 1956. The citrus huang lung bin (Greening) disease in China. *Acta Phytopathologica Sinica* **2**: 14-38.

Lin H, Pietersen G, Han C, Read DA, Lou B, Gupta G & Civerolo EL, 2015. Complete genome sequence of '*Candidatus* Liberibacter africanus', a

bacterium associated with citrus Huanglongbing. *Genome Announcement* 12-13.

Lopes SA & Frare GF, 2008. Graft transmission and cultivar reaction of citrus to '*Candidatus* Liberibacter americanus'. *Plant Disease* **92**: 21-24.

Lopes SA, Frare GF, Camatgo LEA, Wulff NA, Teixeira DC, Bassanezi RB, Beattie GAC & Ayres AJ, 2010. Liberibacters associated with orange jasmine in Brazil: incidence in urban areas and relatedness to citrus Liberibacters. *Plant Pathology* **59**: 1044-1053.

Manicom BQ & van Vuuren SP, 1990. Symptoms of greening disease with special emphasis on African greening. *In* B Aubert, S Tontyaporn and D Buabgsuwon (eds.), In Proceedings of the 4th International Asia Pacific Conference on Citrus Rehabilitation. Chiang Mai, Thailand, 4-10th Feb. 1990.

Martinelli F, Uratsu SL, Albrecht U, Reagan RL, Phu ML, Britton M, Buffalo V, Frass J, Leicht E, Zhao W, Lin D, D'Souza R, Davis CE, Bowman KD & Dandekar AM, 2012. Transcriptome profiling of citrus fruit response to Huanglongbing disease. *PLoS One* **7**: e3839.

Martinez AL & Wallace JM, 1967. Citrus leaf-mottle-yellows disease in the Philippines and transmission of the causal virus by a psyllid, *Diaphorina citri. Plant Disease Reporter* **51**: 692-695.

Mayikawa T, 1980. Experimentally induced symptoms and host range of citrus Likubin (greening disease). *Annals of the Phytopathological Society of Japan* **46**: 224-230.

McClean APD & Oberholzer PCJ, 1965a. Greening disease of the sweet orange: Evidence that it is caused by a transmissible virus. *South African Journal of Agricultural Science* **8**: 253-276.

McClean APD & Oberholzer PCJ, 1965b. Citrus psylla, a vector of the greening disease of sweet orange. *South African Journal of Agricultural Science* **8**: 297-298.

McClean APD & Schwarz RE, 1970. Greening or blotchy-mottle disease of citrus. *Phytophylactica* **2**: 177-194.

Moll JN & Martin MM, 1973. Electron microscope evidence that citrus psylla (*Trioza erytreae*) is a vector of greening disease in South Africa. *Phytophylactica* **5**: 41-44.

Moran VC, 1968a. The development of the citrus psylla, *Trioza erytreae* (Del Guercio) (Homoptera: Psyllidae), on *Citrus limon* and four indigenous hosts plants. *Journal of the Entomological Society of South Africa* **31**: 391-402.

Moran VC, 1968b. Preliminary observations on the choice of host plants by adults of the citrus psylla, *Trioza erytreae* (Del Guercio) (Homoptera: Psyllidae). *Journal of the entomological Society of South Africa* **31**: 404-410.

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

Moreno P, da Graça JV & Yokomi RK, 1996. Preface. Pp. v-vi *In* P Moreno, JV da Graça and RK Yokomi (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Morris J, Shiller J, Mann R, Smith G, Yen A & Rodoni B, 2017. Novel '*Candidatus* Liberibacter' species identified in the Australian eggplant psyllid, *Acizzia solanicola*. *Microbial Biotechnology* **10**: 833-844.

Munyaneza JE, Fisher TW, Sengoda VG, Garczynski SF, Nissinen A & Lemmetty A, 2010. First report of '*Candidatus* Liberibacter solanacearum' associated with psyllid-affected carrots in Europe. *Plant Disease* **94**: 639.

Munyaneza JE, Sengoda VG, Crosslin JM, Garzón-Tiznado JA & Cardenas-Valenzuela OG, 2009. First report of '*Candidatus* Liberibacter solanacearum' in tomato plants in México. *Plant Disease* **93**: 1076.

Munyaneza JE, Sengoda VG, Sundheim L & Meadow R, 2014. Survey of '*Candidatus* Liberibacter solanacearum' in carrot crops affected by the psyllid *Trioza apicalis* (Hemiptera: Triozidae) in Norway. *Journal of Plant Pathology* **96**: 397-402.

Oberholzer PCJ, von Staden DFA & Basson WJ, 1963. Greening disease of sweet orange in South Africa. Pp. 213-219 *In* WC Price (ed.), In Proceedings of the 3rd Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Outi Y, Cortese P, Santinoni L, Palma l, Adostini J, Preusler C, Gastaminza G, Perez G & Dominguez E, 2013. HLB in Argentina: a new disease outbreak. *In* Proceedings of the 3rd International Research Conference of Huanglongbing. Orlando, Florida. 2013.

Phahladira MNB, Viljoen R & Pietersen G, 2012. Widespread occurrence of '*Candidatus* Liberibacter africanus subspecies capensis' in *Calodendrum capense* in South Africa. *European Journal of Plant Pathology* **134**: 39-47.

Pietersen G, Arrebola E, Breytenbach JHJ, Korsten L, le Roux HF, la Grange H, Lopes SA, Meyer JB, Pretorius MC, Schwerdtfeger M, van Vuuren SP & Yamamoto P, 2010. A survey for '*Candidatus* Liberibacter' species in South Africa confirms the presence of only '*Ca.* L. africanus' in commercial citrus. *Plant Disease* **94**: 244-249.

Planet P, Jagoueix S, Bové JM & Garnier M, 1995. Detection and characterization of the African citrus greening Liberobacter by amplification, cloning and sequencing of the *rpl*KAJL-*rpo*BC operon. *Current Microbiology* **30**: 137-144.

Pourreza A, November 17, 2016. New early detection of citrus HLB [online]. Kearney Research and Extension Center, University of California Cooperative Extension. Available from: Topics in subtropics https://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=22551 [accessed 26 March 2019].

Raddadi N, Gonella E, Camerota C, Pizzinat A, Tedeschi R, Crotti E, Mandrioli M, Bianco PA, Daffonchio D & Alma A, 2011. '*Candidatus* Liberibacter europaeus' sp. nov. that is associated with and transmitted by the psyllid *Cacopsylla pyri* apparently behaves as an endophyte rather than a pathogen. *Environmental Microbiology* **13**: 414-426.

Roberts R, Cook G, Grout TG, Khamis F, Rwomushana I, Nderitu PW, Seguni ZS, Materu CL, Steyn C, Pietersen G, Ekesi S & le Roux HF, 2017. Resolution of the identity of '*Candidatus* Liberibacter' species from Huanglongbing-affected citrus in East Africa. *Plant Disease* doi: 10.1094/PDIS-11-16-1655-RE.

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

Roberts R, Steenkamp ET & Pietersen G, 2015. Novel lineages of '*Candidatus* Liberibacter africanus' associated with native rutaceous hosts of *Trioza erytreae* in South Africa. *International Journal of Systematics and Evolutionary Microbiology* **65**: 723-731.

Salibe AA & Cortez RE, 1968. Leaf mottling- a serious virus disease of citrus in the Philippines. Pp. 131-136 *In* JFL Childs (ed.), In Proceedings of the 4th International Conference of Citrus Virologists. University of California, Riverside, CA, USA.

Saponari M, De Bac G, Breithaupt J, Loconsole G, Yokomi RK & Catalano L, 2010. First report of '*Candidatus* Liberibacter asiaticus' associated with Huanglongbing in sweet orange in Ethiopia. *Plant Disease* **94**: 482.

Schneider H, 1968. Anatomy of Greening-diseases sweet orange shoots. *Phytopathology* **58**: 1155-1160.

Schwarz RE & van Vuuren SP, 1971. Decrease in fruit greening of sweet orange by trunk injection of tetracycline. *Plant Disease Reporter* **55**: 747-750.

Sechler A, Schuenzel EL, Cooke P, Donnua S, Thaveechai N, Postnikova E, Stone AL, Schneider WL, Damsteegt VD & Schaad NW, 2009. Cultivation of '*Candidatus* Liberibacter asiaticus', '*Ca*. L. africanus', and '*Ca*. L. americanus' associated with Huanglongbing. *Phytopathology* **99**: 480-486.

Secor GA, Rivera VV, Abad JA, Lee IM, Clover GRG, Liefting LW, Li X & De Boet SH, 2009. Association of '*Candidatus* Liberibacter solanacearum'

with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. *Plant Disease* **93**: 574-583.

Shimwela MM, Narouei-Khadan HA, Halbert SE, Keremane ML, Minsavage GV, Timilsina S, Massawe DP, Jones JB & van Bruggern AHC, 2016. First occurrence of *Diaphorina citri* in East Africa, characterization of the *Ca.* Liberibacter species causing Huanglongbing (HLB) in Tanzania, and potential further spread of *D. citri* and HLB in Africa and Europe. *European Journal of Plant Pathology* **146**: 349-368.

Shokrollah H, Abdullah TL, Sijam K & Abdullah SNA, 2009. Determination of the presence of Huanglongbing in seeds and movement of the pathogen in *Citrus reticulata. American Journal of Applied Sciences* **6**: 1180-1185.

Slisz AM, Breska AP, Mishchuk DO, McCollum G & Slupsky CM, 2012. Metabolic analysis of citrus infection by '*Candidatus* Liberibacter' reveals insight into pathogenicity. *Journal of Proteome Research* **11**: 4223-4230.

Stokstad E, 2012. Dread citrus disease turns up in California, Texas. *Science* **336**: 283-284.

Tahzima R, Maes M, Achbani EH, Swisher KD, Munyaneza JE & De Jonghe K, 2014. First report of '*Candidatus* Liberibacter solanacearum' on carrot in Africa. *Plant Disease* **98**: 1426.

Tatineni S, Sagaram US, Gowda S, Robertson CJ, Dawson W, Iwanami T & Wang N, 2008. In planta distribution of '*Candidatus* Liberibacter asiaticus' as revealed by polymerase chain reaction (PCR) and real-time PCR. *Phytopathology* **98**: 592-599.

Teixeira DC, Eveillard S, Sirand-Pugnet P, Wulff A, Saillard C, Ayres AJ & Bové JM, 2008. The *tufB-secE-nusG-rplKAJL-rpoB* gene cluster of the Liberibacters: sequence comparisons, phylogeny and speciation. *International Journal of Systematic and Evolutionary Microbiology* **58**: 141-1421.

Teixeira DC, Saillard C, Eveillard S, Danet JL, da Costa PI, Ayres AJ & Bové JM, 2005. '*Candidatus* Liberibacter americanus', associated with citrus

Huanglongbing (greening disease) in São Paulo state, Brazil. *International Journal of Systematic and Evolutionary Microbiology* **55**: 1857-1862.

Van den Berg MA, 1990. The citrus pyslla, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae): A review. *Agriculture, Ecosystems & Environment* **30**: 171-194.

Van den Berg MA, van Vuuren SP & Deacon VE, 1991-1992. Studies on greening transmission by citrus psylla, *Trioza eytreae* (Hemiptera: Triozidae). *Israel Journal of Entomology* **25-26**: 51-56.

Van der Merwe AJ & Andersen FG, 1937. Chromium and manganese toxicity. Is it important in Transvaal citrus greening? *Farming South Africa* **12**: 439-440.

Van Vuuren SP, Cook G & Pietersen G, 2011. Lack of evidence for seed transmission of '*Candidatus* Liberibacter africanus' associated with greening (Huanglongbing) in citrus in South Africa. *Plant Disease* **95**: 1026.

Villechanoux S, Garnier M, Laigret F, Renaudin J & Bové JM, 1993. The Genome of the Non-Cultured, Bacterial-Like Organism Associated with Citrus Greening Disease Contains the nusG- rplKAJL-rpoBC Gene Cluster and the Gene for a Bacteriophage Type DNA Polymerase. *Current Microbiology* **26**: 161-166.

Walter AJ, Hall DG & Duan YP, 2012, Low incidence of '*Candidatus* Liberibacter asiaticus' in *Murraya paniculata* and associated *Diaphorina citri. Plant Disease* **96**: 827-832.

Wirawan IGP, Simanjuntak S, Sritamin M & Wijaya N, 2017. Detection of Citrus Vein Phloem Degeneration (CVPD) disease and the quality of healthy fruits in nutrient deficiency of citrus. *Bali Medical Journal* **3**: S117-S120.

Wulff NA, Zhang S, Setubal JC, Alemida NF, Martins EC, Harakava R, Kumar D, Rangel LT, Foissac X, Bové JM & Gabriel DW, 2014. The complete genome sequence of '*Candidatus* Liberibacter americanus', associated with citrus huanglongbinh. *Molecular Plant-Microbe Interactions* **27**: 163-176.

Xu CF, Xia YH, Li KB & Ke C, 1988. Further study of the transmission of citrus Huanglongbing by a psyllid, *Diaphorina citri* Kuwayama. Pp. 243-248 *In* LW Timmer, SM Garnsey and L Navarro (eds.), In Proceedings of the 10th Conference of the International organization of Citrus Virologists. University of California, Riverside, CA, USA.

Zhang M, Duan Y, Zhou L, Turechek WW, Stover E & Powell CA, 2010. Screening molecules for control of citrus Huanglongbing using an optimised regeneration system for "*Candidatus* Liberibacter asiaticus"-infected periwinkle (*Catharanthus roseus*) cuttings. *Phytopathology* **100**: 239-245.

Zhao XY, 1981. Citrus yellow shoot (Huanglongbing) in China: A Review. *Proceedings of the International Society of Citriculture* **1**: 466-469.

Zhou LJ, Gabriel DW, Duan YP, Halbert SE & Dixon WN, 2007. First report of dodder transmission of Huanglongbing from naturally infected *Murraya paniculata* to citrus. *Plant Disease* **91**: 227.

Chapter 3

'*Candidatus* Liberibacter africanus' in non-rutaceous alternate host species from South Africa

3.1. Introduction

The citrus trade plays an important role in South Africa's economy. In 2018 South Africa exported 33% of its total citrus production to Europe, 18% to the Middle East, 16% to South-East Asia, 9% to the United Kingdoms, 9% to Russia, 8% to Asia, 6% to North America, and the last 1% to other countries around the world (CGA, 2018). The South African citrus industry is however under continuous pressure from a variety of diseases. One of these diseases considered a tremendous threat to the production of citrus in South Africa is locally known as 'Citrus Greening disease' (CG).

CG is associated with a phloem-limited bacterium, '*Candidatus* Liberibacter africanus' (Laf), that represents a unique lineage within the class Alphaproteobacteria, from the phylum Proteobacteria (Jagoueix *et al.,* 1994). CG is similar to but milder than Huanglongbing (HLB) which is associated with '*Ca.* Liberibacter asiaticus' (Las) that causes decline and ultimately death of citrus trees in all countries where Las occurs (Lin & Lin, 1956; Bové & Garnier, 1984; Aubert *et al.,* 1988; Garnier & Bové, 1996; Garnier *et al.,* 1996; Coletta-Filho *et al.,* 2004). Members of the Alphaproteobacteria are typically transmitted via insect vectors that commonly fall within the Psylloidea (Hemiptera: Sternorrhyncha) (McClean & Oberholzer, 1965; Capoor *et al.,* 1967; Teixeira *et al*., 2005; Secor *et al.*, 2009; Raddadi *et al*., 2011; Burckhardt & Ouvrard, 2012). Bacteria from this family are predominantly fastidious (Garnier & Bové, 1983). The unculturable characteristic of these organisms has contributed to the prolonged characterisation of the bacteria, but in 2009 a newly designed medium (Liber A) has allowed the cultivation of all three citrus infecting Liberibacter species (Sechler *et al*., 2009). The bacteria have reduced genomes with an average size of 1.2 Mb (Duan *et al.,* 2009; Lin *et al*., 2015).

The primary transmission of Laf amongst citrus orchards in South Africa occurs via the feeding and flight actions of the vector, *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) (McClean & Oberholzer, 1965; Burckhardt & Ouvrard, 2012). It has been demonstrated that Laf cannot be transmitted vertically through infected seeds (van Vuuren *et al*., 2011). CG has some symptoms that can be used to identify infected citrus plants, e.g. mottled

appearance of infected leaves. The mottled leaves however are similar to that of a nutrient deficiency (McClean & Oberholzer, 1965). The fruit produced from infected citrus branches are smaller in size than those from healthy citrus plants. They are often lopsided and have a characteristic bitter taste (McClean & Oberholzer, 1965). These fruits are unfit for exportation, therefore, large-scale infections of citrus orchards can have a negative impact on the citrus industry of South Africa.

Stringent control strategies are implemented to limit the spread of CG among orchards to reduce the impact of the disease on the industry. These include the removal of infected branches and sometimes even the removal of the entire tree, planting of disease-free plant material, and chemical control to reduce population levels of *T. erytreae* within citrus orchards in South Africa (Buitendag & von Broembsen, 1993). In spite of this, the disease remains an on-going problem, especially in production areas in cooler region. It has previously been suggested that Laf may be continually introduced into orchards, even if control strategies have been followed, from alternative reservoir host plants amongst the natural vegetation in the vicinity of the orchards (van den Berg *et al.,* 1991).

A number of studies have been done to determine whether alternative rutaceous host species for '*Ca.* Liberibacter' species exist, using both natural and experimental transmission (Moran, 1968; Mayikawa, 1980; van den Berg *et al.,* 1991-1992; Korsten *et al.,* 1996; Damsteegt *et al.,* 2010). Previous studies have shown that the rutaceous *Clausena anisata, Vepris lanceolata* and *Zanthoxylum capense* serve as natural native hosts of *T. erytreae* and are capable of supporting all the developmental stages of the vector (Moran, 1968). *Cl. anisata* may serve as an alternative host for Laf as demonstrated with graft inoculation techniques (van den Berg *et al.,* 1991-1992). Laf infections of *V. lanceolata* have also been identified previously through hybridization (Korsten *et al.,* 1996). However, no studies that attempted to identify alternative non-rutaceous host species as reservoir sources for Laf have been published.

The Cape Floristic Region (CFR) of the Western Cape, South Africa is famous for its diversity hotspots with a large amount of endemic plant species associated with the region. An important region of citrus production, Citrusdal is also located in the vicinity of the CFR. The CFR primarily consists of Fynbos species that are indigenous to South Africa. Recent research focussed on the diversity of the bacterial communities of plant species within the Fynbos and Succulent Karoo biomes of South Africa has shown that novel members within the Alphaproteobacteria class are associated with Fynbos species of the CFR (Steenkamp *et al.,* 2015, Miyambo *et al.,* 2016). Therefore, during this study we attempted to identify non-rutaceous potential host species from both indigenous and other non-indigenous plant species found in the natural vegetation in the CFR. The aim is to identify alternative plant species that may act as reservoir plant hosts to explain the reintroduction of Laf in South African citrus orchards after stringent control strategies have been implemented to limit Laf infections in these orchards. We do this even though Laf *sensu stricto* has not yet been found to infect alternative host species outside of the commercial citrus species.

3.2. Materials and Methods

3.2.1. Sampling sites and plant samples

Leaf and petiole samples of 989 plant specimens of various indigenous and non-indigenous other plant species were collected from nine sites in three regions within the CFR in the Western Cape. The plant samples were collected from 20 randomly selected plants per species for testing for the presence of Laf. If less than 20 specimens were observed for a plant species at a given site, plant samples were collected from all available specimens. Samples were collected mainly within the natural distributions of the plant species but often close to citrus or grapevines (see Appendix A, Table A.1). Permission from the landowners were obtained to sample within these areas. The GPS locations were recorded for each sample collected and a unique accession number was given to each sample (e.g. 17-0605 or 18-0250, based on the year the plant sample was collected and the number of samples collected during that year). At each site, where possible, branches and flowers of representative species were collected for morphological identification.

3.2.2. Insect samples

Collection of psyllids (Hemiptera: Sternorrhyncha: Psylloidea) was done using vacuum sampling with a modified cordless leaf blower (set to suck the air in, instead of blowing air out) with a stocking attached with an elastic band at the inlet (Krüger & Fiore, 2019). The duration of the insect collection varied depending on the size of the plant sampled. Plants smaller than 30 cm were sampled for 10 seconds, plants equal to or larger than 30 cm and smaller than 1 m were sampled for 30 seconds, and plants equal to or larger than 1 m for 90 seconds. After collection of insects, the content of the stocking was transferred to a 125 ml honey jar filled with approximately 30-50 ml of absolute alcohol to preserve the collected insects. The respective containers were labelled with the same accession number given to the corresponding plant sample from which the insects were collected. The jars were transported to the laboratory where insects were sorted and submitted for identification to the Biosystematics Division of the Agricultural Research Council – Plant Protection Research (ARC-PPR) institute.

3.2.3. Detection of 'Candidatus Liberibacter africanus'

Total DNA was extracted from leaves and petioles from the individual plant samples following the CTAB protocol of Doyle and Doyle (1990). The extraction of total DNA from individual insects was adapted from a protocol provided by J. Peccoud and N. Sauvion (INRA Montpellier, France) based on Sambrook and Russell (2001) which was used for the non-destructive sample preparation by Proteinase-K digestion in a TNES buffer (1 M Tris-HCl, pH 7.4, 5 M NaCl, 0.5 M EDTA, 10 % SDS). The TNES crude extract was added to 300 ul CTAB buffer [2% CTAB, 50mM EDTA (pH 8), 0.2% 2-ME, 100mM Tris-HCl (pH 8), 1.5M NaCl, 1% PVP-40], incubated (65°C, 15 min) and purified of organics using 400 µl of chloroform. The aqueous phase was transferred to a new tube and precipitation was conducted at -20ºC for 1 hour with sodium acetate (0.3M NaOAc) and two volumes of ethanol (66%). The extracted DNA was pelleted by centrifugation (13,000 rpm for15 min) and washed with 70% ethanol twice, discarding the supernatant and drying the pellet in open tubes at room temperature. Thereafter the DNA was resuspended in 30 µl TE buffer and stored at -20°C.

The DNA extracts were subjected to a Liberibacter 'Universal' real-time PCR assay using a modified version of the Li *et al*. (2006) protocol (Roberts *et al*., 2015). The reactions were set up with 10 µl KAPA Probe Fast qPCR Master Mix (Sigma-Aldrich, St. Louis, MO, USA), 400 nM per primer with LibUF forward primer (Roberts *et al*., 2015) and HLBr reverse primer (Li *et al.,* 2006), 200 nm probe HLBp (Li *et al.,* 2006), made up to a final volume of 20 µl with nuclease-free water. Amplification was performed on a Roto-Gene Q with the following conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 10 s and combined annealing and acquisition at 60°C for 20 s. Fluorescence and crossing thresholds (Ct) value per sample was determined using the Rotor-Gene Q software version 2.3.1.49 (Qiagen, Hilden, Germany).

Samples with a Ct value lower than 31 (selected as the positive/negative threshold based on previous studies) were further assessed for the presence of Laf by conventional PCR of 16S rRNA, *rplJ* and *omp* genes (Roberts *et al.,* 2015), which respectively encode the 16S ribosomal RNA (rRNA) subunit, 50S ribosomal subunit protein L10 (*rplJ*) and the outer membrane protein (*omp*). For 16S rRNA amplification, the primer set used was the generic Liberibacter genus primer pair, LG774F/LG1463R, developed by Morris *et al.* (2017). The amplification reactions were set up using DreamTaq Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) as follows: 12µl DreamTaq Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 200nm of both forward and reverse primer, and the reaction was made up to a final reaction volume of 25µl with nuclease-free water. The reactions were set up on a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) thermo-cycler. Cycling conditions were set up as follows: initial denaturation at 92°C for 3 min, followed by 35 cycles of denaturation at 92°C for 20 s, annealing at 62°C for 20 s and extension at 72°C for 45 s, with a final extension at 72°C for 5 min.

For the amplification of the *rplJ* gene the Laf and Las specific A2/J5 primer set was used, as described by Hocquellet *et al*. (1999). The reaction was set up as described above using DreamTaq Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and the cycling conditions were set up as

follows: initial denaturation at 92°C for 3 min, followed by 35 cycles of denaturation at 92°C for 20 s, annealing at 58°C for 20 s and elongation at 72°C for 30 s, with final elongation at 72°C for 5 min.

For the amplification of the *omp* gene the Laf specific HP1inv/OMP8inv primer set was used, as described by Bastianel *et al*. (2005). The reaction was set up as described above using DreamTaq Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions were set up as follows: initial denaturation at 92°C for 3 min, followed by 35 cycles of denaturation at 92°C for 20 s, annealing at 50°C for 20 s and elongation at 72°C for 30 s, with final elongation at 72°C for 5 min.

Amplification products of all PCRs were viewed on a 1% agarose gel (stained with ethidium bromide) following electrophoresis. Amplicons obtained after 16S rRNA amplification were purified enzymatically using exonuclease I (Thermo Scientific) and Fast AP (Thermo scientific) according to manufacturers' instructions. Purified products were submitted to the sequencing facility of the University of Pretoria (Pretoria, South Africa) for Sanger sequencing using the forward primer, LG774F. The quality of the sequences obtained were assessed in Chromas V2.6. After the quality assessment, the sequences were combined into datasets along with those Liberibacter 16S rRNA reference sequences available in GenBank. The datasets were aligned using the MAFFT online tool (Katoh *et al*., 2002). The alignments were subsequently trimmed in BioEdit V 2.7.5 (Hall, 1999) to ensure that cognate gene regions were assessed. The trimmed datasets were imported into Mega X (Kumar *et al*., 2018) in which best-fit evolutionary models and maximum phylogenies were determined for each barcoding dataset. The primer sequences for Laf detection are listed in Table 1.

Table 1: *Primer and probe sequences for 'Candidatus Liberibacter africanus' gene amplification.*

Primer Name	Primer sequence (5'-3')	Target gene	Reference
LibUF	GGCAGGCCTAACACATGC	16S	Roberts *et al.,* 2015
HLBr	GCGTTATCCCGTAGAAAAAGGTAG	16S	Li *et al.,* 2006
HLBp	AGACGGGTGAGTAACGCG	16S	Li *et al.,* 2006
LG774F	GTAAACGATGAGTGCTAGCTGTTGGG	16S	Morris *et al.,* 2017
LG1463R	CTGACCRTACCGTGGCCGG	16S	Morris *et al.,* 2017
A2	TATAAAGGTTGACCTTTCGAGTTT	*rplJ*	Hocquellet *et al.,* 1999
J5	ACAAAAGCAGAAATAGCAACAA	*rplJ*	Hocquellet *et al*., 1999
HP1inv	ATGAATTTGCCTATTCC	*omp*	Bastianel *et al.,* 2005
OMP8inv	TCACGAATCACAGAATC	*omp*	Bastianel *et al.,* 2005

3.2.4. Barcoding of plant host DNA

DNA from the collected plant samples (at least one specimen of each species) were subjected to DNA barcoding of the ribulose-1,5-bisphosphate (*rbcL*) gene in order to confirm the morphological plant host species identification. The primer sets used are listed in Table 2 below. Amplification of *rbcL* were carried out using DreamTaq Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), as described above. The PCR cycling conditions were set up as follows: initial denaturation at 92°C for 3 min, followed by 35 cycles of denaturation at 92°C for 20 s, annealing at 55°C for 20 s and elongation at 72°C for 90 s, with final elongation at 72°C for 5 min.

Amplification products of all barcoding PCRs were viewed on a 1% agarose gel (stained with ethidium bromide) following electrophoresis. The amplicons of *rbcL* were submitted to the sequencing facility of the University of Pretoria (Pretoria, South Africa) for Sanger sequencing using the barcode forward primer. The quality of the sequences obtained were assessed in Chromas V2.6. After the quality assessment, the sequences were compiled into datasets including members of the same families of the plant hosts and those which had the greatest identity with BLAST for easier analysis. The datasets were aligned using the MAFFT online tool (Katoh *et al*., 2002). The alignments were subsequently trimmed in BioEdit V 2.7.5 (Hall, 1999) to ensure that cognate gene regions were assessed. The trimmed datasets were imported into Mega X (Kumar *et al*., 2018) in which best-fit evolutionary models and maximum phylogenies were determined for each barcoding dataset.

Table 2: *Primer sequences used for DNA barcoding of plant host species.*

Primer name	Primer sequence (5'-3')	Target gene	Reference
rbcLa F	ATGTCACCACAAACAGAGACTAAAGC	*rbcL*	Levin *et al.,* 2003
rbcLa R	GTAAAATCAAGTCCACCRCG	*rbcL*	Kress & Erickson, 2007

3.2.5. Next-generation Sequencing

A single sample of *Atriplex semibaccata* was selected for next-generation sequence (NGS) analysis of the amplicon using an Illumina HiSeq 2000 platform at Life Sequencing (*Instituto Valenciano de Investigaciones Agrarias* (IVIA), *Centro de Protección Vegetal y Biotecnología*, Valencia, Spain). The sample selected had a Ct value below 31 within the Liberibacter 'Universal PCR', the DNA concentration of the sample was above 250 ng/µl, and the sample yielded an amplicon (albeit at low concentration) in the 16S rRNA gene during conventional PCR test. The pair-ended sequence reads obtained from the sequencing facility in Spain were imported into CLC Genomics Workbench 9 software (Qiagen Bioinformatics, Hilden, Germany) at ARC-

PPRI, Pretoria, South Africa and assembled into a single pair-ended sequence.

3.2.6. de novo Assembly

The quality of the NGS pair-ended sequence reads was assessed using the FastQC (v0.11.8) quality control analysis tool for high-throughput sequencing data and it was determined whether trimming of the sequence was required. Thereafter *de novo* assembly of the pair-ended sequence reads (with a total of 64,154,824 reads) was carried out by means of the CLC Genomics Workbench 9 program, using the following conditions: length fraction of 0.5; similarity fraction of 0.7; minimum contig length of 10,000. The contigs obtained from *de novo* assembly were analysed using the nucleotide BLAST analysis tool.

3.2.7. Reference mapping

The pair-ended sequence reads were uploaded onto the Kaiju web server (fast and sensitive taxonomic classification for metagenomics, http://kaiju.binf.ku.dk) and a metagenomic overview was obtained. Additionally, the trimmed reads were mapped against available Liberibacter 16S rRNA reference sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) by using the CLC Genomics Workbench 9 program, to determine whether Liberibacter spp. were present within the original sample.

The read mappings for the dataset was carried out using low stringency conditions (length fraction of 0.5; similarity fraction of 0.8; 'ignore' non-specific match handling). A consensus sequence from each reference mapping to the known Liberibacter 16S rRNA genes was extracted. The consensus sequences were aligned with the available 16S rRNA Liberibacter sequences, as well as other Alpha-, Beta-, and Gammaproteobacteria 16S rRNA reference sequences using the MAFFT online tool (Katoh *et al.,* 2002). Thereafter the aligned dataset was trimmed in BioEdit version 7.2.5 (Hall, 1999) to assess the cognate region within the represented 16S rRNA sequences. Subsequently the phylogenetic relationships of the aligned sequences were assessed by producing best-fit DNA evolutionary model and

maximum-likelihood phylogenies of the trimmed alignment using Mega version X (Kumar *et al.,* 2018).

Thereafter the read mappings for the dataset was carried out using the same conditions as mentioned above and mapped against the complete genome of Laf (GenBank accession: CP004021.1). A consensus sequence from the reference mapping was extracted and analysed on CLC Genomics Workbench 9 software (Qiagen Bioinformatics, Hilden, Germany). Contigs were obtained from the derived consensus sequence. Based on the lengths of the contigs, four contigs were selected for nucleotide BLAST analysis and subsequent phylogenetic analysis to attempt to identify the bacterial entity that may have caused the non-target amplification of the 16S rRNA Liberibacter gene during conventional PCR tests.

3.3. Results

3.3.1. Insect PCR tests

Vacuum sampling resulted in the collection of 1,288 individual insects. However, psyllids were collected only from one plant species. Two out of 35 plant samples from *Roepera foetida* (Zygophyllaceae) collected in the Robertson region contained psyllids (accession numbers 18-0042 and 18-0046; Table 3). The psyllids were collected in summer (January 2018). No indication of previous psyllid infestation (typical nymph induced galls) on the plants surveyed were observed during the study. DNA extractions were conducted and accession numbers were given to the psyllid DNA samples: two psyllid samples from the 18-0042 plant sample (accession numbers: 18-0042 Psyllid A and 18-0042 Psyllid B), and three psyllid samples from the 18-0046 plant sample (accession numbers: 18-0046 Psyllid A, 18-0046 Psyllid B and 18-0046 Psyllid C). None of the psyllid DNA samples subjected to a *Liberibacter* 'Universal' real-time PCR assay yielded a Ct value below 31.

Table 3: *Psyllid samples collected and tested using a Liberibacter 'Universal' real-time PCR assay.*

Plant acc. number	Plant species	Insect type	Insect acc. number	Ct value
18-0042	*R. foetida*	Psyllid	18-0042 Psyllid A	-
		Psyllid	18-0042 Psyllid B	-
18-0046	*R. foetida*	Psyllid	18-0046 Psyllid A	-
		Psyllid	18-0046 Psyllid B	36.08
		Psyllid	18-0046 Psyllid C	-

- = no Ct values obtained after 40 cycles of amplification.

3.3.2. Plant PCR tests

A total of 989 plant samples were collected representing 42 non-rutaceous plant species (Table 4; see also Appendix A, Table A.2 and Table A.3). None of the non-rutaceous samples displayed yellow mottling symptoms, but the single citrus (Rutaceous) control sample collected did display CG symptoms. Of the plant samples collected, 142 samples yielded a Ct value below 31 following the Liberibacter 'Universal' real-time PCR assay (see Appendix A, Table A.4). A total of 79 of the plant samples with Ct values less than 31 (i.e. 79 out of 142) were from the three *Atriplex* species [*Atriplex semibaccata, A. nummularia* and *A. lindleyi* (most related to *Atriplex farinosa* – based on barcode)] collected, while 15 from *Rapistrum rugosum,* 15 were from *Lycium ferocissimum*, and fewer specimens were from a number of other species. PCR of neither the *rplJ* nor the *omp* gene yielded any amplification products for these samples, even under reduced stringency conditions. Seven of the samples with Ct<31 yielded 16S rRNA gene amplification products (684 bp in size) (Table 5; see also Appendix A, Table A.4).

Table 4: *Plant species collected, location, number of specimens collected and number of samples that yielded a Ct<31 during real-time PCR tests.*

Host species based on morphology	Location (Western Cape)	No. of specimens sampled	No. of specimens with Ct<31*
Aizoaceae			
Aizoon africanum (basionym: *Galenia africana*)	Slanghoek Robertson Worcester	72	4
Disphyma australe subsp. *australe*	Robertson Worcester	18	1
Drosanthemum hispidum	Robertson Lutzville	20	1
Drosanthemum speciosum	Robertson	10	1
Mesembryanthemum crystallinum	Vredendal	20	-

Amaranthaceae			
Atriplex lindleyi	Robertson	32	8
	Vredendal		
Atriplex nummularia	Lutzville	54	1
	Vredendal		
Atriplex semibaccata	Robertson	93	70
	Lutzville		
	Vredendal		
Salsola kali	Robertson	60	4
Asteraceae			
Conyza scabrida	Robertson	20	1
Elytropappus (Dicerothamnus) rhinocerotis	Robertson	12	1
Eriocephalus brevifolius	Vredendal	6	1
Euryops speciosissimus	Slanghoek	20	2

Helichrysum cymosum	Robertson	20	-
Hymenolepis crithmifolia	Robertson	10	-
Oncosiphon grandiflorum	Robertson	59	-
	Vredendal		
Oncosiphon suffruticosum	Lutzville	20	-
	Vredendal		
Osteospermum oppositifolium	Lutzville	10	-
Pteronia incana	Slanghoek	55	-
	Robertson		
Senecio burchellii	Robertson	19	-
Boraginaceae			
Amsinckia menziesii	Robertson	30	-
	Vredendal		

Brassicaceae			
Brassica tournefortii	Vredendal	20	4
Raphanus raphanistrum	Robertson	25	4
Rapistrum rugosum	Robertson	25	15
	Vredendal		
Malvaceae			
Hermannia grossularifolia	Robertson	10	1
Menispermaceae			
Cissampelos capensis	Worcester	12	2
Montiniaceae			
Montinia caryophyllacea	Slanghoek	51	-

Oxalidaceae			
Oxalis pes-caprae	Lutzville	9	1
Poaceae			
Cynodon dactylon	Robertson	1	-
Polygalaceae			
Muraltia heisteria	Slanghoek	10	-
Proteaceae			
Hakea sericea	Slanghoek	10	-
Leucadendron tinctum	Slanghoek	5	-
Protea cynaroides	Slanghoek	20	-
Sorocephalus pinifolius	Slanghoek	24	1

Restionaceae			
Unidentified *Restio*	Slanghoek	10	-
Rosaceae			
Cliffortia odorata	Slanghoek	10	-
Santalaceae			
Thesium lineatum	Slanghoek	5	-
Sapindaceae			
Dodonaea viscosa	Slanghoek	5	-
Solanaceae			
Lycium ferocissimum	Vredendal	20	15

Thymelaeaceae			
Passerina corymbose (synonym: *Passerina vulgaris*)	Slanghoek	20	-
Vitaceae			
Vitis vinifera	Robertson	2	-
Zygophyllaceae			
Roepera foetida	Robertson	35	4
Total		**989**	**142**

*Samples with a Ct<31 following Liberibacter 'Universal' real-time PCR assay were considered potentially positive for Liberibacters.

- = no Ct values obtained after 40 cycles of amplification.

Table 5: *Amplification of 16S rRNA Liberibacter gene.*

Sample	Band present	Host species
17-6320	Very faint	*Raphanus raphanistrum*
18-0114	Very faint	*Atriplex lindleyi*
18-0122	Very faint	*Atriplex lindleyi*
18-0151	Very faint	*Atriplex semibaccata*
18-0156	Very faint	*Atriplex semibaccata*
18-0157	Very faint	*Atriplex semibaccata*
18-0164	Very faint	*Atriplex semibaccata*

3.3.3. Amplicon sequencing and analysis

Sanger sequencing analysis, subsequent testing of the quality of the obtained sequences and nucleotide BLAST analysis against the NCBI GenBank database were performed on the 7 samples that yielded amplification products after the 16S rRNA conventional PCRs. The nucleotide BLAST results are listed below in Table 6. All of the sequenced amplicons matched with unidentified uncultured bacteria on the GenBank database, but the BLAST results could not be used to accurately identify the bacterial entity within the samples used for the amplification of the 16S rRNA gene, because the sequence similarities (identity percentages) of six of the seven amplicon sequences were below 98%, and bacterial 16S rRNA sequences within a genus typically share 98-100% sequence similarities. Therefore, further analysis was required to indicate whether the amplicons did show the presence of Laf.

Table 6: *Nucleotide BLAST results of 16S rRNA amplification products.*

Sample	Nucleotide BLAST result		
	BLAST result	**GenBank acc.**	**Identity %**
17-6320	Uncultured bacterium clone BACd-4Fp 16S ribosomal RNA gene, partial sequence	GQ127973.1	92%*
18-0114	Uncultured bacterium clone HWGB-17 16S ribosomal RNA gene, partial sequence	JQ684275.1	94%*
18-0122	Uncultured bacterium isolate 112934649217 16S ribosomal RNA gene, partial sequence	HQ118435.1	85%*
18-0151	Uncultured bacterium clone TX2_7D16 16S ribosomal RNA gene, partial sequence	JN178621.1	99%
18-0156	Uncultured bacterium clone TX2_6F11 16S ribosomal RNA gene, partial sequence	JN178475.1	97%
18-0157	Uncultured bacterium clone FCPT613 16S ribosomal RNA gene, complete sequence	JX282244.1	93%*
18-0164	Uncultured bacterium clone TX2_2M11 16S ribosomal RNA gene, partial sequence	JN178033.1	97%

* Bacterial sequences within a genus typically share 98-100% sequence similarity.

The amplicon sequences were then compiled into a dataset along with all known 16S rRNA sequences available in GenBank for members within the genus Liberibacter. The dataset was aligned and subsequently trimmed to ensure that the cognate 16S rRNA gene regions were assessed. The trimmed dataset was imported into Mega X in which the best-fit evolutionary model and maximum phylogeny was determined for the dataset.

None of the sampled amplicon sequences were closely related to any of the known Liberibacter species, with samples 18-0114 and 18-0122 the least related to Liberibacters. Therefore, another dataset was prepared, as described above, including all the known 16S rRNA sequences for members within the genus Liberibacter as well as available representative sequences

for phytobacteria of the Alpha-, Beta-, and Gammaproteobacteria to attempt to identify whether the samples may be closely related to Liberibacters – in order to explain why 142 samples tested positive in the real-time PCR tests. The dataset was aligned and subsequently trimmed (as described above) to ensure that the cognate gene regions were assessed. The best-fit evolutionary model and maximum phylogeny was determined for the dataset as previously described (Figure 1 below). The 16S rRNA sequences amplified from samples 17-6320, 18-0122, 18-0151, 18-0156, 18-0157 and 18-0164 were identified as Alphaproteobacteria, and the sequence amplified from sample 18-0114 did not belong to Alpha-, Beta-, or Gammaproteobacteria.

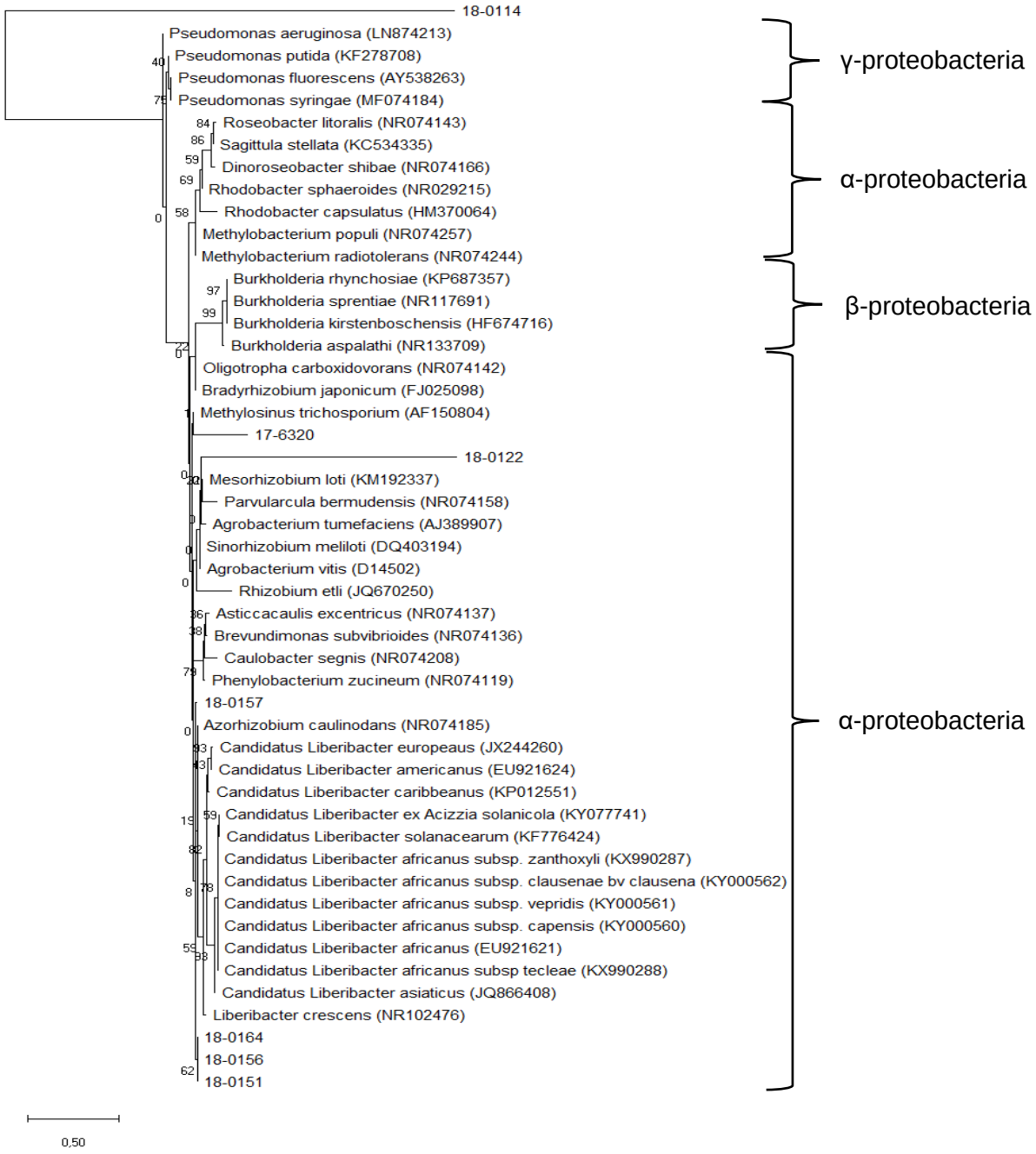


Figure 1: Maximum-likelihood phylogeny of members within the Liberibacter genus, as well as representative sequences for phytobacteria of the Alpha-, Beta- and Gammaproteobacteria, based on 16S rRNA sequences. Samples within this study are indicated by their accession number **-**** while the reference samples have the GenBank accession numbers presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980) with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,50 substitutions per nucleotide position.

3.3.4. Plant DNA Barcoding

One sample per plant species in all locations collected were selected for barcoding PCR with the amplification of the *rbcL* gene which was used to confirm the identity of the plant species selected for this study. Out of 48 plant species collected 42 were positively identified using Sanger sequencing analysis and nucleotide BLAST analysis against the NCBI GenBank database. Most of the plant species identities were determined based on the morphology (Appendix A, Table A.3) and *rbcL* as barcode (Table 7). The *rbcL* gene could not be used to correctly identify all the plant species, therefore the remaining 5 plant species were identified based on their morphological properties and characteristics. The families of the plant species are listed in Table 4 (see also Appendix B for Phylogenetic trees of families).

Table 7: *Identification of plant species using rbcL as barcode, indicating the identity percentage of the plant species, in combination with the morphologies.*

Sample	***BLAST***	Identity %	GenBank accession	Morphology	Plant species identification
17-6063	*Protea cynaroides*	99%	DQ875837.1	*Protea* sp.	*P. cynaroides*
17-6070	*Montinia caryophyllacea*	99%	L11194.2	*M. caryophyllacea*	*M. caryophyllacea*
17-6091	*Atriplex semibaccata*	99%	MF668602.1	*A. semibaccata*	*A. semibaccata*
17-6115	*Euryops speciosissimus*	99%	AM234870.1	*E. speciosissimus*	*E. speciosissimus*
17-6125	*Helichrysum cymosum*	99%	AM234877.1	*H. cymosum*	*H. cymosum*
17-6144	*Muraltia heisteria*	99%	AJ829698.1	*M. heisteria*	*M. heisteria*
17-6153	*Sorocephalus pinifolius*	99%	EU676077.1	*S. pinifolius*	*S. pinifolius*
17-6179	*Hakea archaeoides*	99%	EU676114.1	*H. sericea*	*H. sericea*
17-6184	*Thesium fruticosum*	99%	EF584609.1	*T. lineatum*	*T. lineatum*
17-6188	*Leucadendron tinctum*	99%	DQ875836.1	*L. tinctum*	*L. tinctum*
17-6211	*Passerina vulgaris*	99%	AM162538.1	*P. vulgaris*	*P. corymbosa* (synonym: *P. vulgaris*)

17-6228	-	-	-	*Cliffortia* sp.	*C. odorata*
17-6238	*Dodonaea viscosa*	99%	MF155892.1	*D. viscosa*	*D. viscosa*
17-6252	*Spatalla incurve*	99%	EU676078.1	Unidentified *Restio*	Unidentified *Restio*
17-6304	*Raphanus sativus*	99%	KJ16483.1	*R. raphanistrum*	*R. raphanistrum*
17-6334	*Drosanthemum hispidum*	99%	AM234790.1	*D. speciosum*	*D. speciosum*
17-6376	*Disphyma australe* subsp. *australe*	99%	KT626694.1	*D. australe* subsp. *austral*	*D. australe* subsp. *australe*
17-6403	*Zygophyllum hirticaule*	99%	AJ133869.1	*Roepera foetida*	*R. foetida*
17-6413	*Drosanthemum hispidum*	99%	AM234790.1	*D. hispidum*	*D. hispidum*
17-6432	*Hymenolepis gnidioides*	99%	AM234882.1	*H. crithmifolia*	*H. crithmifolia*
17-6443	*Hermannia angularis*	99%	KP110334.1	*H. grossularifolia*	*H. grossularifolia*
18-0013	*Aizoon africanum (basionym: G. africana)*	99%	JQ025048.1	*Galenia Africana*	*A. africanum (*basionym: *G. africana*)
18-0030	*Cissampelos capensis*	99%	FJ026471.1	*C. capensis*	*C. capensis*
18-0073	*Salsola kali*	99%	HM850332.1	*S. kali*	*S. kali*
18-0253	*Dicerothamnus rhinocerotis*	98%	KP110256.1	*Elytropappus rhinocerotis*	*E. rhinocerotis*

18-0129	*Vitis vinifera*	99%	MG946878.1	*V. vinifera*	*V. vinifera*
18-0131	-	-	-	*Senecio burchellii*	*S. burchellii*
18-0138	*Cynodon dactylon*	99%	KY024482.1	*Cynodon dactylon*	*C. dactylon*
18-0265	*Conyza scabrida*	99%	AM234861.1	*C. scabrida*	*C. scabrida*
18-0286	-	-	-	*Pteronia* sp.	*P. incana*
18-0305	*Brassica tournefortii*	99%	KX298998.1	*B. tournefortii*	*B. tournefortii*
18-0337	*Oncosiphon grandiflorum*	99%	EU385002.1	*O. grandiflorum*	*O. grandiflorum*
18-0351	*Mesembryanthemum crystallinum*	99%	HM850175.1	*M. crystallinum*	*M. crystallinum*
18-0379	*Atriplex farinose*	99%	KY656734.1	*A. lindleyi*	*A. lindleyi*
18-0381	*Atriplex nummularia*	99%	MF590079.1	*A. nummularia*	*A. nummularia*
18-0395	*Bassia diffusa*	99%	AM234799.1	*Eriocephalus brevifolius*	*E. brevifolius*
18-0421	-	-	-	*Osteospermum oppositifolium*	*O. oppositifolium*
18-0433	*Oxalis pes-caprae*	99%	JQ412403.1	*O. pes-caprae*	*O. pes-caprae*

18-0461	-	-	-	*Oncosiphon suffruticosum*	*O. suffruticosum*
18-0511	*Lycium ferocissimum*	99%	AM235152.1	*L. ferocissimum*	*L. ferocissimum*
18-0556	*Amsinckia menziesii*	99%	MG223443.1	*A. menziesii*	*A. menziesii*
18-0582	*Rapistrum rugosum*	99%	MG247086.1	*R. rugosum*	*R. rugosum*

3.3.5. Phylogenetic analysis

Phylogenetic analysis (Figure 2) of the plant samples were done to indicate the phylogeny of the different plant families (see Table 4) collected during this study. For some plant samples no GenBank sequence of the *rbcL* gene is available. Therefore, the *rbcL* gene of the plant species closest related to the respective samples, based on barcoding, was used instead for the phylogenetic analysis (Figure 2). Sample 18-0395 contained DNA extracted from *E. brevifolius* (Asteraceae) (based on morphology) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *B. diffusa* (Amaranthaceae) was used. Sample 17-6252 was extracted from an unidentified *Restio* species (Restionaceae), but during barcoding and nucleotide BLAST analysis the closest relative was identified as *S. incurva* (Proteaceae), therefore the *rbcL* sequence of *S. incurva* was used for phylogenetic analysis. Sample 17-6403 contained DNA extracted from *R. foetida* (Zygophyllaceae) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *Z. hirticaule* (Zygophyllaceae) was used during the phylogenetic analysis.

The Phylogenetic analysis (Figure 2) also indicated that the barcode gene, *rbcL,* cannot be used to differentiate between the different species within the same genus, for instance the different *Atriplex* species, therefore morphology was used in combination with the *rbcL* barcode to identify the plant species.

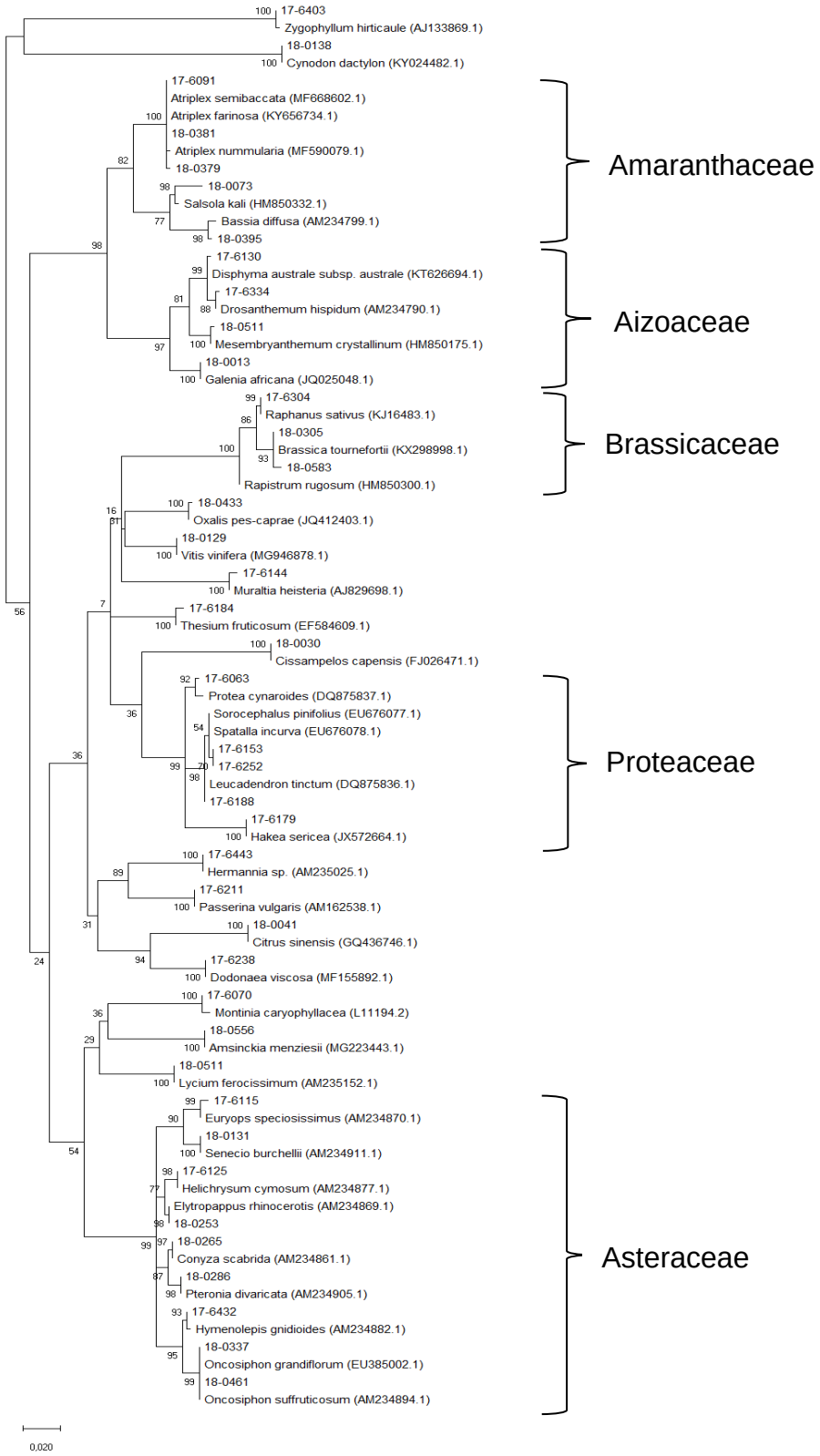


Figure 2: Maximum-likelihood phylogeny of the plant species based on *rbcL* barcoding sequences. The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980) with gamma distribution with invariant sites (G+I). Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,02 substitutions per nucleotide position. The Family groups are also indicated.

3.3.6. Next-generation Sequencing, de novo assembly and reference mapping

Sample 18-0151 was submitted for NGS at Life Sequencing IVIA (Spain). This sample contained the DNA extracted from an *Atriplex semibaccata* plant, as described above. The Ct value of this sample obtained from the Liberibacter 'Universal' real-time PCR assay was 29.51 and had a DNA concentration of 263.70 ng/µl. The sample yielded a very faint band during gel electrophoresis analysis after the amplification of the 16S rRNA Laf gene via the conventional end-point PCR test. NGS of sample 18-0151 obtained from Life Sequencing facility in Spain were imported into CLC Genomics Workbench 9 and assembled into a single pair-ended sequence and returned over 64 million (64,154,824) sequence reads. The quality of the pair-ended sequence reads was assessed with FastQC quality control tool. No trimming was required as the 'adapter content' and the quality of the sequence reads were acceptable. *de novo* Assembly of the total reads were done and 164 contigs were produced. Multi-BLAST analysis of the contigs performed revealed no Liberibacter related sequences.

The results obtained after uploading the 64 million sequence reads onto the Kaiju web server can be seen in Appendix B (Figure B.7). This was analysed using highly stringent conditions, which were set up as follows: run mode: greedy, minimum match length: 7, minimum match score: 50, allowed mismatched: 5. A total of 61,079,898 reads out of the total 64,154,824 reads (95.21%) were classified. The taxa present in the sample were identified, as seen in Figure B.7, Appendix B. Assessment of the trimmed NGS data reads indicated that out of the 64 million reads assessed, 37,172,292 (58%) reads matched known sequences from the Proteobacteria phylum and represented 72% of all the bacterial matches. We assessed the portion of the reads that matched Alphaproteobacteria, the class to which Liberibacter spp. belong. Thereafter we assessed those reads within the Rhizobiales order, and then those of the Rhizobiaceae family (order and family of Liberibacter spp.) to attempt to identify the potential presence of divergent Liberibacter spp. within the NGS data obtained. Only 0.0025% (1607 reads) of the total reads were

classified as Liberibacter spp.-like, as shown in Table 8 below (Figure B.9, Appendix B).

Based on the original analysis of the data using the Krona chart from the Kaiju web server, it was shown that the majority of the sequence reads contained Gammaproteobacteria, although Betaproteobacteria sequences were also present. Therefore, the Beta- and Gammaproteobacteria present within sample 18-0151 were also assessed. The total number of reads that matched Alpha-, Beta-, and Gammaproteobacteria are indicated in Table 8. The assembled consensus sequences were aligned with the available 16S rRNA Liberibacter sequences, as well as other Alpha-, Beta-, and Gammaproteobacteria 16S rRNA sequences using the MAFFT online tool. Thereafter the aligned dataset was trimmed in BioEdit to assess the cognate region within the represented 16S rRNA sequences. Subsequently the phylogenetic relationships of the aligned sequences were assessed by producing a best-fit DNA evolutionary model and maximum-likelihood phylogenies of the trimmed alignment using Mega version X (Figure 3).

Table 8: *Percentage sequence reads of Alpha-, Beta-, Delta-, and Gammaproteobacteria from data obtained from NGS of Atriplex semibaccata sample (accession number 18-0151), including the Liberibacter genus from Alphaproteobacteria.*

		Total reads matched to known sequences	Percentage of total reads (%)	Percentage of Proteobacteria (%)
Phylum	Proteobacteria	37,172,292	57.942	-
Class	Alphaproteobacteria	2,571,687	4.0086	6.9183
Order	Rhizobiales	1,046,416	1.6311	2.8150
Family	Rhizobiaceae	268,581	0.4186	0.7225
Genus	Liberibacter	1607	0.0025	0.0043
Other Classes	Betaproteobacteria	1,652,961	2.5765	4.4468
	Deltaproteobacteria	620,654	0.9674	1.6697
	Gammaproteobacteria	31,891,415	49.7101	85.7935

The pair-ended sequence reads obtained from the sequencing facility were then subjected to reference mapping against all known 16S rRNA Liberibacter reference sequences (Table 9) using low stringency conditions (length fraction of 0.5; similarity fraction of 0.8; 'ignore' non-specific match handling). An assembled consensus sequence from each reference mapping was extracted. The lengths of the Liberibacter derived consensus sequences from sample 18-0151 and the number of reads mapped to the reference Liberibacter spp. sequences are listed in Table 9 below. These assembled consensus sequences had an average sequence coverage of 96.53% against all the reference sequences (Table 8).

The lengths of the assembled consensus sequences produced when 16S rRNA reference sequences of various Liberibacters were used for reference mapping was generally high and often almost exactly the same length as the reference sequences. As this was found for most reference species this

clearly illustrates that this part of the genome is not useful for resolving the species of Liberibacters.

The Liberibacter 16S rRNA reference assembled contig sequences obtained from sample 18-0151 were subjected to nucleotide BLAST analysis against the NCBI GenBank database, but none matched any Liberibacter sequences. The consensus sequences shared 90-94% sequence similarity with a variety of different bacterial entries on GenBank. The BLAST analyses suggest that *Atriplex semibaccata* does in fact contain unique bacterial communities, but the presence of Liberibacter spp. within the sample could not be confirmed. Given the stated aim of finding Liberibacter alternate hosts identification of other bacteria was not pursued.

Table 9: *Read mapping results of Illumina HiSeq reads obtained from NGS of DNA extracted from Atriplex semibaccata sample (accession number 18-0151) mapped against available Liberibacter 16S rRNA sequences. The length of the assembled consensus sequences obtained per reference mapping is presented in the base pairs (bp).*

Reference sequence (GenBank Acc.)	Reference sequence (bp)	No. reads mapped to reference	Assembled consensus sequence from 18-0151 (bp)	Sequence coverage (%)
Laf (EU921619.1)	1432	258,822	1427	99.65
LafC (KY000560.1)	1500	271,507	1490	99.33
LafCl (KY000562.1)	1500	271,507	1488	99.20
LafT (KX990288.1)	1501	284,936	1494	99.53
LafV (KY000561.1)	1500	271,507	1490	99.33
LafZ (KX990287.1)	1500	285,149	1496	99.73
Lam (FJ036892.1)	1417	201,666	1189	83.91
Las (JQ866401.1)	1122	201,155	1104	98.40
Lbr (KY077741.1)	1464	275,349	1453	99.25
Lcar (KP012551.1)	1125	225,714	1127	100.00
Lcr (NR_102476.2)	1482	294,320	1490	100.00
Leu (JX244260.1)	2072	310,252	1868	90.15
Lso (MF041968.1)	1180	172,344	1019	86.36

Maximum-likelihood phylogeny performed on the reference guided assembled sequences showed that these were not Liberibacter-like sequences. This supports the conventional end-point PCR results obtained from amplification of the 16S rRNA Liberibacter gene which amplified various other bacteria from the plants. Phylogenetic analysis revealed that the assembled consensus sequences obtained from the Liberibacter-like 16S rRNA read mappings represented a distinct clade within the Proteobacteria

phylum. Analysis also indicated that these consensus sequences may be most closely related to members of the Gammaproteobacteria.

While we did not wish to identify other bacteria found, we did wish to identify the bacterial entity resulting in the cross-reactivity of the 'Universal' Liberibacter specific PCR which yielded Ct values of less than 31 during the real-time PCR assays, and with weak amplification of the 16S rRNA Liberibacter gene during the conventional PCR tests. We therefore analysed the Gammaproteobacteria reads as these were the most numerous. They represented almost half (49.71%) of the total amount of reads when analysed using the Kaiju web server.

Reads from the NGS data of sample 18-0151 matching Gammaproteobacteria are shown in Figure B.10 (Appendix B). The majority of the reads matched *Kushneria avicenniae* (60% of Gammaproteobacteria, or 30% of the total reads). Other species from the *Kushneria* genus also present were *Kushneria aurantia, Kushneria indalinina* and *Kushneria marisflavi.* The available GenBank 16S rRNA sequences of these *Kushneria* species were therefore included during the phylogenetic analysis of the assembled consensus sequences (Figure 3).

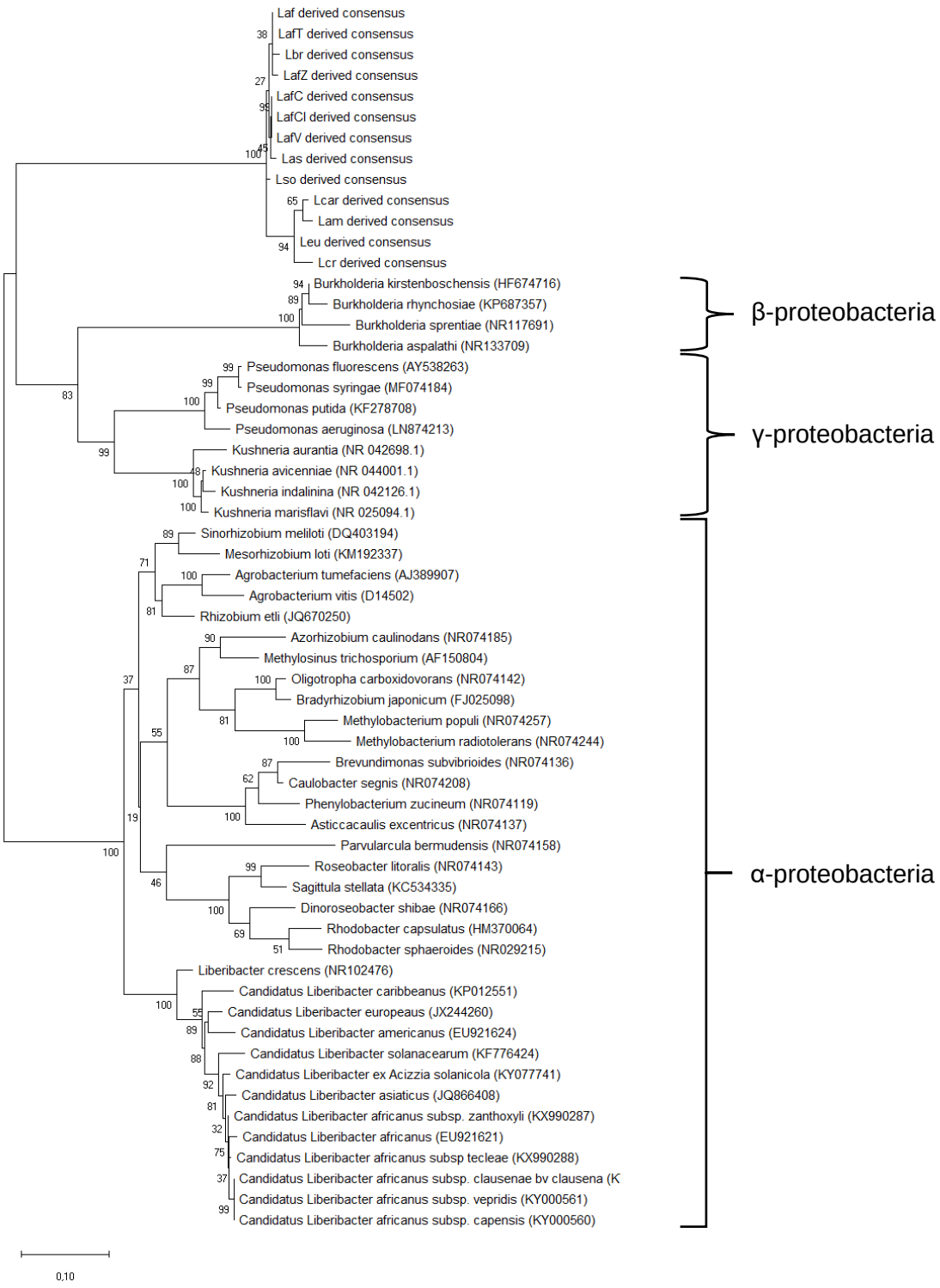


Figure 3: Maximum-likelihood phylogeny of the derived consensus sequences from the Liberibacter-like 16S rRNA read mappings obtained from the NGS data (*Atriplex semibaccata* sample, accession number 18-0151). The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980) with gamma distribution with invariant sites (G+I). The Alpha- (α), Beta- (β), and Gammaproteobacteria (γ), from the Proteobacteria phylum, are indicated, including other *Kushneria* species. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,10 substitutions per nucleotide position.

To confirm the absence of a bacteria related to Laf we also used the complete genome of '*Ca.* Liberibacter africanus' (GenBank accession: CP004021.1) during reference mapping of the NGS data of sample 18-0151 (see Appendix B, Figure B.11 and B.12). The amount of reads that matched to the complete genome of Laf was 35,314 out of the total 64,154,824 reads from the NGS data. No contigs of any significant lengths were obtained. Contigs were selected from the consensus sequence derived from the Laf genome for nucleotide BLAST analysis, due to the fact that the mapped portion of the genome (derived from the complete genome of Laf) was almost entirely incomplete with a high number of unknown bases (substituted with N's) (see Appendix B, Figure B.12), and the contigs were selected based on the sequence lengths – as these were the longest contigs obtained. Four contigs were selected, with sizes above 300 bp, for nucleotide BLAST analysis.

***Table 10**: Contigs derived from complete genome of 'Candidatus Liberibacter africanus' from reference mapping.*

Laf genome derived contig number	Sequence length (bp)	Number of non-matching bases
1	345	0
2	842	155
3	539	111
4	351	51

None of the derived contigs matched with any Liberibacter species during the BLAST analysis (see Table 11 below). The only derived contig that matched with a significant identity percentage to a known species via BLAST analysis was contig 4. This derived contig matched to *Novosphingobium tardaugens*, which is a bacterium belonging to the Alphaproteobacteria class. The presence of this bacteria within the plant species tested may explain the non-target amplification of the 16S rRNA gene and yield of Ct values below 31. But this cannot be said with certainty, therefore further analysis is required which was not within the scope of this study.

Table 11: *Nucleotide BLAST results of contigs derived from complete genome of 'Candidatus Liberibacter africanus' from reference mapping.*

Laf genome derived contig number	Nucleotide BLAST result		
	BLAST result	**GenBank acc.**	**Identity %**
1	*Psychrobacter* sp. KH172YL61 DNA, complete genome	AP019516.1	88.70%
2	*Kushneria konosiri* strain X49, complete genome	CP021323.1	86.57%
3	*Chenopodium quinoa* chloroplast, complete genome	MK159176.1	93.06%
4	*Novosphingobium tardaugens* NBRC 16725 chromosome, complete genome	CP034179.1	99.34%

* Bacterial sequences within a genus typically share 98-100% sequence similarity.

The derived contigs were aligned with available Liberibacter and other Proteobacteria sequences, including *Kushneria* spp. and *Novosphingobium tardaugens*, using the MAFFT online tool. Thereafter the aligned dataset was trimmed in BioEdit to assess the cognate region within the represented sequences. Subsequently the phylogenetic relationships of the aligned sequences were assessed by producing a best-fit DNA evolutionary model and maximum-likelihood phylogenies of the trimmed alignment using Mega version X (Figure 4). Phylogenetic analysis once again verified the presence of an as yet unidentified bacterial species, as two contigs were completely unrelated to any of the known Proteobacteria and the other two contigs were closely related to the other Proteobacteria.

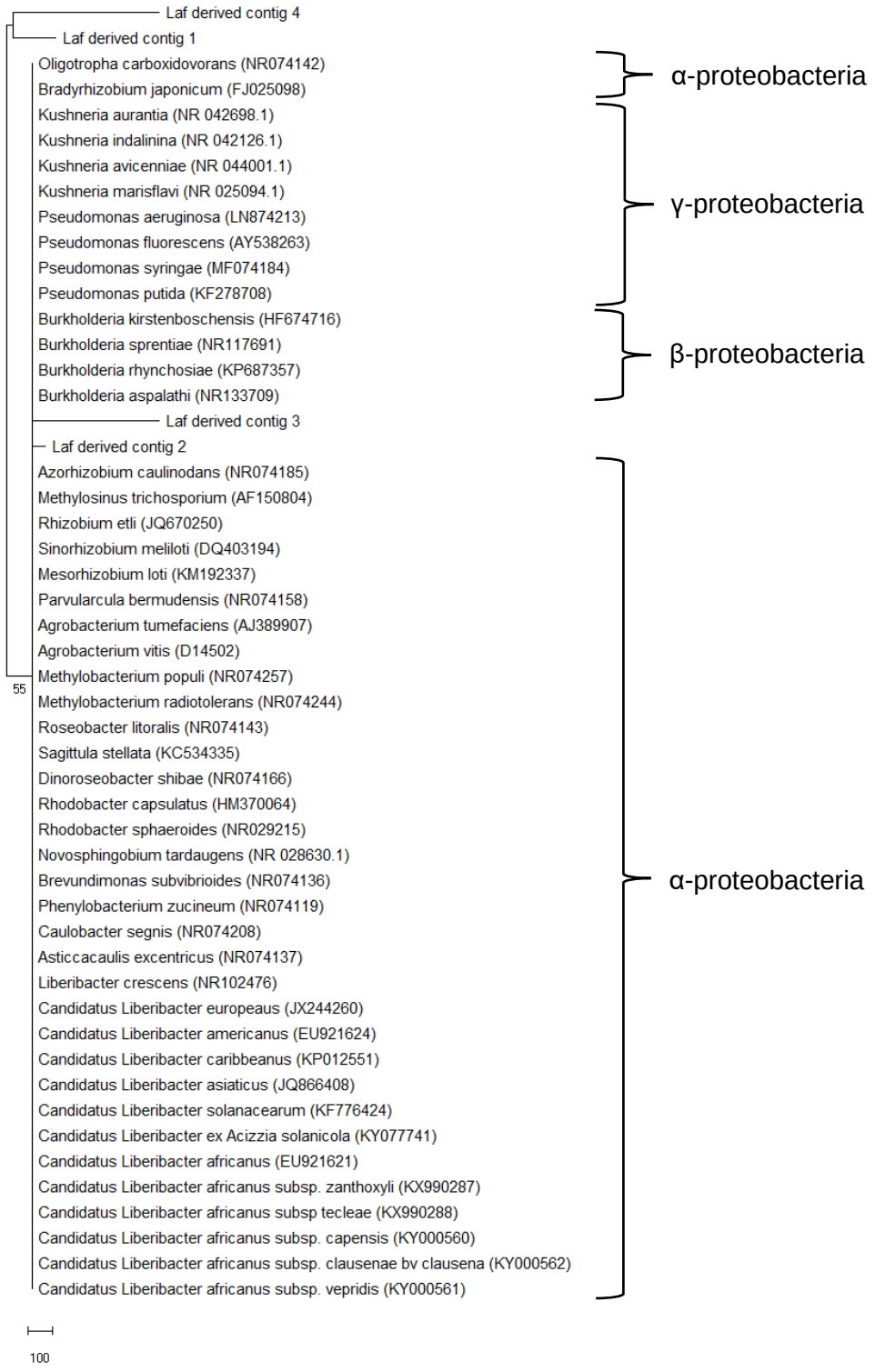


Figure 4: Maximum-likelihood phylogeny of the '*Candidatus* Liberibacter africanus' genome derived contig sequences from read mappings obtained from the NGS data (*Atriplex semibaccata* sample, accession number 18-0151). The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980) with gamma distributions (G). The Alpha- (α), Beta- (β), and Gammaproteobacteria (γ), from the Proteobacteria phylum, are indicated, including other *Kushneria* species. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 100 substitutions per nucleotide position.

3.4. Discussion

In the current study, no evidence was found for the presence of '*Ca.* Liberibacter africanus' in any of the indigenous or other non-rutaceous potential plant host species collected in the Western Cape. Out of the 989 potential alternative host plant species sampled, 142 samples yielded Ct values of less than 31, the set threshold, following a Liberibacter 'Universal' the real-time PCR assay. The primer set used was designed to detect Laf, Las and Lam infections, by utilising the same probe and reverse primer and a different forward primer for each of the three Liberibacter species (Li *et al.,* 2006). However, the primer set has subsequently been shown to non-specifically amplify *Bradyrhizobium* populations from roots of citrus trees during real-time PCR tests (Shin & van Bruggen, 2018). Liberibacters and *Bradyrhizobium* both belong to the Proteobacteria. The modification to the Li *et al.* (2006) primers by Roberts *et al.* (2015) was done in order to specifically increase the probability of detecting more divergent Liberibacters. The modified primer set therefore has an increased probability of detecting other members of the Alphaproteobacteria or related bacteria. Amplification of bacteria other than Liberibacters in the real-time PCR tests would not necessarily occur with the conventional PCR systems (Hocquellet *et al*., 1999; Bastianel *et al.,* 2005) employed, which have higher specificity for Liberibacters. This was confirmed during the current study.

The 142 samples that yielded Ct values of less than 31 appeared to have non-target, non-specific amplification by the 16S rDNA primer and probe set utilised for the initial screening of the plant species for Liberibacters. During this study we utilised the amplification of a highly conserved gene such as 16S rRNA and protein-coding genes such as *omp* and *rplJ* genes. While no amplification was obtained with the Laf specific *omp* gene as well as the Laf and Las specific ribosoma*l rplJ* gene, seven of the 142 samples did yield very faint bands during gel electrophoresis analysis after the amplification of the 16S rRNA Laf gene. Sanger sequencing and phylogenetic analysis of these samples showed that none of the samples were related to any of the known Liberibacters. The utilisation of the universal 16S rRNA PCR primers, which typically allows for the favoured product to be sequenced, therefore allowed

for the detection of as many Liberibacter species as possible against non-specific amplification from non-Liberibacters. Therefore by *in silico* analysis, it was determined that the region between the LG774F/LG1463R conventional PCR primer pair of Morris *et al*. (2017) can differentiate between Liberibacters and non-Liberibacters.

Similarly, rutaceous plant species from the CFR did not yield any Liberibacter positive results (Roberts, per comm). In addition, no obvious signs of psyllids were observed (Roberts, per comm). Despite the successful detection of Laf-subspecies from rutaceous hosts by utilising the same Liberibacter 'Universal' real-time PCR assay (Roberts *et al.,* 2015; Roberts & Pietersen, 2017), the current study suggests that it is important to reassess its utilisation in view of the non-specific, non-target amplification of unrelated bacteria observed here.

The characteristic symptoms associated with Liberibacter infection of citrus, which include yellowing and mottling, were not observed on any of the collected non-rutaceous plant specimens sampled during the study. A single rutaceous (citrus) sample collected from an orchard in Worcester did display yellowing and mottling and yielded a Ct value of below 31 in the real-time PCR test but did not test positive for the presence of Laf infection during any of the conventional PCR tests. The observed symptoms in this specific collected plant sample could possibly have been caused by environmental conditions such as physiological stress. This is possible as many of the samples were collected during 2017 and early 2018, when the Western Cape was still experiencing the negative effects of a severe three-year drought. Another possible cause may be the presence of other biological agents other than Liberibacters, such as phytoplasms or viral infections. It is noteworthy however that a number of Liberibacters do not appear to cause visible disease symptoms on the hosts they infect (Raddadi *et al.* 2010; Roberts *et al.* 2015), and the absence of disease symptoms does not necessarily indicate the absence of Liberibacter infections.

The transmission of Liberibacters is highly dependent on the insect vectors within the Psylloidae (McClean & Oberholzer, 1965; Capoor *et al.,*

1967). A total of five psyllids were collected from *R. foetida* plants. The identity of these psyllids was from the basis of a parallel independent study. The absence of psyllids from the remainder of the plant species sampled may be due to the severe three-year drought or that the non-rutaceous plant species sampled during this study are unsuitable hosts for psyllids. Future sampling and testing of different Fynbos species and other plant species found in the Western Cape of South Africa is required to assess this.

It should also be noted that the plants selected for sampling was done based on the presence of large numbers of a given species in an area and all were from the CFR of the Western Cape, South Africa. The plants sampled were not necessarily in the vicinity of citrus orchards as Roberts *et al.* (2015) demonstrated that '*Ca.* Liberibacter africanus subspecies' infected plants occur in non-citrus areas as well. However, the absence of citrus in the vicinity of some of the sampling sites would reduce the probability of finding Laf *sensu stricto* in such samples.

Based on the results, none of the non-rutaceous Fynbos and other plant species collected from the various areas in the Western Cape, South Africa, contained Laf. To identify whether the bacterial population that amplified during the real-time PCR assays belongs to the Alpha-, Beta-, or Gammaproteobacteria group, further analysis of the NGS data obtained from a single *A. semibaccata* plant sample was conducted. This confirmed the absence of Liberibacter spp.

de novo Assembly of the NGS data of the sample from *A. semibaccata*, which yielded a Ct value of less than the set threshold of 31 during the real-time PCR assays, failed to identify the bacterial entity that may have caused the non-specific real-time PCR amplification obtained during this study. A total of 164 contigs were obtained via *de novo* assembly, with none matching any Liberibacter species during nucleotide BLAST analyses. Therefore, further phylogenetic analysis was required to attempt to identify the bacterial organism that was present in the *A. semibaccata* sample. The analysis showed that the sample did not contain a bacterial entity closely related to Alphaproteobacteria, the class of '*Ca.* Liberibacter' species, but rather one

more closely related to Gammaproteobacteria based on the positioning of the consensus sequences derived from all known 16S rRNA Liberibacter sequences to the rest of the Alpha-, Beta-, and Gammaproteobacteria. An analysis of the total reads (over 64 million reads) within the NGS dataset suggests that 49.71% of the total reads belong to the Gammaproteobacteria. While a total of 60% of the Gammaproteobacteria reads matched with *Kushneria avicenniae*, further phylogenetic analysis, now including four *Kushneria* spp. whose 16S rRNA sequences were obtained from GenBank, indicated that the Liberibacter derived consensus sequences were not closely related to any of the four species. The derived consensus sequences once again belonged to the Gammaproteobacteria. After reference mapping of the NGS data to the complete genome of Laf four contig sequences were selected from the Laf derived consensus sequence for nucleotide BLAST analysis and subsequent phylogenetic analysis. None of the contigs matched any Liberibacter spp. and the phylogenetic analysis indicated that the bacterial entity present within the *A. semibaccata* sample possibly belongs to the Proteobacteria group.

The 16S rRNA gene sequence was used to attempt to identify the bacterial entity being amplified in the real-time PCR. The small-subunit ribosomal RNA has been previously considered a valuable tool for prokaryotic phylogeny as it is believed to be one of the most constrained and universal molecules available (Woese, 1987; Daubin *et al.,* 2002), and there are now hundreds of thousands of sequences available online (GenBank) from both environmental and cultured organisms (Lang *et al.,* 2013). However, phylogenetic trees inferred using the 16S rRNA gene will most likely differ from phylogenetic trees inferred using other phylogenetic marker genes (Lang *et al.,* 2013). It is therefore desirable to use multiple genes when analysing prokaryotic phylogeny (Eisen, 1995; Daubin *et al.,* 2002; Lang *et al.,* 2013). Another bacterial gene utilised extensively, and that is potentially useful to a study as this one is the gene that encodes the RecA protein (*recA*) (Eisen, 1995). The *recA* gene is a bacterial gene that appears to be conserved in all bacteria (Eisen, 1995). This gene can thus be used in future studies, alongside the

16S rRNA gene, to attempt to identify the bacterial entity amplified during the real-time PCR.

The plant species selected for this study were identified based on the morphological properties of the plants, and barcoding PCR tests for the amplification of the *rbcL* gene were used to confirm the identities of the plant species or to identify their closest relatives. Some of the species could not be identified using the amplification of the *rbcL* gene, therefore those plants were identified based only on their morphology.

Other barcodes typically proposed for plant species barcoding are the plastid genes, such as the most conserved *rpoB, rpoC1* and *rbcL* genes, or a section of the *matK* gene, but in some plant families these genes identified amplification problems. The Consortium for the Barcode of Life (CBoL) Plant Working Group therefore recommended that a 2-locus combination of *rbcL* and *matK* that should be used as the standard plant barcode (CBoL, 2009; Casiraghi *et al.,* 2010). Therefore, during future studies it is suggested that *matK* be used in conjunction with the *rbcL* gene for better identification of the host species and their closest relatives. It is advised to use multiple barcodes in order to correctly and more accurately identify plant species, especially when interspecies differentiation is also needed. Depending on the gene utilised DNA barcoding can efficiently identify species within taxa, but this is not always the case (Sbordoni, 2010). It has been shown that general barcoding techniques using universal primers provided mixed results with regard to data accuracy, and that DNA barcoding of processed plant material as a stand-alone means of identification of plant species is not recommended (Parveen *et al.,* 2016). Therefore, DNA barcoding is not an ideal method for the accurate identification of species (Casiraghi *et al.,* 2010) and the inherent limitations of using DNA barcoding exclusively as a means of identifying species makes it unsuitable for identifying plant species (Parveen *et al.,* 2016).

In conclusion, it is clear that the bacterial entity amplified by the Liberibacter ‘Universal’ real-time PCR in sample 18-0151 was not Liberibacter spp., but an, as yet, unidentified member of the Gammaproteobacteria. This

bacterium may also be present in a number of other *Atriplex* samples which yielded amplicons in the real-time PCR as well as in some of the other plant species. This could be explored in future studies.

The overall aim of this study was to identify possible alternative plant species that may act as reservoir hosts to explain the reintroduction of Laf in South African citrus orchards after stringent control strategies have been implemented to limit Laf infections in these orchards. This study failed to identify such plant species. One possible explanations for the reintroduction of Laf into these citrus orchards may include the re-infestation of the citrus plants by *T. erytreae* insect vectors carrying Laf from surrounding Laf infected orchards or possible alternative host plants of the vector. This can also be explored in future studies.

3.5. References

Aubert B, Garnier M, Cassin JC & Bertin Y, 1988. Citrus greening disease survey in East and West African countries south of Sahara. Pp. 231-237 *In* LW Timmer, SM Garnsey and L Navarro (eds.), In Proceedings of the 10th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Bastianel C, Garnier-Semancik G, Renaudin J, Bové JM & Eveillard S, 2005. Diversity of '*Candidatus* Liberibacter asiaticus' based on the *omp* gene sequence. *Applied and Environmental Microbiology* **71**: 6473-6478.

Bové JM & Garnier M, 1984. Citrus greening and psylla vectors of the disease in the Arabian Peninsula. Pp. 258-263 *In* P Moreno, JV da Graça and LW Timmer (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Buitendag CH & von Broembsen LA, 1993. Living with citrus greening in South Africa. Pp. 269-273 *In* P Moreno, JV da Graça and LW Timmer (eds.), In Proceedings of the 12th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Burckhardt D & Ouvrard D, 2012. A revised classification of the jumping plant-lice (Hemiptera: Psylloidea). *Zootaxa* **3509**: 1-34.

Capoor SP, Rao DG & Viswanath SM, 1967. *Diaphorina citri* Kuwayama, a vector of the greening disease of citrus in India. *Indian Journal of Agricultural Science* **37**: 572-575.

Casiraghi M, Labra M, Ferri E, Galimberti A & De Mattia F, 2010. DNA barcoding: theoretical aspects and practical applications. Pp. 269-273 *In* PL Nimis and LR Vignes (eds.), In Tools for Identifying Biodiversity: Progress and Problems. Eindhoven University of Technology, Eindhoven, North Brabant, Netherlands.

Citrus Growers Association (CGA), Key industry statistics for citrus growers 2018. http://www.citrusresourcewarehouse.org.za/home/document-

home/information/cga-key-industry-statistics/5475-cga-key-industry-statistics-2018/file [accessed 30 March 2019].

Coletta-Filho HD, Targon MLPN, Takita MA, De Negri JD, Pompeu JJ & Machado MA, 2004. First report of the causal agent of Huanglongbing (''*Candidatus* Liberibacter asiaticus'') in Brazil. *Plant Disease* **88**: 1382.

Consortium for the Barcode of Life (CBoL), 2009. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of USA* **106**: 12794-12797.

Damsteegt VD, Postnikova EN, Stone AL, Kuhlmann M, Wilson C, Schaad NW, Brlansky RH & Schneider WL, 2010. *Murraya paniculata* and related species as potential host and inoculums reservoirs of '*Candidatus* Liberibacter asiaticus', causal agent of Huanglongbing. *Plant Disease* **94**: 528-533.

Daubin V, Gouy M & Perrière G, 2002. A phylogenomic approach to bacterial phylogeny: Evidence of a core of genes sharing a common history. *Genome Research* **12**: 1080-1090.

Doyle JJ & Doyle JL, 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Duan Y, Zhou L, Hall DG, Li W, Doddapaneni H, Lin H, Liu L, Vahling M, Gabriel DW, Williams KP, Dickerman A, Sun Y & Gottwald T, 2009. Complete genome sequence of citrus Huanglongbing bacterium, '*Candidatus* Liberibacter asiaticus' obtained through metagenomics. *Molecular Plant-Microbe Interactions* **22**: 1011-1020.

Eisen JA, 1995. The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. *Journal of Molecular Evolution* **41**: 1105-1123.

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

Garnier M & Bové JM, 1996. Distribution of the Huanglongbing (Greening) Liberibacter species in fifteen African and Asian countries. Pp. 388-391 *In* JV da Graça, RF Lee and RK Yokomi (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Garnier M, Jagoueix S, Toorawa P, Grisoni M, Mallessard R, Dookun A, Saumtally S, Autrey JC and Bové JM, 1996. Both Huanglongbing (Greening) Liberibacter species are present in Mauritius and Reunion. Pp. 271-275 *In* JV da Graça, P Moreno and RK Yokomi (eds.), In Proceedings of the 13th Conference of the International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hocquellet A, Toorawa P, Bové JM & Garnier M, 1999. Detection and identification of the two '*Candidatus* Liberobacter species' associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the β operon. *Molecular and Cellular Probes* **13**: 373-379.

Jagoueix S, Bové JM & Garnier M, 1994. The phloem-limited bacterium of greening is a member of the alpha subdivision of the proteobacteria. *International Journal of Systematic Bacteriology* **44**: 379-386.

Katoh K, Misawa K, Kuma K & Miyata T, 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059-3066.

Kimura M, 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120.

Korsten L, Jagoueix S, Bové JM & Garnier M, 1996. Huanglongbing (Greening) detection in South Africa. Pp. 395-398 *In* JV da Graça, P Moreno and RK Yokomi (eds.), In Proceedings of the 13th Conference of the

International Organization of Citrus Virologists. University of California, Riverside, CA, USA.

Kress J & Erickson LL, 2007. A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding trnH-psb spacer region. *PLoS One* **6**: 1-10.

Krüger K & Fiore N, 2019. Sampling methods for leafhopper, planthopper and psyllid vectors. *Methods in Molecular Biology* **1875**: 37-52.

Kumar S, Stecher G, Li M, Knyaz C & Tamura K, 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**: 1547-1549.

Lang JM, Darling AE & Eisen JA, 2013. Phylogeny of bacterial and archaeal genomes using conserved genes: supertrees and supermatrices. *PLoS One* **8**: e62510.

Levin RA, Wagnet WL, Hoch PC, Nepokroeff M, Pires JC, Zimmer EA & Sytsma KJ, 2003. Family-level relationships of Onagraceae based on chloroplast *rbcL* and *ndhF* data. *American Journal of Botany* **90**: 107-115.

Li W, Hartung JS & Levy L, 2006. Quantitative real-time PCR for detection and identification of '*Candidatus* Liberibacter species' associated with citrus huanglongbing. *Journal of Microbial Methods* **66**: 104-115.

Lin KH & Lin KH, 1956. The citrus huang lung bin (Greening) disease in China. *Acta Phytopathologica Sinica* **2**: 14-38.

Lin H, Pietersen G, Han C, Read DA, Lou B, Gupta G & Civerolo EL, 2015. Complete genome of '*Candidatus* Liberibacter africanus', a bacterium associated with citrus huanglongbing, *Genome Announcement* **3**: e00733-15.

Mayikawa T, 1980. Experimentally induced symptoms and host range of citrus Likubin (greening disease). *Annals of the Phytopathological society of Japan* **46**: 224-230.

McClean APD & Oberholzer PCJ, 1965. *Citrus psylla*, a vector of the greening disease of sweet orange. *South African Journal of Agricultural Science* **8**: 297-298.

Miyambo T, Makhalanyane TP, Cowan DA & Valverde A, 2016. Plants of the fynbos biome harbour host species-specific bacterial communities. *FEMS Microbiology Letters* **363**: fnw122.

Moran VC, 1968. The development of the citrus psylla, *Trioza erytreae* (Del Guercio) (Homoptera: Psyllidae), on *Citrus limon* and four indigenous host plants. *Journal of the Entomological Society of Southern Africa* **31**: 391-402.

Morris J, Shiller J, Mann R, Smith G, Yen A & Rodoni B, 2017. Novel '*Candidatus* Liberibacter' species identified in the Australian eggplant psyllid*, Acizzia solanicola. Microbial Biotechnology* **10**: 833-844.

Parveen I, Gafner S, Techen N, Murch SJ & Khan IA, 2016. DNA Barcoding for the Identification of Botanicals in Herbal Medicine and Dietary Supplements: Strengths and Limitations. *Planta Medica* **82**: 1225-1235.

Raddadi N, Gonella E, Camerota C, Pizzinat M, Tedeschi R, Crotti E, Mandrioli M, Bianco PA, Daffonchio D & Alma A, 2011. '*Candidatus* Liberibacter europaeus' sp. nov. that is associated with and transmitted by the psyllid *Cacopsylla pyri* apparently behaves as an endophyte rather than a pathogen. *Environmental Microbiology* **13**: 414-426.

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

Roberts R, Steenkamp ET & Pietersen G, 2015. Novel lineages of '*Candidatus* Liberibacter africanus' associated with native rutaceous hosts of *Trioza erytreae* in South Africa. *International Journal of Systematics and Evolutionary Microbiology* **65**: 723-731.

Sambrook J & Russell DW, 2001. Molecular cloning: a laboratory manual. Vol. 2, 3rd edition. Cold Spring Harbor Laboratory Press, New York.

Sbordoni V, 2010. Strength and Limitations of DNA Barcode under the Multidimensional Species Perspective. Pp. 275-280 *In* PL Nimis and LR

Vignes (eds.), In Tools for Identifying Biodiversity: Progress and Problems. Eindhoven University of Technology, Eindhoven, North Brabant, Netherlands.

Sechler A, Schuenzel EL, Cooke P, Donnua S, Thaveechai N, Postnikova E, Stone AL, Schneider WL, Damsteegt VD & Schaad NW, 2009. Cultivation of '*Candidatus* Liberibacter asiaticus', '*Ca*. L. africanus', and '*Ca*. L. americanus' associated with Huanglongbing. *Phytopathology* **99**: 480-486.

Secor GA, Rivera VV, Abad JA, Lee I-M, Clover GRG, Liefting LW, Li X & De Boer SH, 2009. Association of '*Candidatus* Liberibacter solanacearum' with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy and PCR. *Plant Disease* **93**: 574-583.

Shin K & van Bruggen AHC, 2018. Bradyrhizobium isolated from Huanglongbing (HLB) affected citrus trees reacts positively with primers for *Candidatus* Liberibacter asiaticus. *European Journal of Plant Pathology* **151**: 291-306.

Steenkamp ET, van Zyl E, Beukes CW, Avontuur JR, Chan WY, Palmer M, Mthombeni LS, Phalane FL, Sereme TK & Venter SN, 2015. *Burkholderia kirstenboschensis* sp. nov. nodulates papilionoid legumes indigenous to South Africa. *Systematic and Applied Microbiology* **38**: 545-554.

Teixeira DC, Saillard C, Eveillard S, Danet JL, da Costa PI, Ayres AJ & Bové JM, 2005. '*Candidatus* Liberibacter americanus', associated with citrus Huanglongbing (greening disease) in São Paulo state, Brazil. *International Journal of Systematic and Evolutionary Microbiology* **55**: 1857-1862.

Van den Berg MA, Deacon VE & Steenkamp PJ, 1991. Dispersal within and between citrus orchards and native hosts, and nymphal mortality of citrus psylla, *Trioza eytreae* (Hemiptera: Triozidae). *Agriculture, Ecosystems and Environment* **35**: 297-309.

Van den Berg MA, van Vuuren SP & Deacon VE, 1991-1992. Studies on greening transmission by citrus psylla, *Trioza eytreae* (Hemiptera: Triozidae). *Israel Journal of Entomology* **25-26**: 51-56.

Van Vuuren SP, Cook G & Pietersen G, 2011. Lack of evidence for seed transmission of '*Candidatus* Liberibacter africanus' associated with greening (Huanglongbing) in citrus in South Africa. *Plant Disease* **95**: 1026.

Woese C, 1987. Bacterial evolution. *Microbiological Reviews* **51**: 221-271.

Appendices

Appendix A – Sample collection and test results

Figure A.1: Locations surveyed in the Western Cape (red markers), South Africa, for alternative host plants of '*Candidatus* Liberibacter africanus' in natural vegetation. Nine sites in the Western Cape were selected and the regions surveyed fall into the winter rainfall region where most of the rain falls during the winter from June to August. Three surveys were carried out, the first during September (spring) in 2017 (**A**), the second during January (summer) in 2018 (**B**), and the third during August (winter) in 2018 (**C**).

Figure A.2: Collection and sampling of alternative host plant species in the Western Cape, South Africa. (**A**) Measurement of the plant sizes with a measuring stick. The measurement of the black parts is 5 cm, and the space between the black parts is 10 cm. (**B**) Taking a photo record, (**C**) as well as labelling the plant, (**D**) sampling and (**E**) taking the GPS coordinates.

Figure A.3: Suction device which consisted of a leaf blower (set to suck the air in, instead of blowing air out) and attaching a stocking to the front of the leaf blower with an elastic band to collect the insects inhabiting the plants sampled. The device was usef for 10 seconds to collect insects from small plants, 30 seconds for plants larger than 30 cm, and 90 seconds for plants larger than 1 meter in size.

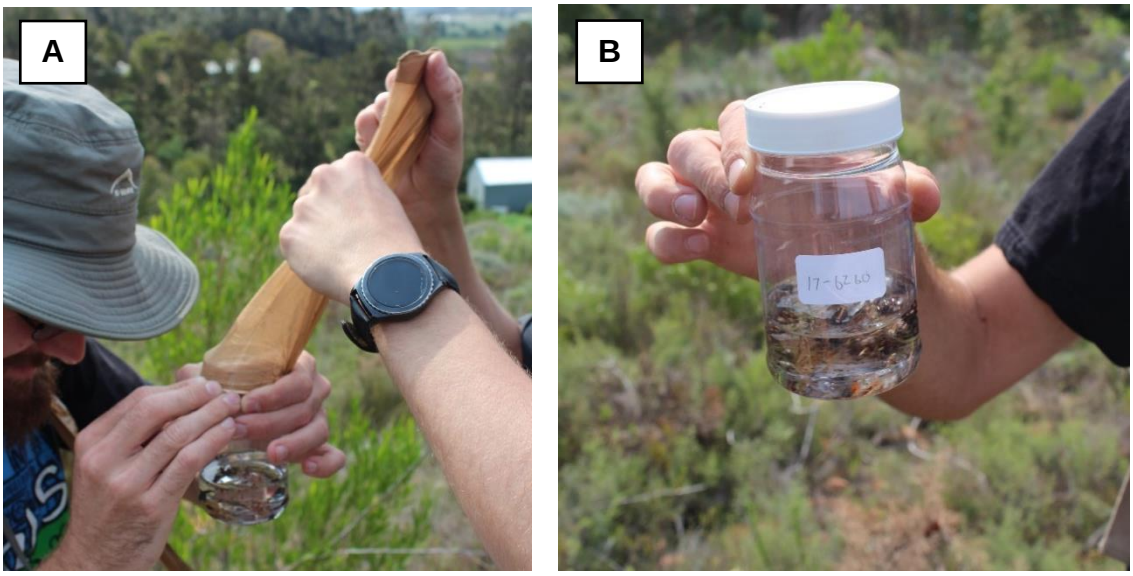
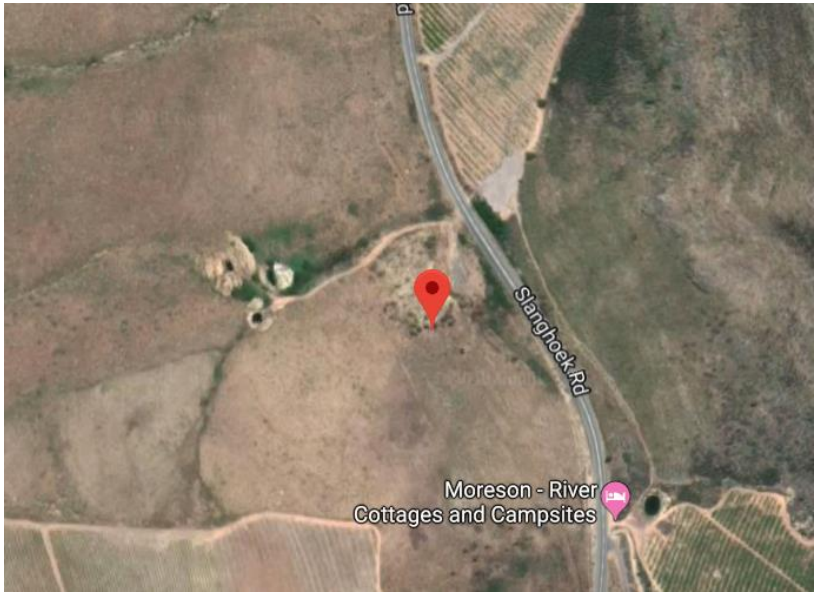


Figure A.4: (**A**) Insects collected via the suction device, in the stockings, are placed into containers with 30-50 ml absolute alcohol to preserve the samples. (**B**) Containers were labelled according to the corresponding plant samples from which the insects were collected.

Table A.1: *Sampling locations and descriptions.*

<table>
<tr><td colspan="2">Location (Fieldtrip A, Site 2)</td><td rowspan="2">General description of site</td></tr>
<tr><td colspan="2">Slanghoek, Western Cape</td></tr>
<tr><td colspan="2">Sampling date</td><td rowspan="2">Plant and insect samples were collected from natural vegetation growing in between vineyard rows from a vineyard from Slanghoek.</td></tr>
<tr><td colspan="2">11/09/2017</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Plant species sampled
Protea cynaroides, Montinia caryophyllacea, Atriplex semibaccata, Aizoon africanum, Euryops speciosissimus.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
<tr><td>-33.554403249</td><td>19.204532589</td></tr>
<tr><td colspan="2">Aerial view</td><td>Ground view</td></tr>
<tr><td colspan="2"> 
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<table>
<tr><td colspan="2">Location (Fieldtrip A, Site 2)</td><td>General description of site</td></tr>
<tr><td colspan="2">Slanghoek, Western Cape</td><td rowspan="2">Plant and insect samples were collected from natural vegetation on a hill that was in the vicinity of different vineyards in Slanghoek.</td></tr>
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">12/09/2017</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Montinia caryophyllacea, Muraltia heisteria, Sorocephalus pinifolius, Pteronia incana, Hakea sericea, Thesium lineatum, Leucadendron tinctum, Passerina carymbosa, Cliffortia odorata, Dodonaea viscosa, Unidentified Restio.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
<tr><td>-33.555231157</td><td>19.206587272</td></tr>
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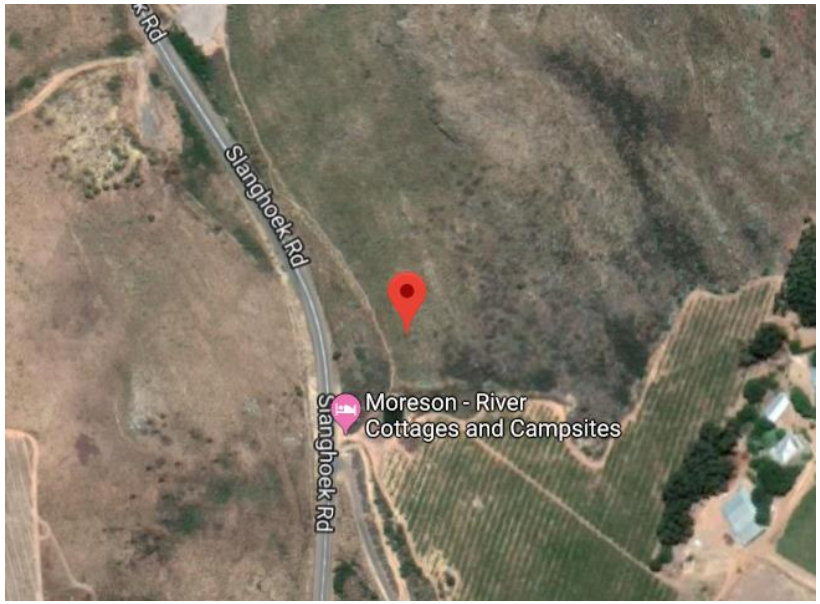
<tr><td colspan="2"> 
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<table>
<tr><td colspan="2">Location (Fieldtrip A, Site 3)</td><td>General description of site</td></tr>
<tr><td colspan="2">Robertson, Western Cape</td><td rowspan="2">Plant and insect samples were collected near vineyards in Robertson, located near a river/dam in Robertson.</td></tr>
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">13/09/2017 – 14/09/2017</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Oncosiphon grandiflorum, Amsinckia menziesii, Raphanus raphanistrum, Raphanus rugosum, Drosanthemum speciosum, Atriplex lindleyi, Pteronia incana, Helichrysum cymosum, Disphyma australe subsp. australe, Aizoon africanum, Roepera foetida, Drosanthemum hispidum, Hymenolepis crithmifolia, Hermannia grossularifolia.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
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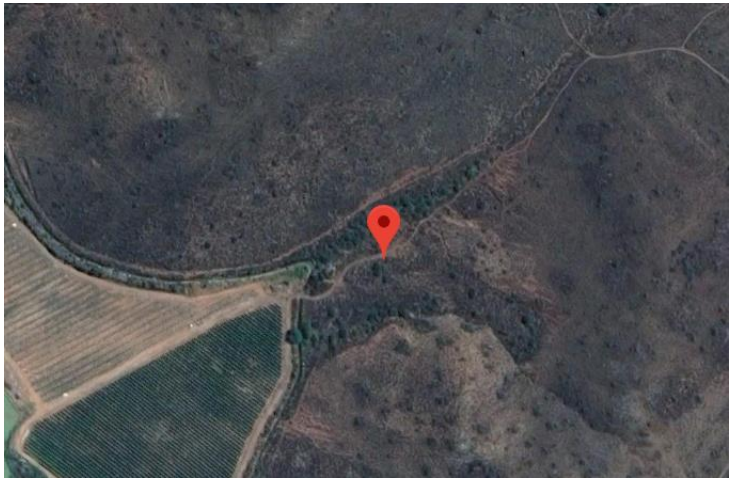
<tr><td colspan="2">  </td><td>  </td></tr>
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<table>
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<tr><td colspan="2">Worcester [Over Hex (N1)], Western Cape</td><td rowspan="2">Plant and insect samples were collected from rocky area on a hill and located near citrus orchards, next to the N1.</td></tr>
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">22/01/2018</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Aizoon africanum, Disphyma australe subsp. australe, Cissampelos capensis, Citrus sinensis.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
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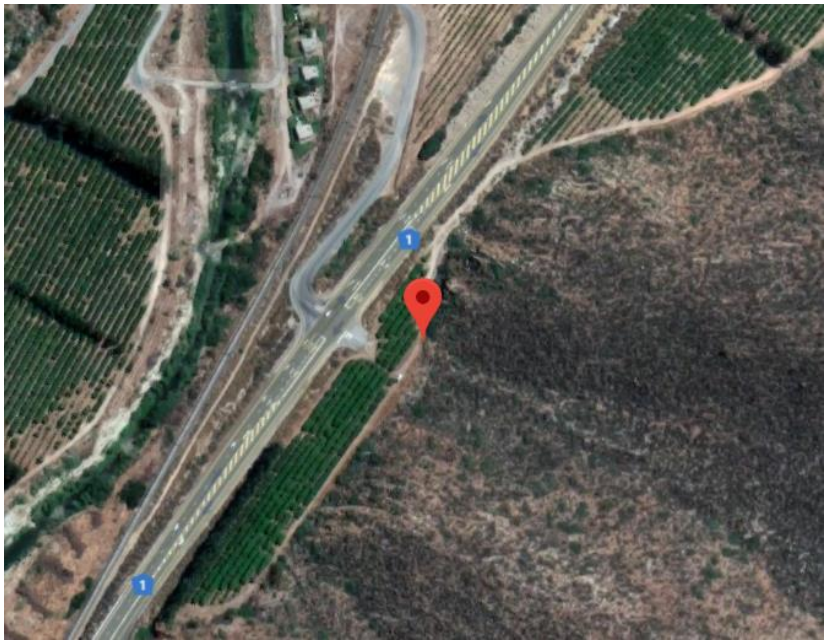
<tr><td colspan="2">  </td><td>  </td></tr>
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<tr><td colspan="2">Robertson (Norree), Western Cape</td><td rowspan="3">Plant and insect samples were collected from natural vegetation growing in between this vineyard located in Norree, Robertson.</td></tr>
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">22/01/2018</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Plant species sampled
Roepera foetida, Salsola kali, Elytropappus rhinocerotis.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
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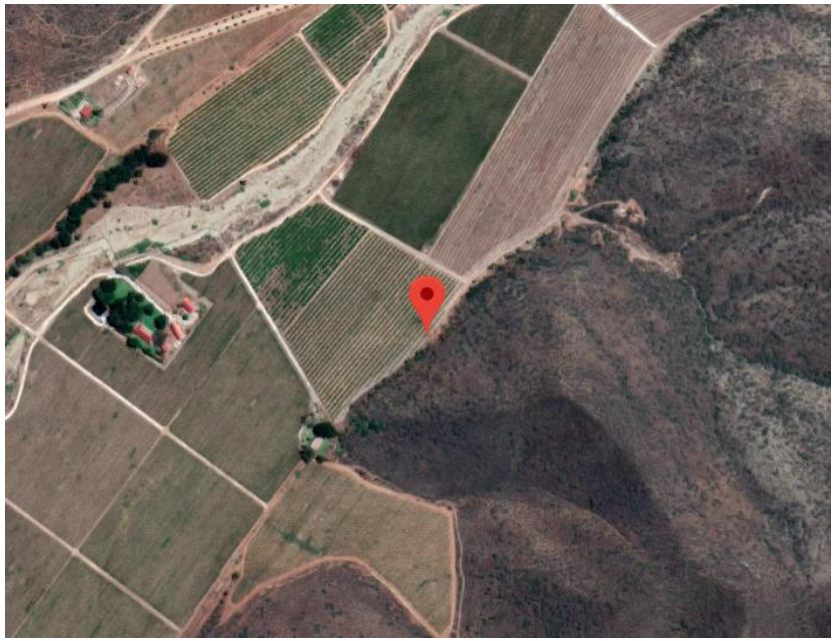
<tr><td colspan="2">  </td><td>  </td></tr>
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<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">23/01/2018</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Plant species sampled
Aizoon africanum, Atriplex lindleyi, Vitis vinifera, Senecio burchellii, Cynodon dactylon, Atriplex semibaccata, Salsola kali.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
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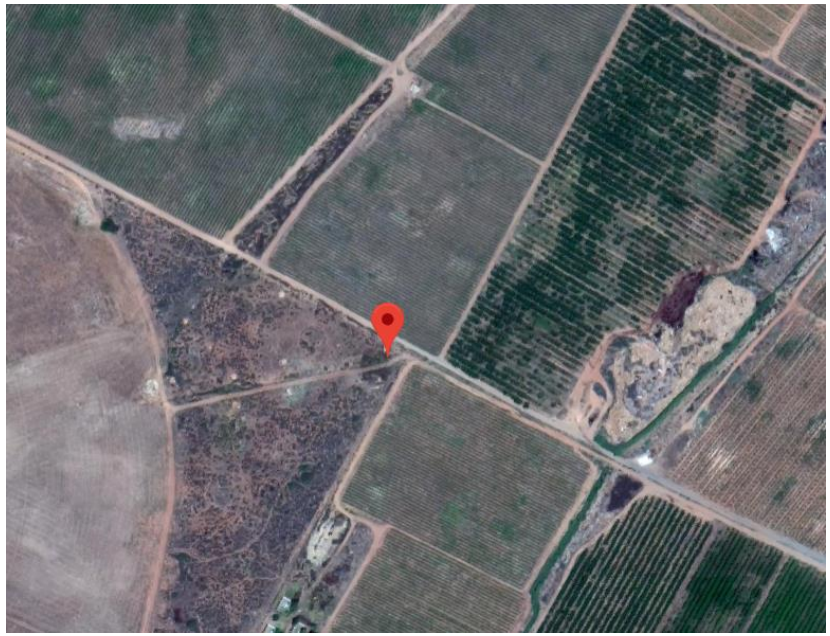
<tr><td colspan="2">  </td><td>  </td></tr>
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<tr><td colspan="2">Robertson, Western Cape</td><td rowspan="2">Plant and insect samples were collected along the riverbank/dam, as well as from plants growing near the vineyard (Merlot block).</td></tr>
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<tr><td colspan="2">24/01/2018</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Atriplex semibaccata, Salsola kali, Aizoon africanum, Elytropappus rhinocerotis, Conyza scabrida, Helichrysum cymosum, Pteronia incana.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
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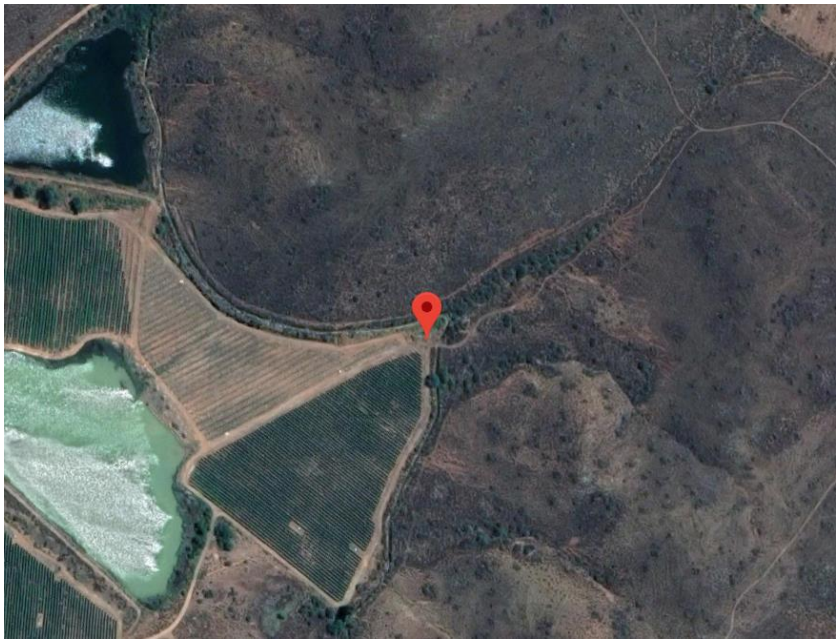
<tr><td colspan="2">  </td><td>  </td></tr>
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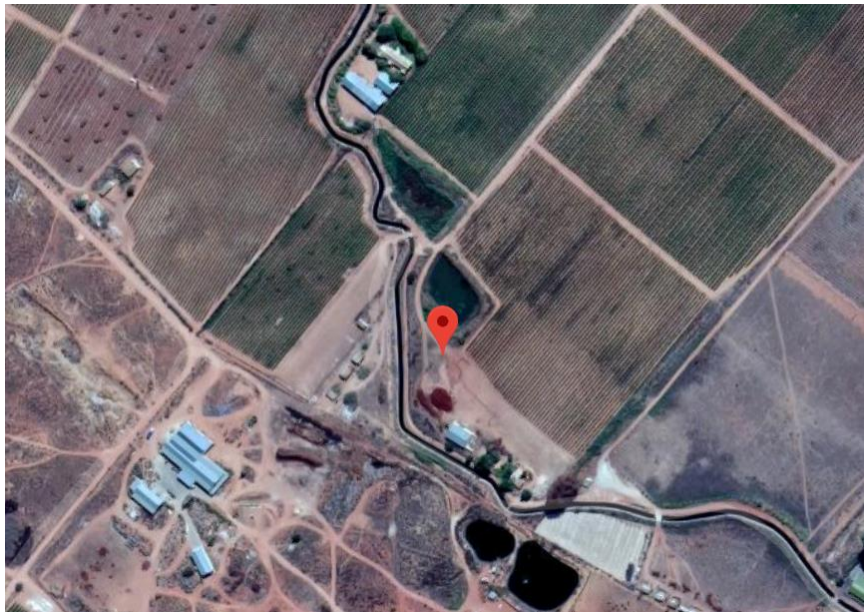
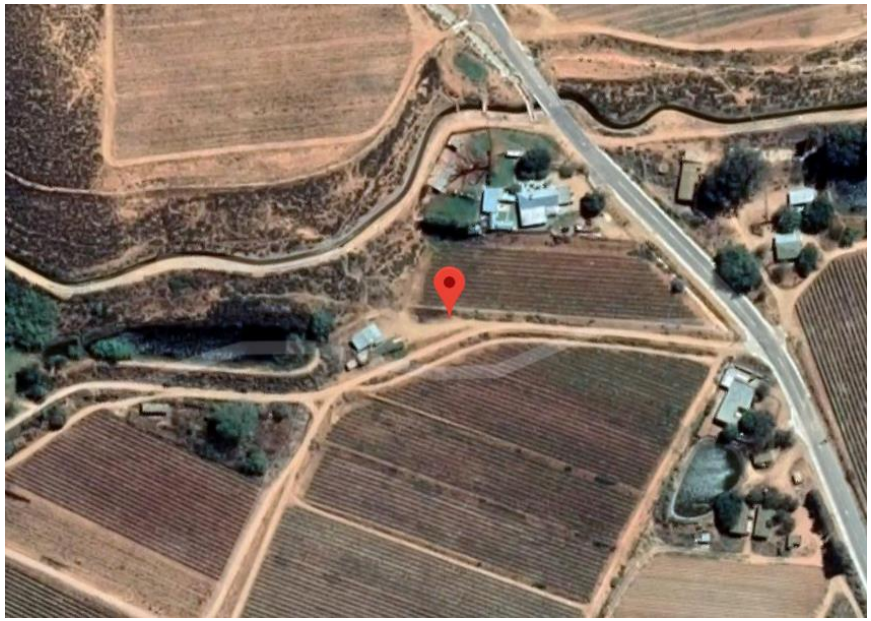
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">27/08/2018</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Brassica tournefortii, Oncosiphon grandiflorum, Atriplex semibaccata, Mesembryanthemum crystallinum, Atriplex lindleyi. Atriplex nummularia, Eriocephalus brevifolius.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
<tr><td>-31.674208377</td><td>18.471305000</td></tr>
<tr><td colspan="2">Aerial view</td><td>Ground view</td></tr>
<tr><td colspan="2">  </td><td>  </td></tr>
</table>

<table>
<tr><td colspan="2">Location (Fieldtrip C, Site 2)</td><td>General description of site</td></tr>
<tr><td colspan="2">Lutzville, Western Cape</td><td rowspan="2">Plant and insect samples were collected from a hill and along a dirt road next to a vineyard located in Lutzville.</td></tr>
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">28/08/2018</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Atriplex nummularia, Osteospermum oppositofolium, Oxalis pes-caprae, Atriplex semibaccata, Oncosiphon suffruticosum, Drosanthemum hispidum.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
<tr><td>-31.557976251</td><td>18.326710374</td></tr>
<tr><td colspan="2">Aerial view</td><td>Ground view</td></tr>
<tr><td colspan="2">  </td><td>  </td></tr>
</table>

<table>
<tr><td colspan="2">Location (Fieldtrip C, Site 3)</td><td>General description of site</td></tr>
<tr><td colspan="2">Klawer, Western Cape</td><td rowspan="2">Plant and insect samples were collected along a dirt road, near different vineyards and a river in Klawer, near Vredendal.</td></tr>
<tr><td colspan="2">Sampling date</td></tr>
<tr><td colspan="2">29/08/2018</td><td>Plant species sampled</td></tr>
<tr><td colspan="2">Coordinates</td><td rowspan="3">Atriplex nummularia, Lycium ferocissimum, Oncosiphon grandiflorum, Amsinckia menziesii, Atriplex semibaccata, Rapistrum rugosum.</td></tr>
<tr><td>Latitude (S)</td><td>Longitude (E)</td></tr>
<tr><td>-31.721943127</td><td>18.526613733</td></tr>
<tr><td colspan="2">Aerial view</td><td>Ground view</td></tr>
<tr><td colspan="2">  </td><td>  </td></tr>
</table>

Table A.2: *Plant species sampled from the Western Cape, South Africa.*

Species number	Plant species	Species number	Plant species
1	*Aizoon africanum*	22	*Hymenolepis crithmifolia*
2	*Amsinckia menziesii*	23	*Leucadendron tinctum*
3	*Atriplex lindleyi*	24	*Lycium ferocissimum*
4	*Atriplex nummularia*	25	*Mesembryanthemum crystallinum*
5	*Atriplex semibaccata*	26	*Montinia caryophyllacea*
6	*Brassica tournefortii*	27	*Muraltia heisteria*
7	*Cissampelos capensis*	28	*Oncosiphon grandiflorum*
8	*Citrus sinensis* (control)	29	*Oncosiphon suffruticosum*
9	*Cliffortia odorata*	30	*Osteospermum oppositifolium*
10	*Conyza scabrida*	31	*Oxalis pes-caprae*
11	*Cynodon dactylon*	32	*Passerina corymbose*
12	*Disphyma australe* subsp.	33	*Protea cynaroides*
	australe	34	*Pteronia incana*
13	*Dodonaea viscosa*	35	*Raphanus raphanistrum*
14	*Drosanthemum hispidum*	36	*Rapistrum rugosum*
15	*Drosanthemum speciosum*	37	Unidentified *Restio*
16	*Elytropappus rhinocerotis*	38	*Roepera foetida*
17	*Eriocephalus brevifolius*	39	*Salsola kali*
18	*Euryops speciosissimus*	40	*Senecio burchellii*
19	*Hakea sericea*	41	*Sorocephalus pinifolius*
20	*Helichrysum cymosum*	42	*Thesium lineatum*
21	*Hermannia grossularifolia*	43	*Vitis vinifera*

Table A.3: *Details of the plant species collected, in alphabetical order, including the distribution and sample locations.*

Plant species		**Total samples collected:**
Aizoon africanum (L.) Klak [basionym: *Galenia Africana* (L.)]		72
Common name		**Sampled from:**
Yellow bush Kraalbos, Geelbos, Perdebos		Slanghoek Robertson Worcester
Distribution		**Family**
South Africa (Free State, Northern Cape, Western Cape, Eastern Cape), Namibia, South West Angola.		Aizoaceae

Plant species		**Total samples collected:**
Amsinckia menziesii (Lehm.) A. Nelson & J.F. Macbr.		32
Common name		**Sampled from:**
Fiddleneck, Yellow Burrweed Vioolnek		Robertson Vredendal
Distribution		**Family**
North America (Alaska, Canada, Western USA, Mexico), South Africa (Western Cape).		Boraginaceae

Plant species		**Total samples collected:**
Atriplex lindleyi Moq.		32
Common name		**Sampled from:**
Lindley's saltbush Soutbos		Robertson Vredendal
Distribution		**Family**
Southern Australia, South Africa (Western Cape), USA (California).		Amaranthaceae

Plant species		**Total samples collected:**
Atriplex nummularia (Lind.)		54
Common name		**Sampled from:**
Old man saltbush Oumansoutbos		Lutzville Vredendal
Distribution		**Family**
Southern Australia, South Africa (Western Cape), North & South America (USA, Mexico, Chile), Spain, Taiwan, Oceania.		Amaranthaceae

Plant species		**Total samples collected:**
Atriplex semibaccata R.Br.		93
Common name		**Sampled from:**
Creeping saltbush, berry saltbush Rooibessiebos		Robertson Lutzville Vredendal
Distribution		**Family**
Southern Australia, South Africa (Western Cape), Northern & Western Africa, Arabian Peninsula, Southern Europe, USA (California), Chile.		Amaranthaceae

Plant species		**Total samples collected:**
Brassica tournefortii Guoan		20
Common name		**Sampled from:**
Sahara mustard, Asian mustard, African mustard, Mediterranean turnip, Tournefort's birdrape, wild turnip		Vredendal
Distribution		**Family**
Southern Australia, USA (California, Nevada, Arizona, New Mexico, Texas), Southern Europe, Northern Africa, South Africa (Western Cape), Western Asia, Pakistan, UK, New Zealand.		Brassicaceae

Plant species		**Total samples collected:**
Cissampelos capensis L.f.		12
Common name		**Sampled from:**
Dawidjies, Dawidjieswortel, Fynblaarklimop		Worcester
Distribution		**Family**
South Africa (Western Cape, Northern Cape, Eastern Cape), Namibia.		Menispermaceae

Plant species		**Total samples collected:**
Citrus sinensis L. * (*used as control)		1
Common name		**Sampled from:**
Sweet orange Soet lemoen		Worcester
Distribution		**Family**
Originated near the border between China & Vietnam, cultivated everywhere in the subtropics & tropics.		Rutaceae

Plant species		**Total samples collected:**
Cliffortia odorata L.f.		10
Common name		**Sampled from:**
Wildewingerd		Slanghoek
Distribution		**Family**
South Africa (Western Cape, Eastern Cape).		Rosaceae

Plant species		**Total samples collected:**
Conyza scabrida DC		20
Common name		**Sampled from:**
Horseweed, butterweed, fleabane		Robertson
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, KwaZulu-Natal, Free State, Mpumalanga, Lesotho, Swaziland.		Asteraceae

Plant species		**Total samples collected:**
Cynodon dactylon (L.) Pers		1
Common name		**Sampled from:**
Couch grass, quick grass, finger grass Kweekgras, fynkweek, kruisgras, vingergras		Robertson
Distribution		**Family**
Southern Africa (found in all Grassland, Savanna, Nama-Karoo & Fynbos biomes).		Poaceae

Plant species		**Total samples collected:**
Disphyma australe (Aiton) N.E.Br. subsp*. australe*		18
Common name		**Sampled from:**
Horokaka, round-leaved pigface, New Zealand iceplant, purple dewplant		Robertson Worcester
Distribution		**Family**
New Zealand, South Africa, Australia.		Aizoaceae

Plant species		**Total samples collected:**
Dodonaea viscosa Jacq.		5
Common name		**Sampled from:**
Native Hops, Florida hopbush, switch sorrel		Slanghoek
Distribution		**Family**
Tropical, subtropical & warm temperate regions of Africa (i.e. South Africa), North & South America, Southern Asia & Australia.		Sapindaceae

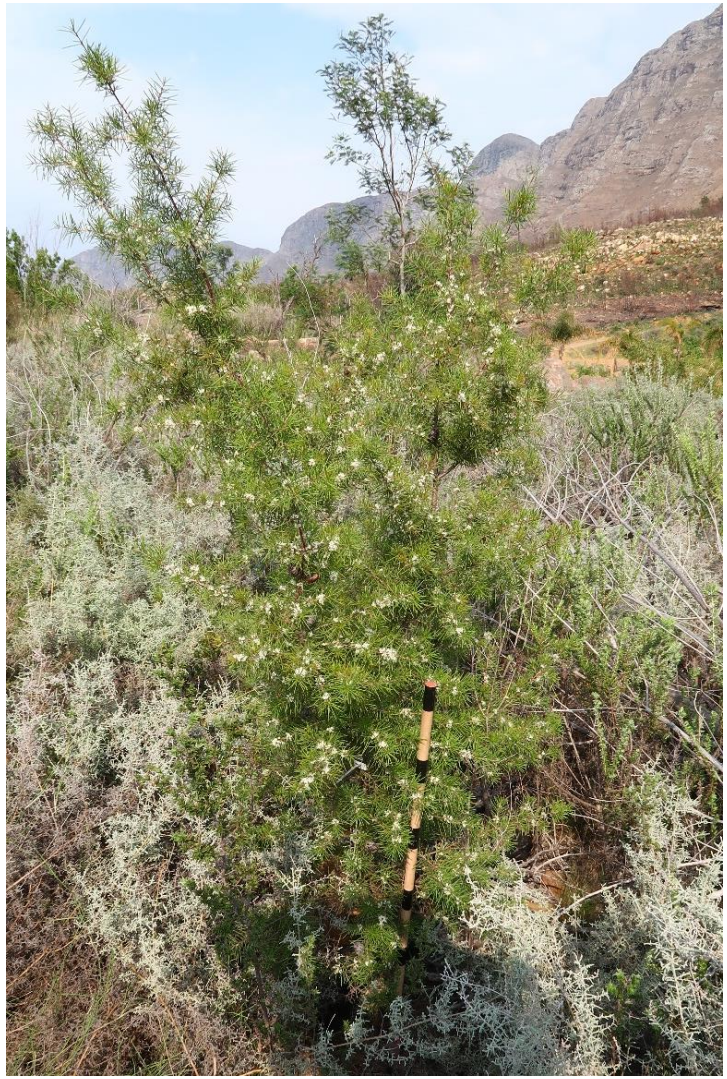
Plant species		**Total samples collected:**
Drosanthemum hispidum (L.) Schwantes		20
Common name		**Sampled from:**
Miniature pigs face Plat pers vygie, Fyn T'nouroebos		Robertson Lutzville
Distribution		**Family**
South Africa (Western Cape, Northern Cape, Eastern Cape, Free State), Namibia, Spain, Italy.		Aizoaceae

Plant species		**Total samples collected:**
Drosanthemum speciosum (Haw.) Schwantes		10
Common name		**Sampled from:**
Royal dewflower, dewflower, red ice plant Worcester-Robertson vygie		Robertson
Distribution		**Family**
South Africa (Western Cape), Namibia.		Aizoaceae

Plant species		**Total samples collected:**
Elytropappus rhinocerotis (L.f.) Koekemoer		12
Common name		**Sampled from:**
Rhinoceros bush Renosterbos		Robertson
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, Northern Cape).		Asteraceae

Plant species		**Total samples collected:**
Eriocephalus brevifolius (DC.) M.A.N.Müll.		6
Common name		**Sampled from:**
Kapok bush Kapokbos		Vredendal
Distribution		**Family**
South Africa (Western Cape, Northern Cape).		Asteraceae

Plant species		**Total samples collected:**
Euryops speciosissimus DC		20
Common name		**Sampled from:**
Giant resinbush, Clanwilliam euryops Pronkharpuisbos, grootharpuisbos		Slanghoek
Distribution		**Family**
South Africa (Western Cape).		Asteraceae

Plant species		**Total samples collected:**
Hakea sericea Schrad. & J.C.Wendl.		10
Common name		**Sampled from:**
Silky hakea, needlebush Syerige hakea		Slanghoek
Distribution		**Family**
Australia, South Africa (Western Cape, Eastern Cape), South Western Europe.		Proteaceae

Plant species	 	**Total samples collected:**
Helichrysum cymosum (L.) D. Don		20
Common name		**Sampled from:**
Gold carpet Goue tapyt		Robertson
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, KwaZulu-Natal).		Asteraceae

Plant species		**Total samples collected:**
Hermannia grossularifolia L.		10
Common name		**Sampled from:**
Doll’s rose Poprosie		Robertson
Distribution		**Family**
South Africa (Western Cape)		Malvaceae

Plant species		**Total samples collected:**
Hymenolepis crithmifolia (L.) Greuter, M.V. Agab. & Wagenitz		10
Common name		**Sampled from:**
Coulter-bush Koulterbos, pokbos		Robertson
Distribution	 	**Family**
South Africa (Western Cape, Northern Cape).		Asteraceae

Plant species		**Total samples collected:**
Leucadendron tinctum I.Williams		5
Common name		**Sampled from:**
Spicy conebush Bergroos		Slanghoek
Distribution		**Family**
South Africa (Western Cape – Fynbos biome).		Proteaceae

Plant species		**Total samples collected:**
Lycium ferocissimum Miers		20
Common name		**Sampled from:**
Cape box thorn, honey thorn, snake-berry, African box thorn Slangbessie, karriedoring, bokdoring		Vredendal
Distribution		**Species number:**
South Africa (Western Cape, Eastern Cape, Free State, Mpumalanga), Australia, New Zealand, USA.		Solanaceae

Plant species		**Total samples collected:**
Mesembryanthemum crystallinum L.		20
Common name		**Sampled from:**
Ice plant Soutslaai, brakslaai, slaaibossie, volstruisslaai, olifantslaai, brakvy		Vredendal
Distribution		**Family**
Africa [i.e. South Africa (Western Cape, Northern Cape)], Sinai, Southern Europe, North & South America.		Aizoaceae

Plant species		**Total samples collected:**
Montinia caryophyllacea Thunb.		51
Common name		**Sampled from:**
Pepper bush, wild clove bush Pepperbos, bergklapper		Slanghoek
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, Northern Cape), Namibia.		Montiniaceae

Plant species		**Total samples collected:**
Muraltia heisteria (L.) DC		10
Common name		**Sampled from:**
Spiky purple gorse Persdoringbos, kastybos, boeldokdoring, Voëltjie-kan-nie-sit-nie		Slanghoek
Distribution		**Family**
South Africa (Western Cape, Eastern Cape).		Polygalaceae

Plant species		**Total samples collected:**
Oncosiphon grandiflorum (Thunb.) Källersjö		59
Common name		**Sampled from:**
Matricaria Groot stinkkruid, knoppies-stinkkruid, stinkkruid		Robertson Vredendal
Distribution		**Family**
South Africa (Western Cape, Northern Cape), Southern Namibia.		Asteraceae

Plant species		**Total samples collected:**
Oncosiphon suffruticosum (L.) Källersjö		20
Common name		**Sampled from:**
Stinkingweed, Columbia daisy, shrubby mayweed Wurmkruid, wurmbos, miskruid, stinkkruid		Lutzville Vredendal
Distribution		**Family**
South Africa (Western Cape, Northern Cape), Southern Australia, parts of USA.		Asteraceae

Plant species		**Total samples collected:**
Osteospermum oppositifolium (Aiton) Norl.		10
Common name		**Sampled from:**
Stinkskaapbos, skaapbos, bietou		Lutzville
Distribution		**Family**
Southern & tropical Africa, north to Egypt, Arabian Peninsula & Jordan, South Africa (Western Cape, Northern Cape), Namibia.		Asteraceae

Plant species		**Total samples collected:**
Oxalis pes-caprae L.		9
Common name		**Sampled from:**
Bermuda buttercup, African wood-sorrel, buttercup oxalis, yellow sorrel, yellow oxalis, Cape sorrel, English weed, sourgrass, soursob Klawersuring, geelsuring, wildesuring, suring, tuinsuring		Lutzville
Distribution		**Family**
South Africa, Namibia, Australia, Europe, UK, tropical Asia, New Zealand, southern USA (California, Arizona, Florida).		Oxalidaceae

Plant species		**Total samples collected:**
Passerina corymbosa Eckl. ex C.H.Wright [synonym: *Passerina vulgaris* L.]		20
Common name		**Sampled from:**
Gonna bush, common cluster-flower Gonna Gonnabos, bakkerbos		Slanghoek
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, KwaZulu-Natal).		Thymelaceae

Plant species		**Total samples collected:**
Protea cynaroides (L.)		20
Common name		**Sampled from:**
King protea Koningsprotea, grootsuikerkan		Slanghoek
Distribution		**Family**
South Africa (Western Cape, Eastern Cape).		Proteaceae

Plant species		**Total samples collected:**
Pteronia incana (Burm.) DC.		55
Common name		**Sampled from:**
Blue bush, wild lavender Asbossie, bitterbos, bloubos, laventelbossie, perdebossie, ribbokbos		Slanghoek Robertson
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, Northern Cape).		Asteraceae

Plant species		**Total samples collected:**
Raphanus raphanistrum L.		25
Common name		**Sampled from:**
Wild radish, jointed charlock, runch Wilde radyse, ramenas		Robertson
Distribution		**Family**
Australia, Europe, Azores, Madeira, Canary Islands, Northern Africa, Southern Africa (i.e. South Africa), Western Asia.		Brassicaceae

Plant species		**Total samples collected:**
Rapistrum rugosum (L.) All.		25
Common name		**Sampled from:**
Annual bastard cabbage, common giant mustard, short fruited wild turnip, turnip weed, wrinkled gold-of-pleasure		Robertson Vredendal
Distribution		**Family**
Australia, Southern Europe, Azores, Madeira, Canary Islands, Northern Africa, Southern Africa (i.e. South Africa), Western Asia.		Brassicaceae

Plant species		**Total samples collected:**
Unidentified *Restio*		10
Common name		**Sampled from:**
N/a		Slanghoek
Distribution		**Family**
Restio genus: South Africa (Western Cape, Eastern Cape, Northern Cape, KwaZulu-Natal).		Restionaceae

Plant species		**Total samples collected:**
Roepera foetida (Schrad. & J.C.Wendl.) Beier & Thulin		35
Common name		**Sampled from:**
Syrian Beancaper, scrambling twinleaf Jakkalspisbos, slymbos, skilpadkos		Robertson
Distribution		**Family**
South Africa (Northern Cape, Western Cape, Eastern Cape), Namibia.		Zygophyllaceae

Plant species		**Total samples collected:**
Salsola kali L.		60
Common name		**Sampled from:**
Tumbleweed, roly-poly, saltwort, windwitch, prickly glasswort Kakiebos, taaibos, tolbos, steekblom		Robertson
Distribution		**Family**
Europe, Asia, Australia, North & South America, Northern Africa, Southern Africa (i.e. South Africa), New Zealand, etc.		Amaranthaceae

Plant species		**Total samples collected:**
Senecio burchellii DC.		19
Common name		**Sampled from:**
Ragwort, Burchell-senecio, Guanobush, Molteno disease plant, Madagascar grounsel, fireweed Geelgifbos, gifbossie, springkaanbossie		Robertson
Distribution		**Family**
South Africa (Western Cape, Eastern Cape, Northern Cape).		Asteraceae

Plant species		**Total samples collected:**
Sorocephalus pinifolius (Salisb. ex Knight) Rourke		24
Common name		**Sampled from:**
Long-leaf clusterhead Witkoppie		Slanghoek
Distribution		**Family**
South Africa (Western Cape).		Proteaceae

Plant species		**Total samples collected:**
Thesium lineatum L.f.		5
Common name		**Sampled from:**
Vaalstorm, witstorm		Slanghoek
Distribution		**Family**
South Africa (Western Cape, Northern Cape, Eastern Cape, Free State, North West), Namibia.		Santalaceae

Plant species		**Total samples collected:**
Vitis vinifera L.		2
Common name		**Sampled from:**
Common grapevine		Robertson
Distribution		**Family**
Mediterranean, central Europe, South Western Asia, Southern Germany, East & Northern Iran, South Africa (Western Cape), etc.		Vitaceae

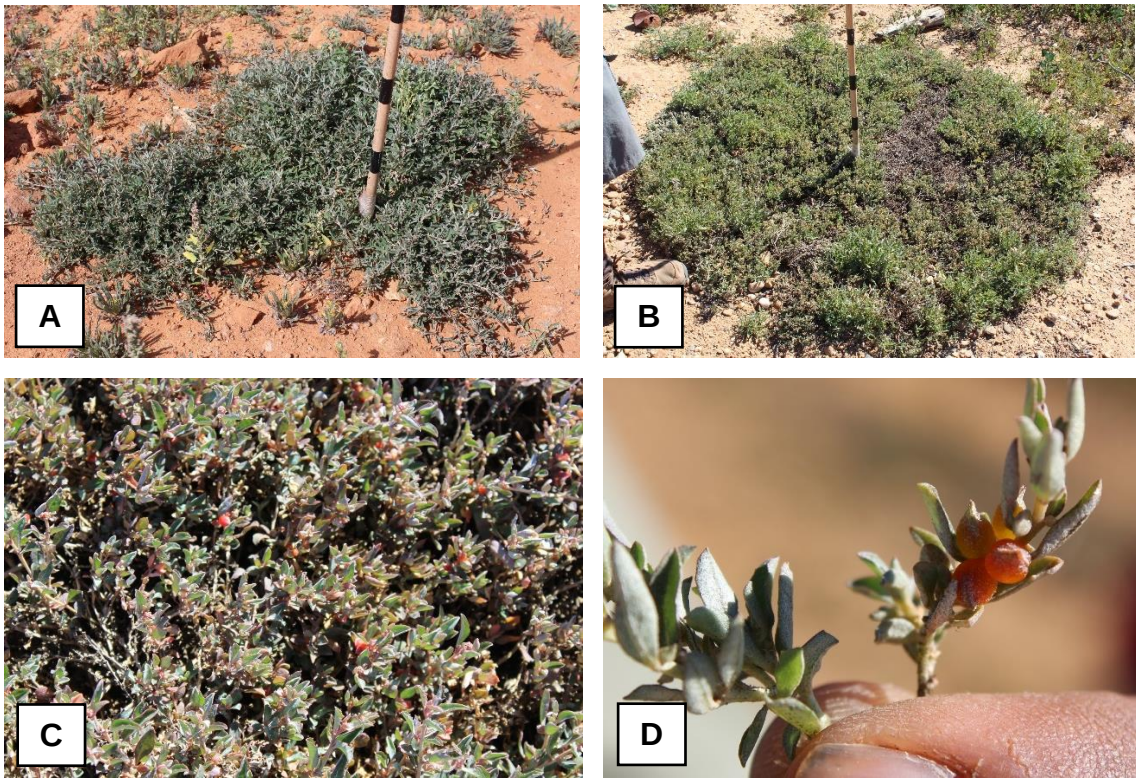


Figure A.5: *Atriplex semibaccata*, a plant species with the highest number of specimens yielding low Ct values in '*Candidatus* Liberibacter' specific real-time PCR tests during the study. (**A**) and (**B**) indicates the plant growth, close to the ground and in an outward direction. (**C**) and (**D**) indicates the red berries, the shape of the leaves and the colour of the leaves which are characteristic of *Atriplex semibaccata*.

Figure A.6: A plant species with a high number of specimens yielding low Ct values in '*Candidatus* Liberibacter' specific real-time PCR tests. The plant species is *Rapistrum rugosum.*

Figure A.7: A plant species with a high number of specimens yielding low Ct values in '*Candidatus* Liberibacter' specific real-time PCR tests. The plant species is *Lycium ferocissimum.*

Figure A.8: *Atriplex lindleyi* specimens with a high number of individuals yielding low Ct values in '*Candidatus* Liberibacter' specific real-time PCR tests. This species was shown to be most closely related to *A. farinosa* based on the *rbcL* sequence.

Figure A.9: *Atriplex nummularia* containing a high number of specimens yielding low Ct values in '*Candidatus* Liberibacter' specific real-time PCR tests. These plants grew next to and in-between vineyards in Lutzville.

Table A.4: *Specimen information and results obtained from real-time PCR assays and conventional PCR tests.*

Acc. Nr.	Host Species	GPS			Ct*	Conventional PCRs		
		Longitude (E)	Latitude (S)	Altitude		*16S*	*omp*	*rplJ*
17-6050	*P. cynaroides*	19.204532589	-33.554403249	326.350	-	-	-	-
17-6051	*P. cynaroides*	19.204420577	-33.554497529	328.289	-	-	-	-
17-6052	*P. cynaroides*	19.204373572	-33.554480666	330.112	-	-	-	-
17-6053	*P. cynaroides*	19.204390749	-33.554504941	329.426	-	-	-	-
17-6054	*P. cynaroides*	19.204371300	-33.554523092	332.645	-	-	-	-
17-6055	*P. cynaroides*	19.204380185	-33.554543491	332.547	-	-	-	-
17-6056	*P. cynaroides*	19.204480229	-33.554581283	333.104	-	-	-	-
17-6057	*P. cynaroides*	19.204513215	-33.554577637	332.285	-	-	-	-
17-6058	*P. cynaroides*	19.204526210	-33.554548116	328.697	-	-	-	-
17-6059	*P. cynaroides*	19.204536244	-33.554529749	329.276	-	-	-	-
17-6060	*P. cynaroides*	19.204549485	-33.554610060	332.408	-	-	-	-
17-6061	*P. cynaroides*	19.204577049	-33.554618812	331.484	-	-	-	-
17-6062	*P. cynaroides*	19.204573416	-33.554640976	331.815	-	-	-	-
17-6063	*P. cynaroides*	19.204555463	-33.554655705	332.792	-	-	-	-
17-6064	*P. cynaroides*	19.204497255	-33.554615328	335.132	-	-	-	-
17-6065	*P. cynaroides*	19.204477039	-33.554607934	334.067	-	-	-	-

17-6066	*P. cynaroides*	19.204434459	-33.554691966	337.200	-	-	-	-
17-6067	*P. cynaroides*	19.204398130	-33.554700609	337.273	-	-	-	-
17-6068	*P. cynaroides*	19.204418885	-33.554598392	332.671	-	-	-	-
17-6069	*P. cynaroides*	19.204485405	-33.554512360	328.880	-	-	-	-
17-6070	*M. caryophyllacea*	-	-	-	-	-	-	-
17-6071	*M. caryophyllacea*	19.204657626	-33.554429207	323.198	-	-	-	-
17-6072	*M. caryophyllacea*	19.204720054	-33.554464942	322.241	-	-	-	-
17-6073	*M. caryophyllacea*	19.204709347	-33.554500931	323.983	-	-	-	-
17-6074	*M. caryophyllacea*	19.204742632	-33.554565078	325.170	-	-	-	-
17-6075	*M. caryophyllacea*	19.204695653	-33.554550943	326.451	-	-	-	-
17-6076	*M. caryophyllacea*	19.204541587	-33.554566398	329.820	-	-	-	-
17-6077	*M. caryophyllacea*	19.204550894	-33.554579473	330.162	-	-	-	-
17-6078	*M. caryophyllacea*	19.204648106	-33.554608612	328.959	-	-	-	-
17-6079	*M. caryophyllacea*	19.204619015	-33.554485975	326.022	-	-	-	-
17-6080	*M. caryophyllacea*	19.204622171	-33.554500277	326.810	-	-	-	-
17-6081	*M. caryophyllacea*	19.204596226	-33.554496450	327.422	-	-	-	-
17-6082	*M. caryophyllacea*	19.204594337	-33.554505385	327.636	-	-	-	-
17-6083	*M. caryophyllacea*	19.204568430	-33.554511715	326.565	-	-	-	-
17-6084	*M. caryophyllacea*	19.204588035	-33.554520207	328.233	-	-	-	-
17-6085	*M. caryophyllacea*	-	-	-	-	-	-	-

17-6086	*M. caryophyllacea*	19.204575853	-33.554553050	328.562	-	-	-	-
17-6087	*M. caryophyllacea*	19.204612479	-33.554558718	327.768	-	-	-	-
17-6088	*M. caryophyllacea*	19.204606741	-33.554578954	328.128	-	-	-	-
17-6089	*M. caryophyllacea*	19.204637214	-33.554627089	329.498	-	-	-	-
17-6090	*M. caryophyllacea*	-	-	-	-	-	-	-
17-6091	*A. semibaccata*	19.684432412	-33.831866680	233.503	-	-	-	-
17-6092	*A. semibaccata*	19.684464045	-33.831797996	229.727	-	-	-	-
17-6093	*A. africanum*	19.684395345	-33.831766963	232.479	-	-	-	-
17-6094	*A. africanum*	19.684414987	-33.831706272	231.792	-	-	-	-
17-6095	*A. africanum*	19.684428961	-33.831676990	230.407	-	-	-	-
17-6096	*A. africanum*	19.684451384	-33.831605308	231.457	-	-	-	-
17-6097	*A. africanum*	19.684495403	-33.831575571	231.155	-	-	-	-
17-6098	*A. africanum*	19.684615822	-33.831637608	229.516	-	-	-	-
17-6099	*A. africanum*	19.684582261	-33.831666821	230.029	-	-	-	-
17-6100	*A. africanum*	19.684560029	-33.831726287	230.586	-	-	-	-
17-6101	*A. africanum*	19.684502604	-33.831689067	232.910	-	-	-	-
17-6102	*A. africanum*	19.684451515	-33.831757279	232.451	-	-	-	-
17-6103	*A. africanum*	19.684425863	-33.831885855	234.232	-	-	-	-
17-6104	*E. speciosissimus*	19.206547525	-33.555214660	320.524	29,72	-	-	-
17-6105	*E. speciosissimus*	19.206561296	-33.555237487	321.161	-	-	-	-

17-6106	*E. speciosissimus*	19.206578979	-33.555233849	322.103	-	-	-	-
17-6107	*E. speciosissimus*	19.206574332	-33.555172350	322.227	-	-	-	-
17-6108	*E. speciosissimus*	19.206515457	-33.555182067	322.580	-	-	-	-
17-6109	*E. speciosissimus*	19.206507311	-33.555167830	322.840	-	-	-	-
17-6110	*E. speciosissimus*	19.206473582	-33.555175680	321.235	-	-	-	-
17-6111	*E. speciosissimus*	19.206463063	-33.555108393	323.420	-	-	-	-
17-6112	*E. speciosissimus*	19.206378159	-33.555093401	323.340	-	-	-	-
17-6113	*E. speciosissimus*	19.206334371	-33.555145494	323.681	-	-	-	-
17-6114	*E. speciosissimus*	19.206424444	-33.555145347	323.289	-	-	-	-
17-6115	*E. speciosissimus*	19.206450328	-33.555246601	321.627	-	-	-	-
17-6116	*E. speciosissimus* (sick)	19.206359802	-33.554984304	323.170	-	-	-	-
17-6117	*E. speciosissimus*	19.206406069	-33.555274768	321.744	-	-	-	-
17-6118	*E. speciosissimus*	19.206390279	-33.555281113	322.798	-	-	-	-
17-6119	*E. speciosissimus*	19.206584761	-33.555246326	321.085	-	-	-	-
17-6120	*E. speciosissimus*	19.206617910	-33.555255353	320.669	26,81	-	-	-
17-6121	*E. speciosissimus*	19.206583667	-33.555296767	320.646	-	-	-	-
17-6122	*E. speciosissimus*	19.206324063	-33.555096541	323.300	-	-	-	-
17-6123	*E. speciosissimus*	19.206567575	-33.555206006	322.337	-	-	-	-
17-6124	*M. caryophyllacea*	19.206694446	-33.555209256	321.949	-	-	-	-

17-6125	*M. caryophyllacea*	19.206678648	-33.555189240	323.796	-	-	-	-
17-6126	*M. caryophyllacea*	19.206679782	-33.555196421	321.486	-	-	-	-
17-6127	*M. caryophyllacea*	19.206629138	-33.555201653	322.309	-	-	-	-
17-6128	*M. caryophyllacea*	19.206612079	-33.555212172	322.096	-	-	-	-
17-6129	*M. caryophyllacea*	19.206628474	-33.555256866	320.880	-	-	-	-
17-6130	*M. caryophyllacea*	19.206603263	-33.555244120	322.670	-	-	-	-
17-6131	*M. caryophyllacea*	19.206587272	-33.555231157	322.599	-	-	-	-
17-6132	*M. caryophyllacea*	19.206587009	-33.555207052	322.278	-	-	-	-
17-6133	*M. caryophyllacea*	19.206523612	-33.555207232	321.768	-	-	-	-
17-6134	*M. caryophyllacea*	19.206546166	-33.555177950	323.503	-	-	-	-
17-6135	*M. caryophyllacea*	19.206595977	-33.555114432	325.300	-	-	-	-
17-6136	*M. caryophyllacea*	19.206579275	-33.555106624	325.568	-	-	-	-
17-6137	*M. caryophyllacea*	19.206470884	-33.555208711	321.781	-	-	-	-
17-6138	*M. caryophyllacea*	19.206473991	-33.555201840	321.714	-	-	-	-
17-6139	*M. caryophyllacea*	19.206444279	-33.555188254	323.689	-	-	-	-
17-6140	*M. caryophyllacea*	19.206440684	-33.555156625	323.052	-	-	-	-
17-6141	*M. caryophyllacea*	19.206416305	-33.555142398	324.037	-	-	-	-
17-6142	*M. caryophyllacea*	19.206466822	-33.555098153	323.359	-	-	-	-
17-6143	*M. caryophyllacea*	19.206419361	-33.555114770	323.780	-	-	-	-
17-6144	*M. heisteria*	19.206733182	-33.555229647	321.607	-	-	-	-

17-6145	*M. heisteria*	19.206755442	-33.555269755	320.395	-	-	-	-
17-6146	*M. heisteria*	19.206750131	-33.555277830	320.618	-	-	-	-
17-6147	*M. heisteria*	19.206736891	-33.555302298	319.143	-	-	-	-
17-6148	*M. heisteria*	19.206730643	-33.555287275	319.536	-	-	-	-
17-6149	*S. pinifolius*	19.206473926	-33.555355412	319.824	-	-	-	-
17-6150	*S. pinifolius*	19.206468945	-33.555364106	319.740	-	-	-	-
17-6151	*S. pinifolius*	19.206465231	-33.555394166	319.692	-	-	-	-
17-6152	*S. pinifolius*	19.206483205	-33.555429367	318.787	-	-	-	-
17-6153	*S. pinifolius*	19.206478161	-33.555475733	315.940	-	-	-	-
17-6154	*S. pinifolius*	19.206459065	-33.555498781	315.913	-	-	-	-
17-6155	*S. pinifolius*	19.206484347	-33.555509977	315.839	-	-	-	-
17-6156	*S. pinifolius*	19.206552181	-33.555513111	315.325	-	-	-	-
17-6157	*S. pinifolius*	19.206569497	-33.555495296	315.177	-	-	-	-
17-6158	*S. pinifolius*	19.206591821	-33.555506041	314.785	-	-	-	-
17-6159	*S. pinifolius*	19.206591144	-33.555520382	314.942	-	-	-	-
17-6160	*S. pinifolius*	19.206531489	-33.555540537	314.850	-	-	-	-
17-6161	*S. pinifolius*	19.206474214	-33.555548131	316.940	28,66	-	-	-
17-6162	*S. pinifolius*	19.206432640	-33.555574633	316.010	-	-	-	-
17-6163	*P. incana*	19.208774144	-33.557733223	286.477	-	-	-	-
17-6164	*P. incana*	19.208783070	-33.557710927	285.449	-	-	-	-

17-6165	*P. incana*	19.208801566	-33.557755080	287.318	-	-	-	-
17-6166	*P. incana*	19.208831196	-33.557737147	287.042	-	-	-	-
17-6167	*P. incana*	19.208848840	-33.557756238	287.811	-	-	-	-
17-6168	*P. incana*	19.208884329	-33.557758026	287.986	-	-	-	-
17-6169	*P. incana*	19.208872123	-33.557733746	287.056	-	-	-	-
17-6170	*P. incana*	19.208865631	-33.557678188	285.398	-	-	-	-
17-6171	*P. incana*	19.208829308	-33.557691494	284.843	-	-	-	-
17-6172	*P. incana*	19.208719432	-33.557700682	285.289	-	-	-	-
17-6173	*H. sericea*	19.208802880	-33.557695674	285.876	-	-	-	-
17-6174	*H. sericea*	19.208831304	-33.557735418	286.611	-	-	-	-
17-6175	*H. sericea*	19.208830934	-33.557714193	286.439	-	-	-	-
17-6176	*H. sericea*	19.208842333	-33.557673919	285.273	-	-	-	-
17-6177	*H. sericea*	19.208846762	-33.557773980	289.078	-	-	-	-
17-6178	*H. sericea*	19.208940213	-33.557830882	291.177	-	-	-	-
17-6179	*H. sericea*	19.208984615	-33.557840150	290.962	-	-	-	-
17-6180	*H. sericea*	19.209043143	-33.557861283	292.703	-	-	-	-
17-6181	*H. sericea*	19.209030129	-33.557839983	290.894	-	-	-	-
17-6182	*H. sericea*	19.209003782	-33.557858752	292.299	-	-	-	-
17-6183	*T. lineatum*	19.208753696	-33.558031704	294.688	-	-	-	-
17-6184	*T. lineatum*	19.208729134	-33.558024056	293.171	-	-	-	-

17-6185	*T. lineatum* (witches' broom)	19.208720929	-33.558025682	293.218	-	-	-	-
17-6186	*T. lineatum*	19.208688145	-33.558106822	292.711	-	-	-	-
17-6187	*T. lineatum*	19.208668152	-33.558095197	294.844	-	-	-	-
17-6188	*L. tinctum*	19.208730335	-33.558100340	294.307	-	-	-	-
17-6189	*L. tinctum*	19.208704045	-33.558117780	295.666	-	-	-	-
17-6190	*L. tinctum*	19.208671717	-33.558166205	294.725	-	-	-	-
17-6191	*L. tinctum*	19.208871868	-33.558107772	299.432	-	-	-	-
17-6192	*L. tinctum* (female)	19.208885740	-33.558079386	300.975	-	-	-	-
17-6193	*M. caryophyllacea*	19.208847191	-33.558045595	297.256	-	-	-	-
17-6194	*M. caryophyllacea*	19.208888479	-33.558103147	297.704	-	-	-	-
17-6195	*M. caryophyllacea*	19.208829160	-33.558152520	297.086	-	-	-	-
17-6196	*M. caryophyllacea*	19.208822579	-33.558192697	298.937	-	-	-	-
17-6197	*M. caryophyllacea*	19.208814057	-33.558239097	299.956	-	-	-	-
17-6198	*M. caryophyllacea*	19.208826915	-33.558249872	299.594	-	-	-	-
17-6199	*M. caryophyllacea*	19.208897289	-33.558219756	300.898	-	-	-	-
17-6200	*M. caryophyllacea*	19.208933391	-33.558244916	301.029	-	-	-	-
17-6201	*M. caryophyllacea*	19.208978629	-33.558249352	301.422	-	-	-	-
17-6202	*M. caryophyllacea*	19.208994754	-33.558273756	302.171	-	-	-	-

17-6203	*M. heisteria*	19.208980454	-33.558272357	302.417	-	-	-	-
17-6204	*M. heisteria*	19.208868378	-33.558304319	301.213	-	-	-	-
17-6205	*M. heisteria*	19.208859194	-33.558287156	299.501	-	-	-	-
17-6206	*M. heisteria*	19.208850194	-33.558282487	299.316	-	-	-	-
17-6207	*M. heisteria*	19.208783162	-33.558025239	294.374	-	-	-	-
17-6208	*P. corymbosa*	19.208716405	-33.557231110	280.641	-	-	-	-
17-6209	*P. corymbosa*	19.208748140	-33.557253654	278.783	-	-	-	-
17-6210	*P. corymbosa*	19.208794411	-33.557269691	279.425	-	-	-	-
17-6211	*P. corymbosa*	19.208697888	-33.557313188	280.438	-	-	-	-
17-6212	*P. corymbosa*	19.208709296	-33.557331014	280.120	-	-	-	-
17-6213	*P. corymbosa*	19.208744164	-33.557333273	280.116	-	-	-	-
17-6214	*P. corymbosa*	19.208739788	-33.557266441	279.319	-	-	-	-
17-6215	*P. corymbosa*	19.208668676	-33.557323320	280.933	-	-	-	-
17-6216	*P. corymbosa*	19.208674995	-33.557427499	282.405	-	-	-	-
17-6217	*P. corymbosa*	19.208645483	-33.557454438	282.018	-	-	-	-
17-6218	*P. corymbosa*	19.208581432	-33.557501787	282.755	-	-	-	-
17-6219	*P. corymbosa*	19.208588977	-33.557556428	282.943	-	-	-	-
17-6220	*P. corymbosa*	19.208538944	-33.557563152	282.686	-	-	-	-
17-6221	*P. corymbosa*	19.208514334	-33.557632313	283.548	-	-	-	-
17-6222	*P. corymbosa*	19.208509642	-33.557588424	283.807	-	-	-	-

17-6223	*P. corymbosa*	19.208500444	-33.557886297	286.962	-	-	-	-
17-6224	*P. corymbosa*	19.208447650	-33.557877976	287.372	-	-	-	-
17-6225	*P. corymbosa*	19.208451285	-33.557921461	287.620	-	-	-	-
17-6226	*P. corymbosa*	19.208457546	-33.557953604	285.917	-	-	-	-
17-6227	*P. corymbosa*	19.208423719	-33.557960429	286.666	-	-	-	-
17-6228	*C. odorata*	19.208428464	-33.557969594	288.050	-	-	-	-
17-6229	*C. odorata*	19.208507174	-33.557897336	287.142	-	-	-	-
17-6230	*C. odorata*	19.208534836	-33.557819076	287.131	-	-	-	-
17-6231	*C. odorata*	19.208532639	-33.557706747	286.315	-	-	-	-
17-6232	*C. odorata*	19.208539296	-33.557680112	284.612	-	-	-	-
17-6233	*C. odorata*	19.208522860	-33.557597481	284.493	-	-	-	-
17-6234	*C. odorata*	19.208548716	-33.557554521	284.648	-	-	-	-
17-6235	*C. odorata*	19.208601007	-33.557560562	283.054	-	-	-	-
17-6236	*C. odorata*	19.208642598	-33.557511962	282.515	-	-	-	-
17-6237	*C. odorata*	19.208656771	-33.557441039	281.785	-	-	-	-
17-6238	*D. viscosa*	19.209667830	-33.557504836	281.206	-	-	-	-
17-6239	*D. viscosa*	19.209671203	-33.557495026	281.825	-	-	-	-
17-6240	*D. viscosa*	19.209635740	-33.557461189	280.961	-	-	-	-
17-6241	*D. viscosa*	19.209594269	-33.557491317	280.640	-	-	-	-
17-6242	*D. viscosa*	19.209583070	-33.557408918	279.398	-	-	-	-

17-6243	Unidentified *Restio*	19.209715835	-33.557544882	281.234	-	-	-	-
17-6244	Unidentified *Restio*	19.209696100	-33.557532773	281.171	-	-	-	-
17-6245	Unidentified *Restio*	19.209700945	-33.557549234	281.685	-	-	-	-
17-6246	Unidentified *Restio*	19.209679406	-33.557613955	283.562	-	-	-	-
17-6247	Unidentified *Restio*	19.209645584	-33.557573612	283.130	-	-	-	-
17-6248	Unidentified *Restio*	19.209653174	-33.557604783	283.032	-	-	-	-
17-6249	Unidentified *Restio*	19.209759998	-33.557589650	282.070	-	-	-	-
17-6250	Unidentified *Restio*	19.209823714	-33.557555746	281.725	-	-	-	-
17-6251	Unidentified *Restio*	19.209850174	-33.557605822	283.764	-	-	-	-
17-6252	Unidentified *Restio*	19.209880077	-33.557560520	281.430	-	-	-	-
17-6253	*S. pinifolius*	19.209981325	-33.557537928	280.322	-	-	-	-
17-6254	*S. pinifolius*	19.209984099	-33.557563675	281.479	-	-	-	-
17-6255	*S. pinifolius* (yellow)	19.210023876	-33.557614835	284.651	-	-	-	-
17-6256	*S. pinifolius*	19.210017406	-33.557627836	283.752	-	-	-	-
17-6257	*S. pinifolius*	19.210051240	-33.557621444	283.756	-	-	-	-
17-6258	*S. pinifolius*	19.210074500	-33.557612792	283.607	-	-	-	-
17-6259	*S. pinifolius* (yellow)	19.210050442	-33.557666860	284.837	-	-	-	-
17-6260	*S. pinifolius*	19.210069148	-33.557681134	286.653	-	-	-	-

17-6261	*S. pinifolius*	19.210123581	-33.557665119	286.002	-	-	-	-
17-6262	*S. pinifolius*	19.692364412	-33.828341050	199.365	-	-	-	-
17-6263	*O. grandiflorum*	19.692375206	-33.828286758	200.777	-	-	-	-
17-6264	*O. grandiflorum*	19.692353560	-33.828193990	200.880	-	-	-	-
17-6265	*O. grandiflorum*	19.692345971	-33.828162058	200.295	-	-	-	-
17-6267	*O. grandiflorum*	19.692353543	-33.828122578	200.327	-	-	-	-
17-6268	*O. grandiflorum*	19.692376323	-33.828066784	200.489	-	-	-	-
17-6269	*O. grandiflorum*	19.692430172	-33.828020794	200.533	-	-	-	-
17-6270	*O. grandiflorum*	19.692473301	-33.828083524	200.826	-	-	-	-
17-6271	*O. grandiflorum*	19.692498966	-33.828129890	200.829	-	-	-	-
17-6272	*O. grandiflorum*	19.692494647	-33.828150157	201.011	-	-	-	-
17-6273	*O. grandiflorum*	19.692489931	-33.828180290	200.876	-	-	-	-
17-6274	*O. grandiflorum*	19.692388728	-33.828138202	200.996	-	-	-	-
17-6275	*O. grandiflorum*	19.692416526	-33.828357133	201.754	-	-	-	-
17-6276	*O. grandiflorum*	19.692402589	-33.828426450	201.446	-	-	-	-
17-6277	*O. grandiflorum*	19.692460040	-33.828542755	201.533	-	-	-	-
17-6278	*O. grandiflorum*	19.692464248	-33.828568313	200.796	-	-	-	-
17-6279	*O. grandiflorum*	19.692480155	-33.828638012	200.575	-	-	-	-
17-6280	*O. grandiflorum*	19.692440122	-33.828662741	201.888	-	-	-	-
17-6281	*O. grandiflorum*	19.692487711	-33.828798950	200.458	-	-	-	-

17-6282	*O. grandiflorum*	19.692510445	-33.828923516	200.377	-	-	-	-
17-6283	*A. menziesii*	19.692441443	-33.828980547	200.128	-	-	-	-
17-6284	*A. menziesii*	19.692515089	-33.829048091	201.685	-	-	-	-
17-6285	*A. menziesii*	19.692474530	-33.829102013	200.742	-	-	-	-
17-6286	*A. menziesii*	19.692534002	-33.829097506	201.099	-	-	-	-
17-6287	*A. menziesii*	19.692529517	-33.829123062	200.890	-	-	-	-
17-6288	*A. menziesii*	19.692551376	-33.829187809	199.650	-	-	-	-
17-6289	*A. menziesii*	19.692564936	-33.829224175	199.937	-	-	-	-
17-6290	*A. menziesii*	19.692493183	-33.829293728	199.020	-	-	-	-
17-6291	*A. menziesii*	19.692599984	-33.829357682	197.730	-	-	-	-
17-6292	*A. menziesii*	19.692597567	-33.829401000	200.711	-	-	-	-
17-6293	*A. menziesii*	19.692606277	-33.829444694	201.692	-	-	-	-
17-6294	*A. menziesii*	19.692555401	-33.829509698	201.253	-	-	-	-
17-6295	*A. menziesii*	19.692551305	-33.829554861	200.700	-	-	-	-
17-6296	*A. menziesii*	19.692645285	-33.829552083	200.573	-	-	-	-
17-6297	*A. menziesii*	19.692690776	-33.829838094	200.502	-	-	-	-
17-6298	*A. menziesii*	19.692749726	-33.829971857	200.115	-	-	-	-
17-6299	*A. menziesii*	19.692686897	-33.830094447	200.033	-	-	-	-
17-6300	*A. menziesii*	19.692713007	-33.830314846	200.009	-	-	-	-
17-6301	*A. menziesii*	19.692956082	-33.831272603	200.391	-	-	-	-

17-6302	*A. menziesii*	19.693436600	-33.831674364	201.104	-	-	-	-
17-6303	*R. raphanistrum*	19.692302989	-33.828312614	199.172	-	-	-	-
17-6304	*R. raphanistrum*	19.692268079	-33.828286839	199.950	-	-	-	-
17-6305	*R. raphanistrum*	19.692322730	-33.828364013	199.757	-	-	-	-
17-6306	*R. raphanistrum*	19.692182145	-33.828414598	200.147	-	-	-	-
17-6307	*R. raphanistrum*	19.692315324	-33.828541348	200.159	-	-	-	-
17-6308	*R. raphanistrum*	19.692413491	-33.829021514	200.627	-	-	-	-
17-6309	*R. raphanistrum*	19.692412169	-33.829265331	200.355	29,53	-	-	-
17-6310	*R. raphanistrum*	19.692537577	-33.829574463	201.438	-	-	-	-
17-6311	*R. raphanistrum*	19.692468033	-33.829536139	201.208	28,48	-	-	-
17-6312	*R. raphanistrum*	19.692570261	-33.829598367	200.587	-	-	-	-
17-6313	*R. raphanistrum* (water)	19.692787465	-33.831026596	199.751	-	-	-	-
17-6314	*R. raphanistrum* (water)	19.692818673	-33.831056954	199.774	-	-	-	-
17-6315	*R. raphanistrum* (water)	19.692983209	-33.831070799	197.585	-	-	-	-
17-6316	*R. raphanistrum* (water)	19.692905987	-33.831129854	198.365	27,44	-	-	-
17-6317	*R. raphanistrum*	19.692832616	-33.831026306	200.747	-	-	-	-

	(water)							
17-6318	*R. raphanistrum* (AY block)	19.689265959	-33.827886736	201.479	-	-	-	-
17-6319	*R. raphanistrum* (AY block)	19.689325095	-33.827885321	201.799	-	-	-	-
17-6320	*R. raphanistrum* (AY block)	19.689132637	-33.827941806	202.519	27,69	+**	-	-
17-6321	*R. raphanistrum* (AY block)	19.689228334	-33.828014100	202.030	-	-	-	-
17-6322	*R. raphanistrum* (AY block)	19.689301967	-33.828030246	202.431	-	-	-	-
17-6323	*R. raphanistrum* (AY block)	19.689320525	-33.828052526	201.610	-	-	-	-
17-6324	*R. raphanistrum* (AY block)	19.689170258	-33.828028386	201.653	-	-	-	-
17-6325	*R. raphanistrum* (AY block)	19.689117194	-33.828068296	203.067	-	-	-	-
17-6326	*R. raphanistrum* (AY block)	19.689160898	-33.828165286	203.656	-	-	-	-
17-6327	*R. raphanistrum*	19.688871696	-33.828185844	203.811	-	-	-	-

	(AY block)							
17-6328	*R. raphanistrum*	19.688802743	-33.827980549	203.148	-	-	-	-
17-6329	*R. rugosum*	19.688821427	-33.827913401	201.736	-	-	-	-
17-6330	*R. rugosum*	19.688829562	-33.827870679	202.835	-	-	-	-
17-6331	*R. rugosum*	19.688843111	-33.827801251	201.686	-	-	-	-
17-6332	*R. rugosum*	19.688938117	-33.827460140	201.870	-	-	-	-
17-6333	*D. speciosum*	19.684618077	-33.831575497	227.667	-	-	-	-
17-6334	*D. speciosum*	19.684555927	-33.831567131	229.041	-	-	-	-
17-6335	*D. speciosum*	19.684467674	-33.831596557	231.090	-	-	-	-
17-6336	*D. speciosum*	19.684442942	-33.831633004	231.182	-	-	-	-
17-6337	*D. speciosum*	19.684303394	-33.831990997	234.814	-	-	-	-
17-6338	*D. speciosum*	19.684245033	-33.832095658	235.722	16,42	-	-	-
17-6339	*D. speciosum*	19.684242309	-33.832154256	237.079	-	-	-	-
17-6340	*D. speciosum*	19.684230438	-33.832187203	237.033	-	-	-	-
17-6341	*D. speciosum*	19.684201176	-33.832233207	237.349	-	-	-	-
17-6342	*D. speciosum*	19.684181881	-33.832302442	238.029	-	-	-	-
17-6343	*H. cymosum*	19.684153500	-33.832339451	237.899	-	-	-	-
17-6344	*H. cymosum*	19.684100431	-33.832438519	238.569	-	-	-	-
17-6345	*H. cymosum*	19.684062548	-33.832534779	238.854	-	-	-	-
17-6346	*H. cymosum*	19.684166547	-33.832314171	239.272	-	-	-	-

17-6347	*H. cymosum*	19.684246750	-33.832137394	237.794	-	-	-	-
17-6348	*A. lindleyi*	19.684344737	-33.832104906	235.639	-	-	-	-
17-6349	*A. lindleyi*	19.684365169	-33.831946920	236.502	-	-	-	-
17-6350	*A. lindleii*	19.684405276	-33.831921977	233.873	-	-	-	-
17-6351	*P. incana*	19.862443557	-33.797398186	247.144	-	-	-	-
17-6352	*P. incana*	19.862391700	-33.797393580	247.014	-	-	-	-
17-6353	*P. incana*	19.862348887	-33.797410013	246.299	-	-	-	-
17-6354	*P. incana*	19.862324319	-33.797419170	246.424	-	-	-	-
17-6355	*P. incana*	19.862606292	-33.797278075	248.081	-	-	-	-
17-6356	*P. incana*	19.862572889	-33.797346393	249.599	-	-	-	-
17-6357	*P. incana*	19.862556419	-33.797371726	248.821	-	-	-	-
17-6358	*P. incana*	19.862638136	-33.797185752	249.468	-	-	-	-
17-6359	*P. incana*	19.862698678	-33.797162594	250.103	-	-	-	-
17-6360	*P. incana*	19.862743624	-33.797157188	250.901	-	-	-	-
17-6361	*P. incana*	19.862851468	-33.797172578	251.976	-	-	-	-
17-6362	*P. incana*	19.862316038	-33.797447250	246.399	-	-	-	-
17-6363	*P. incana*	19.862347442	-33.797475197	247.762	-	-	-	-
17-6364	*P. incana*	19.862405116	-33.797428191	246.529	-	-	-	-
17-6365	*P. incana*	19.862360075	-33.797527572	246.775	-	-	-	-
17-6366	*P. incana*	19.862400432	-33.797521665	247.389	-	-	-	-

17-6367	*P. incana*	19.862372802	-33.797369872	248.176	-	-	-	-
17-6368	*P. incana*	19.862564271	-33.797190607	249.068	-	-	-	-
17-6369	*P. incana*	19.862672356	-33.797165019	249.567	-	-	-	-
17-6370	*P. incana*	19.862625028	-33.797191622	248.741	-	-	-	-
17-6371	*H. cymosum*	19.862159877	-33.797730031	246.409	-	-	-	-
17-6372	*H. cymosum*	19.862161056	-33.797770868	245.332	-	-	-	-
17-6373	*H. cymosum*	19.862174865	-33.797797066	246.612	-	-	-	-
17-6374	*H. cymosum*	19.862182792	-33.797750541	248.118	-	-	-	-
17-6375	*H. cymosum*	19.862177409	-33.797561586	247.682	-	-	-	-
17-6376	*D. australe* subsp. *australe*	19.861937503	-33.798274277	245.664	-	-	-	-
17-6377	*D. australe* subsp. *australe*	19.861938116	-33.798241290	244.407	-	-	-	-
17-6378	*D. australe* subsp. *australe*	19.861936967	-33.798247874	242.548	-	-	-	-
17-6379	*D. australe* subsp. *australe*	19.861967275	-33.798204653	246.853	-	-	-	-
17-6380	*D. australe* subsp. *australe*	19.862088487	-33.798051662	246.755	-	-	-	-

17-6381	*D. australe* subsp. *australe*	19.862119499	-33.797952288	244.199	-	-	-	-
17-6382	*D. australe* subsp. *australe*	19.862141561	-33.797951469	246.368	-	-	-	-
17-6383	*D. australe* subsp. *australe*	19.862132939	-33.797915235	245.304	-	-	-	-
17-6384	*D. australe* subsp. *australe*	19.862140507	-33.797673199	244.679	-	-	-	-
17-6385	*D. australe* subsp. *australe*	19.862166834	-33.797938103	244.826	-	-	-	-
17-6386	*A. africanum*	19.861914754	-33.798270834	245.187	-	-	-	-
17-6387	*A. africanum*	19.861897690	-33.798301949	244.534	-	-	-	-
17-6388	*A. africanum*	19.861884622	-33.798329406	245.183	-	-	-	-
17-6389	*A. africanum*	19.861873613	-33.798394292	245.066	-	-	-	-
17-6390	*A. africanum*	19.861814697	-33.798462842	244.259	-	-	-	-
17-6391	*A. africanum*	19.861826057	-33.798488157	245.470	-	-	-	-
17-6392	*A. africanum*	19.861786029	-33.798556721	244.074	-	-	-	-
17-6393	*A. africanum*	19.861728128	-33.798781065	244.259	-	-	-	-
17-6394	*A. africanum*	19.861696812	-33.798912003	243.220	-	-	-	-
17-6395	*A. africanum*	19.861630452	-33.799040379	242.722	-	-	-	-

17-6396	*R. foetida*	19.861564027	-33.799129378	243.168	-	-	-	-
17-6397	*R. foetida*	19.861349637	-33.799355408	243.892	-	-	-	-
17-6398	*R. foetida*	19.861652109	-33.799141677	248.145	-	-	-	-
17-6399	*R. foetida*	19.861709792	-33.799075684	245.280	-	-	-	-
17-6400	*R. foetida*	19.861707399	-33.799047269	244.739	-	-	-	-
17-6401	*R. foetida*	19.861824213	-33.798522629	245.891	-	-	-	-
17-6402	*R. foetida*	19.861743969	-33.798575018	244.943	-	-	-	-
17-6403	*R. foetida*	19.861818460	-33.798561996	246.729	-	-	-	-
17-6404	*R. foetida*	19.861867105	-33.798465013	245.940	-	-	-	-
17-6405	*R. foetida*	19.861867245	-33.798451152	244.401	-	-	-	-
17-6406	*R. foetida*	19.861854331	-33.798425747	243.689	-	-	-	-
17-6407	*R. foetida*	19.861932927	-33.798323788	244.032	-	-	-	-
17-6408	*R. foetida*	19.862109985	-33.797998085	243.307	-	-	-	-
17-6409	*R. foetida*	19.862167054	-33.797847411	244.731	-	-	-	-
17-6410	*R. foetida*	19.862163178	-33.797858635	246.517	-	-	-	-
17-6411	*D. hispidum*	19.859276050	-33.799229081	227.771	-	-	-	-
17-6412	*D. hispidum*	19.859286513	-33.799213441	227.506	-	-	-	-
17-6413	*D. hispidum*	19.859331560	-33.799205042	229.947	-	-	-	-
17-6414	*D. hispidum*	19.859313760	-33.799196737	230.280	-	-	-	-
17-6415	*D. hispidum*	19.859335605	-33.799244064	228.992	-	-	-	-

17-6416	*D. hispidum*	19.859393495	-33.799278091	230.062	-	-	-	-
17-6417	*D. hispidum*	19.859432365	-33.799351987	229.907	-	-	-	-
17-6418	*D. hispidum*	19.859438466	-33.799372314	228.548	25,76	-	-	-
17-6419	*D. hispidum*	19.859444409	-33.799386599	229.793	-	-	-	-
17-6420	*D. hispidum*	19.859408982	-33.799464685	229.447	-	-	-	-
17-6421	*P. incana*	19.859445470	-33.799496392	230.661	-	-	-	-
17-6422	*P. incana*	19.859520838	-33.799334957	231.234	-	-	-	-
17-6423	*P. incana*	19.859527369	-33.799348579	231.331	-	-	-	-
17-6424	*P. incana*	19.859498684	-33.799308636	232.097	-	-	-	-
17-6425	*P. incana*	19.859468811	-33.799228682	232.340	-	-	-	-
17-6426	*P. incana*	19.859389982	-33.799218012	231.604	-	-	-	-
17-6427	*P. incana*	19.859402150	-33.799168332	231.355	-	-	-	-
17-6428	*P. incana*	19.859358559	-33.799185682	232.901	-	-	-	-
17-6429	*P. incana*	19.859331719	-33.799107383	232.323	-	-	-	-
17-6430	*P. incana*	19.859185943	-33.799046972	232.308	-	-	-	-
17-6431	*H. crithmifolia*	19.858177447	-33.798301921	232.186	-	-	-	-
17-6432	*H. crithmifolia*	19.858219819	-33.798318067	232.224	-	-	-	-
17-6433	*H. crithmifolia*	19.858216397	-33.798285505	230.576	-	-	-	-
17-6434	*H. crithmifolia*	19.858132970	-33.798279003	231.835	-	-	-	-
17-6435	*H. crithmifolia*	19.858156064	-33.798341649	231.469	-	-	-	-

17-6436	*H. crithmifolia*	19.858024178	-33.798186690	233.137	-	-	-	-
17-6437	*H. crithmifolia*	19.857965575	-33.798164691	231.341	-	-	-	-
17-6438	*H. crithmifolia*	19.857907628	-33.798110317	231.483	-	-	-	-
17-6439	*H. crithmifolia*	19.857846192	-33.798073975	232.606	-	-	-	-
17-6440	*H. crithmifolia*	19.857837704	-33.798017784	231.034	-	-	-	-
17-6441	*H. grossularifolia*	19.857922751	-33.798081435	232.681	-	-	-	-
17-6442	*H. grossularifolia*	-	-	-	-	-	-	-
17-6443	*H. grossularifolia*	19.857891925	-33.798103727	233.232	-	-	-	-
17-6444	*H. grossularifolia*	19.858031624	-33.798185368	232.214	-	-	-	-
17-6445	*H. grossularifolia*	19.858115893	-33.798234878	232.369	-	-	-	-
17-6446	*H. grossularifolia*	19.858587983	-33.798592610	233.005	18,92	-	-	-
17-6447	*H. grossularifolia*	19.858599435	-33.798595046	232.859	-	-	-	-
17-6448	*H. grossularifolia*	19.858795173	-33.798755259	231.814	-	-	-	-
17-6449	*H. grossularifolia*	19.859164106	-33.799019266	230.158	-	-	-	-
17-6450	*H. grossularifolia*	19.859368894	-33.799205808	232.572	-	-	-	-
18-0001	*G. africana*	19.540496661	-33.533081689	381.836	-	-	-	-
18-0002	*G. africana*	19.540482381	-33.533120052	381.394	-	-	-	-
18-0003	*G. africana*	19.540465592	-33.533152129	381.607	-	-	-	-
18-0004	*G. africana*	19.540465928	-33.533157324	381.328	-	-	-	-
18-0005	*G. africana*	19.540426548	-33.533205274	381.197	-	-	-	-

18-0006	*G. africana*	19.540439152	-33.533230526	383.859	-	-	-	-
18-0007	*G. africana*	19.540468159	-33.533260027	384.185	-	-	-	-
18-0008	*G. africana*	19.540507432	-33.533265484	384.063	-	-	-	-
18-0009	*G. africana*	19.540420401	-33.533298158	384.048	17,35	-	-	-
18-0010	*G. africana*	19.540396352	-33.533309829	383.311	-	-	-	-
18-0011	*G. africana*	19.540346405	-33.533327377	382.997	16,77	-	-	-
18-0012	*G. africana*	19.540306246	-33.533343744	382.866	-	-	-	-
18-0013	*G. africana*	19.540285521	-33.533373936	381.153	-	-	-	-
18-0014	*G. africana*	19.540278755	-33.533442011	381.940	-	-	-	-
18-0015	*G. africana*	19.540274884	-33.533473389	382.740	-	-	-	-
18-0016	*G. africana*	19.540242572	-33.533488668	382.951	-	-	-	-
18-0017	*G. africana*	19.540370292	-33.533457739	383.300	-	-	-	-
18-0018	*G. africana*	19.540396601	-33.533478059	386.214	-	-	-	-
18-0019	*G. africana*	19.540362463	-33.533510109	386.344	-	-	-	-
18-0020	*G. africana*	19.540395108	-33.533522193	388.844	-	-	-	-
18-0021	*D. australe* subsp. *australe*	19.540369708	-33.533491882	386.810	-	-	-	-
18-0022	*D. australe* subsp. *australe*	19.540408835	-33.533478614	389.653	-	-	-	-

18-0023	*D. australe* subsp. *australe*	19.540439503	-33.533479550	385.882	-	-	-	-
18-0024	*D. australe* subsp. *australe*	19.540483897	-33.533497467	391.647	-	-	-	-
18-0025	*D. australe* subsp. *australe*	19.540638247	-33.533611839	395.372	-	-	-	-
18-0026	*D. australe* subsp. *australe*	19.540667939	-33.533666916	396.247	17,58	-	-	-
18-0027	*D. australe* subsp. *australe*	19.540723192	-33.533662825	399.295	-	-	-	-
18-0028	*D. australe* subsp. *australe*	19.540816008	-33.533615618	399.573	-	-	-	-
18-0029	*C. capensis*	19.540880350	-33.533503013	398.512	-	-	-	-
18-0030	*C. capensis*	19.540951969	-33.533478649	400.292	20,33	-	-	-
18-0031	*C. capensis*	19.540775851	-33.533636077	400.527	-	-	-	-
18-0032	*C. capensis*	19.540745016	-33.533665287	398.982	-	-	-	-
18-0033	*C. capensis*	19.540731068	-33.533679706	397.976	-	-	-	-
18-0034	*C. capensis*	19.540702476	-33.533710046	400.944	-	-	-	-
18-0035	*C. capensis*	19.540609132	-33.533684640	396.857	-	-	-	-
18-0036	*C. capensis*	19.540607198	-33.533660878	396.065	-	-	-	-

18-0037	*C. capensis*	19.540556946	-33.533655275	397.070	-	-	-	-
18-0038	*C. capensis*	19.540512495	-33.533632640	395.343	-	-	-	-
18-0039	*C. capensis*	19.540447811	-33.533553734	389.957	19,17	-	-	-
18-0040	*C. capensis*	19.540369055	-33.533560362	388.711	-	-	-	-
18-0041	*Ci. sinensis* (possibly CG)	19.540299609	-33.533247962	381.343	29,75	-	-	-
18-0042	*R. foetida*	19.782747649	-33.751976896	309.475	-	-	-	-
18-0043	*R. foetida*	19.782696159	-33.752020843	314.417	-	-	-	-
18-0044	*R. foetida*	19.782649947	-33.752073879	308.737	-	-	-	-
18-0045	*R. foetida*	19.782570759	-33.752110843	308.903	-	-	-	-
18-0046	*R. foetida*	19.782376907	-33.752257038	307.081	-	-	-	-
18-0047	*R. foetida*	19.782338903	-33.752282527	307.355	-	-	-	-
18-0048	*R. foetida*	19.782284838	-33.752343527	306.556	-	-	-	-
18-0049	*R. foetida*	19.782234294	-33.752357969	307.968	27,48	-	-	-
18-0050	*R. foetida*	19.782114558	-33.752438427	307.290	-	-	-	-
18-0051	*R. foetida*	19.782084097	-33.752442991	308.482	-	-	-	-
18-0052	*R. foetida*	19.782052021	-33.752473166	307.873	-	-	-	-
18-0053	*R. foetida*	19.782017912	-33.752487242	306.864	-	-	-	-
18-0054	*R. foetida*	19.781993196	-33.752508543	305.538	-	-	-	-
18-0055	*R. foetida*	19.781926635	-33.752538482	305.854	30,07	-	-	-

18-0056	*R. foetida*	19.781802217	-33.752626273	305.348	-	-	-	-
18-0057	*R. foetida*	19.781734187	-33.752680144	305.507	24,58	-	-	-
18-0058	*R. foetida*	19.781714000	-33.752700570	307.986	29,76	-	-	-
18-0059	*R. foetida*	19.781670516	-33.752783182	299.332	-	-	-	-
18-0060	*R. foetida*	19.781644000	-33.752846459	307.224	-	-	-	-
18-0061	*R. foetida*	19.781665365	-33.752793868	305.221	-	-	-	-
18-0062	*S. kali*	19.781430610	-33.752864477	306.118	-	-	-	-
18-0063	*S. kali*	19.781468768	-33.752840612	304.003	-	-	-	-
18-0064	*S. kali*	19.781486717	-33.752831336	302.983	-	-	-	-
18-0065	*S. kali*	19.781494796	-33.752804191	303.307	-	-	-	-
18-0066	*S. kali*	19.781557189	-33.752758500	304.257	-	-	-	-
18-0067	*S. kali*	19.781586647	-33.752699955	305.179	-	-	-	-
18-0068	*S. kali*	19.781622250	-33.752676785	304.281	-	-	-	-
18-0069	*S. kali*	19.781658022	-33.752634639	304.516	-	-	-	-
18-0070	*S. kali*	19.781700905	-33.752595170	305.319	-	-	-	-
18-0071	*S. kali*	19.781726319	-33.752579147	304.412	-	-	-	-
18-0072	*S. kali*	19.781757965	-33.752537699	306.782	-	-	-	-
18-0073	*S. kali*	19.781825082	-33.752479117	304.831	-	-	-	-
18-0074	*S. kali*	19.781841412	-33.752449921	305.203	-	-	-	-
18-0075	*S. kali*	19.781908747	-33.752394353	304.236	28,43	-	-	-

18-0076	*S. kali*	19.781964784	-33.752330819	305.337	-	-	-	-
18-0077	*S. kali*	19.781998773	-33.752296201	306.361	-	-	-	-
18-0078	*S. kali*	19.782040802	-33.752266241	305.119	-	-	-	-
18-0079	*S. kali*	19.782108857	-33.752178230	306.267	-	-	-	-
18-0080	*S. kali*	19.782291616	-33.752004679	306.664	-	-	-	-
18-0081	*S. kali*	19.782386210	-33.751901386	308.123	-	-	-	-
18-0082	*E. rhinocerotis*	19.782975581	-33.751735946	309.784	-	-	-	-
18-0083	*E. rhinocerotis*	19.782978084	-33.751716643	308.293	-	-	-	-
18-0084	*E. rhinocerotis*	19.783014211	-33.751705216	310.817	-	-	-	-
18-0085	*E. rhinocerotis*	19.783034171	-33.751728124	309.847	-	-	-	-
18-0086	*E. rhinocerotis*	19.783033363	-33.751760113	311.278	-	-	-	-
18-0087	*E. rhinocerotis*	19.783100661	-33.751698686	311.248	-	-	-	-
18-0088	*E. rhinocerotis*	19.783035219	-33.751670593	309.881	-	-	-	-
18-0089	*G. africana*	19.963177199	-33.833068651	202.516	-	-	-	-
18-0090	*G. africana*	19.963147934	-33.833109192	203.116	-	-	-	-
18-0091	*G. africana*	19.963113233	-33.833087203	202.044	-	-	-	-
18-0092	*G. africana*	19.963076166	-33.833119516	203.656	-	-	-	-
18-0093	*G. africana*	19.963017292	-33.833127928	204.090	-	-	-	-
18-0094	*G. africana*	19.962961855	-33.833114052	202.348	-	-	-	-
18-0095	*G. africana*	19.962891245	-33.833094601	203.419	-	-	-	-

18-0096	*G. africana*	19.962916750	-33.833037225	200.482	-	-	-	-
18-0097	*G. africana*	19.962897135	-33.832975615	205.841	-	-	-	-
18-0098	*G. africana*	19.962925759	-33.832955623	203.652	-	-	-	-
18-0099	*G. africana*	19.962835086	-33.832940471	204.185	-	-	-	-
18-0100	*G. africana*	19.962769886	-33.832941251	204.413	-	-	-	-
18-0101	*G. africana*	19.962716996	-33.833001792	204.917	-	-	-	-
18-0102	*G. africana*	19.962767887	-33.832828117	204.882	-	-	-	-
18-0103	*G. africana*	19.962710204	-33.832791246	205.131	-	-	-	-
18-0104	*G. africana*	19.962584614	-33.832809517	203.769	-	-	-	-
18-0105	*G. africana*	19.962490843	-33.832880099	203.477	-	-	-	-
18-0106	*G. africana*	19.962553394	-33.832946843	203.825	-	-	-	-
18-0107	*G. africana*	19.962557744	-33.833022181	204.401	-	-	-	-
18-0108	*G. africana*	19.962481774	-33.832986572	204.977	-	-	-	-
18-0109	*G. africana*	19.962494951	-33.833077694	204.933	-	-	-	-
18-0110	*A. lindleyi*	19.962461979	-33.833105655	205.007	-	-	-	-
18-0111	*A. lindleyi*	19.962483523	-33.833117581	204.317	-	-	-	-
18-0112	*A. lindleyi*	19.962541086	-33.833091942	203.580	-	-	-	-
18-0113	*A. lindleyi*	19.962568578	-33.833090314	204.646	-	-	-	-
18-0114	*A. lindleyi*	19.962612413	-33.833072161	205.442	30,29	+**	-	-
18-0115	*A. lindleyi*	19.962627236	-33.833038376	204.642	25,31	-	-	-

18-0116	*A. lindleyi*	19.962627752	-33.833082417	205.154	30,67	-	-	-
18-0117	*A. lindleyi*	19.962561561	-33.833120899	204.929	-	-	-	-
18-0118	*A. lindleyi*	19.962614599	-33.833175075	206.233	-	-	-	-
18-0119	*A. lindleyi*	19.962553290	-33.833180074	205.960	-	-	-	-
18-0120	*A. lindleyi*	19.962490849	-33.833194494	204.115	26,64	-	-	-
18-0121	*A. lindleyi*	19.962468778	-33.833233716	202.953	-	-	-	-
18-0122	*A. lindleyi*	19.962478700	-33.833269378	204.937	30,29	+**	-	-
18-0123	*A. lindleyi*	19.962520228	-33.833257127	205.144	-	-	-	-
18-0124	*A. lindleyi*	19.962540068	-33.833233057	204.876	-	-	-	-
18-0125	*A. lindleyi*	19.962357924	-33.833253620	203.673	-	-	-	-
18-0126	*A. lindleyi*	19.962287636	-33.833255066	203.851	30,78	-	-	-
18-0127	*A. lindleyi*	19.962223793	-33.833243471	204.002	-	-	-	-
18-0128	*A. lindleyi*	19.961993227	-33.833280585	204.857	30,48	-	-	-
18-0129	*V. vinifera*	19.962011436	-33.833312971	204.125	-	-	-	-
18-0130	*V. vinifera*	19.961978545	-33.833352417	204.140	-	-	-	-
18-0131	*S. burchellii*	19.962000630	-33.833344943	204.487	-	-	-	-
18-0132	*S. burchellii*	19.961992906	-33.833412323	204.157	-	-	-	-
18-0133	*S. burchellii*	19.961992923	-33.833425918	203.752	-	-	-	-
18-0134	*S. burchellii*	19.961994152	-33.833448891	203.845	-	-	-	-
18-0135	*S. burchellii*	19.962012521	-33.833466531	204.382	-	-	-	-

18-0136	*S. burchellii*	19.961991339	-33.833466664	204.415	-	-	-	-
18-0137	*S. burchellii*	19.961989350	-33.833496219	204.203	-	-	-	-
18-0138	*C. dactylon*	19.961961495	-33.833482335	204.178	-	-	-	-
18-0139	*S. burchellii*	19.961960214	-33.833442069	204.351	-	-	-	-
18-0140	*S. burchellii*	19.961979189	-33.833450427	204.317	-	-	-	-
18-0141	*S. burchellii*	19.961921769	-33.833441832	204.927	-	-	-	-
18-0142	*S. burchellii*	19.961906094	-33.833409718	204.255	-	-	-	-
18-0143	*S. burchellii*	19.961872945	-33.833376749	205.315	-	-	-	-
18-0144	*S. burchellii*	19.961875712	-33.833484743	204.473	-	-	-	-
18-0145	*S. burchellii*	19.961876152	-33.833542604	204.430	-	-	-	-
18-0146	*S. burchellii*	19.961922618	-33.833576797	204.587	-	-	-	-
18-0147	*S. burchellii*	19.961949837	-33.833582395	204.763	-	-	-	-
18-0148	*S. burchellii*	19.962180500	-33.833196284	204.432	-	-	-	-
18-0149	*S. burchellii*	19.962199188	-33.833220185	205.495	-	-	-	-
18-0150	*S. burchellii*	19.962249447	-33.833202610	201.350	-	-	-	-
18-0151	*A. semibaccata*	19.962177678	-33.833119748	205.778	29,51	+**	-	-
18-0152	*A. semibaccata*	19.962287211	-33.833107990	205.062	30,06	-	-	-
18-0153	*A. semibaccata*	19.962250277	-33.833066830	204.738	28,91	-	-	-
18-0154	*A. semibaccata*	19.962255208	-33.833047784	203.091	-	-	-	-
18-0155	*A. semibaccata*	19.962176871	-33.833028260	203.783	27,99	-	-	-

18-0156	*A. semibaccata*	19.962270251	-33.833001123	205.542	28,22	+**	-	-
18-0157	*A. semibaccata*	19.962238507	-33.832976799	205.230	25,20	+**	-	-
18-0158	*A. semibaccata*	19.962315802	-33.833019797	205.419	26,79	-	-	-
18-0159	*A. semibaccata*	19.962325724	-33.833053622	205.615	28,49	-	-	-
18-0160	*A. semibaccata*	19.962381944	-33.833041240	204.973	22,63	-	-	-
18-0161	*A. semibaccata*	19.962353032	-33.832992707	204.999	27,24	-	-	-
18-0162	*A. semibaccata*	19.962403734	-33.833073355	204.493	27,74	-	-	-
18-0163	*A. semibaccata*	19.962497717	-33.833123613	204.005	27,27	-	-	-
18-0164	*A. semibaccata*	19.962514711	-33.833137427	203.974	25,09	+**	-	-
18-0165	*A. semibaccata*	19.962517259	-33.833170259	203.936	27,32	-	-	-
18-0166	*A. semibaccata*	19.962558428	-33.833179533	203.625	27,04	-	-	-
18-0167	*A. semibaccata*	19.962628680	-33.833171100	203.613	25,82	-	-	-
18-0168	*A. semibaccata*	19.963561035	-33.833021528	199.638	26,17	-	-	-
18-0169	*A. semibaccata*	19.963438357	-33.833061706	200.456	25,25	-	-	-
18-0170	*A. semibaccata*	19.963388187	-33.833042917	201.835	27,35	-	-	-
18-0171	*S. kali*	19.963349385	-33.833032407	200.880	-	-	-	-
18-0172	*S. kali*	19.963540786	-33.833109932	200.111	-	-	-	-
18-0173	*S. kali*	19.963717187	-33.833148996	198.318	-	-	-	-
18-0174	*S. kali*	19.963915023	-33.833205076	199.537	-	-	-	-
18-0175	*S. kali*	19.963897351	-33.833240148	196.694	-	-	-	-

18-0176	*S. kali*	-	-	-	-	-	-	-
18-0177	*S. kali*	-	-	-	-	-	-	-
18-0178	*S. kali*	-	-	-	-	-	-	-
18-0179	*S. kali*	-	-	-	-	-	-	-
18-0180	*S. kali*	-	-	-	-	-	-	-
18-0181	*S. kali*	-	-	-	-	-	-	-
18-0182	*S. kali*	-	-	-	-	-	-	-
18-0183	*S. kali*	-	-	-	-	-	-	-
18-0184	*S. kali*	-	-	-	-	-	-	-
18-0185	*S. kali*	-	-	-	-	-	-	-
18-0186	*S. kali*	-	-	-	-	-	-	-
18-0187	*S. kali*	-	-	-	-	-	-	-
18-0188	*S. kali*	-	-	-	-	-	-	-
18-0189	*S. kali*	-	-	-	-	-	-	-
18-0190	*S. kali*	-	-	-	-	-	-	-
18-0201	*A. semibaccata*	19.862106017	-33.797382671	243.661	-	-	-	-
18-0202	*A. semibaccata*	19.862073953	-33.797420068	243.429	-	-	-	-
18-0203	*A. semibaccata*	19.862098987	-33.797361126	243.963	-	-	-	-
18-0204	*A. semibaccata*	19.862107067	-33.797357371	243.918	-	-	-	-
18-0205	*A. semibaccata*	19.862126881	-33.797344363	244.441	27,82	-	-	-

18-0206	*A. semibaccata*	19.862107873	-33.797343279	242.464	-	-	-	-
18-0207	*A. semibaccata*	19.862087694	-33.797333988	243.498	-	-	-	-
18-0208	*A. semibaccata*	19.862073387	-33.797320020	243.482	-	-	-	-
18-0209	*A. semibaccata*	19.862041836	-33.797318579	243.543	-	-	-	-
18-0210	*A. semibaccata*	19.862044176	-33.797290252	243.070	-	-	-	-
18-0211	*A. semibaccata*	19.862129942	-33.797279631	243.726	-	-	-	-
18-0212	*A. semibaccata*	19.862173661	-33.797278429	242.710	-	-	-	-
18-0213	*A. semibaccata*	19.862134399	-33.797310512	244.011	-	-	-	-
18-0214	*A. semibaccata*	19.862142450	-33.797446368	243.189	22,43	-	-	-
18-0215	*A. semibaccata*	19.862061811	-33.797422490	244.839	-	-	-	-
18-0216	*A. semibaccata*	19.862044765	-33.797419762	243.994	-	-	-	-
18-0217	*A. semibaccata*	19.862029997	-33.797407345	243.461	-	-	-	-
18-0218	*A. semibaccata*	19.861975153	-33.797429415	242.386	-	-	-	-
18-0219	*A. semibaccata*	19.861940972	-33.797437898	241.699	-	-	-	-
18-0220	*A. semibaccata*	19.861922661	-33.797447905	241.254	-	-	-	-
18-0221	*S. kali*	19.861379835	-33.797551788	238.978	27,34	-	-	-
18-0222	*S. kali*	19.861545961	-33.797476065	239.433	-	-	-	-
18-0223	*S. kali*	19.861609499	-33.797442008	240.711	-	-	-	-
18-0224	*S. kali*	19.861638752	-33.797433944	240.786	-	-	-	-
18-0225	*S. kali*	19.861661523	-33.797426703	240.626	-	-	-	-

18-0226	*S. kali*	19.861778239	-33.797292043	242.749	-	-	-	-
18-0227	*S. kali*	19.861981592	-33.797285282	243.750	-	-	-	-
18-0228	*S. kali*	19.862106721	-33.797254739	243.695	-	-	-	-
18-0229	*S. kali*	19.862124370	-33.797254576	242.615	-	-	-	-
18-0230	*S. kali*	19.862177665	-33.797203264	244.928	-	-	-	-
18-0231	*S. kali*	19.862196312	-33.797196666	245.085	-	-	-	-
18-0232	*S. kali*	19.862190319	-33.797181265	244.200	28,64	-	-	-
18-0233	*S. kali*	19.862241642	-33.797170623	246.813	-	-	-	-
18-0234	*S. kali*	19.862279944	-33.797201288	244.661	-	-	-	-
18-0235	*S. kali*	19.862247635	-33.797193402	243.855	28,42	-	-	-
18-0236	*S. kali*	19.862248406	-33.797205374	244.973	-	-	-	-
18-0237	*S. kali*	19.862266283	-33.797264509	245.629	-	-	-	-
18-0238	*S. kali*	19.862234658	-33.797305139	246.062	-	-	-	-
18-0239	*S. kali*	19.862214101	-33.797285091	245.427	-	-	-	-
18-0240	*S. kali*	19.862133988	-33.797315793	244.684	-	-	-	-
18-0241	*G. africana*	19.862150932	-33.797541020	244.729	-	-	-	-
18-0242	*G. africana*	19.862143184	-33.797515527	244.077	-	-	-	-
18-0243	*G. africana*	19.862141252	-33.797478563	245.821	-	-	-	-
18-0244	*G. africana*	19.862155273	-33.797399658	245.620	-	-	-	-
18-0245	*G. africana*	19.862190958	-33.797400596	246.118	-	-	-	-

18-0246	*G. africana*	19.862241041	-33.797404054	243.098	-	-	-	-
18-0247	*G. africana*	19.862403480	-33.797356314	247.244	-	-	-	-
18-0248	*G. africana*	19.862447769	-33.797388775	249.365	-	-	-	-
18-0249	*G. africana*	19.862475855	-33.797288759	248.535	30,63	-	-	-
18-0250	*G. africana*	19.862537618	-33.797214100	246.625	27,35	-	-	-
18-0251	*E. rhinocerotis*	19.862502504	-33.797225757	248.780	24,23	-	-	-
18-0252	*E. rhinocerotis*	19.862577189	-33.797273165	250.475	-	-	-	-
18-0253	*E. rhinocerotis*	19.862454040	-33.797259337	248.240	-	-	-	-
18-0254	*E. rhinocerotis*	19.862417380	-33.797264439	246.791	-	-	-	-
18-0255	*E. rhinocerotis*	19.862279545	-33.797323783	245.826	-	-	-	-
18-0256	*C. scabrida*	19.862196527	-33.797432994	245.371	-	-	-	-
18-0257	*C. scabrida*	19.862235564	-33.797423080	244.935	-	-	-	-
18-0258	*C. scabrida*	19.862353765	-33.797447050	247.427	-	-	-	-
18-0259	*C. scabrida*	19.862257428	-33.797281125	245.002	29,48	-	-	-
18-0260	*C. scabrida*	19.862270489	-33.797259340	245.163	-	-	-	-
18-0261	*C. scabrida*	19.862294484	-33.797200034	245.542	-	-	-	-
18-0262	*C. scabrida*	19.862267716	-33.797172362	245.351	-	-	-	-
18-0263	*C. scabrida*	19.862260055	-33.797160200	245.033	-	-	-	-
18-0264	*C. scabrida*	19.862206140	-33.797158921	246.018	-	-	-	-
18-0265	*C. scabrida*	19.862179869	-33.797166235	245.172	-	-	-	-

18-0266	*C. scabrida*	19.862104002	-33.797184316	246.156	-	-	-	-
18-0267	*C. scabrida*	19.862088232	-33.797192756	245.197	-	-	-	-
18-0268	*C. scabrida*	19.862065073	-33.797198629	245.105	-	-	-	-
18-0269	*C. scabrida*	19.862026059	-33.797209380	244.715	-	-	-	-
18-0270	*C. scabrida*	19.862012475	-33.797214712	244.819	-	-	-	-
18-0271	*C. scabrida*	19.861935079	-33.797227270	245.268	-	-	-	-
18-0272	*C. scabrida*	19.861711955	-33.797264554	245.190	-	-	-	-
18-0273	*C. scabrida*	19.861642923	-33.797270741	245.271	-	-	-	-
18-0274	*C. scabrida*	19.861605902	-33.797267748	245.532	-	-	-	-
18-0275	*C. scabrida*	19.861416555	-33.797276766	244.658	-	-	-	-
18-0276	*H. cymosum*	19.861410124	-33.797307429	244.024	-	-	-	-
18-0277	*H. cymosum*	19.861432552	-33.797298326	244.496	-	-	-	-
18-0278	*H. cymosum*	19.861446638	-33.797312807	243.638	-	-	-	-
18-0279	*H. cymosum*	19.861457632	-33.797302186	243.759	-	-	-	-
18-0280	*H. cymosum*	19.861517216	-33.797288838	245.436	-	-	-	-
18-0281	*H. cymosum*	19.861542565	-33.797296276	243.414	-	-	-	-
18-0282	*H. cymosum*	19.861547581	-33.797292513	244.688	-	-	-	-
18-0283	*H. cymosum*	19.861572069	-33.797294271	244.967	-	-	-	-
18-0284	*H. cymosum*	19.861554276	-33.797284700	245.962	-	-	-	-
18-0285	*H. cymosum*	19.861466984	-33.797351575	242.284	-	-	-	-

18-0286	*P. incana*	19.857632637	-33.797891904	231.353	-	-	-	-
18-0287	*P. incana*	19.857671483	-33.797931450	231.565	-	-	-	-
18-0288	*P. incana*	19.857793770	-33.798019393	231.796	-	-	-	-
18-0289	*P. incana*	19.857813567	-33.798032568	233.761	-	-	-	-
18-0290	*P. incana*	19.857826899	-33.798044689	232.941	-	-	-	-
18-0291	*P. incana*	19.857852764	-33.798060070	232.579	-	-	-	-
18-0292	*P. incana*	19.857913086	-33.798064314	232.508	-	-	-	-
18-0293	*P. incana*	19.857942723	-33.798081692	231.840	-	-	-	-
18-0294	*P. incana*	19.857963111	-33.798101650	232.388	-	-	-	-
18-0295	*P. incana*	19.857996072	-33.798122773	231.251	-	-	-	-
18-0296	*P. incana*	19.858002793	-33.798175733	232.080	-	-	-	-
18-0297	*P. incana*	19.858040972	-33.798202440	230.690	-	-	-	-
18-0298	*P. incana*	19.858084890	-33.798224711	231.716	-	-	-	-
18-0299	*P. incana*	19.858176914	-33.798265124	232.088	-	-	-	-
18-0300	*P. incana*	19.858208965	-33.798317509	232.355	-	-	-	-
18-0301	*B. tournefortii*	18.471305000	-31.674208377	68.932	-	-	-	-
18-0302	*B. tournefortii*	18.471313454	-31.674192478	69.517	-	-	-	-
18-0303	*B. tournefortii*	18.471342013	-31.674175493	69.618	-	-	-	-
18-0304	*B. tournefortii*	18.471377206	-31.674160771	69.695	-	-	-	-
18-0305	*B. tournefortii*	18.471354423	-31.674118045	69.325	30,08	-	-	-

18-0306	*B. tournefortii*	18.471369076	-31.674104471	69.331	-	-	-	-
18-0307	*B. tournefortii*	18.471327796	-31.674054202	69.541	-	-	-	-
18-0308	*B. tournefortii*	18.471292958	-31.674029162	69.397	-	-	-	-
18-0309	*B. tournefortii*	18.471341117	-31.674004303	69.068	-	-	-	-
18-0310	*B. tournefortii*	18.471276678	-31.674005097	69.690	29,93	-	-	-
18-0311	*B. tournefortii*	18.471308448	-31.673973915	69.364	30,35	-	-	-
18-0312	*B. tournefortii*	18.471254224	-31.673960072	69.172	-	-	-	-
18-0313	*B. tournefortii*	18.471229482	-31.673953176	69.832	-	-	-	-
18-0314	*B. tournefortii*	18.471198655	-31.673897822	70.728	-	-	-	-
18-0315	*B. tournefortii*	18.471184527	-31.673873622	69.836	-	-	-	-
18-0316	*B. tournefortii*	18.471165311	-31.673850179	69.494	-	-	-	-
18-0317	*B. tournefortii*	18.471138752	-31.673803766	69.512	-	-	-	-
18-0318	*B. tournefortii*	18.471126171	-31.673795060	69.224	-	-	-	-
18-0319	*B. tournefortii*	18.471144834	-31.673792581	69.135	-	-	-	-
18-0320	*B. tournefortii*	18.471096101	-31.673731264	69.723	30,07	-	-	-
18-0321	*O. grandiflorum*	18.471350825	-31.674354281	68.043	-	-	-	-
18-0322	*O. grandiflorum*	18.471351939	-31.674363129	67.926	-	-	-	-
18-0323	*O. grandiflorum*	18.471359719	-31.674371676	67.592	-	-	-	-
18-0324	*O. grandiflorum*	18.471395992	-31.674321090	67.399	-	-	-	-
18-0325	*O. grandiflorum*	18.471397943	-31.674309651	67.310	-	-	-	-

18-0326	*O. grandiflorum*	18.471405449	-31.674294017	67.618	-	-	-	-
18-0327	*O. grandiflorum*	18.471387747	-31.674279561	67.584	-	-	-	-
18-0328	*O. grandiflorum*	18.471394394	-31.674269286	68.287	-	-	-	-
18-0329	*O. grandiflorum*	18.471434753	-31.674260050	67.722	-	-	-	-
18-0330	*O. grandiflorum*	18.471442091	-31.674226017	68.300	-	-	-	-
18-0331	*O. grandiflorum*	18.471436791	-31.674145723	68.138	-	-	-	-
18-0332	*O. grandiflorum*	18.471434253	-31.674121200	68.097	-	-	-	-
18-0333	*O. grandiflorum*	18.471646542	-31.674315571	67.993	-	-	-	-
18-0334	*O. grandiflorum*	18.471661162	-31.674325621	67.419	-	-	-	-
18-0335	*O. grandiflorum*	18.471689793	-31.674329522	67.833	-	-	-	-
18-0336	*O. grandiflorum*	18.471702326	-31.674373170	67.675	-	-	-	-
18-0337	*O. grandiflorum*	18.471736597	-31.674418078	67.820	-	-	-	-
18-0338	*O. grandiflorum*	18.471799296	-31.674453630	67.829	-	-	-	-
18-0339	*O. grandiflorum*	18.471830649	-31.674481294	67.619	-	-	-	-
18-0340	*O. grandiflorum*	18.471876647	-31.674527480	68.164	-	-	-	-
18-0341	*A. semibaccata*	18.471364307	-31.674294848	68.480	30,43	-	-	-
18-0342	*A. semibaccata*	18.471345016	-31.674279159	69.226	27,11	-	-	-
18-0343	*A. semibaccata*	18.471317150	-31.674324061	68.522	-	-	-	-
18-0344	*A. semibaccata*	18.471294246	-31.674321177	68.010	28,02	-	-	-
18-0345	*A. semibaccata*	18.471271919	-31.674297463	68.742	27,09	-	-	-

18-0346	*A. semibaccata*	18.471357299	-31.674250858	68.751	27,08	-	-	-
18-0347	*A. semibaccata*	18.471318046	-31.674230882	69.939	30,76	-	-	-
18-0348	*A. semibaccata*	18.471383935	-31.674217120	69.713	26,52	-	-	-
18-0349	*A. semibaccata*	18.471351316	-31.674190730	70.292	29,01	-	-	-
18-0350	*A. semibaccata*	18.471272211	-31.674204066	70.001	30,26	-	-	-
18-0351	*M. crystallinum*	18.471363739	-31.674158367	71.142	-	-	-	-
18-0352	*M. crystallinum*	18.471380024	-31.674127734	70.336	-	-	-	-
18-0353	*M. crystallinum*	18.471355037	-31.674087179	69.903	-	-	-	-
18-0354	*M. crystallinum*	18.471351254	-31.674050942	69.419	-	-	-	-
18-0355	*M. crystallinum*	18.471346602	-31.674049166	69.811	-	-	-	-
18-0356	*M. crystallinum*	18.471330640	-31.674057557	69.336	-	-	-	-
18-0357	*M. crystallinum*	18.471323236	-31.674034769	69.406	-	-	-	-
18-0358	*M. crystallinum*	18.471285722	-31.674020978	69.591	-	-	-	-
18-0359	*M. crystallinum*	18.471266960	-31.673976539	69.263	-	-	-	-
18-0360	*M. crystallinum*	18.471257618	-31.673954910	69.294	-	-	-	-
18-0361	*M. crystallinum*	18.471239824	-31.673942704	69.279	-	-	-	-
18-0362	*M. crystallinum*	18.471226187	-31.673915901	69.281	-	-	-	-
18-0363	*M. crystallinum*	18.471201963	-31.673896712	69.294	-	-	-	-
18-0364	*M. crystallinum*	18.471192240	-31.673874443	69.327	-	-	-	-
18-0365	*M. crystallinum*	18.471178570	-31.673864711	69.862	-	-	-	-

18-0366	*M. crystallinum*	18.470999577	-31.673901915	69.959	-	-	-	-
18-0367	*M. crystallinum*	18.470992294	-31.673834390	69.921	-	-	-	-
18-0368	*M. crystallinum*	18.470986732	-31.673746633	69.484	-	-	-	-
18-0369	*M. crystallinum*	18.470988961	-31.673697559	69.334	-	-	-	-
18-0370	*M. crystallinum*	18.471047380	-31.673644033	68.670	-	-	-	-
18-0371	*A. lindleyi*	18.471268433	-31.673331697	68.539	-	-	-	-
18-0372	*A. lindleyi*	18.471282576	-31.673309003	68.030	-	-	-	-
18-0373	*A. lindleyi*	18.471313510	-31.673280875	68.797	-	-	-	-
18-0374	*A. lindleyi*	18.471333083	-31.673260319	68.142	-	-	-	-
18-0375	*A. lindleyi*	18.471496071	-31.673090035	68.606	-	-	-	-
18-0376	*A. lindleyi*	18.471545490	-31.673107888	67.315	-	-	-	-
18-0377	*A. lindleyi*	18.471521282	-31.673076819	68.029	-	-	-	-
18-0378	*A. lindleyi*	18.471509765	-31.673014904	68.842	-	-	-	-
18-0379	*A. lindleyi*	18.471489972	-31.672994989	68.298	30,30	-	-	-
18-0380	*A. lindleyi*	18.471523778	-31.672971838	68.306	-	-	-	-
18-0381	*A. nummularia*	18.471494114	-31.672938202	67.660	-	-	-	-
18-0382	*A. nummularia*	18.471481644	-31.672941486	67.901	-	-	-	-
18-0383	*A. nummularia*	18.471493567	-31.672918009	67.340	-	-	-	-
18-0384	*A. nummularia*	18.471531606	-31.672882913	67.425	-	-	-	-
18-0385	*A. nummularia*	18.471524844	-31.672916375	68.073	-	-	-	-

18-0386	*A. nummularia*	18.471540099	-31.672890241	67.488	-	-	-	-
18-0387	*A. nummularia*	18.471469341	-31.672887267	67.957	-	-	-	-
18-0388	*A. nummularia*	18.471539774	-31.672990179	67.798	-	-	-	-
18-0389	*A. nummularia*	18.471620963	-31.673024722	68.449	-	-	-	-
18-0390	*A. nummularia*	18.471497773	-31.672997073	67.356	-	-	-	-
18-0391	*E. brevifolius*	18.471603388	-31.672992579	67.744	-	-	-	-
18-0392	*E. brevifolius*	18.471609080	-31.672972026	67.653	-	-	-	-
18-0393	*E. brevifolius*	18.471753026	-31.672828121	67.905	-	-	-	-
18-0394	*E. brevifolius*	18.471772997	-31.672805339	67.306	-	-	-	-
18-0395	*E. brevifolius*	18.471791222	-31.672775435	68.028	-	-	-	-
18-0396	*E. brevifolius*	18.472426243	-31.672207829	66.490	30,90	-	-	-
18-0397	*A. nummularia*	18.326710374	-31.557976251	50.371	-	-	-	-
18-0398	*A. nummularia*	18.326783234	-31.557950620	50.915	-	-	-	-
18-0399	*A. nummularia*	18.326851693	-31.557954401	50.934	-	-	-	-
18-0400	*A. nummularia*	18.326899419	-31.557962457	50.323	-	-	-	-
18-0401	*A. nummularia*	18.326952812	-31.557960738	49.986	-	-	-	-
18-0402	*A. nummularia*	18.327031785	-31.557962599	50.221	-	-	-	-
18-0403	*A. nummularia*	18.327038082	-31.557945953	51.208	-	-	-	-
18-0404	*A. nummularia*	18.327122153	-31.557965107	50.140	-	-	-	-
18-0405	*A. nummularia*	18.327167507	-31.557975951	49.566	-	-	-	-

18-0406	*A. nummularia*	18.327200369	-31.557978532	49.926	-	-	-	-
18-0407	*A. nummularia*	18.327245266	-31.557975525	50.097	-	-	-	-
18-0408	*A. nummularia*	18.327319811	-31.557983617	50.105	-	-	-	-
18-0409	*A. nummularia*	18.327362658	-31.557988936	49.730	-	-	-	-
18-0410	*A. nummularia*	18.327428771	-31.557990716	49.945	-	-	-	-
18-0411	*A. nummularia*	18.327489284	-31.557995594	50.106	-	-	-	-
18-0412	*A. nummularia*	18.327650453	-31.558074008	48.937	-	-	-	-
18-0413	*A. nummularia*	18.327535714	-31.558058657	48.965	-	-	-	-
18-0414	*A. nummularia*	18.327521878	-31.558095840	48.237	-	-	-	-
18-0415	*A. nummularia*	18.327477092	-31.558058687	48.653	-	-	-	-
18-0416	*A. nummularia*	18.327402002	-31.558052520	48.676	-	-	-	-
18-0417	*A. nummularia*	18.327353652	-31.558050103	48.659	-	-	-	-
18-0418	*A. nummularia*	18.327248731	-31.558041144	48.110	-	-	-	-
18-0419	*A. nummularia*	18.327116604	-31.558035578	48.602	29,41	-	-	-
18-0420	*A. nummularia*	18.327101671	-31.558067293	49.022	-	-	-	-
18-0421	*O. oppositifolum*	18.326559381	-31.557670784	51.594	-	-	-	-
18-0422	*O. oppositifolum*	18.326564223	-31.557657357	53.637	-	-	-	-
18-0423	*O. oppositifolum*	18.326524848	-31.557674195	53.237	-	-	-	-
18-0424	*O. oppositifolum*	18.326512833	-31.557702768	52.950	-	-	-	-
18-0425	*O. oppositifolum*	18.326495122	-31.557733508	52.955	-	-	-	-

18-0426	*O. oppositifolum*	18.326459719	-31.557747977	53.573	-	-	-	-
18-0427	*O. oppositifolum*	18.326428441	-31.557759245	54.564	-	-	-	-
18-0428	*O. oppositifolum*	18.326386125	-31.557743457	54.921	-	-	-	-
18-0429	*O. oppositifolum*	18.326352844	-31.557746651	56.317	-	-	-	-
18-0430	*O. oppositifolum*	18.326343252	-31.557710620	57.001	-	-	-	-
18-0431	*O. pes-caprae*	18.327316258	-31.557962999	50.715	-	-	-	-
18-0432	*O. pes-caprae*	18.327344097	-31.557965760	51.127	-	-	-	-
18-0433	*O. pes-caprae*	18.327372202	-31.557971421	51.023	-	-	-	-
18-0434	*O. pes-caprae*	18.327401451	-31.557970614	50.863	-	-	-	-
18-0435	*O. pes-caprae*	18.327417704	-31.557975572	50.814	-	-	-	-
18-0436	*O. pes-caprae*	18.327458906	-31.557990001	49.299	29,27	-	-	-
18-0437	*O. pes-caprae*	18.327483546	-31.557995525	49.632	-	-	-	-
18-0438	*O. pes-caprae*	18.327542138	-31.558002310	49.882	-	-	-	-
18-0439	*O. pes-caprae*	18.327597548	-31.558000789	49.690	-	-	-	-
18-0440	*A. semibaccata*	18.327684433	-31.558010602	49.291	-	-	-	-
18-0441	*A. semibaccata*	18.328041807	-31.558114560	47.860	23,53	-	-	-
18-0442	*A. semibaccata*	18.327882221	-31.558103484	47.518	21,69	-	-	-
18-0443	*A. semibaccata*	18.327829803	-31.558099668	48.244	26,04	-	-	-
18-0444	*A. semibaccata*	18.327819129	-31.558088154	48.350	23,19	-	-	-
18-0445	*A. semibaccata*	18.327802102	-31.558114330	48.453	22,39	-	-	-

18-0446	*A. semibaccata*	18.327622520	-31.558099483	48.238	26,20	-	-	-
18-0447	*A. semibaccata*	18.327589014	-31.558075943	48.239	22,10	-	-	-
18-0448	*A. semibaccata*	18.327593764	-31.558098816	48.139	22,46	-	-	-
18-0449	*A. semibaccata*	18.327608987	-31.558111542	48.286	27,86	-	-	-
18-0450	*A. semibaccata*	18.327514964	-31.558060801	49.234	23,08	-	-	-
18-0451	*A. semibaccata*	18.327417060	-31.558048475	48.800	19,52	-	-	-
18-0452	*A. semibaccata*	18.327379622	-31.558047518	48.158	21,14	-	-	-
18-0453	*A. semibaccata*	18.327369108	-31.558043601	48.278	20,96	-	-	-
18-0454	*A. semibaccata*	18.327227313	-31.558031291	48.387	19,80	-	-	-
18-0455	*A. semibaccata*	18.327187502	-31.558060704	48.173	21,18	-	-	-
18-0456	*A. semibaccata*	18.327089832	-31.558028175	47.561	19,10	-	-	-
18-0457	*A. semibaccata*	18.327064516	-31.558029925	47.745	30,07	-	-	-
18-0458	*A. semibaccata*	18.327050027	-31.558065909	47.654	21,43	-	-	-
18-0459	*A. semibaccata*	18.327031602	-31.558033373	48.006	21,90	-	-	-
18-0460	*A. semibaccata*	18.327002910	-31.558034912	48.432	22,42	-	-	-
18-0461	*O. suffruticosum*	18.326262524	-31.557962334	52.372	-	-	-	-
18-0462	*O. suffruticosum*	18.326244270	-31.557969555	52.052	-	-	-	-
18-0463	*O. suffruticosum*	18.326232782	-31.557983905	53.424	-	-	-	-
18-0464	*O. suffruticosum*	18.326203714	-31.557996267	52.395	-	-	-	-
18-0465	*O. suffruticosum*	18.326183727	-31.558007786	51.272	-	-	-	-

18-0466	*O. suffruticosum*	18.326173991	-31.557980753	51.483	-	-	-	-
18-0467	*O. suffruticosum*	18.326196467	-31.557974497	51.727	-	-	-	-
18-0468	*O. suffruticosum*	18.326184937	-31.557968002	51.846	-	-	-	-
18-0469	*O. suffruticosum*	18.326213464	-31.557965680	52.234	-	-	-	-
18-0470	*O. suffruticosum*	18.326246744	-31.557948434	52.617	-	-	-	-
18-0471	*O. suffruticosum*	18.326264963	-31.557930095	53.180	-	-	-	-
18-0472	*O. suffruticosum*	18.326284561	-31.557922286	53.563	-	-	-	-
18-0473	*O. suffruticosum*	18.326275094	-31.557903904	53.835	-	-	-	-
18-0474	*O. suffruticosum*	18.326235569	-31.557898750	54.061	-	-	-	-
18-0475	*O. suffruticosum*	18.326175979	-31.557897032	54.548	-	-	-	-
18-0476	*O. suffruticosum*	18.326416424	-31.557923614	51.871	-	-	-	-
18-0477	*O. suffruticosum*	18.326502733	-31.557878375	52.165	-	-	-	-
18-0478	*O. suffruticosum*	18.326524489	-31.557857127	51.481	-	-	-	-
18-0479	*O. suffruticosum*	18.326555366	-31.557810191	52.235	-	-	-	-
18-0480	*O. suffruticosum*	18.326559105	-31.557754757	52.055	-	-	-	-
18-0481	*D. hispidum*	18.326411026	-31.557807377	54.828	-	-	-	-
18-0482	*D. hispidum*	18.326397149	-31.557814594	55.214	-	-	-	-
18-0483	*D. hispidum*	18.326337048	-31.557833401	55.465	-	-	-	-
18-0484	*D. hispidum*	18.326337175	-31.557842514	55.201	-	-	-	-
18-0485	*D. hispidum*	18.326294861	-31.557846615	56.089	-	-	-	-

18-0486	*D. hispidum*	18.326288379	-31.557805287	55.998	-	-	-	-
18-0487	*D. hispidum*	18.326279457	-31.557781247	57.324	-	-	-	-
18-0488	*D. hispidum*	18.326224413	-31.557778027	58.349	-	-	-	-
18-0489	*D. hispidum*	18.326313422	-31.557729596	57.766	-	-	-	-
18-0490	*D. hispidum*	18.326323234	-31.557723416	57.301	-	-	-	-
18-0491	*A. nummularia*	18.526613733	-31.721943127	56.529	-	-	-	-
18-0492	*A. nummularia*	18.526642599	-31.721931329	56.028	-	-	-	-
18-0493	*A. nummularia*	18.526762653	-31.721915258	54.553	-	-	-	-
18-0494	*A. nummularia*	18.526776464	-31.721897393	54.095	-	-	-	-
18-0495	*A. nummularia*	18.526841182	-31.721869262	53.189	-	-	-	-
18-0496	*A. nummularia*	18.526883749	-31.721857667	52.834	-	-	-	-
18-0497	*A. nummularia*	18.526908070	-31.721855103	51.869	-	-	-	-
18-0498	*A. nummularia*	18.526951666	-31.721840832	52.032	-	-	-	-
18-0499	*A. nummularia*	18.527008798	-31.721828699	51.490	-	-	-	-
18-0500	*A. nummularia*	18.527031283	-31.721801204	51.129	-	-	-	-
18-0501	*A. nummularia*	18.527064588	-31.721822550	51.032	-	-	-	-
18-0502	*A. nummularia*	18.527093813	-31.721813442	49.967	-	-	-	-
18-0503	*A. nummularia*	18.527124557	-31.721814824	50.451	-	-	-	-
18-0504	*A. nummularia*	18.527196661	-31.721867113	50.274	-	-	-	-
18-0505	*A. nummularia*	18.527250093	-31.721806116	49.247	-	-	-	-

18-0506	*A. nummularia*	18.527299695	-31.721830843	49.497	-	-	-	-
18-0507	*A. nummularia*	18.527342100	-31.721817220	49.494	-	-	-	-
18-0508	*A. nummularia*	18.527408217	-31.721797263	48.912	-	-	-	-
18-0509	*A. nummularia*	18.527461284	-31.721782672	48.623	-	-	-	-
18-0510	*A. nummularia*	18.527520027	-31.721764406	48.709	-	-	-	-
18-0511	*L. ferocissimum*	18.527213592	-31.721814864	49.469	26,70	-	-	-
18-0512	*L. ferocissimum*	18.527175182	-31.721799415	49.309	-	-	-	-
18-0513	*L. ferocissimum*	18.527315739	-31.721747776	49.500	-	-	-	-
18-0514	*L. ferocissimum*	18.527223414	-31.721777966	48.870	-	-	-	-
18-0515	*L. ferocissimum*	18.527206903	-31.721784043	49.445	-	-	-	-
18-0516	*L. ferocissimum*	18.527233591	-31.721797984	50.044	30,99	-	-	-
18-0517	*L. ferocissimum*	18.527383975	-31.721851864	48.014	-	-	-	-
18-0518	*L. ferocissimum*	18.527298334	-31.722013218	49.402	25,23	-	-	-
18-0519	*L. ferocissimum*	18.527302985	-31.722028874	48.307	25,35	-	-	-
18-0520	*L. ferocissimum*	18.527317369	-31.722022370	46.879	24,20	-	-	-
18-0521	*L. ferocissimum*	18.527357798	-31.721978214	48.228	30,24	-	-	-
18-0522	*L. ferocissimum*	18.527347608	-31.721964165	47.930	30,86	-	-	-
18-0523	*L. ferocissimum*	18.527347049	-31.721950570	47.963	25,77	-	-	-
18-0524	*L. ferocissimum*	18.527368707	-31.721928907	47.960	30,19	-	-	-
18-0525	*L. ferocissimum*	18.527395930	-31.721946932	48.165	21,01	-	-	-

18-0526	*L. ferocissimum*	18.527380747	-31.721908957	48.245	29,98	-	-	-
18-0527	*L. ferocissimum*	18.527386212	-31.722010893	48.417	29,23	-	-	-
18-0528	*L. ferocissimum*	18.527438240	-31.721992340	48.313	29,89	-	-	-
18-0529	*L. ferocissimum*	18.527388949	-31.722076458	48.304	25,05	-	-	-
18-0530	*L. ferocissimum*	18.527480260	-31.722098955	48.075	30,44	-	-	-
18-0531	*O. grandiflorum*	18.527222412	-31.722008486	48.545	-	-	-	-
18-0532	*O. grandiflorum*	18.527219418	-31.721990482	49.294	-	-	-	-
18-0533	*O. grandiflorum*	18.527217941	-31.721971095	49.181	-	-	-	-
18-0534	*O. grandiflorum*	18.527205713	-31.721980600	48.343	-	-	-	-
18-0535	*O. grandiflorum*	18.527205390	-31.721971320	48.212	-	-	-	-
18-0536	*O. grandiflorum*	18.527236738	-31.721965290	48.664	-	-	-	-
18-0537	*O. grandiflorum*	18.527235980	-31.721957665	48.600	-	-	-	-
18-0538	*O. grandiflorum*	18.527222132	-31.721953517	48.534	-	-	-	-
18-0539	*O. grandiflorum*	18.527250987	-31.721938672	48.754	-	-	-	-
18-0540	*O. grandiflorum*	18.527185073	-31.721903861	49.244	-	-	-	-
18-0541	*O. grandiflorum*	18.527165145	-31.721907268	49.674	-	-	-	-
18-0542	*O. grandiflorum*	18.527054130	-31.721855851	50.782	-	-	-	-
18-0543	*O. grandiflorum*	18.527072782	-31.721849791	50.788	-	-	-	-
18-0544	*O. grandiflorum*	18.527157562	-31.721828122	50.034	-	-	-	-
18-0545	*O. grandiflorum*	18.527166927	-31.721823703	50.177	-	-	-	-

18-0546	*O. grandiflorum*	18.527189684	-31.721817627	50.432	-	-	-	-
18-0547	*O. grandiflorum*	18.527245433	-31.721846558	49.891	-	-	-	-
18-0548	*O. grandiflorum*	18.527268028	-31.721848172	50.152	-	-	-	-
18-0549	*O. grandiflorum*	18.527307631	-31.721836111	49.497	-	-	-	-
18-0550	*O. grandiflorum*	18.527318701	-31.721850300	48.537	-	-	-	-
18-0551	*A. menziesii*	18.527262772	-31.721806172	49.558	-	-	-	-
18-0552	*A. menziesii*	18.527368600	-31.721770989	49.457	-	-	-	-
18-0553	*A. menziesii*	18.527375370	-31.721766556	49.040	-	-	-	-
18-0554	*A. menziesii*	18.527384163	-31.721766494	49.375	-	-	-	-
18-0555	*A. menziesii*	18.527394503	-31.721765197	48.974	-	-	-	-
18-0556	*A. menziesii*	18.527406720	-31.721761554	49.587	-	-	-	-
18-0557	*A. menziesii*	18.527432285	-31.721758958	49.535	-	-	-	-
18-0558	*A. menziesii*	18.527445972	-31.721756585	49.754	-	-	-	-
18-0559	*A. menziesii*	18.527460136	-31.721749610	49.504	-	-	-	-
18-0560	*A. menziesii*	18.527481045	-31.721740768	49.414	-	-	-	-
18-0561	*A. semibaccata*	18.476252296	-31.655402856	44.685	26,58	-	-	-
18-0562	*A. semibaccata*	18.476227068	-31.655416397	43.724	24,03	-	-	-
18-0563	*A. semibaccata*	18.476222577	-31.655434789	43.400	27,06	-	-	-
18-0564	*A. semibaccata*	18.476239091	-31.655379682	43.880	28,16	-	-	-
18-0565	*A. semibaccata*	18.476252015	-31.655364318	43.580	24,95	-	-	-

18-0566	*A. semibaccata*	18.476277161	-31.655331718	44.052	24,27	-	-	-
18-0567	*A. semibaccata*	18.476279386	-31.655308007	43.750	21,19	-	-	-
18-0568	*A. semibaccata*	18.476297247	-31.655274876	44.120	-	-	-	-
18-0569	*A. semibaccata*	18.476307096	-31.655239842	43.754	29,94	-	-	-
18-0570	*A. semibaccata*	18.476340994	-31.655192381	43.604	24,40	-	-	-
18-0571	*A. semibaccata*	18.476321747	-31.655184469	43.462	19,91	-	-	-
18-0572	*A. semibaccata*	18.476339215	-31.655162889	43.613	21,66	-	-	-
18-0573	*A. semibaccata*	18.476330751	-31.655146743	43.222	26,23	-	-	-
18-0574	*A. semibaccata*	18.476350711	-31.655136876	43.940	20,39	-	-	-
18-0575	*A. semibaccata*	18.476349877	-31.655123499	44.110	20,41	-	-	-
18-0576	*A. semibaccata*	18.476353529	-31.655097809	43.763	20,75	-	-	-
18-0577	*A. semibaccata*	18.476363715	-31.655076430	43.329	18,40	-	-	-
18-0578	*A. semibaccata*	18.476375097	-31.655051194	44.420	18,36	-	-	-
18-0579	*A. semibaccata*	18.476377528	-31.655030836	43.315	19,43	-	-	-
18-0580	*A. semibaccata*	18.476426657	-31.654950995	43.525	19,68	-	-	-
18-0581	*R. rugosum*	18.476454422	-31.655090365	44.877	26,48	-	-	-
18-0582	*R. rugosum*	18.476448056	-31.655110212	45.058	21,01	-	-	-
18-0583	*R. rugosum*	18.476431727	-31.655143802	44.814	27,82	-	-	-
18-0584	*R. rugosum*	18.476407982	-31.655218220	45.776	29,15	-	-	-
18-0585	*R. rugosum*	18.476401560	-31.655231381	44.665	29,32	-	-	-

18-0586	*R. rugosum*	18.476392343	-31.655251704	44.931	29,89	-	-	-
18-0587	*R. rugosum*	18.476376224	-31.655280279	44.773	30,16	-	-	-
18-0588	*R. rugosum*	18.476355645	-31.655348207	45.191	29,98	-	-	-
18-0589	*R. rugosum*	18.476340893	-31.655381289	45.195	30,00	-	-	-
18-0590	*R. rugosum*	18.476328501	-31.655401501	44.674	30,29	-	-	-
18-0591	*R. rugosum*	18.476298386	-31.655479263	44.766	30,89	-	-	-
18-0592	*R. rugosum*	18.476282614	-31.655514626	45.090	30,99	-	-	-
18-0593	*R. rugosum*	18.476251600	-31.655596805	45.409	29,83	-	-	-
18-0594	*R. rugosum*	18.476229476	-31.655637620	45.205	-	-	-	-
18-0595	*R. rugosum*	18.476185390	-31.655741633	44.891	30,99	-	-	-
18-0596	*R. rugosum*	18.476106481	-31.655925990	45.258	-	-	-	-
18-0597	*R. rugosum*	18.476092962	-31.655767751	44.631	-	-	-	-
18-0598	*R. rugosum*	18.476095376	-31.655670460	44.575	-	-	-	-
18-0599	*R. rugosum*	18.476154484	-31.655605119	44.137	-	-	-	-
18-0600	*R. rugosum*	18.476213735	-31.655429424	43.899	29,72	-	-	-

*Only the Ct values of the samples with a Ct value below 31 are indicated.
**Samples that yielded amplicons of the 16S rRNA gene, but sequencing and phylogenetic analysis indicated these were not Liberibacters.
- = no Ct values obtained after 40 cycles of amplification.

Appendix B – Phylogenetic analysis

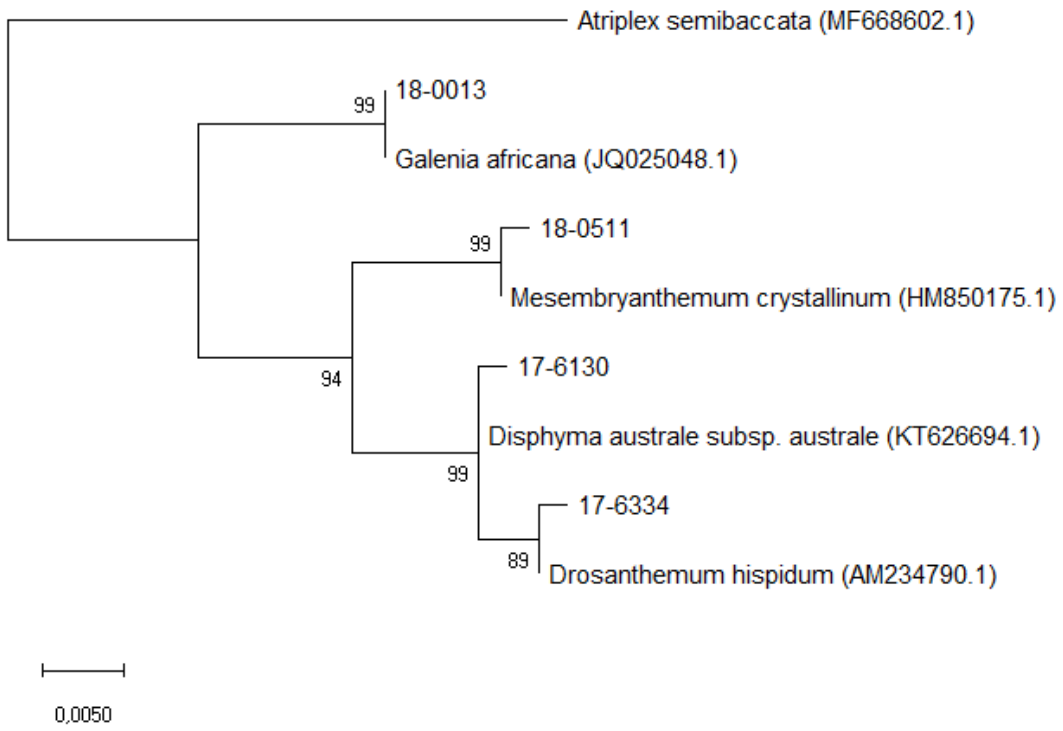


Figure B.1: Maximum-likelihood phylogeny of the plant species from Aizoaceae based on rbcL barcoding sequence. *Atriplex semibaccata rbcL* sequence obtained from GenBank is included as an outgroup. The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Tamura-3-parameter model (Tamura, 1992). Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,005 substitutions per nucleotide position.

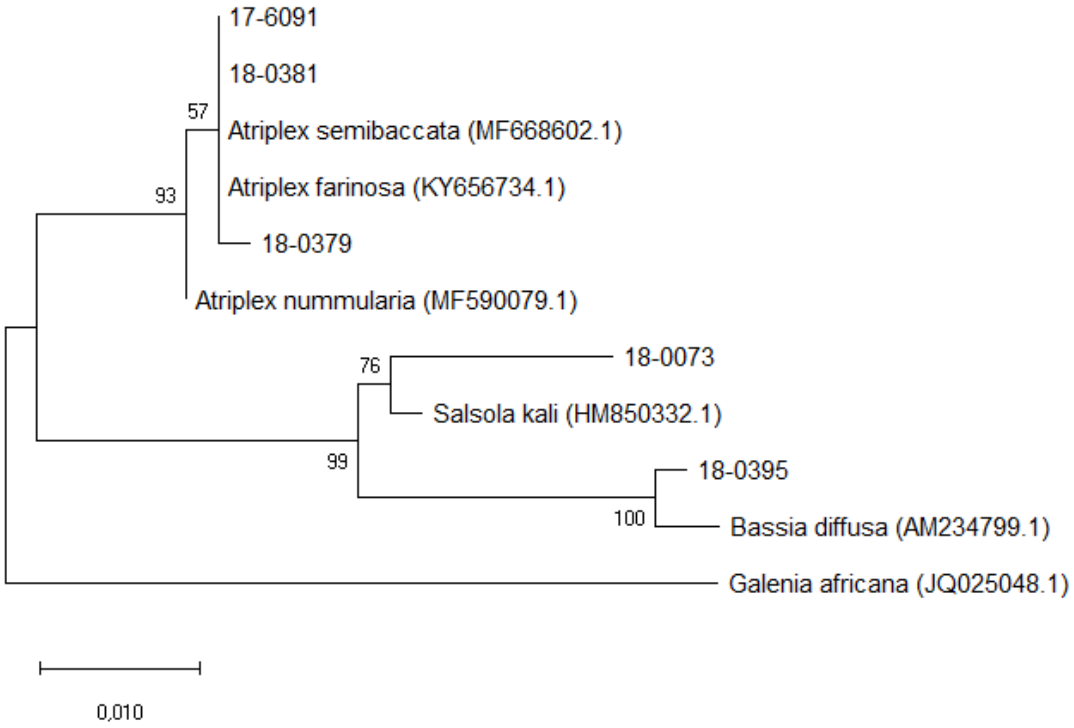


Figure B.2: Maximum-likelihood phylogeny of the plant species from Amaranthaceae based on rbcL barcoding sequence. *Galenia africanum* (*Aizoon africanum*) *rbcL* sequence obtained from GenBank is included as an outgroup. The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980). Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,01 substitutions per nucleotide position. Sample 18-0379 contained DNA extracted from *Atriplex lindleyi* (based on morphology) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *A. farinosa* was used (closest related to *A. lindleyi* based on barcoding and BLAST). Sample 18-0395 contained DNA extracted from *Eriocephalus brevifolius* (based on morphology) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *B. diffusa* was used (closest related to *E. brevifolius* based on barcoding and BLAST).

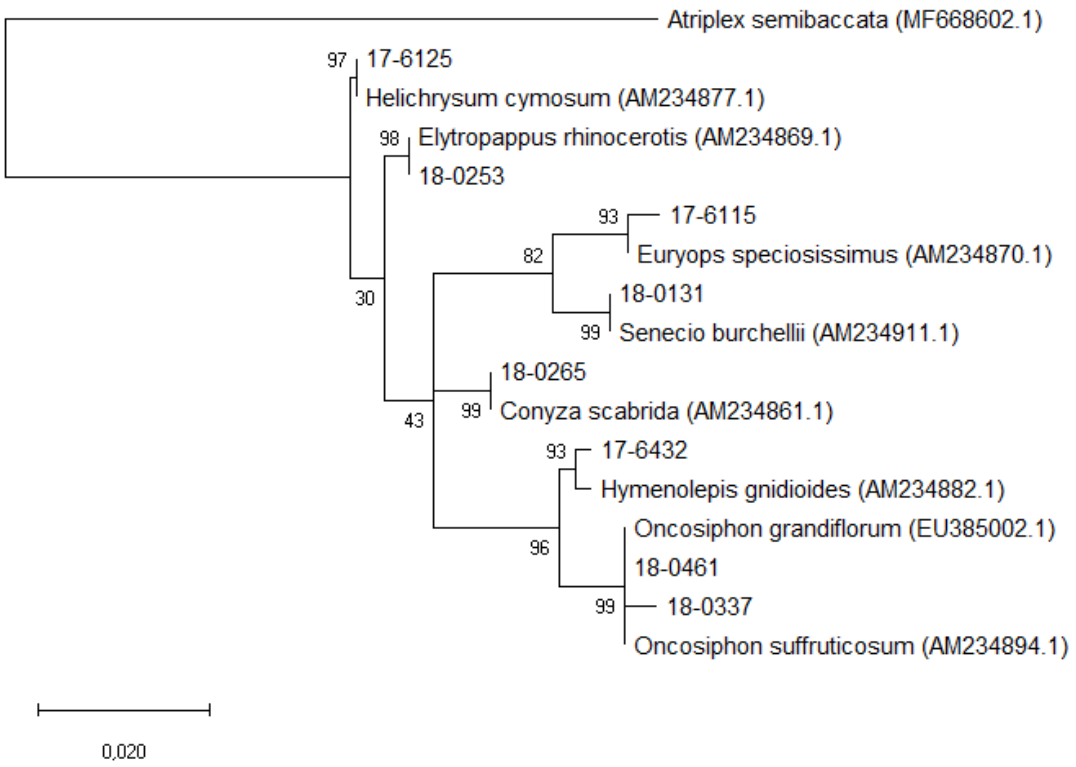


Figure B.3: Maximum-likelihood phylogeny of the plant species from Asteraceae based on rbcL barcoding sequence. Atriplex semibaccata rbcL sequence obtained from GenBank is included as an outgroup. The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Tamura-3-parameter model (Tamura, 1992) with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,02 substitutions per nucleotide position. Sample 17-6125 contained DNA extracted from *Helichrysum crithmifolia* (based on morphology) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *H. gnidioides* was used (closest related to *H. crithmifolia* based on barcoding and BLAST).

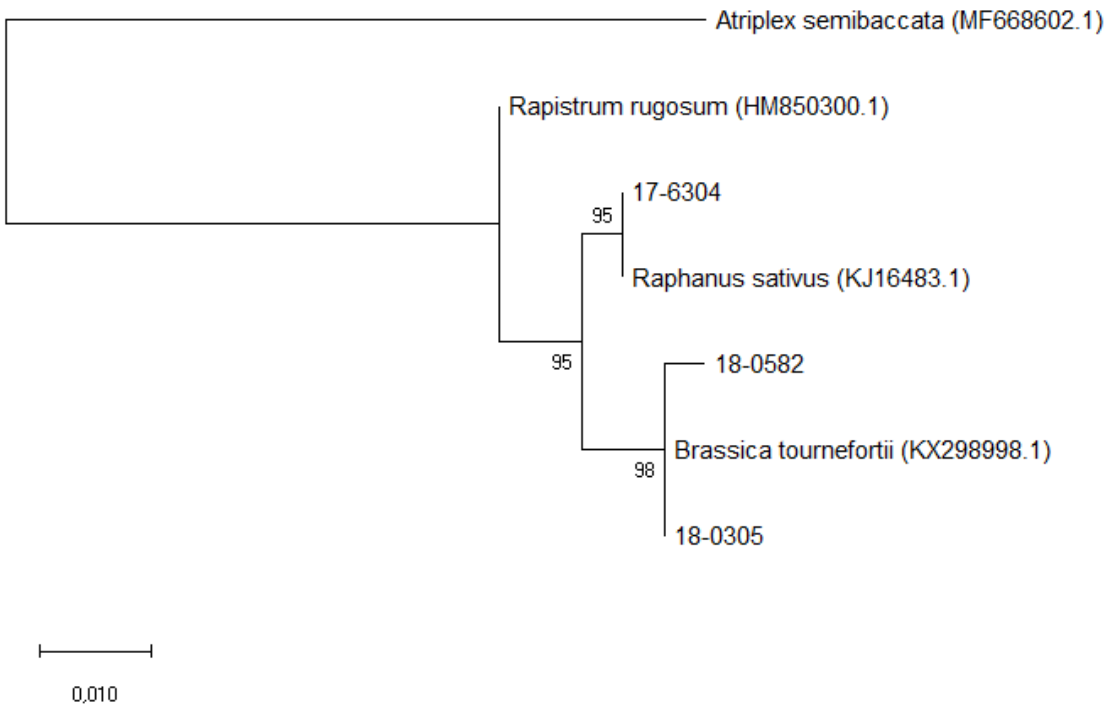


Figure B.4: Maximum-likelihood phylogeny of the plant species from Brassicaceae based on rbcL barcoding sequence. Atriplex *semibaccata rbcL* sequence obtained from GenBank is included as an outgroup. The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Tamura-3-parameter model (Tamura, 1992). Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,01 substitutions per nucleotide position. Sample 17-6304 contained DNA extracted from *Raphanus raphanistrum* (based on morphology) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *R. sativus* was used (closest related to *R. raphanistrum* based on barcoding and BLAST). Sample 18-0582 was extracted from *Rapistrum rugosum.*

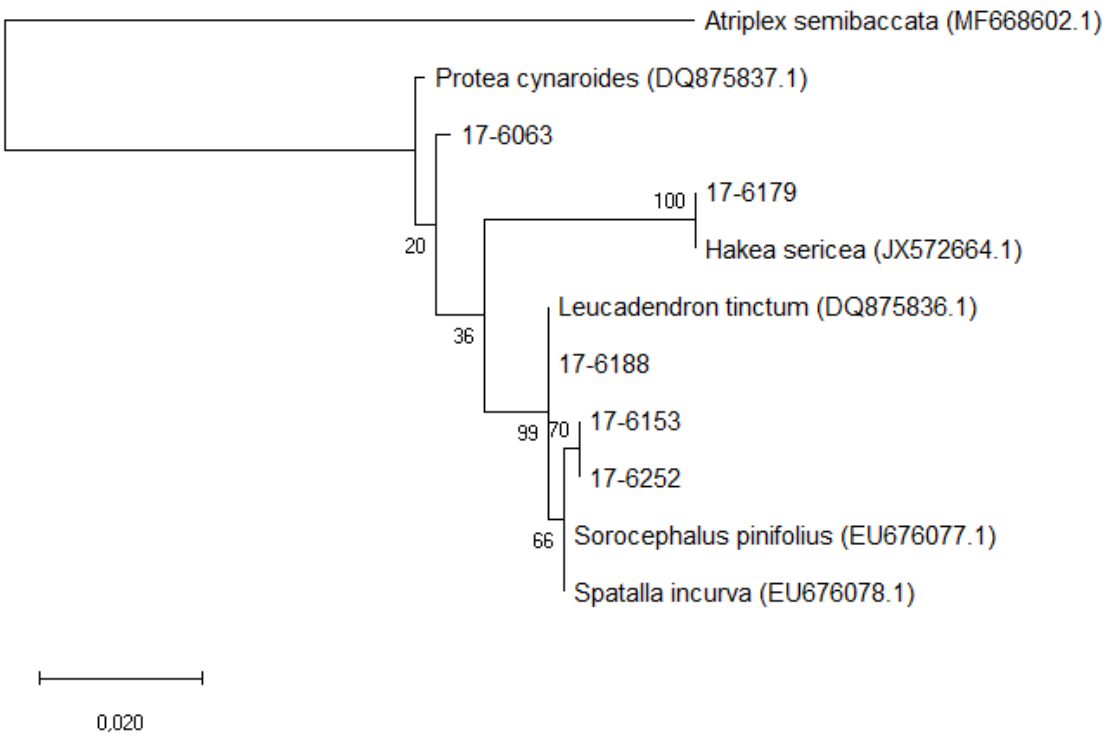


Figure B.5: Maximum-likelihood phylogeny of the plant species from Proteaceae based on rbcL barcoding sequence. *Atriplex semibaccata rbcL* sequence obtained from GenBank is included as an outgroup. The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980) with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,02 substitutions per nucleotide position. Sample 17-6179 contained DNA extracted from *Hakea sericea* (based on morphology) for which the GenBank sequence of the *rbcL* gene is not available, therefore the *rbcL* gene of *H. sericea* was used instead of the sequence identified via BLAST (closest related to *H. archaeoides* based on barcoding and BLAST). Sample 17-6252 contained DNA extracted from an Unidentified *Restio* (based on morphology), therefore the *rbcL* gene of *S. incurva* was used (closest related to *E. brevifolius* based on barcoding and BLAST).

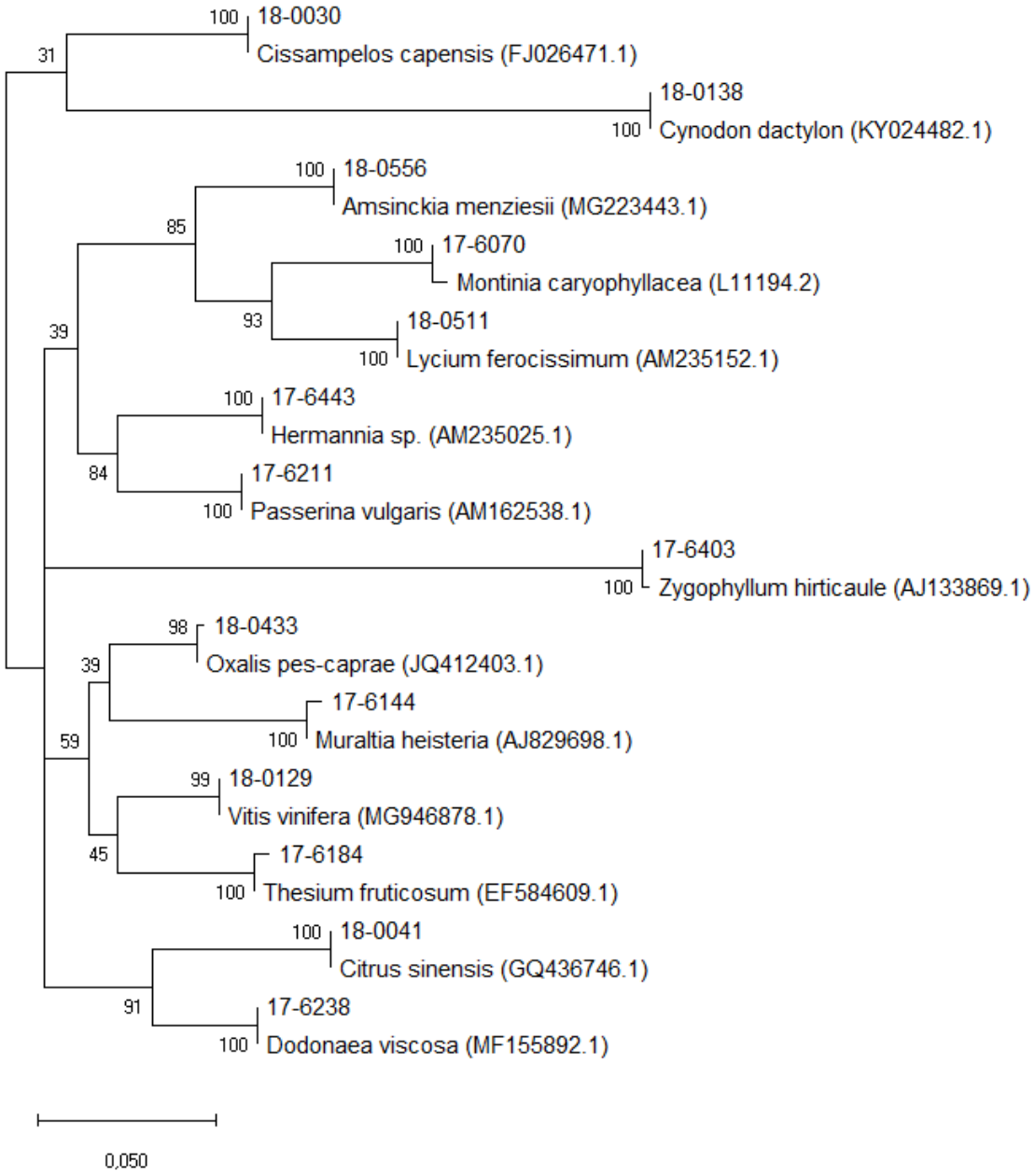


Figure B.6: Maximum-likelihood phylogeny of the plant species from different families based on *rbcL* barcoding sequences (see Table 7). The GenBank accession numbers are presented in brackets. The phylogeny was inferred using the Kimura-2-parameter model (Kimura, 1980) with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,05 substitutions per nucleotide position. Please see Table 4 for the identities of the plant species (based on morphology), as the *rbcL* sequence of closest relative (based on barcoding and BLAST) of the some of the plant species were used in the phylogenetic tree above. Sample 17-6403 contained DNA extracted from *Roepera foetida* (based on morphology) for which no GenBank sequence of the *rbcL* gene is available, therefore the *rbcL* gene of *Z. hirticaule* was used instead of the sequence identified via BLAST (closest related to *R. foetida* based on barcoding and BLAST).

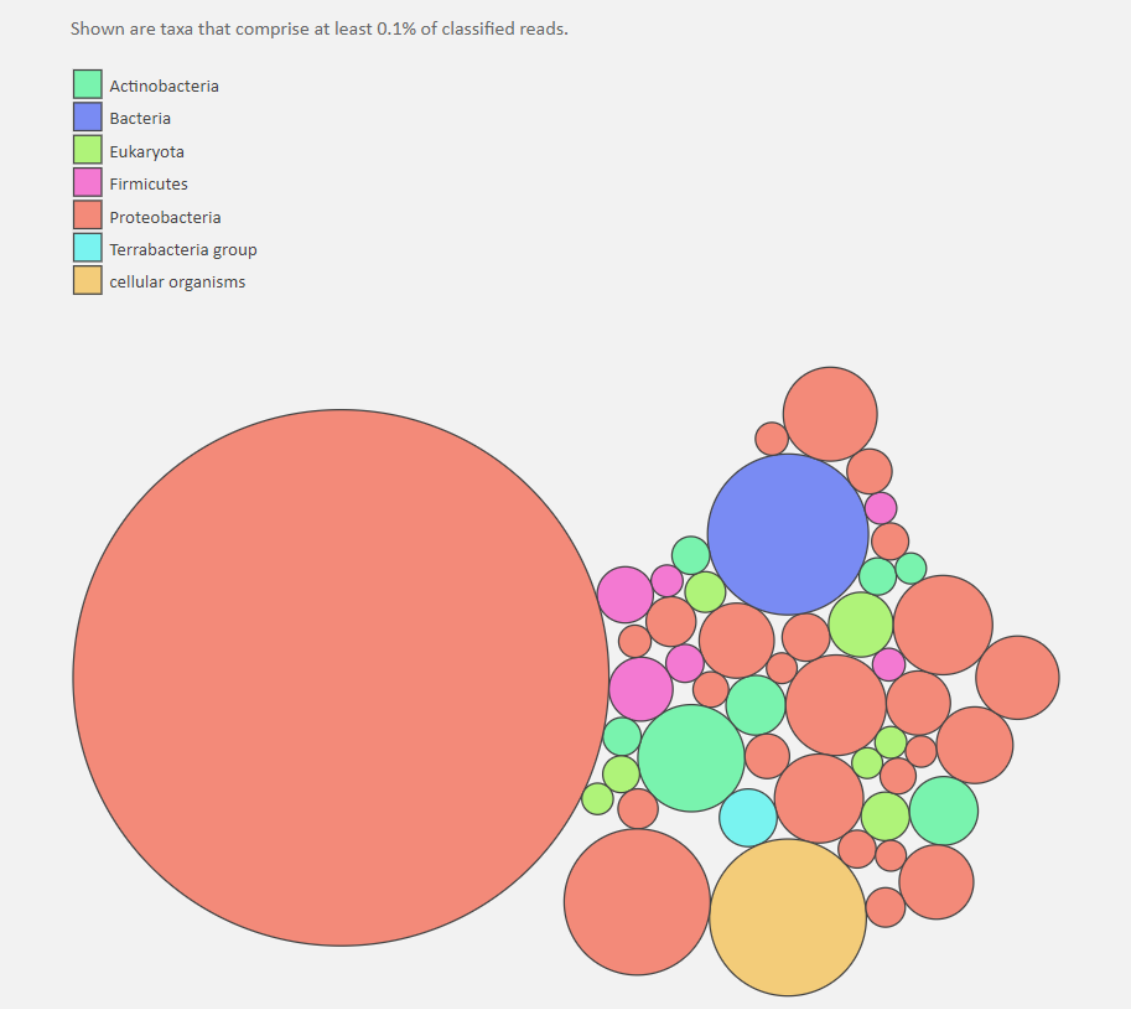


Figure B.7: Results obtained from Kaiju web server (http://kaiju.binf.ku.dk/results/114472-6041362856) indicating the taxa present within the reads obtained after NGS of sample 18-0151. The colour indication is listed at the top left. The majority reads were identified as Proteobacteria (red). Other taxa present: Actinobacteria (light blue), Bacteria (dark blue), Eukaryota (light green blue), Firmicutes (pink), Terrabacteria group (light blue), and other cellular organisms (orange).

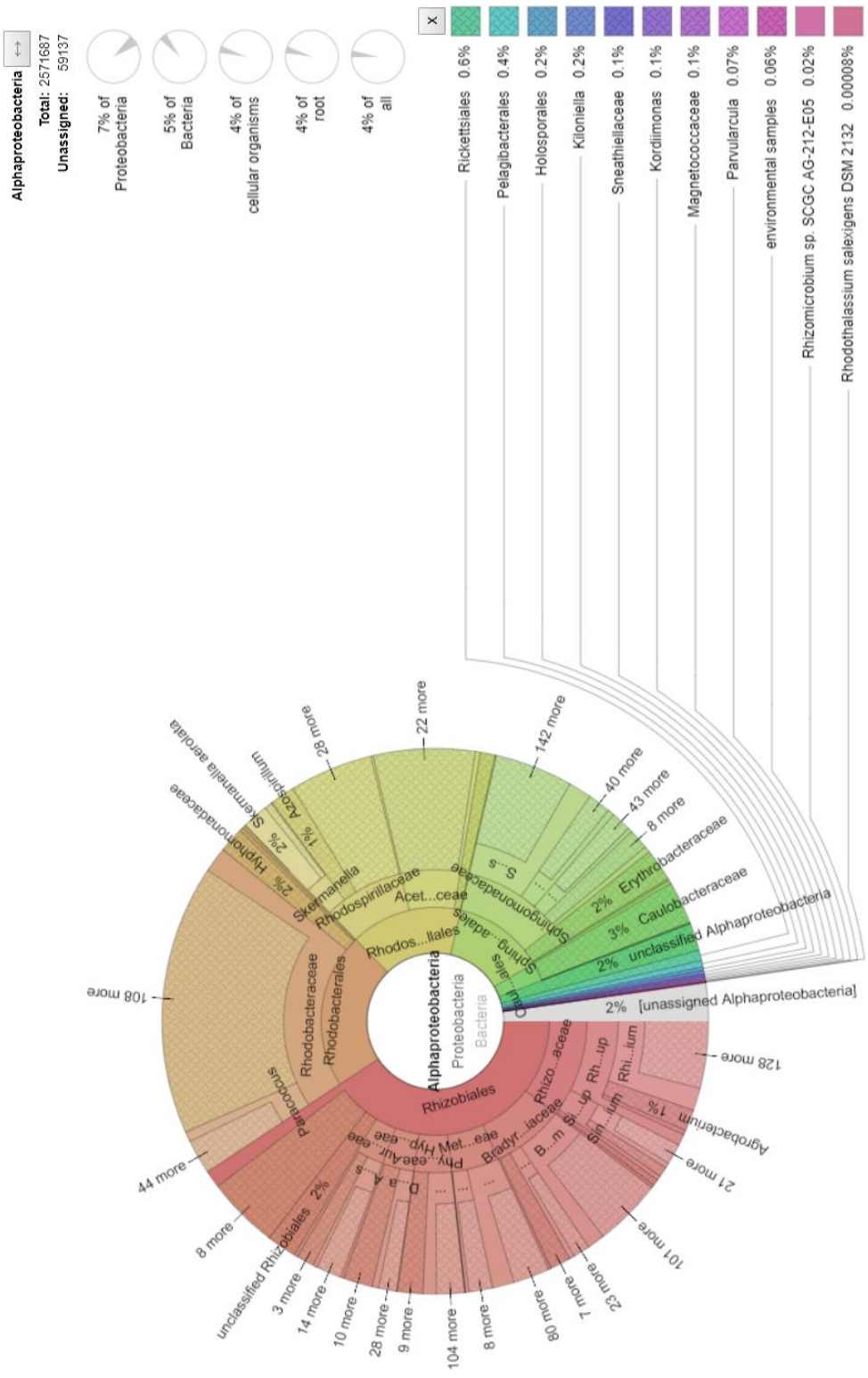


Figure B.8: Krona chart of the dataset obtained from NGS of sample 18-0151. The Krona chart is an in-browser interactive visualisation of the taxon abundances present within the NGS dataset. A total of 64,154,824 reads were present in the dataset that was analysed via the Krona chart. The chart was used to attempt to identify the presence of Liberibacter spp. Out of the 64 million reads, 4% of the reads (2,571,687 reads) matched known Alphaproteobacteria, which was 6.92% of the total Proteobacteria present within the NGS data.

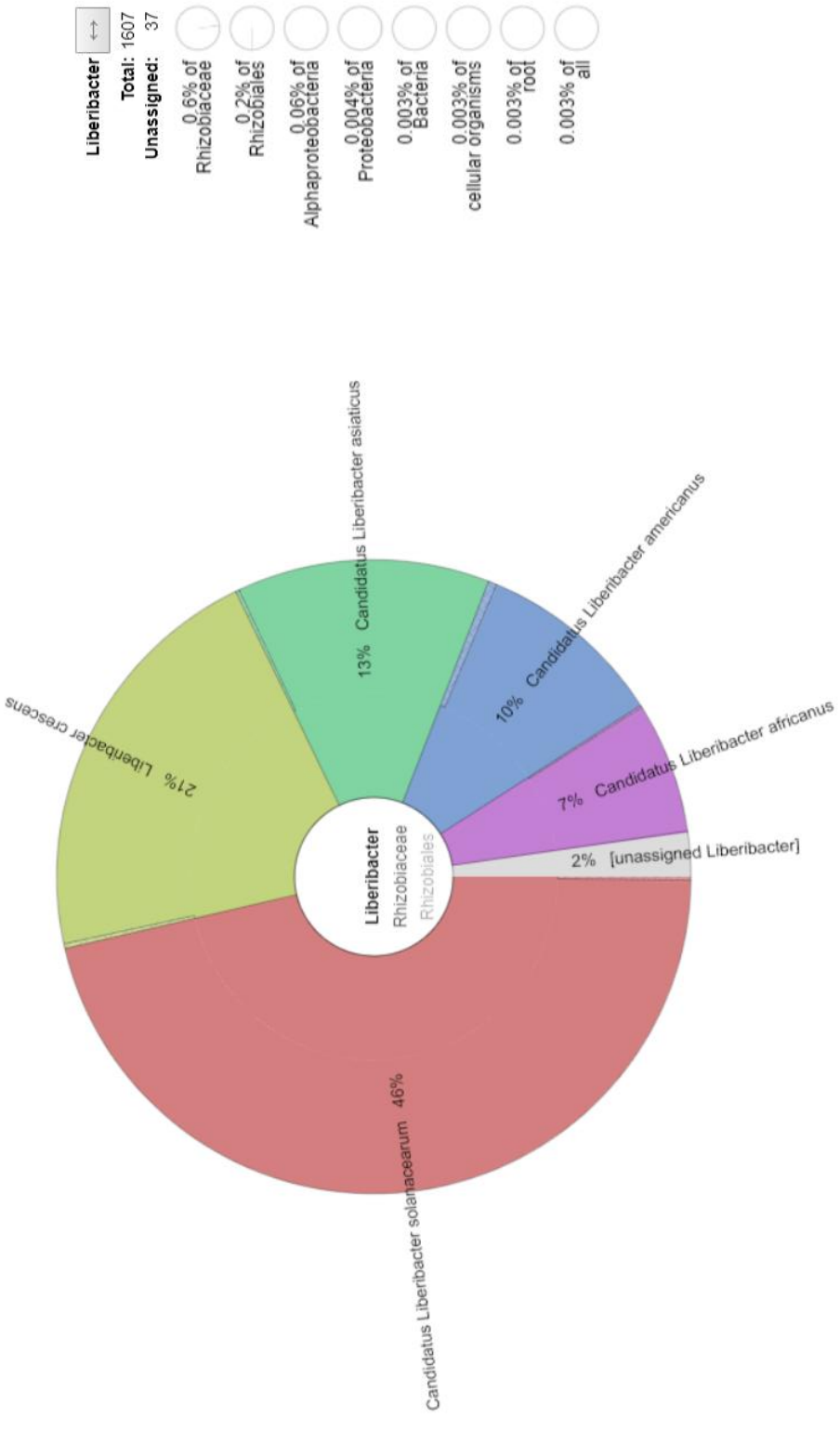


Figure B.9: Krona chart of the dataset obtained from NGS of sample 18-0151. Out of the 64 million reads, only 1607 reads (0.0025%) matched with Liberibacter spp. (Rhizobiales; Rizobiaceae). Of the 0.0025% sequences that matched Liberibacter spp., 46% sequences matched '*Ca.* L. solanacearum' (red), 21% matched *Liberibacter crescens* (light green), 13% matched '*Ca.* L. asiaticus' (dark green), 10% matched '*Ca.* L. americanus' (blue), 7% matched '*Ca.* L. africanus' (purple), and only 2% remained unidentified (grey).

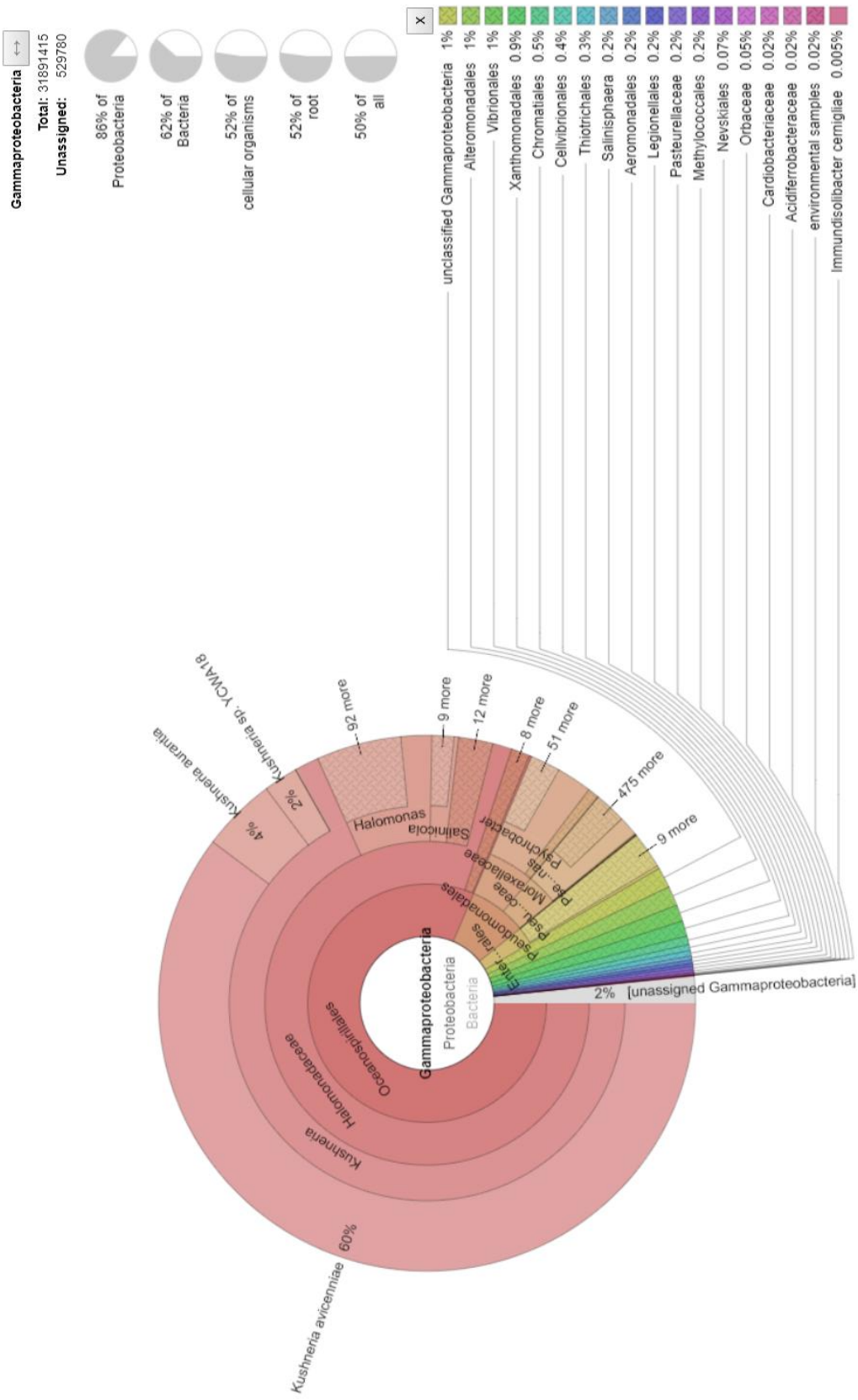


Figure B.10: Krona chart of the dataset obtained from NGS of sample 18-0151 indicating the Gammaproteobacteria present within the dataset. Nearly half of the 64 million reads (49.71%) were identified as Gammaproteobacteria. A total of 60% of the Gammaproteobacteria reads matched to *Kushneria avicenniae*.

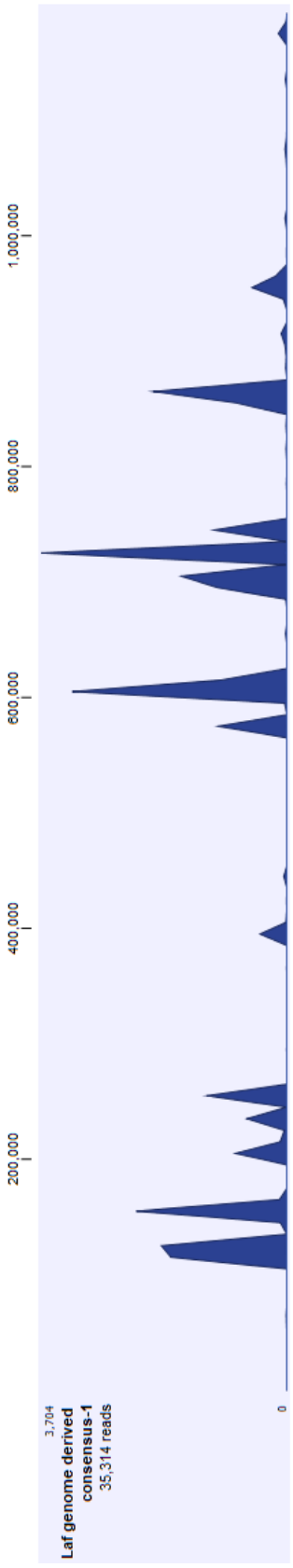


Figure B.11: Read mapping of NGS data from sample 18-0151 against complete genome of Laf (GenBank accession: CP004021.1). The amount of reads that matched to the complete genome of Laf was 35,314 out of the total 64,154,824 reads. Dark blue indicates the sequence regions matching the Laf genome.

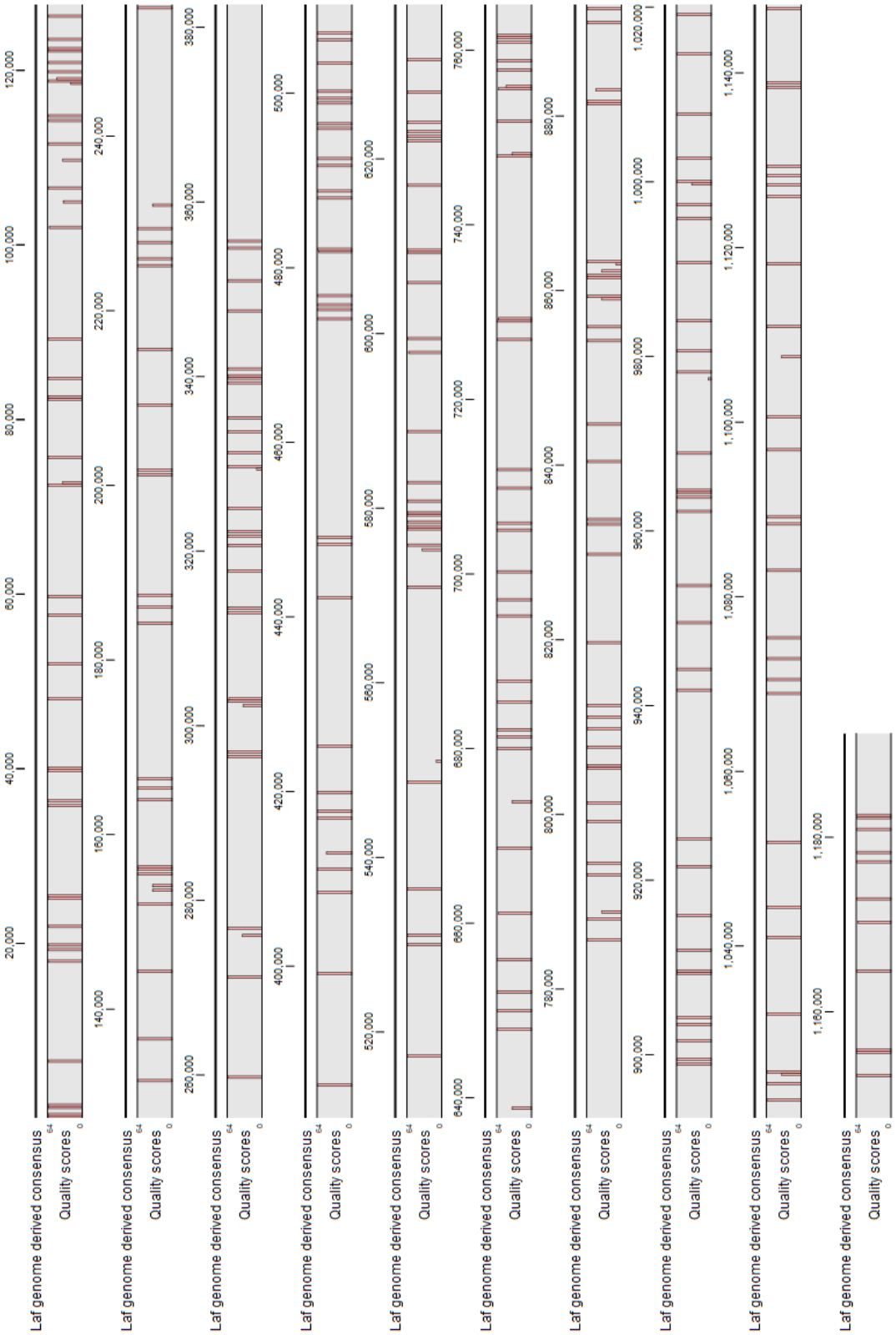


Figure B.12: Laf derived consensus sequence obtained from read mapping of NGS data from sample 18-0151 against complete genome of Laf (GenBank accession: CP004021.1). The amount of reads that matched to the complete genome of Laf was 35,314 out of the total 64,154,824 reads. Pink indicates sequence regions that matched the Laf genome, and grey indicates unmatched regions.

References

Kimura M, 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120.

Tamura K, 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution* **9**: 678-687.