# Genetic and biological diversity of '*Candidatus* Liberibacters' from South Africa

By

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

SIGNATURE: ..……..

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**List of Abbreviations**

| | |
|---|---|
| **˚C** | Degrees Celsius |
| **µl** | Microliter |
| **BLAST** | Basic Local Alignment Search Tool |
| **bp** | Base pair |
| **bv.** | Biovar |
| **Ca. L.** | *Candidatus* Liberibacter |
| **CFB** | Citrus Foundation Block |
| **CFR** | Cape Floristic Region |
| **CG** | Citrus Greening |
| **CGA** | Citrus Growers Association |
| **CIP** | Citrus Improvement Programme |
| **CRI** | Citrus Research International |
| **Ct** | Crossing Threshold |
| **CTAB** | Hexadecyl-trimethyl-ammonium bromide |
| **DAFF** | Department of Agriculture Forest and Fisheries |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxyribonucleotide Triphosphate |
| **dsRNA** | Double-Stranded RNA |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **GDP** | Gross Domestic Produce |
| **GPS** | Global Positioning System |
| **H** | Haploid Genetic Diversity |

| | |
|---|---|
| **Ha** | Hectare |
| **HLB** | Huanglongbing |
| **HTS** | High-Throughput Sequencing |
| **Laf** | *Candidatus* Liberibacter africanus |
| **LafC** | *Candidatus* Liberibacter africanus subsp. capensis |
| **LafCl** | *Candidatus* Liberibacter africanus subsp. clausenae |
| **LafT** | *Candidatus* Liberibacter africanus subsp. tecleae |
| **LafV** | *Candidatus* Liberibacter africanus subsp. vepridis |
| **LafZ** | *Candidatus* Liberibacter africanus subsp. zanthoxyli |
| **Lam** | *Candidatus* Liberibacter americanus |
| **LAMP** | Loop-mediated isothermal Amplification |
| **Las** | *Candidatus* Liberibacter asiaticus |
| **Lbr** | *Candidatus* Liberibacter brunswickensis |
| **Lcr** | *Liberibacter crescens* |
| **Leu** | *Candidatus* Liberibacter europaeus |
| **Lso** | *Candidatus* Liberibacter solanacearum |
| **Mb** | Mega bases |
| **min** | Minute |
| **MLO** | Mycoplasma-Like Organism |
| **mM** | Milli Mole |
| **Myr** | Million years |
| **Na** | Number of Alleles |
| **Ne** | Number of Effective Alleles |
| **PCoA** | Principle Coordinate Analysis |

| | |
|---|---|
| **PCR** | Polymerase Chain Reaction |
| **RFLP** | Restriction Fragment Length Polymorphism |
| **RNA** | Ribonucleic Acid |
| **RNAi** | RNA interference |
| **RPA** | Recombinase Polymerase Amplification |
| **rDNA** | Ribosomal DNA |
| **s** | Seconds |
| **SANBI** | South African National Biodiversity Institute |
| **SNP** | Single Nucleotide Polymorphism |
| **spp** | Species |
| **SSR** | Simple Sequence Repeats |
| **subsp** | Subspecies |
| **UPGMA** | Unweighted Pair Group Method with Arithmetic Mean |
| **USA** | United States of America |
| **VNTR** | Variable Number of Tandem Repeats |

**Summary**

Citrus greening disease (CG) in South Africa is associated with the phloem-limited bacterium, '*Candidatus* Liberibacter africanus' (Laf). This disease has been known to cause yellowing leaf symptoms as well as the formation of unprofitable fruit for nearly a century in this country. In addition to Laf, Liberibacters have been described from indigenous trees belonging to the Rutaceae family in this country. The first of these to be described was '*Ca.* L. africanus subsp. capensis' and was followed by the description of '*Ca.* L. africanus subsp. clausenae' (LafCl), *'Ca.* L. africanus subsp. vepridis' (LafV) and *'Ca.* L. africanus subsp. zanthoxyli' (LafZ). It has been speculated that either one of these Laf-subspecies may have given rise to Laf *senso stricto* associated with commercial citrus in South Africa. This dissertation aimed to expand on the indigenous rutaceous host species evaluated for the presence of Laf either as alternative hosts, or harbouring close relatives of Laf. From *Oricia, Teclea* and *Agathosma spp* sampled, a novel Liberibacter was only described from a *Teclea gerrardii* collected in Southern KwaZulu-Natal. This Liberibacter was named '*Ca.* L. africanus subsp. tecleae (LafT)'. It was further assessed whether any of these Laf-subspecies infect commercial citrus species in South Africa. Previous epidemiological studies on CG in South Africa found that Laf was the sole agent associated with this disease, with the current study supporting these findings. Finally, the genetic diversity of Laf was assessed across four provinces from different citrus types using microsatellite markers. This analysis indicated that the genetic diversity of Laf in South Africa is comparatively high, and that the genetic populations observed were mainly influenced by geographic distribution rather than citrus type. This study gave support to the hypothesis that Laf originated on the African continent from an indigenous species present in the country. The study further supports that Laf is well adapted to its commercial citrus host as genetically distinct populations are formed based on geographical populations.

# Chapter 1

# Introduction and overview of the study

## 1.1 Introduction

Citrus Greening disease (CG) in South Africa is associated with the phloem-limited bacterium, '*Candidatus* Liberibacter africanus' (Laf), within the Alphaproteobacteria (Garnier and Bové 1983, Jagoueix et al. 1994). Typical symptoms related with this disease include the appearance of a yellow mottle on affected mature leaves and the formation of deformed, bitter fruit (Manicom and van Vuuren 1991). During the late 1980s, CG was responsible for the near-elimination of citrus production in the lowveld, prompting extensive research into the control of this disease (Pretorius and van Vuuren 2006). Through the implementation of a three-pronged control strategy involving the planting of disease free material, the control of *Trioza erytrea* Del Geurcio (Hemiptera: Triozidae), the vector of Laf (McClean and Oberholzer 1965) and the removal of inoculum sources (Buitendag and von Broembsen 1993), CG incidence has been significantly reduced.

With this reduction, recent studies pertaining to CG from South Africa have mainly focused on the existence of alternative hosts of Laf which may act as reservoirs for this bacterium. Three native hosts of *T. erytreae,* i.e. *Clausena anisata, Vepris lanceolata* and *Zanthoxylum capense* were surveyed for the presence of Laf, however, Laf *senso stricto* was not identified from these hosts, but rather subspecies of Laf. Each of these subspecies were named for the host from which they were characterised i.e. '*Ca.* L. africanus subsp. clausenae' (LafCl), *'Ca.* L. africanus subsp. vepridis' (LafV) and '*Ca.* L. africanus subsp. zanthoxyli' (LafZ) (Roberts et al. 2015). These subspecies were described subsequent to the discovery of '*Ca.* L. africanus subsp. capensis' (LafC) (Garnier et al. 2000), identified from a *Calodendrum capense* tree grown in close proximity to a CG affected citrus orchards in the Western Cape. *C. capense* does not support the development of *T. erytreae* (Moran 1968), a factor likely to contribute to the lack of evidence of the involvement of LafC in the epidemiology of CG in South Africa (Pietersen et al. 2010).

Despite LafC not being implicated in CG in South Africa, the significance of these subspecies were demonstrated when it was found that a biovar of LafCl naturally infected commercial citrus species in East Africa (Roberts et al. 2017). This study shed light on the ability of Laf-subspecies to jump hosts, should the environment favour such a shift. It has been suggested that Laf evolved on the African continent from a Liberibacter which was present within an indigenous host prior to the introduction of commercial citrus species (Phahladira et al. 2012, Roberts et al. 2015). While Roberts et al. 2017 provided some evidence that CG evolved on

the African continent, not enough is known on the diversity of Liberibacter present within indigenous Rutaceous species in South Africa and whether any of these infect commercial citrus in South Africa. Also, the genetic make-up is of Laf populations in South Africa is unknown. Answers to these questions will help fill the knowledge gap which exists for Laf and potentially give insight into how Laf *senso stricto* came to be associated with a once locally devastating disease of citrus.

**1.2 Study overview**

The current study aimed to answer these questions in four research chapters:

*Chapter 3:*

The aim in Chapter 3 was to identify alternative hosts of Laf from indigenous *Teclea* and *Oricia* species. These species have a limited distribution and are mainly found in Southern KwaZulu-Natal. Liberibacters detected by real-time PCR were further described through end-point PCR, Sanger sequencing and phylogenetic analyses of core genes. The study further characterised the full 16S rDNA sequences of the Laf-subspecies identified from South Africa.

*Chapter 4:*

This chapter expanded on the indigenous Rutaceae hosts studied for the presence of Laf, or related Liberibacters within the genus *Agathosma*. *Agathosma* is a unique genus within the fynbos biome of the Cape floristic region. In contrast to the woody species previously studied for the presence of Laf, *Agathosma* species are aromatic shrubs found only in the Western Cape. The presence of Liberibacter from this genus was determined by means of a combination of real-time PCR, end-point PCR and high-throughput sequence analyses.

*Chapter 5:*

Knowing that a number of Laf-subspecies are present in South Africa, specific primers were designed for each of these subspecies for the purpose of identifying Laf-subspecies from commercial citrus. These primer sets were assessed for their ability to be used within a multiplex end-point PCR reaction. The primers were further utilised to determine whether any of the Laf-subspecies were present in historic DNA extracts collected from commercial citrus across South Africa.

*Chapter 6:*

Knowing the association of Laf-subspecies, thus far described, with indigenous rutaceous species, the final research chapter aimed to determine the genetic variability of Laf populations in South Africa in commercial citrus. This was achieved through a microsatellite approach. Populations studied originated from different geographical localities as well as from different citrus types.

*Chapter 7:*

The final chapter of this dissertation looks at the study holistically and attempts to make conclusions on the importance of describing Laf-subspecies from different indigenous hosts as well as making sense on the possible evolutionary processes that resulted in CG disease in South Africa.

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# Chapter 2

# Literature Review:

# An overview of Citrus Greening disease and its associated bacterium in South Africa

## 2.1 Introduction

The first documented record of citrus being introduced onto South African shores was noted in the diary of Jan van Riebeek in 1654. By 1661, van Riebeek's private orchard contained 1,172 citrus trees including sweet oranges, lemons and pomelos (Anonymous 1926) indicating the suitability of the Southern African climate to sustain this crop. Citrus production expanded throughout the country and by 1907, the first locally produced citrus was exported to international markets (Moore 1962). The expansion of citrus in South Africa was further demonstrated by the clearing of natural vegetation in Zebediela in the Limpopo province in 1918 to accommodate the planting of over half a million citrus trees over a six year period (Mathews 1986). Today, 77,708 ha of land in 7 of the 9 provinces, with the exception of the Free State and Gauteng, is dedicated to commercial citrus production with more than half of this land being used for the production of sweet oranges (CGA 2018). Citrus production accounts for R14,8 million of the country's gross domestic product (GDP) making up an astounding 25% of the total gross value contribution from horticulture (DAFF 2017). Globally, South Africa is ranked as the third largest exporter of citrus, with the main export markets being Europe and Asia (DAFF 2017).

Due to the economic importance of this industry, various measures have been put in place to ensure that citrus thrives in South Africa with special attention being given to a number of biosecurity risks. The 2018 annual report by the Citrus Growers Association (CGA), lists citrus black spot, false codling moth, *Bacterocera dorsalis* and Huanglongbing (HLB) as the most important phytosanitary risks to the citrus industry. Of these, HLB which has caused severe economic losses in Asia and the Americas (Gottwald et al. 2007, Zhang et al. 2010), has not yet been reported in South Africa. Despite this, a disease with similar disease expression, locally known as citrus greening (CG), has been documented here since the late 1920s (Oberholzer et al. 1965).

The first incidence in which CG caused significant losses to citrus crops was between 1932 and 1946 from White River, Mpumalanga. This was succeeded by losses being attributed to CG from citrus producers in Rustenburg and Tzaneen by 1958, rendering approximately 100 000 trees in Mpumalanga, the North West and Limpopo unprofitable (Oberholzer et al. 1965). The effect of this disease was hardest felt by production areas of Tzaneen, White River, Western Nelspruit and Rustenburg where commercial citrus production was virtually eliminated during the 1960 and 1970s (Pretorius and van Vuuren 2006).

This review will mainly focus on research which has been conducted on CG in South Africa, with comparisons being made to HLB and its associated pathogens. Additionally, where information is lacking for CG, the organism associated with HLB was used as model.

### 2.2 History and description of the causal agent of CG

By 1929, South African citrus producers within the Rustenburg area noticed a disease on citrus which was characterised by the appearance of a yellow mottling symptom on leaves, similar to that of a zinc deficiency. Fruits from branches displaying this yellowing symptom were small, bitter and remained green, giving rise to the name 'Citrus Greening disease'. Similar foliar and fruit symptoms were observed in different provinces of China in the late 1800's, where the disease was named for its yellow appearance as Huang Lung (Yellow shoot/dragon), Wei Huang (Withering yellow), Huang Qi Bin (yellow crab disease) with the most commonly adopted name being Huang Lung Bin – yellow shoot or yellow dragon disease (Huanglongbing or HLB) (Lin and Lin 1956). In the Philippines, the disease associated with these symptoms was known as mottle-leaf disease (Salibe and Cortez 1968) in Taiwan it was known as Likubin (Matsumoto and Wang 1961), and as die-back in India (Fraser and Singh 1968). Calavan 1968, however, proposed that these different diseases may be similar in nature.

Initially, these diseases were attributed to being associated with an array of different causes including nutrient deficiencies, water-logging, an excess of organic matter and the association of nematodes (Lin and Lin 1956, Oberholzer et al. 1965). It was however demonstrated that the diseases form South Africa, China, the Philippines and Taiwan were graft transmissible, giving rise to the idea that a virus was responsible for the symptoms observed (McClean and Oberholzer 1965a, Salibe and Cortez 1968, Matsumoto and Wang 1961, Lin and Lin 1965). Electron microscopy studies conducted by Laflèche and Bové (1970) demonstrated the presence of mycoplasma-like organisms (MLO) in the sieve-tube elements of CG affected citrus from South Africa. However, these MLO's had a thicker, more complex cell membrane and it was proposed that the organism associated with CG was prokaryote-like rather than mycoplasma-like (Garnier et al. 1976). Similar observations were made in Taiwan (Chen et al. 1971), Reunion, India and the Philippines (Garnier et al. 1976) further demonstrating a common cause of the various diseases mentioned above. Moll et al. 1980 alluded to the possibility that these prokaryotic organisms had similar cell walls to gram-negative bacteria, and in 1984, Garnier and co-workers provided evidence of the gram-negative nature of this

causal organism (Garnier et al. 1984). Garnier and Bové (1983) additionally demonstrated that this gram-negative bacterium remained restricted to the sieve tubes of the plant they infect.

It would, however, be another decade before the organisms associated with CG and HLB were described. Villechanoux et al. (1993) sequenced part of the *nusG-rplKAJL-rpoBC* gene cluster from both African and Asian isolates of this disease and concluded that these bacteria belonged to an undescribed bacterial genus. Subsequently, Jagoueix et al. (1994) successfully obtained 16S rDNA sequences, placing these bacteria within the Alphaproteobacteria. It was also clear that the bacteria obtained from South Africa, to that obtained from Asia were different, albeit closely related. Using this information, these bacteria were given the names '*Candidatus* Liberobacter africanum' (Laf) and *'Ca.* Liberobacter asiaticum' (Las). These names were adapted to *'Ca.* Liberibacter africanus' and '*Ca.* Liberibacter asiaticus' to comply with the international rules of nomenclature (Garnier et al. 2000). After the characterisation of the causal organisms associated with CG, HLB, Likubin and die-back, it was decided that the name HLB should be adopted when referring to either of these diseases associated with Laf and Las (Moreno et al. 1996). However, for the sake of this review, CG will be used when referring to Laf and HLB will be used to define the disease associated with Las.

In addition to Laf and Las, a third citrus-associated Liberibacter species which infects citrus and is associated with HLB-like symptoms was described in Brazil which is known as *'Ca.* L. americanus' (Lam) (Teixeira et al. 2005, Lopes et al. 2008). More recently, '*Ca.* L. africanus subsp. clausenae' bv. citrus (LafCl bv citrus) was found to be associated with citrus trees displaying typical CG symptoms in eastern Africa (Roberts et al. 2017). The significance of LafCl will, however, be discussed separately. Both Laf and Lam are heat sensitive with citrus recovering from symptoms at above 27˚C (Garnier and Bové 1983, Lopes et al. 2009), whereas Las can tolerate temperatures of over 30˚C (Garnier and Bové 1983).

### 2.3 Transmission

Citrus-associated Liberibacter species are spread naturally by insect vectors, are graft transmissible and can be transmitted experimentally with dodder (Garnier and Bové 1983). As for seed transmission, no evidence has been found to support the transmission of Laf through seeds (van Vuuren et al. 2011). Las, however, have been found to be present on the seed coats of fruits infected with this Liberibacter (Tatineni et al. 2008, Hilf et al. 2013), despite this, there is limited evidence of transmission and subsequent systemic infection of seedlings with Las when grown from affected seeds (Albrech and Bowman 2009, Hartung et al. 2010a, Hilf 2011).

As graft and vector transmission have been most extensively studied for citrus-associated Liberibacters, these are discussed further.

### *2.3.1 Graft Transmission*

During the early days of CG research, McClean and Oberholzer (1965a) demonstrated that the organism associated with CG in South Africa was graft transmissible. It was however noted that CG positive budwood from different geographical localities had different rates of transmission through grafting (Schwarz 1972, van Vuuren 1993). Van Vuuren (1993) found that the number of buds or length of grafting material made no difference in the percentage of successful graft transmissions for Laf. Contradictory to this, Lam, despite being graft transmissible, showed varied rates of transmission through budwood grafting depending on the size of the buds used, as well as the season in which grafting occurred, with winter grafting resulting in lower transmission rates (Lopes and Frare 2008). It has been demonstrated that Las can be transmitted using a leaf-disk grafting method. This method has higher transmission success when compared to budwood grafting material and can be used to rapidly screen new cultivars for resistance to Las (Zambon et al. 2017) and can be valuable in conducting transmission studies of Laf.

### *2.3.2 Vector Transmission*

As early as 1957 the involvement of *Trioza erytreae* Del Guercio (Hemiptera: Triozidae), has been suggested in the occurrence and natural spread of CG in South Africa (Oberholzer et al 1965). In 1965, McClean and Oberholzer successfully transmitted CG to healthy citrus seedlings though vector transmission studies with adult *T. erytreae* (McClean and Oberholzer 1965b). These results were subsequently supported by the observation of MLO's in the salivary glands of *T. erytreae* adults which had fed on CG affected citrus (Moll and Martin 1973). Soon after identifying *T. erytreae* as the vector transmitting the organism associated with CG, it was demonstrated that Las is transmitted by the liviid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Capoor et al. 1967). Vector transmission of Laf and Las is however not restricted to their initially described vectors as *T. erytreae* is capable of transmitting Las (Massonie et al. 1976) and *D. citri* can transmit Laf (Lallemand et al. 1986). Las has additionally been found to be associated with the nymphs and adults of *Cacopsylla citrisuga* Yang & Li (Hemiptera: Psyllidae) which have fed on HLB diseased trees (Cen et al. 2012). Transmission studies with *C. citrisuga* have not yet been conducted and it remains unknown whether this insect act as a

vector to Las. In Brazil, it was found that *D. citri* was also responsible for the transmission of Lam (Teixeira et al 2005).

Both adult and instar nymph stages of *T. erytreae* and *D. citri* are phytophagous, allowing these vectors to acquire and transmit the pathogens associated with CG and HLB from the stage of 4$^{th}$ instar nymph onwards following an acquisition period of 4 to 24 hours (McClean 1974, Capoor et al. 1974, Xu et al. 1988, Hung et al. 2004). A clear correlation was found in the acquisition rate of Las from citrus hosts by *D. citri,* depending on the life stage at which these insects feed on infected material, with nearly 100% of nymphs acquiring these pathogens compared to about 50% acquisition by adults (Capoor et al. 1974, Pelz-Stelinski et al. 2010). After acquisition, these insects will transmit their associated Liberibacter species in a circulative-persistent manner due to the ability of the Liberibacters to multiply within their insect vectors (Xu et al. 1988, Hung et al. 2004, Inoue et al. 2009, Ammar et al. 2016). Ammar et al. 2016 demonstrated that Las reaches greater titres within *D. citri* nymphs when compared to adults. This correlates with the efficiency at which Las is transmitted to healthy seedlings by the different life stages of this vector. Las has also been found to be transmitted between *D. citri* individuals, both horizontally from males to females during courtship (Mann et al. 2011) and transovarially from females to eggs, a characteristic which is thought to be shared by *T. erytreae* (van den Berg et al. 1991-1992, Pelz-Stelinski et al. 2010).

The maturity of the leaves contributes to the efficiency at which Las is transmitted by *D. citri,* with an increased transmission rate being observed when these liviids fed on new flush (Hall et al. 2016). Liviids are also known to preferentially populate new flush, which can explain why there is such a correlation (Setamou et al. 2016). *T. erytreae* also preferentially feed on new flush (Moran and Buchan 1975) and even though no studies have been conducted on the acquisition and transmission rates of Laf by *T. erytreae* feeding on young flush, it can be assumed that this correlation between tree maturity and Laf transmission is also true.

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

**2.4 Host range, disease progression and symptom expression.**

In South Africa, all commercial citrus species are known to be susceptible to CG with sweet oranges being severely affected, grapefruit and lemon varieties being mildly tolerant and limes and pomelos being the least affected (McClean and Schwarz 1970, Manicom and van Vuuren 1991). In addition to commercial citrus species, Las has been identified from ornamental Rutaceae species such as *Clausena lansium* (Ding et al. 2005) and *Murraya paniculata* (Deng et al. 2007), with Lam additionally being identified from *M. paniculata* (Lopes et al. 2010). A number of alternative hosts have been shown experimentally to support the multiplication of Las, including *Limonia acidissima*, *Severinia buxifolia* (Hung et al. 2000; Hung et al. 2001) and *M. exotica* (Damsteegt et al. 2010) as well as the non-rutaceous hosts, *Catharanthus roseus* (Garnier and Bové 1983), *Nicotiana tabacum* (Garnier and Bové 1993), *Lycopersicum esculentum* (Duan et al 2008) and *Cuscuta indecora* (Hartung et al. 2010b). Laf *senso stricto*, however, has not yet been identified from alternative hosts.

When infected with Laf, symptom expression will remain confined to a single branch in the case where mature trees are infected, however, if young trees are infected the entire tree will be affected by Laf (McClean and Schwarz 1970). The most characteristic foliar symptom associated with CG and HLB is the appearance of mottling which radiates from leaf veins and is mainly observed on mature leaves (Fig 2.2). Younger leaves from new shoots show more uniform chlorosis, are upright and remain small. As the disease progresses, these affected leaves will eventually turn leathery and their veins will appear more prominent. Fruits produced from infected trees are lopsided, tend to remain small, and fail to completely change colour. These fruit are of no economic value as symptomatic fruit has a bitter, salty taste and has an

overall lower juice percentage compared to healthy fruit (Bassanezi et al. 2009). The roots from infected trees are poorly developed (Manicom and van Vuuren 1990), contributing to the overall decline and vigour of infected trees. In a study conducted by Graham et al. (2013) they found that HLB contributes to 27-40% decrease in the density of fibrous roots compared to 30% losses in young trees infected with Las, a phenomenon which occurs even before symptom expression appears within the tree canopy.

Fig 2.2: Typical mottling symptom observed on sweet orange trees infected with Laf (image R Roberts) and Las (image R Roberts).

Once acquired, Las spreads systemically from the point of infection, through the sieve tissue of the infected tree (Kim et al. 2009) to all tree parts, with the exception of endosperm and embryos of affected seed where it is absent (Tatineni et al. 2008). Upon pathogen invasion, the middle lamella between cell walls surrounding the sieve elements start to swell leading to collapse and necrosis of cells, a process which occurs in leaves, stems and roots (Aritua et al. 2013). With Las infection, Las moves downwards to the roots assisted by the flow of phloem sap, where this Liberibacter multiplies before becoming systemic within its host (Johnson et al. 2013). Subsequent infection causes the plugging of sieve pores with callose-like material, excessive starch accumulation and further necrosis of sieve elements and companion cells, leading to the chlorosis symptoms observed for both Laf and Las infections (Schneider 1968, Folimonova and Achor 2010). Interestingly, it seems that infected trees do little to limit the spread of disease as Albrecht and Bowman (2008) found that 5-9 weeks post infection, very few defence related genes are activated. They did, however, find that genes involved in starch accumulation had increased activity in leaves, which leads to the restriction of phloem transport which may act as a defence mechanism to help limit the spread of the bacteria through the tree

(Koh et al. 2012). In contrast, starch is depleted within the roots, attributing to the general decline of citrus trees infected with Las (Aritua et al. 2013). In fruits, there is a noticeable increase in photosynthetic activity which has been attributed to the inability of diseased fruit to change colour from green (Martinelli et al. 2012).

### 2.5 Distribution of citrus-associated Liberibacter species

Of the three described citrus-associated Liberibacter species, Las has the widest distribution being found in Asia, the Americas, Caribbean and the Mascarene Islands, Laf has to date only been detected in Africa and the Mascarene islands (Bové and Garnier 1994), whereas Las has been detected only in the Americas and China (Garnier and Bové 1996, Halbert 2005, Coletta-Filho et al. 2004). Table 2.1 lists the countries in which CG and HLB have been identified as well as the vectors which are present.

Table 2.1: Distribution of CG and HLB and their associated Liberibacter species and vectors.

| **Distribution** | | **Liberibacter(s)** | **Vector/s present** | **Reference/s** |
|---|---|---|---|---|
| *Africa* | Burundi | Laf | *T. erytreae* | Aubert et al. 1988 |
| | Cameroon | Laf | *T. erytreae* | Aubert et al. 1988 |
| | DRC | Laf | *T. erytreae* | Aubert et al. 1988 |
| | Egypt | Unknown | Unknown | Tolba and Soliman 2015 |
| | Ethiopia | Laf, Las | *T. erytreae* | Aubert et al. 1988 |
| | Kenya | Laf | *T. erytreae* and *D. citri* | Aubert et al. 1988 |
| | Malawi | Laf | *T. erytreae* | Aubert et al. 1988 |
| | Rwanda | Laf | *T. erytreae* | Aubert et al. 1988 |
| | Somalia | Laf | *T. erytreae* | Aubert et al. 1988 |
| | South Africa | Laf | *T. erytreae* | Garnier and Bové 1996 |
| | Swaziland | Laf | *T. erytreae* | Catling and Atkinson 1974 |
| | Tanzania | Laf | *T. erytreae* and *D. citri* | Bové and Garnier 1994, Shimwela et al. 2016 |
| | Uganda | Laf | *T. erytreae* and *D. citri* | Kalyebi et al 2015 |
| | Zimbabwe | Laf | *T. erytreae* | Garnier and Bové 1996 |
| *Asia* | Bangladesh | Las | *D. citri* | Bové 2006 |
| | Bhutan | Las | *D. citri* | Bové 2006 |
| | East Timor | Las | *D. citri* | Weinert et al. 2004 |
| | Cambodia | Las | *D. citri* | Garnier and Bové 1996 |
| | China | Las | *D. citri* | Garnier and Bové 1996 |
| | India | Las | *D. citri* | Garnier and Bové 1984 |
| | Indonesia | Las | *D. citri* | Garnier and Bové 1996 |
| | Japan | Las | *D. citri* | Bové 2006 |
| | Laos | Las | *D. citri* | Garnier and Bové 1999 |

| | | | | |
|---|---|---|---|---|
| | Malaysia | Las | *D. citri* | Garnier and Bové 1996 |
| | Myanmar | Las | *D. citri* | Garnier and Bové 1999 |
| | Nepal | Las | *D. citri* | Garnier and Bové 1996 |
| | Pakistan | Las | *D. citri* | Bové 2006 |
| | Papua new Guinea | Las | *D. citri* | Weinert et al. 2004 |
| | Philippines | Las | *D. citri* | Garnier and Bové 1996 |
| | Saudi Arabia | Las | *D. citri* | Bové and Garnier 1984 |
| | Sri Lanka | Las | *D. citri* | Garnier and Bové 1996 |
| | Taiwan | Las | *D. citri* | Garnier and Bové 1996 |
| | Thailand | Las | *D. citri* | Garnier and Bové 1996 |
| | Vietnam | Las | *D. citri* | Garnier and Bové 1996 |
| | Yemen | Las | *D. citri* | Bové and Garnier 1984 |
| *Mascarene* | Madagascar | Laf , Las | *T. erytreae* and *D. citri* | Bové and Garnier 1994 |
| *Islands* | Mauritius | Laf , Las | *T. erytreae* and *D. citri* | Garnier et al. 1996 |
| | Réunion | Laf , Las | *T. erytreae* and *D. citri* | Garnier et al. 1996 |
| *North America* | California | Las | *D. citri* | Stokstad 2012 |
| | Florida | Las | *D. citri* | Halbert 2005 |
| | Mexico | Las | *D. citri* | CABI, 2018 |
| | Texas | Las | *D. citri* | Stokstad 2012, da Graça et al. 2015 |
| *South America* | Brazil | Las, Lam | *D. citri* | Coletta-Filho et al. 2004, Teixeira et al. 2005 |
| | Argentina | Las | *D. citri* | Outi et al. 2013 |
| *Central America* | Belize | Las | *D. citri* | Manjunath et al. 2010 |
| *and Caribbean* | Cuba | Las | *D. citri* | Luis et al. 2009 |
| | Guadeloupe | Las | *D. citri* | Celliers et al. 2014 |
| | Martinique | Las | *D. citri* | Celliers et al. 2014 |

## 2.6 Detection

The detection of citrus-associated Liberibacter species has been a subject of extensive study as the early detection of these causal agents assists in effective control measures being implemented to limit the spread of CG and HLB in the field. Initially, the identification of CG from orchards was conducted through the visual identification of symptoms, which is unreliable due to the similarities between symptom expression of CG and HLB to nutrient deficiencies (Fraser and Singh 1968), especially zinc. A misdiagnosis of CG and HLB as a zinc deficiency can have detrimental consequences as it has been shown that the addition of excess zinc causes an increase in Las populations (Zhang et al. 2016). During the late 1960s, biological indexing became the preferred method for confirming the presence of the organism associated with CG from affected trees. This method too is unreliable as the rate of transmission of the

various Liberibacter species from grafting is not 100% and symptom expression from grafting can take anything from 9 weeks to 3 months to appear. In addition to grafting, CG in South Africa was confirmed by the detection of the fluorescent marker, gentisic acid, through thin layer chromatography methods from the 1960's up until the 1980s (Schwarz 1965, Schwarz 1968, Schwarz and van Vuuren 1970, van Lelyveld et al. 1988). Schwarz (1970) indicated that this compound was present in affected fruit prior to the appearance of CG symptoms, allowing for the early detection of CG in fields. It was however demonstrated that gentisic acid could be detected from trees experiencing other environmental stresses which were not affected by CG, thus leading to the false-positive detection of CG in fields (Feldman and Hanks 1969).

During the 1970s, electron microscopy was used for the detection of CG and HLB (Laflèché and Bové 1970) and during the late 1980s, serological methods which employed the use of monoclonal antibodies such as ELISA were used (Garnier et al. 1991, Varma et al. 1993, Bové et al. 1993). The use of these methods, however, had limited use as not all isolates could be detected, and subsequent DNA probes were developed for use in hybridization assays (Villechanoux et al. 1992). In South Africa, probe In-2.6 and probe As 1.7 became popular for the detection of Laf (Planet et al. 1995, Korsten et al. 1996). The use of serological detection methods has however regained popularity due to the use of in-field detection devices such as lateral-flow devices and due of the inherent economic value of these methods for large-scale screening (Yuan et al. 2016, Pagliaccia et al. 2017).

### *2.6.1 Molecular identification*

During the 1990s, the use of molecular detection methods through polymerase chain reaction (PCR) became widely adopted. Through targeting conserved regions within the genomes of Liberibacters, PCR-based techniques could be developed for the rapid detection and differentiation of Laf, Las and Lam. The first available sequences from which PCR assays could be derived were the *nusG-rplKAJL-rpoB* gene cluster (Villechanoux et al. 1993) and the 16S rDNA gene sequence (Jagoueix et al. 1994). Jagoueix et al. (1996) designed primers spanning 1160 bp within the 16S rDNA sequence of both Laf and Las with subsequent differentiation of Laf and Las being conducted by restriction length polymorphism assay using *xbaI*. This was succeeded by the development of a single primer set targeting ribosomal protein J (*rplJ*) sequences, which distinguished between Laf and Las based on the size of the amplicon produced (Hocquellet et al. 1999a).

Obtaining additional sequence information for these Liberibacters remained challenging due to the unculturable nature of these organisms (Garnier and Bové 1983). In 1997 the 16S/23S ribosomal intergenic spacer region of Laf and Las were obtained (Jagoueix et al. 1997) and in 1999, the *nusG, pgm, omp* and a hypothetical protein gene sequences were characterised for Las (Hocquellet et al. 1999b). Through the use of high-throughput sequencing (HTS) technologies, Duan et al. (2009) obtained the full genome sequence of Las which was subsequently followed by the genome of Lam (Wulff et al. 2014) and Laf (Lin et al. 2015). Despite this, the majority of PCR-based assays, such as real-time PCR, Loop-mediated isothermal amplification (LAMP) and Recombinase Polymerase Amplification (RPA) are still based on 16S rDNA (Li et al. 2006; Urasaki et al. 2008, Fujikawa and Iwanami 2012, Bertolini et al. 2014, Ghosh et al. 2016, Wu et al. 2016, Park et al. 2018, Zhong et al. 2018) and *nusG-rplKAJL-rpoB* sequences (Okuda et al. 2005, Ananthakrishnan et al. 2013, Qian et al. 2018). Laf and Las share high sequence homologies for these two sequences and it has been demonstrated that tests directed against Laf and Las based on 16S rDNA sequences do not discriminate between these two Liberibacters and requires additional confirmation by sequencing (Roberts et al. 2017).

Nageswara-Rao et al. (2013) developed 50 primer pairs across the genome of Las and found that of these primers, those directed against the *tubB,* elongation factor and permease protein genes were able to amplify all of the known citrus infecting Liberibacter species (i.e. Laf, Las and Lam) whereas primers against the *omp,* integral membrane protein and RNA polymerase beta subunit genes amplified only Laf and Las. Despite this cross-amplification, these diagnostic primers still require downstream sequencing to determine the nature of the Liberibacter present, which is both time consuming and expensive. Alternative genes for diagnostic purposes across the Las genome have also been explored by Kongenaru et al. 2014 who identified 18 potential genes for Las-specific detection. The use of Las-associated prophage sequences are another popular diagnostic target for the specific detection of Las (Morgan et al. 2012, Keremane et al. 2015, Qian et al. 2017), however, this sequence has been shown to be absent from certain Las isolates, leading to the false-negative detection of Las. Zheng et al. 2016 developed real-time PCR primers against the β-subunit of the ribonucleotide reductase gene (*nrdB*) of Las and demonstrated that detection with this gene was increased when compared to 16S rDNA targets which are attributed to the higher copy number of this gene (X5) compared to 16S rDNA (X3). The *nrdB* gene has been used subsequently in combination with the 16S rDNA gene sequence in a duplex digital droplet PCR assay for the

rapid detection of Las (Selvaraj et al. 2018). In contrast to protein-coding gene regions, Lou et al. 2018 resorted to targeting tandem repeat loci within the Las genome and developed a tandem repeat-based polymerase chain displacement assay, for the detection of Las.

The source of material used for DNA extractions on which to perform diagnostic assays is another important variable in aiding the early detection of citrus-associated Liberibacters. Most commonly, the midribs and petioles from symptomatic leaves are used for Laf detection in South Africa. These are also commonly used for the detection of Las and it was shown that the concentration of Las is increased in mature leaves when compared to young leaves, aiding in accurate diagnostics (Kunta et al. 2014). Nakanishi et al. 2016 additionally found that Las is optimally detected from lower branch collars. One major draw-back with using above-ground plant material is the delay in symptom expression when CG or HLB infection is suspected, and due to the erratic distribution of citrus-associated Liberibacter species, the likelihood of a false-negative detection of these bacteria is increased. Park et al. 2018, therefore, proposed the use of roots for diagnostic screening as a means of an early-warning symptom as Las can be detected in roots prior to symptom expression. Again, this association and use of roots for the detection of Laf has not yet been explored, however, it is plausible that the same may hold true.

Due to the threat posed by HLB, various disciplines have moved their attention to the early detection of HLB in orchards. A number of articles exists which described the use of spectroscopic techniques, such as visible-near infrared spectroscopy (Sankaran et al. 2011; Garcia-Ruiz et al. 2013) laser-induced fluorescence spectroscopy (Ranulfi et al. 2016, Wetterich et al 2016, Ponce et al. 2018) polarized imaging (Pourreza et al. 2016, Pourreza et al. 2017), Raman spectroscopy (Perez et al. 2016) as well as hyperspectral reflectance imaging (Weng et al. 2018) for the detection of HLB. In Florida, the use of sniffer dogs for the early detection of Las within orchards has proven promising as these canines are able to detect HLB prior to symptom expression.

### 2.7 Control

McClean and Oberholzer (1965a) observed that *'Greening is apparently a permanent disease from which there is no recovery*'. Fifty years after the fact, this is still the case as there is no cure for either CG or HLB. Different citrus cultivars have been assessed to determine whether they confer any resistance to CG and HLB, to use in classical breeding programmes, but this has however been met with limited success (Shokrollah et al. 2011, Albrecht et al. 2012). Researchers in Florida additionally assessed whether citrus relatives displayed resistance

towards Las, for future characterisation of potential resistance genes which can be targeted in breeding programmes and transgenic approaches (Ramadugu et al. 2016). Van Vuuren and Manicom (2009) conducted trials in which embryos from chimeric fruit of CG affected trees were germinated and grafted onto commonly used rootstocks. Typically, chimeric fruit from CG affected trees contain healthy sections, suggesting an innate resistance against Laf. Field trials conducted on these plants gave promising results as some of these trees remained free from Laf, even under high pressure scenario. Unfortunately, this work was halted due to the retirement of the researchers involved. Despite the absence of resistant cultivars, a great effort has gone into managing the disease and implementing control strategies to reduce disease incidence within orchards.

### *2.7.1 Therapeutic control approaches*

Initially, CG in South Africa was managed by the application of tetracycline to infected trees (Schwarz and van Vuuren 1971), either as a foliar spray (Martinez et al. 1970) or by trunk injection (Schwarz et al. 1974). Tetracycline, however, proved to be ineffective, as repeat application of this antibiotic was required for continued suppression of the GC organism, and trees exhibited other physiological side-effects due to treatment, such as a reduced fruit size (Schwarz et al. 1974, Buitendag and von Broembsen 1993). The use of tetracycline to control HLB in Asia was also abandoned during the 1990s, however, recent studies have focused on the use of penicillin G, streptomycin and ampicillin for the control of Las in orchards (Zhang et al. 2010; Zhang et al. 2011, Zhang et al. 2012, Zhang et al. 2013, Yang et al. 2016).

### *2.7.2 The three-pronged approach*

Due to the expensive nature and detrimental effects of antibiotic application to CG affected citrus orchards, South African producers turned to a three-pronged control strategy during the 1990s. This involves the planting of disease free material, vector control and removal of inoculum sources (Buitendag and von Broembsen 1993).

#### *2.7.2.1 The citrus improvement programme (CIP)*

In South Africa, under the citrus improvement programme (CIP) which was established in the 1970s, commercial citrus nurseries are supplied with disease free budwood obtained from the citrus foundation block (CFB) (Grout 2012). The CFB is located in Uitenhage, Eastern Cape within a non-citrus producing region, with the nearest commercial citrus orchards situated 40km from the CBF. Additionally, to limit the risk posed by a number of biosecurity risks, the

Department of Agriculture, Forest and Fisheries (DAFF) declared a 5-km exclusion zone around the CFB in which no citrus may be planted, either commercially or for private use (CRI). The CIP and CFB have proven a pivotal role in the control of CG in South Africa.

#### *2.7.2.2 Vector control*

Once seedlings reach the field, vector control strategies are implemented to control triozid populations by chemical means. It is known that *T. erytreae* prefer to colonize young flush as the physiology of these leaves assist with oviposition (Moran and Buchan 1975), and therefore insecticide application is synchronized with these flushing rhythms (Aubert 1987). De Carli et al. 2018, however, found that the efficacy of insecticides rapidly decreases in young shoots, warranting more frequent insecticide application. This excessive use of insecticides does have a number of drawbacks as it can lead to resistance within the target insects as well as have detrimental effects on both human health and the environment. Therefore, a number of 'environmentally friendly' approaches have been explored to form part of an integrated pest management regime for the control of the insects associated with CG and HLB.

Both *T. erytreae* and *D. citri* nymphs are naturally parasitized by *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) (Catling 1969). Using this parasite as means of a biological control agent, *T. erytreae* and *D. citri* populations in Reunion and Mauritius were significantly reduced (Ettienne and Aubert 1980, Aubert and Quilici 1984, van den Berg 1990), and initial release of this control agent in Brazil proved promising (Parra et al. 2016). Unfortunately, the same success has not been achieved elsewhere (Aubert and Quilicci 1984), and chemical control is still the preferred method of vector control, even in the presence of natural infestations of *T. radiata* (Qureshi et al. 2009, Paiva and Parra 2012). A number of additional, potential biological control agents have been identified for control of *D. citri* such as the parasitic wasp, *Vrachygastra mellifica* Say (Reyes-Rosas et al. 2011), the predatory mites *Neoseiulus cucumeris* Oudemans, *N. barkeri* Hughes and *Amblyseius swirskii* Athias (Acari: Phytoseiidae) (Juan-Blasco et al. 2012, Fang et al. 2013), predatory syrphids, green lacewings (Kistner et al. 2017) and a number of entomopathogenic fungi (Lezama-Gutiérrez et al 2012, Ullah et al 2018). However, the efficiency of these to control *D. citri* has not yet been established under field conditions.

More practical approaches to controlling the vector populations have included studies on interplanting citrus orchards with guava (Zaka et al. 2010, Ichinose et al. 2012, Gottwald et al. 2014, Silva et al. 2016), applying botanical oils and potassium silicate as foliar applications

(Kuhns et al. 2016, Ramírez-Godoy et al. 2018) and erecting mesh fences around the perimeters of orchards to limit infestation by *D. citri* (Sétamou et al. 2018). On the other side of the spectrum, extensive studies have been conducted on controlling vector populations through advanced molecular means such RNA interference (RNAi). Using this mechanism, research teams have studied the foliar application of dsRNA which, when ingested, mediates transcriptional down regulation of target genes. A number of these target genes have already been studies and the use of RNAi have proven to increase the mortality rate of both nymphal and adult *D. citri* (El-Shesheny et al. 2013, Taning et al. 2016, Kishk et al. 2017, Galdeano et al. 2017, Andrade and Hunter 2017, Santos-Ortega and Killiny 2018). These approaches have however not yet been evaluated with *T. erytreae* populations.

#### *2.7.2.3 Inoculum reduction*

Despite vigorous vector control within orchards, there still exists a likelihood that both CG and HLB can infect orchards. Lee et al. 2015 demonstrated that the flush became infectious after 15 days post exposure to Las positive psyllids, with symptom expression only appearing 1-2.5 years after infection, even with a 90% reduction in psyllids (Lee et al. 2015). Therefore, it is important that inoculum sources be removed from citrus orchards, forming the third branch of the three-pronged approach. In South Africa, trees younger than 10 years with an infection covering more than 75% of the tree are removed whereas only branches showing symptoms are removed in older trees (Buitendag and von Broembsen 1993).

In countries affected by HLB, a number of novel approaches have been studied to reduce inoculum sources by reducing the titre of Las populations of infected trees, thus limiting the number of trees being removed from orchards in a bid to increase profitability. One such approach is by applying heat treatment to infected trees. This has proven to effectively eliminate Las from nursery seedlings up to 2 years in age (Hoffman et al. 2013, Fan et al. 2016). However, when conducted in field trials on mature trees, heat treatment merely reduced the titre of Las (Doud et al. 2017). The microbiome of seemingly healthy trees located in orchards with high HLB incidence has been studied to potentially give insights into bacterial species which can be applied as beneficial bacterial formulations for disease management (Trivedi et al. 2011, Reira et al. 2017). In addition to these strategies, attention has been given to the screening of antimicrobial compounds and/or peptides over recent years. A number of promising antimicrobials have already been identified which either act by directly affecting Las (Akula et al. 2012, Stover et al. 2013, Conales et al. 2016), or by up-regulating the defence

response within host trees (Li et al. 2016). These antimicrobials are envisioned to be delivered to their target either through transgenic approaches (Stover et al. 2013), foliar applications combined with an oil-in water nano-emulsion formulation (Canales et al. 2016, Yang et al. 2016), trunk injection delivery systems (Ghosh et al. 2018) or through infectious clones (Dawson et al. 2015).

### 2.8 Laf-subspecies

The potential of alternative non-commercial citrus hosts acting as reservoir for Laf in South Africa has long been considered, as the existence of such hosts would influence how CG is controlled. In the early '90s, bark strips from CG positive trees were graft inoculated onto *Clausena anisata* (Rutaceae) and typical CG symptoms developed on this tree (van den Berg et al. 1991-1992). Subsequently, in 1996, Korsten et al. reportedly identified Laf from *Vepris lanceolata* (Rutaceae) with the aid of a southern blotting assay. During the late '90s, typical mottling symptoms were observed on the indigenous ornamental tree, *Calodendrum capense* (Rutaceae), found in close proximity to citrus orchards in the Western Cape. This observation coincided with the first reports of Laf occurring on commercial citrus in the Western Cape (Garnier et al. 1999), further contributing to the idea that alternative hosts of Laf exist which act as reservoirs for this pathogen. However, upon closer inspection, it was found that *C. capense* contained a novel Liberibacter which was separate, albeit homologous, to Laf. This Liberibacter was subsequently named: '*Ca.* L. africanus subsp. capensis' (LafC) (Garnier et al. 2000).

The work conducted by van den Berg et al (1991-1992) and Korsten et al (1996), as well as the description of LafC from *C. capense*, prompted further research into potential alternative hosts of Laf. The first indigenous host species to be extensively studied in this regard were, *C. anisata, V. lanceolata* and *Zanthoxylum capense*, all of which are native hosts of *T. erytreae* in South Africa. From each of these rutaceous species, a unique Laf-subspecies could be described based on phylogenetic analyses of three gene regions. These subspecies are known as; '*Ca.* L. africanus subsp. clausenae' (LafCl), *'Ca.* L. africanus subsp. vepridis' (LafV) and *'Ca.* L. africanus subsp. zanthoxyli' (LafZ) (Roberts et al. 2015). As part of this study, a further subspecies has subsequently been described from *Teclea gerrardii* (Rutaceae) and is known as '*Ca.* L. africanus subsp. teclea' (Roberts and Pietersen 2017, Chapter 3).

Pietersen et al. (2010) found that LafC did not play a role in the epidemiology of CG disease in South Africa, despite being widely associated with *C. capense* trees, even when grown in

close proximity to citrus orchards (Phahladira et al. 2012). However, *T. erytreae,* the most likely vector to transmit these subspecies to citrus, does not survive on *C. capense* (Moran 1968), and therefore it is more likely that LafCl, LafV or LafZ would be transmitted to citrus under natural conditions. During 2015 it was reported that Las was identified in eastern Africa along with *D. citri* (Kalyebi et al. 2015, Shimwela et al. 2016). Due to the importance of these reports, and the threat posed to South African citriculture, surveys were conducted in Kenya, Uganda and Tanzania to confirm these claims. These surveys showed that the identification of Las was in fact due to the non-target amplification of LafCl with the Li et al. (2006) primers. Further characterisation of the genes studied for LafCl from citrus in eastern Africa, indicated the presence of SNP's at specific base pair positions for a number of isolates when compared to LafCl from *C. anisata* (Roberts et al. 2017). This study shed light and the possible need for certain point mutations to occur within these Laf-subspecies to adjust to new host species under environmental stress should their original hosts not be available, assuming that the native hosts from which they were described are in fact their original hosts. It will, however, be important that the full genomes of both LafCl biovars be obtained and compared to determine whether these point mutations occur across different genes to truly make conclusions on the evolutionary divergence of LafCl bv citrus from LafCl bv clausena.

### *2.8.1 The potential role of Laf-subspecies in the existence of Laf*

Doddapaneni et al. 2008 proposed that the Liberibacter lineage emerged prior to the expansion of the Rhizobiales. One school of thought follows, that after this emergence, the three most studied citrus-associated Liberibacter species i.e. Laf, Las and Lam, evolved independently in Africa, Asia and South America (Bové 2006). Population studies using microsatellite marker has supported the Asian origin of Las, with strong support given for the existence of 'founder haplotypes' being present amongst Las isolates from India (Islam et al. 2012). From here, it is proposed that Las spread eastward to China (Beattie et al. 2008, Teixeira et al. 2008). It is also believed that Las evolved with citrus species which are commercially grown today as these citrus species are known to have originated from Asia (Beattie et al. 2008).

The evolution of Laf is however not as clear cut as merely evolving with its citrus host as, Laf *senso stricto* has to date only been identified from commercial citrus species on the African continent, where citrus was introduced. The absence of this Liberibacter outside of the African continent, further supports an African origin for Laf. The existence of multiple Laf-subspecies in South Africa potentially sheds some insights into the evolution of Laf. As demonstrated by

Roberts et al. 2017, Laf-subspecies can be transmitted to commercial citrus, should the environment favour such a host jump. The clearing of natural vegetation in South Africa to support the production of citrus 100 years ago, may have created the ideal environment for Liberibacters already present on the African continent, either from one of the hosts previously studied or a yet unidentified host, to adapt to a commercial citrus host, giving rise to Laf as it is known today. However, these claims can only be substantiated by conducting population studies on Laf from commercial citrus. As with Las, it is expected that such a study could indicate the presence of founder haplotypes for Laf on the African continent.

## 2.9 Concluding remarks

CG has been a part of the South African citrus landscape for nearly 100 years. During this time, this disease has caused the near destruction of this important industry within the northern production regions of the country. Due to the importance of this disease, extensive research was conducted to save the industry from collapse. However, upon the implementation of effective control strategies against CG, research interest on this disease has declined. In contrast, HLB is probably the most thoroughly studied plant disease existing today.

With the imminent threat posed by HLB to South African citriculture, it is important that a clear understanding of Laf be obtained which could potentially aid in the control of Las. The studies conducted in this dissertation, therefore, aimed to touch on fundamental aspects of CG such as the existence of alternative hosts of Laf, the potential involvement of Laf-subspecies in citriculture as well as establishing whether different populations of Laf are present in South Africa.

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# Chapter 3:

# A novel subspecies of '*Candidatus* Liberibacter africanus' found on native *Teclea gerrardii* (Family: Rutaceae) from South Africa*

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## 3.1 Abstract

‘*Candidatus* Liberibacter africanus’ (Laf), is associated with Citrus Greening (CG) disease in South Africa. This bacterium has been identified solely from commercial citrus in Africa and the Mascarene islands, but it may originally be from an indigenous rutaceous host from Africa. When determining whether alternative hosts of Laf exists amongst the indigenous rutaceous hosts of its triozid vector, *Trioza erytreae,* three novel subspecies of Laf were identified i.e. ‘*Ca.* L. africanus subsp. clausenae’ (LafCl), ‘*Ca.* L. africanus subsp. vepridis’ (LafV) and ‘*Ca.* L. africanus subsp. zanthoxyli’ (LafZ) in addition to the formerly identified ‘*Ca.* L. africanus subsp. capensis’ (LafC). The current study expands upon the range of indigenous rutaceous tree species tested for Liberibacters closely related to Laf and its subspecies. A collective 121 samples of *Teclea* and *Oricia* species were sampled from Oribi Gorge and Umtamvunu nature reserves in KwaZulu-Natal. Total DNA was extracted and the presence of Liberibacters from these samples determined by a generic Liberibacter TaqMan real-time PCR assay. Liberibacters from positive samples were further characterised through amplification and sequencing of the 16S rDNA, outer-membrane protein (*omp*) and 50S ribosomal protein L10 (*rplJ*) genes. A single *Teclea gerrardii* specimen tested positive for a Liberibacter, and through phylogenetic analyses of the three genes sequenced, was shown to be unique, albeit closely related to Laf and LafZ. We propose that this newly identified Liberibacter species be named ‘*Ca.* L. africanus subsp. tecleae’ (te.cle'ae. N.L. gen. n. tecleae, of the plant genus Teclea) (LafT).

## 3.2 Introduction

The bacterial genus Liberibacter (class Alphaproteobacteria, family *Rhizobiaceae*) (Jaqoueix et al. 1994; Garnier et al. 2000) currently comprises six described species including the pathogens associated with huanglongbing (HLB) or Citrus Greening (CG) in commercial citrus i.e. ‘*Candidatus* Liberibacter asiaticus’ (Las) (Jaqoueix et al. 1994; Garnier et al. 2000), ‘*Ca.* L. americanus’ (Lam) (Teixiera et al. 2005) and, ‘*Ca.* L. africanus’ (Laf) (Jaqoueix et al. 1994; Garnier et al. 2000). Other species described within this genus includes the agent associated with psyllid yellows disease of tomato (*Solanum lycopersicum,* family Solanaceae) and zebra chip disease of potatoes (*Solanum tuberosum*) i.e. ‘*Ca.* L. solanacearum’ (Lso) (Liefting et al. 2009; Secor et al. 2009), the pear (family Rosaceae) endophyte, ‘*Ca.* L. europaeus’ (Leu) (Raddadi et al. 2011) and *Liberibacter crescens* which was identified from mountain papaya (family Caricaceae) (Fagen et al. 2014). All the members within this bacterial taxon are

fastidious and to date, only *L. crescens* has successfully been obtained in pure culture (Fagen et al. 2014).

In South Africa, CG is considered an economically important disease of citrus, as fruits produced from infected branches are of a reduced quality (McClean and Oberholzer 1965). The agent associated with this disease, Laf, has thus far been associated only with commercial citrus orchards from Africa (Garnier and Bové 1996, Pietersen et al. 2010) and the Mascarene Islands (Garnier et al. 1996) whereas the nearest known relative of Laf based on 16S rRNA sequence data, Las, has a near worldwide distribution (Garnier and Bové 1996, Coletta-Filho et al. 2004, Halbert 2005, Saporani et al. 2010). As commercial citrus species are not indigenous to Africa, it is hypothesized that Laf either made a direct host jump from an indigenous rutaceous species to citrus (Da Graça 2008) or evolved from a Liberibacter species present on the African continent prior to the introduction of commercial citrus species (Phahladira et al. 2012; Roberts et al. 2015). The evolutionary theory of Laf is supported by the current lack of evidence of Laf occurring in indigenous rutaceous species tested thus far, along with the occurrence of four subspecies to Laf from South Africa identified from indigenous Rutaceae species. The subspecies recognized are; '*Ca.* L. africanus subsp. capensis' (LafC) (Garnier et al. 2000), '*Ca.* L. africanus subsp. clausenae' (LafCl), *'Ca.* L. africanus subsp. vepridis' (LafV), and '*Ca.* L. africanus subsp. zanthoxyli' (LafZ) (Roberts et al. 2015). LafCl, LafV and LafZ were identified from the native hosts of the triozid, *Trioza erytreae* del Guercio (Hemiptera: Triozidae) (Moran 1968, Burkhardt and Ovard 2012), the vector of Laf (McClean and Oberholzer 1965).

During the study in which LafCl, LafV and LafZ were characterised, we proposed that any one of the subspecies could have been placed under selective pressure to adapt to a new host species through the feeding behaviour of *T. erytreae*. We did, however, caution that additional Rutaceae species from Africa must be studied to determine the existence of either an alternative host or, a further possible ancestor of Laf. The aim of the current study, therefore, was to determine whether South African native Rutaceae belonging to the *Teclea* and *Oricia* genera contains Liberibacter sequences either identical or related to other species within this taxon.

### 3.3 Method and Materials

Leaf samples of *Teclea* spp and *Oricia bachmannii* were collected from natural forests in Southern KwaZulu-Natal where these genera are known to occur. The GPS coordinates of each sample were recorded, and a unique accession number was allocated per sample (Appendix A).

Total DNA was extracted from leaf petioles and midribs following the CTAB extraction method previously described (Doyle and Doyle 1990).

All samples were subjected to a generic Liberibacter TaqMan real-time PCR assay to identify Liberibacter-positive samples as previously described (Roberts et al. 2015). Reactions were performed on a LightCycler 1.5 capillary-based thermocycler (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The fluorescence emitted in the presence of a positive result was detected and measured using LightCycler 1.4 software (Roche Diagnostics, Risch-Rotkreuz, Switzerland). A crossing threshold (Ct) of Ct<32 was selected as a positive/negative threshold.

Liberibacters identified from *Teclea* and *Oricia* samples were characterised by amplifying portions of the 16S rDNA, outer-membrane protein (*omp*) and 50S ribosomal protein L10 (*rplJ*) genes as described below. A partial 16S rDNA sequence was amplified using primers OA1/OI2c previously described (Jaqoueix et al. 1996). To obtain the complete 16S rDNA sequence for the various Laf-subspecies, primers Laf16-5F1 (TGTTAGATGCCTTTGGCAAGA) and Laf16-5R1 (ATATTCCCCACTGCTGCCTC) (designed within the current study) were used to amplify the 16S rDNA at the 5' region and primers Laf16-3F8 (5'-TTAATTCGATGCAACGCGCA-3') and Laf16-3R8 (5'-GGACGGCGATCCTCTAAAACC-3') were used to amplify the 3' end of the 16S rDNA sequences. All reactions were set up by adding 0.5µl DNA template to a final reaction volume of 25µl consisting of 12.5µl 2X Dream Taq Green master mix (Thermo Fisher Scientific, Waltham, MA, USA), 10µM per primer per set and made up to a final reaction volume using molecular-grade water (Sigma-Aldrich, St Louis, MO, USA). PCR cycling was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The following cycling conditions were used; initial denaturation of 92˚C for 5 min, followed by 35 cycles of denaturation at 92˚C for 30s, annealing at 65˚C for 30s and elongation at 72˚C for 90s, final elongation was carried out at 72˚C for 10 min.

Partial amplification of the *omp* gene region was achieved by utilising primers omp1/omp8inv as previously described (Bastianel et al. 2005). Reactions were set up as for 16S rDNA amplification using the following cycling conditions; initial denaturation at 92˚C for 5 min, 35 cycles of denaturation at 92˚C for 30s, annealing at 50˚C for 30s, elongation at 72˚C for 2 min, followed by a final extension of 72˚C for 10 min.

A portion of the Liberibacter *rplJ* gene was amplified using primers A2/J5 as previously designed (Hocquellet et al. 1999). The reaction was set up as for the 16S rDNA gene and cycling conditions were performed as previously described (Roberts et al. 2015).

Amplification products of each gene region were purified enzymatically using exonuclease I (Thermo Fisher Scientific, Waltham, MA, USA) and FastAP (Werle et al. 1994). Purified amplicons per gene region were sequenced in both directions with corresponding primers using the Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The amplicon sequences were determined using an ABI 3500xL automated sequencer at the University of Pretoria, South Africa.

The DNA sequences obtained were compiled into different datasets along with relevant reference sequences obtained from Genbank. Reference sequences consisted of known sequences of other citrus infecting members within the Liberibacter genus. Each dataset was aligned using the online alignment tool MAFFT (Katoh et al. 2002). Following alignment, each dataset was trimmed in BioEdit version 7.0.9.0 (Hall 1999) to obtain equal length sequences. The best-fit substitution model for each dataset was determined by jModelTest (Posada 2008) and maximum-likelihood phylogenetic analyses were performed using MEGA software version 6.06 (Tamura et al. 2013).

To verify the identity of the tree species sampled, the extracted DNAs were subjected to DNA barcoding through amplification of two DNA barcodes for plants (*rbcL* gene, the large subunit of ribulose-1,5-biphosphate carboxylase and *psbA-trnH* intergenic spacer) (Chase et al. 2005, Pang et al. 2012). PCR reactions for both regions were set up using the DreamTaq Green system as discussed earlier. Amplification of *rbcL* was performed with primers rbcLa F/rbcLa R (Levin et al. 2003, Kress and Erickson 2007) using conditions previously described (Roberts et al. 2015). The *psbA-trnH* intergenic spacer region was amplified by utilising primers psbA3_f/trnHf_05 (Sang et al. 1997; Tate and Simpson 2003) under the following conditions; initial denaturation at 92˚C for 3 min, 35 cycles of denaturation 92˚C for 20s, annealing 58˚C for 20s, extension 72˚C and final extension at 72˚C for 5 min. Amplification products were purified and sequenced as before and phylogenetic analyses were performed as previously described.

### 3.4 Results

A total of 95 *Teclea* and 27 *O. bachmannii* specimens were sampled. A few samples displayed triozid depression marks similar to those made by *T. erytreae*, however, no triozid specimens were obtained from these samples. Sampling was conducted in the Oribi Gorge and Umtamvunu nature reserve as the distribution of the tree species studied is limited along the eastern seaboard of South Africa (Waffo et al. 2006). A single *Teclea* spp specimen (Accession number 13-2189) yielded a positive result in the generic Liberibacter test and was further analysed.

PCR amplification of the 16S, *rplJ* and *omp* gene regions for the Liberibacter-positive *Teclea* sample yielded amplification products corresponding in size to Liberibacter-positive controls. All healthy and 'no template' controls remained negative. The complete Liberibacter 16S rDNA sequence obtained (Genbank Accession KX990288), shared a nucleotide identity of 99.3% with Laf, 99.6% with LafZ and 98.8% with LafC, LafCl and LafV. When compared to Las and Lam, the newly obtained *Teclea* Liberibacter sequence shared a nucleotide identity of 98.0% and 95.0%, respectively. Phylogenetic analyses of the 16S rDNA confirmed that the sequence obtained from *Teclea* is more closely related to Laf and all its known subspecies than other citrus infecting Liberibacter species (Fig. 3.1). While the 16S rDNA gene of African Liberibacters is highly conserved, as previously demonstrated (Roberts et al. 2015), the 16S rDNA sequence obtained from *Teclea* is found in a separate clade, albeit closely related to LafZ.

For respectively *omp* (Genbank accession KU561668) and *rplJ* (Genbank accession KU561667) sequences, nucleotide similarities between the corresponding Liberibacter ex *Teclea* sequences and Laf (87.4% and 86.9%) and LafZ (89.2% and 84.8%) were greater than that for LafC (78.9% and 78.2%), LafCl (79.0% and 79.2%) and LafV (79.5% and 76.3%). Compared to Las *omp* and *rplJ* sequences, the Liberibacter ex *Teclea* shared 73.3% and 72.0% overall sequence identity, respectively. Phylogenetic analysis of these two genes placed the Liberibacter sequence obtained from *Teclea* into a separate clade, closely related to Laf and LafZ (Fig. 3.2; Fig 3.3).

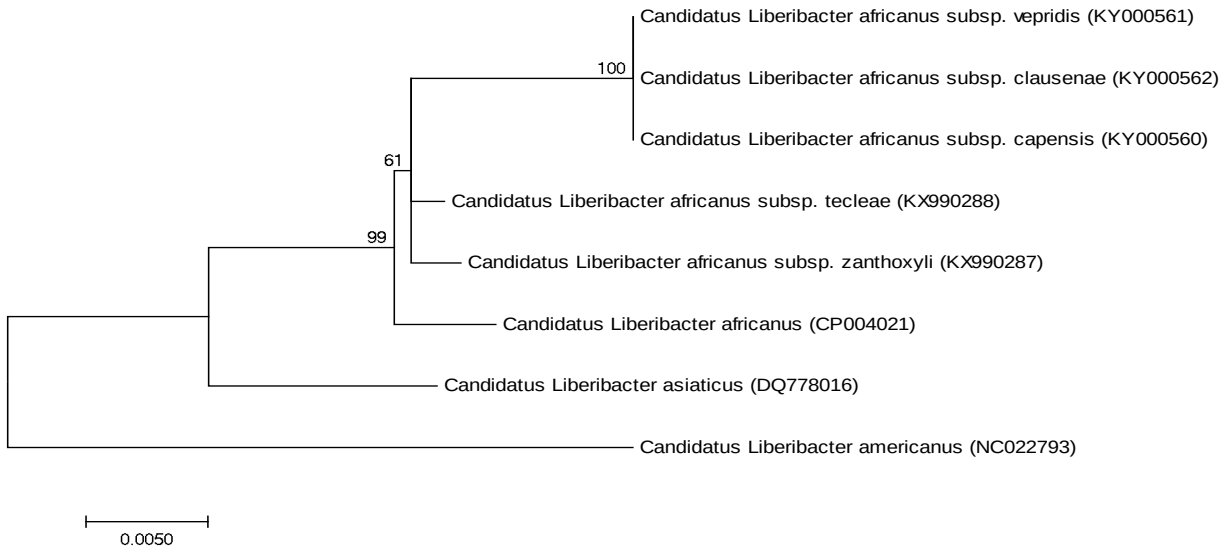


Fig. 3.1**:** Maximum-likelihood phylogeny based on 16S rDNA gene sequences of citrus-associated members within the Liberibacter genus including the sequence obtained from the *Teclea gerrardii* sample examined in this study. Phylogeny was inferred using Hasegawa-Kishino-Yano mode (Hasegawa et al. 1985) with gamma correction to account for site variation. Bootstrap support values based on 1000 replicates are indicated at branches. Genbank accession numbers are shown on the tree for sequences included in this analysis. Bar, 0.005 substitutions per nucleotide position.

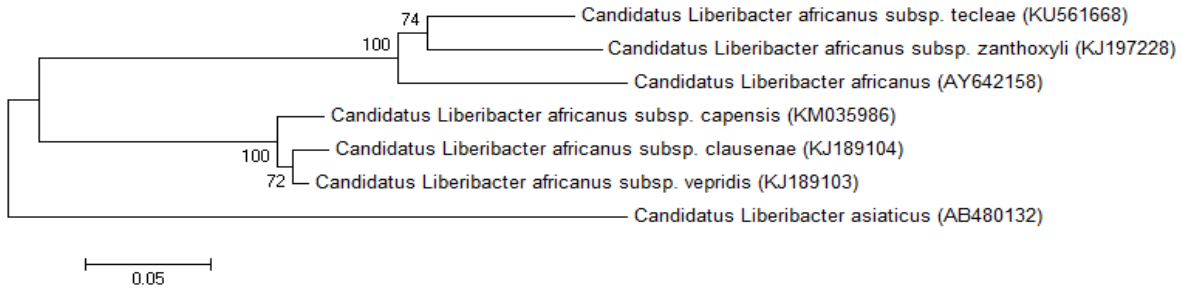


Fig. 3.2: Maximum-likelihood phylogeny based on available *omp* gene sequences of members within the Liberibacter genus which are associated with rutaceous species. The phylogeny was inferred using the general time reversible model with gamma correction to account for site variations. Bootstrap support values based on 1000 replicates are indicated at branches. GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) accession numbers are shown on the tree for sequences included in these analyses. Bar, 0.05 substitutions per nucleotide position.

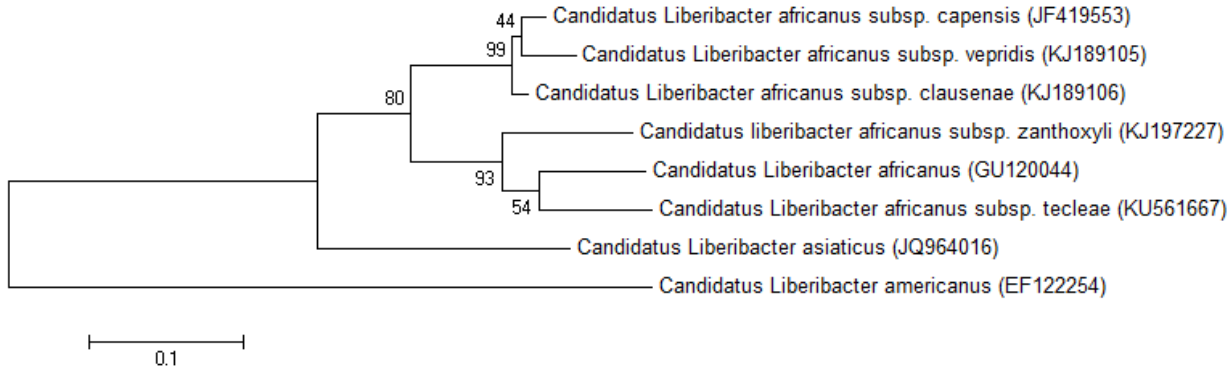


Fig. 3.3: Maximum-likelihood phylogeny based on *rplJ* gene sequences of members within the Liberibacter genus which are associated with rutaceous species. The phylogeny was inferred using the Tamura-Nei model (Tamura and Nei 1993) with gamma correction to account for site variations. Bootstrap support values based on 1000 replicates are indicated at branches. GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) accession numbers are shown on the tree for sequences included in this analysis. Bar, 0.01 substitutions per nucleotide position.

To confirm the identity of the *Teclea* host from which the novel Liberibacter sequences were obtained, all samples collected were subjected to DNA barcoding by sequencing *rbcL* and *psb-trnH* gene region. The *rbcL* gene for all samples collected was successfully amplified, however, this gene sequence could not resolve between *O. bachmannii* and the two *Teclea* species known to occur is South Africa (i.e. *T. natalensis* and *T. gerrardii* I. Verd) (Waffo et al. 2006) (Fig. 3.4). Of the 122 samples subjected to DNA barcoding of the *psb-trnH* gene, only 94 samples were successfully sequenced, with 14 putative *O. bachmannii* and 14 putative *Teclea* spp. failing to amplify. Phylogenetic analyses of this plastid gene region resolved various closely related Rutaceae species within the *Oricia*, *Teclea* and *Vepris* genera into separated clades (Fig. 3.5). Based on these results, it was shown that the tree host of the Liberibacter-positive sample studied here is *T. gerrardii*. *A* cluster of 26 samples formed a clade distinct from, but closely related to other *Oricia* and *Teclea* clades suggesting the presence of hereto undescribed variability in the taxonomy of this genus requiring further studies. A voucher specimen of tree sample 13-2192 was deposited with the KwaZulu-Natal Herbarium (NH) under the Voucher number "A.M. Ngwenya 4433".

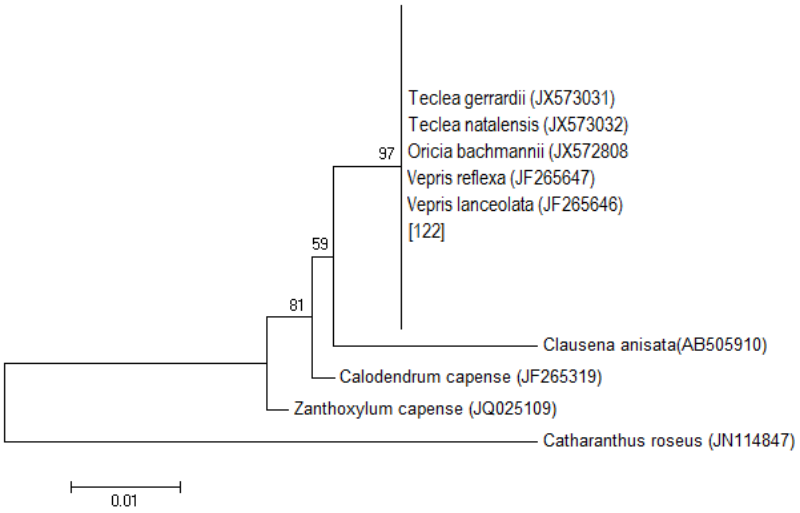


Fig. 3.4: Maximum-likelihood phylogeny of tree host species based on *rbcL* sequences obtained from all *Oricia* and *Teclea* samples collected for this study. The phylogeny was inferred using the Jukes-Cantor model. Bootstrap values based on 1000 replicates are indicated at branch nodes. Branches with >70% bootstrap support for terminal taxa were collapsed. The 122 specimens which were successfully sequenced are indicated in brackets. Bar, 0.01 substitutions per nucleotide position.

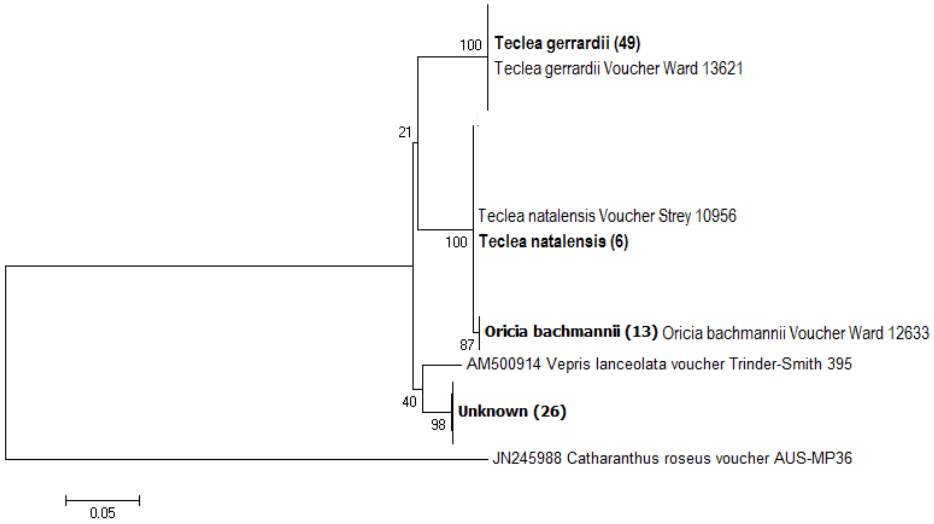


Fig. 3.5: Maximum-likelihood phylogeny of tree host species based on *psb-trnH* sequences obtained from *Oricia* and *Teclea* samples collected in this study (indicated in bold) as well as voucher specimens representing the tree species studied obtained from the South African National Biodiversity Institute (SANBI). The phylogeny was inferred using Tamura's 3-parameter model (Tamura 1992). Bootstrap support values based on 1000 replicates are indicated at branch nodes. Branches with >70% bootstrap support for terminal taxa were collapsed. The number of specimens sequenced per tree species indicated in brackets. Bar, 0.05 substitutions per nucleotide position.

### 3.5 Discussion

The percentage nucleotide identity of the 16S rDNA sequence obtained for the single Liberibacter-positive *T. gerrardii* sample conforms to the >99% nucleotide identities found amongst previously characterised Laf-subspecies (Garnier et al. 2000; Roberts et al. 2015). From the overall sequence similarity described here, and the phylogenies for all gene region studied, it is apparent that the sequences obtained from the single Liberibacter-positive *T. gerrardii* represent a novel Liberibacter sequence closely related to Laf and its subspecies. In maintaining the previous convention, we, therefore, propose that the Liberibacter obtained from *Teclea* also be assigned subspecies status under the proposed name of '*Ca.* L. africanus subsp. tecleae' (te.cle'ae. N.L. gen. n. tecleae, meaning of the plant genus Teclea) (LafT).

It has been suggested that the various subspecies of Laf represent haplotypes of Laf based on 16S rDNA data (Nelson et al. 2015). However, the five Laf-subspecies described (LafC, LafCl, LafV, LafZ and now LafT) were generally identified from multiple specimens of the same specific hosts, LafT being the exception being found in only one specimen, suggesting that gene-flow between these various Liberibacters is limited, supporting the higher taxonomic status afforded by subspecies classification. The high conservation of the 16S rDNA gene of African Liberibacters does, however, suggest that the divergence and isolation of these subspecies within their respective hosts occurred more recently than the divergence of Laf from Las which is estimated at 150 Myr (Teixiera et al. 2008). The subspecies designation thus aims to describe the relatedness of the various subspecies to Laf along with the distinction based on the various rutaceous hosts they occupy.

With the addition of LafT, there are now five recognized subspecies to Laf which have been identified from South Africa. For the sake of the South African citrus industry, it will be important to fully characterise various biological properties of these Laf-subspecies, i.e. vector and host range, to help fully understand the possible impact these Liberibacters may have on commercial citrus crops. Additional sequence information LafC, LafCl, LafV, LafT and LafZ could potentially help clarify the exact taxonomic position of the various subspecies in relation to Laf and give further insight into the divergence of these Liberibacters.

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# Chapter 4:

# Taxonomy and Liberibacter status of *Agathosma* spp.

## 4.1 Abstract

The alphaproteobacterium genus, Liberibacter is characterised by species which are fastidious, insect transmissible and act as both endophytes and pathogens within their host plants. Hosts include members of the Rutaceae, Solanaceae, Apiaceae, Caricaseae and Rosaceae families. In South Africa, Liberibacters have been found to infect both commercial and indigenous Rutaceae species. *Agathosma* (Family Rutaceae), commonly known as buchu, are unique to the Cape floristic region, and are used for medicinal and culinary purposes, with two species, *A. betulina* and *A. crenulata* being farmed commercially. The study aimed to determine whether Liberibacter species are present amongst 826 putative *Agathosma* specimens, representing 19 different morphological species through a generic Liberibacter real-time PCR assay. Positive samples were further assessed for the presence of Liberibacters by end-point PCR of 16S and *rplJ* sequences, with two samples being subjected to high-throughput sequencing. Of these samples, 20 tested positive in real-time PCR reactions, however, subsequent testing could not confirm the presence of Liberibacters. High-throughput sequence analysis further indicated the absence of Liberibacter sequences from the *Agathosma* species assessed. Despite this, 16S sequences from novel Alphaproteobacteria could be obtained through read mapping of the high-throughput data against Liberibacter sequences. Additionally, the molecular taxonomy of *Agathosma* species from this study was assessed by DNA barcoding of *rbcL, matK* and ITS sequences. Barcoding suggested that a high level of hybridization occurs between *Agathosma* species and that morphological characteristics do not reflect true speciation. From this study, it can be concluded that *Agathosma* species harbours unique bacterial sequences.

## 4.2 Introduction

*'Candidatus* Liberibacter species' represents a unique lineage within the Alphaproteobacteria (Jagoueix et al. 1994). Members of this genus are transmitted by insect vectors, commonly within the Psyllidae family (McClean and Oberholzer 1965, Capoor et al. 1967, Secor et al. 2009, Raddadi et al. 2011, Teixeira et al. 2005b), have reduced genomes of an average 1.2Mb (Duan et al. 2009, Wulff et al. 2014, Lin et al. 2015), and are predominantly non-culturable which is attributed to the fastidious nature of these bacteria (Garnier and Bové 1983). The first members within this lineage to be described, i.e. '*Ca.* L. asiaticus' (Las) and '*Ca.* L. africanus' (Laf), were shown to be associated with the diseases citrus Huanglongbing (HLB) in Asia and Citrus Greening (CG) in Africa, respectively (Jagoueix et al. 1994). Since then, a number of

disease associated Liberibacters have been described. In Brazil, '*Ca.* L. americanus' (Lam) was found to be associated with HLB symptoms in citrus (Teixeira et al. 2005a) and in New Zealand it was shown that '*Ca.* L. solanacearum' (Lso), was associated with Zebra chip disease of potato and psyllid yellows disease on tomato and pepper (Family: Solanaceae) (Liefting et al. 2009, Secor et al. 2009). *L. crescens*, the only member within this genus to be cultured, was described from papaya (Family: Caricaceae) showing Papaya bunchy top disease symptoms, (Fagen et al. 2014). However, '*Ca.* L. europaeus' (Leu), does not cause disease in pears (Family: Rosaceae) in Italy and potentially acts as an endosymbiont (Raddadi et al. 2011), suggesting that members within this genus can act as either pathogen or symbiont on host plants.

Non-agricultural hosts may serve as reservoirs for disease associated pathogens and have therefore been studied widely. Las has been found to naturally infect *Murraya paniculata* (Deng et al. 2007), *Severinia buxifolia* (Hung et al. 2001, Deng et al. 2008, Hu et al. 2014), and *Clausena lansium* (Ding et al. 2005) (Family: Rutaceae), all three of which additionally serve as hosts for the vector of Las, *Diaphorina citri* Kuwayama (Capoor et al. 1967). *M. paniculata* (Family: Rutaceae) was additionally found to serve as an alternative host of Lam in Brazil (Lopes et al. 2010). Las has also been identified from the weeds; *Cleome rutidosperma, Pisonia aculeate* and *Trichostigma octandrum* in Jamaica (Brown et al. 2011). Leu was found to infect *Malus pumila, Prunus spinosa*, and *Crataegus monogyna* (Family: Rosaceae) (Camerota et al. 2012) and Lso has been identified from a number of Solanaceous as well as Apiaceous hosts (Thinakaran et al. 2015). The non-specific host nature of Liberibacters is not just limited to the range of host plants which they infect as Leu has been detected in nine different species of psyllids within the *Cacopsylla* genera (Camerota et al. 2012), and *Cacopsylla citrisuga* shown to contain Las in China (Cen et al. 2012). Recently, another Liberibacter species was found to be associated with the eggplant psyllid, *Acizzia solanicola* in Australia, which was subsequently named '*Ca.* L. brunswickensis' (Lbr) (Morris et al. 2017).

Laf however, has not yet been found to infect hosts outside of commercial citrus. Despite this, and more interestingly, a number of subspecies to Laf have been characterised from indigenous Rutaceae trees in South Africa. The first of these subspecies to be described is '*Ca.* L. africanus subsp. capensis' (LafC) and was identified from *Calodendrum capense* trees showing mottling symptoms in the Western Cape (Garnier et al. 2000). It has since been shown that LafC is widely associated with *C. capense* trees in the country (Phahladira et al. 2010). In recent years, an additional four subspecies to Laf have been described in South Africa from indigenous Rutaceae hosts. Three of these i.e. '*Ca.* L. africanus subsp. clausenae' (LafCl), *'Ca.* L. africanus

subsp. vepridis' (LafV) and '*Ca.* L. africanus subsp. zanthoxyli' (LafZ), were described from known native hosts of the triozid vector of Laf, *Trioza erytreae* Del Guercio (McClean and Oberholzer 1965, Moran 1968, Roberts et al. 2015), with the fourth *'Ca.* L. africanus subsp. teclea' LafT being described from *Teclea gerrardii* (This study; Roberts and Pietersen 2017). When describing the latter four Laf-subspecies, it was noted that trees testing positive for the respective subspecies did not display typical mottling symptoms associated with Laf infection on citrus, suggesting that, as with Leu on pears, these Laf-subspecies may act as endophytes within their plant hosts.

The Cape Floristic Region (CFR) of the Western Cape, South Africa, is known as a diversity hotspot with a wealth of endemic plant species being associated with this region. A number of aromatic shrubs are associated with the CFR, including *Agathosma* species, (Family: Rutaceae) which are commonly known as buchu. There are more than 150 *Agathosma* species in South Africa, with highly variable morphologies being observed, between and even within species (Pilans 1950). Two species within this group, in particular, *A. betulina* and *A. crenulata* are becoming of agricultural importance as they are being cultivated for both their medicinal properties and culinary applications (Moolla and Viljoen 2008). The majority of studies on *Agathosma* have focused on the pharmaceutical properties of *A. betulina* and *A. crenulata*, with no information being available on the potential bacterial diversity, and only limited information available on the molecular taxonomy of *Agathosma* species (Trinder-Smith et al. 2007).

As a number of Rutaceae species indigenous to South Africa have been shown to contain Liberibacter populations closely related to Laf, it has been proposed that Laf may have its origin on the African continent from an indigenous Rutaceae host (Phahladira et al. 2012, Roberts et al. 2015). Within the current study, we explore this possibility further. *Agathosma* specimens were collected and tested for the presence of Liberibacter species and the molecular taxonomy of the collected *Agathosma* species represented were studied.

## 4.3 Method and Materials

### *4.3.1 Sample collection*

*Agathosma* species were collected from various sites in the Western Cape Province and KwaZulu-Natal, South Africa. Permits (reference number: CRC/2016-2017/021--2016/) and land owner permission were obtained to sample within these areas. The GPS localities, disease symptoms, and presence of psyllid-like insects were recorded for each sample collected, which were given a unique accession number. Because of the illegal harvesting of these plants for

medicinal purposes, the GPS data is kept confidential (Appendix B). Branches and flowers of selected known and unknown specimens were collected for morphological identification and deposited as voucher specimens within the National Herbarium at the South African National Biodiversity Institute (SANBI), Pretoria.

### *4.3.2 Detection of* Liberibacter *species*

Total DNA was extracted from the stems of collected samples following the protocol of Doyle and Doyle 1990. These extracts were subjected to a Liberibacter universal real-time PCR assay (Li et al 2006, Roberts et al 2015), of which reactions were set up with 10ul KAPA Probe Fast qPCR Master Mix (Sigma-Aldrich, St. Louis, MO, USA), 400nM per primer LibUF (Roberts et al 2015) and HLBr (Li et al 2006), 200nM probe HLBp (Li et al 2006) made up to a final volume of 20µl with nuclease-free water. Amplification was performed on a Rotor-Gene Q as follow; initial denaturation of 5 min at 94˚C followed by 40 cycles of denaturation at 94˚C for 10s and combined annealing and acquisition for 20s at 60˚C. Fluorescence and crossing thresholds (Ct) value per sample was determined using Rotor-Gene Q software version 2.3.1.49 (Qiagen, Hilden, Germany).

Samples with a Ct<32 were further assessed for the presence of Liberibacters by end-point PCR of Liberibacter ribosomal J (*rplJ*) and 16S rDNA sequences. This cut-off was selected based on previous experience in that no amplification is obtained following end-point PCR for samples with a Ct>32 when performing subsequent Liberibacter analyses. For *rplJ* amplification, the A2/J5 primer set described by Hocquellet et al. 1999 was used in reactions containing 12µl of DreamTaq Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 200nM of each primer, and made up to a final reaction volume of 25µl with nuclease-free water. Reactions were set up on a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) thermocycler as follows; 3 min of initial denaturation at 92˚C, followed by 35 cycles of denaturation for 20 s at 92˚C, annealing for 20s at 62˚C and elongation at 72˚C for 30s, with final elongation at 72˚C for 5 min.

Two primer sets were used for the amplification of Liberibacter 16S rDNA sequences within samples, primers set OA1/OI2c (Jagoueix et al. 1996) and set LG774F/LG1463R (Morris et al. 2017). Both reactions were set up as described above using DreamTaq Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and cycling conditions were as follows; 5 min of initial denaturation at 92˚C followed by 35 cycles of denaturation at 92˚C for 30s, annealing

at 65˚C for 30s and extension at 72˚C for 1 min, with a final extension at 72˚C for 10 min. Primer sequences for Liberibacter detection are listed in Table 4.1.

Table 4.1: Primer and probe sequences utilised within this study for Liberibacter amplification. The references for each primer is given.

| **Primer name** | **Primer sequence (5'-3')** | **Target region** | **Reference** |
|---|---|---|---|
| LibUF | GGCAGGCCTAACACATGC | 16S | Roberts et al. 2015 |
| HLBr | GCGTTATCCCGTAGAAAAAGGTAG | 16S | Li et al. 2006 |
| HLBp | AGACGGGTGAGTAACGCG | 16S | Li et al. 2006 |
| A2 | TATAAAGGTTGACCTTTCGAGTTT | *rplJ* | Hocquellet et al. 1999 |
| J5 | ACAAAAGCAGAAATAGCAACAA | *rplJ* | Hocquellet et al. 1999 |
| OA1 | GCGCGTATTTTATACGAGCGGCA | 16S | Jagoueix et al. 1996 |
| OI2c | GCCTCGCGACTTCGCAACCCAT | 16S | Jagoueix et al. 1996 |
| LG774F | GTAAACGATGAGTGCTAGCTGTTGGG | 16S | Morris et al. 2017 |
| LG1463R | CTGACCRTACCGTGGCCGG | 16S | Morris et al. 2017 |

Two samples denoted as 16/8025 and 16/8127 from *A. betulina* and *A. apiculata*, respectively, were selected for high-throughput sequencing (HTS) analysis using an Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA) at the Agricultural Research Council Biotechnology Platform (ARC-BTP, Pretoria, South Africa). These samples were selected as they had low Ct values (22.49 and 27.52 respectively) and originated from different *Agathosma* hosts and different localities (Clanwilliam and Knysna). The pair-ended sequence reads obtained were imported into CLC genomics workbench 9 software (Qiagen Bioinformatics, Hilden, Germany), and trimmed for quality using default settings. To determine the initial proportion of Alphaproteobacteria reads within these datasets, trimmed unassembled reads were subjected to taxonomic identification using Kaiju software package (Menzel et al. 2016). *De novo* assemblies were performed on the pair-ended reads and resulting contigs were mapped against available Liberibacter 16S rDNA reference sequences obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Trimmed reads were additionally mapped to these reference sequences to determine which dataset, contigs or reads, are best suited to determine the presence of Liberibacter sequences from HTS data. A consensus sequence from each mapping was extracted. These consensus sequences were then aligned with available 16S rDNA Liberibacter sequences as well as other Alpha-, Beta- and Gammaproteobacteria 16S rDNA reference sequences using MAFFT online tool (Katoh et al 2002). The aligned dataset was then trimmed in BioEdit version 7.2.5 (Hall 1999) so the cognate region within the represented 16S rDNA sequences were assessed. The best-fit DNA evolutionary model and

maximum–likelihood phylogenies of the trimmed alignment was determined using MEGA version X (Kumar et al. 2018).

*4.3.3 Barcoding of Agathosma species.*

DNA from voucher samples, as well as samples which represents the full scope of *Agathosma* spp, collected, including some, where no voucher specimen had been deposited, were subjected to DNA barcoding of the ribulose-biphosphate (*rbcL*), maturase K (*matK*), internal transcriber space 2 (ITS2) and chloroplast *psb-trnH* spacer region. The primer sets used are listed in Table 4.2.

PCR of *rbcL, matK* and ITS2 barcodes were carried out using GoTaq® G2 Flexi DNA polymerase (Promega, Madison, WI, USA). PCR reactions were set up as follow; 5µl of 5X Green GoTaq® Flexi buffer, 2µl 25mM $MgCl_2$, 0,13 µl GoTaq® G2 Flexi DNA polymerase (5U/µl), 200nM per primer, 200nM dNTP mix, 0,5µl DNA and made up to a final reaction volume of 25µl with distilled water. Cycling conditions for *rbcL* and ITS2 were as follows; initial denaturation at 94˚C for 5min followed by 35 cycles of denaturation at 94˚C for 20s, annealing at 55˚C for *rbcL* and 56˚C for ITS2 for 20s followed by extension at 72˚C for 40s with a final extension step at 72˚C for 10min. For *matK*, a nested-PCR was performed to achieve amplification. First round amplification conditions for *matK* were as follows, initial denaturation at 94˚C for 5min followed by 35 cycles at 94˚C for 30s, 52˚C for 40s and 72˚C for 50s, with a final extension at 72˚C for 10 min. Amplification products from the first round of PCR were used as template for second round amplification which was carried out under the following conditions; initial denaturation at 94˚C for 5min followed by 35 cycles at 94˚C for 30s, 50˚C for 40s and 72˚C for 40s with a final extension at 72˚C for 10min.

Amplification of *psb-trnH* sequences were conducted with Phusion hot start II DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were prepared by the addition of 1µl of DNA to a reaction volume of 24µl containing 5µl 5X Phusion HF buffer, 0,32µl Phusion hot start II DNA polymerase (2U/µL), 0,14µl 10mM dNTP mix, 0,25µl per 10mM primer, 0,75µl DMSO and distilled water. Amplification was performed per the following thermocycling condition; initial denaturation at 95˚C for 1min, followed by 35 cycles of 95˚C for 20s, 64˚C for 30s and 72˚C for 40s, with a final elongation step at 72˚C for 5min.

Amplification products of all barcoding PCRs were viewed on a 1% agarose gel following electrophoresis. Samples which were successfully amplified for the various barcoding genes were submitted to Inqaba Biotech (Pretoria, South Africa) for bi-directional Sanger sequencing

using the respective forward and reverse primers per barcode. The sequences obtained were assessed in Chromas V2.6 for quality. Following the quality assessment, sequences were compiled into datasets per barcode amplified. Datasets were subsequently aligned using MAFFT online tool (Katoh et al. 2002). Alignments were then trimmed in BioEdit V 2.7.5 (Hall 1999) to ensure that cognate gene regions were assessed. 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. A concatenated dataset consisting of all barcodes successfully amplified and sequenced per sample was additionally compiled and assessed as described above.

Table 4.2: Primer sequences used within this study for DNA barcoding of *Agathosma* spp. Primer references are listed.

| **Primer name** | **Primer sequence (5'-3')** | **Target gene** | **Primer Reference** |
|---|---|---|---|
| rbcLa F | ATGTCACCACAAACAGAGACTAAAGC | *rbcL* | Levin 2003 |
| rbcLa R | GTAAAATCAAGTCCACCRCG | *rbcL* | Kress and Erickson 2007 |
| MatK-1RKIM-f | ACCCAGTCCATCTGGAAATCTTGGTTC | *MatK* (round 1) | CCDB protocols |
| MatK-3FKIM-r | CGTACAGTACTTTTGTGTTTACGAG | *MatK* (round 1) | CCDB protocols |
| MatK_390f | CGATCTATTCATTCAATATTTC | *MatK* (round 2) | Cuenoud et al. 2002 |
| MatK_1326r | TCTAGCACACGAAAGTCGAAGT | *MatK* (round 2) | Cuenoud et al. 2002 |
| ITS2-S2F | ATGCGATACTTGGTGTGAAT | ITS2 | Chen et al. 2010 |
| ITS4 | TCCTCCGCTTATTGATATGC | ITS2 | White et al. 1990 |
| psbA3_f | GTTATGCATGAACGTAATGCTC | *psb-trnH* | Sang et al. 1997 |
| trnHf_05 | CGCGCATGGTGGATTCACAATCC | *psb-trnH* | Tate and Simpson 2003 |

## 4.4 Results

### *4.4.1 Detection of* Liberibacters

In total, 826 putative *Agathosma* samples were collected representing 19 different known species (Table 4.3). A few samples displayed yellow mottling symptoms (Appendix B), however, the visual diagnosis of leaf symptoms on the majority of samples collected was difficult due to the minute size of the leaves. Symptoms were best observed on *Agathosma* spp with broader leaves, such as *A. crenulata* and *A. betulina.* No psyllid-like insects or evidence of previous infestation of plants with psyllids were observed on samples collected.

Of the collected samples, only 20 samples had a Ct<32 following real-time PCR assay (Appendix B). PCR of both *rplJ* and 16S rDNA sequences yielded no amplification product for these samples even under reduced stringencies.

Table 4.3: Location and number of *Agathosma* spp collected which were tested with real-time PCR for the presence of Liberibacter sequences.

| **Areas Surveyed** | ***Agathosma* species represented** | **Number of specimens sampled** | **Number of specimens with Ct<32**** |
|---|---|---|---|
| ***KwaZulu Natal*** | | | |
| Port Edward | *A. ovata* | 12 | - |
| ***Garden Route*** | | | |
| Sedgefield | *A. apiculata* | 70 | 1 |
| | *A. capensis* | 1 | - |
| | *A. muirii* | 21 | - |
| | *A.* spp | 2 | - |
| Knysna | *A. capensis* | 2 | - |
| | *A. ovata* | 1 | - |
| Hoekwil | *A. apiculata* | 2 | - |
| | *A. ovata* | 10 | 2 |
| Heralds Bay | *A. apiculate* | 10 | 1 |
| | *A. ovata* | 15 | - |
| Wilderness | *A. acutissima* | 14 | 1 |
| | *A. apiculata* | 5 | - |
| | *A.* spp | 26 | - |
| Ballots Bay | *A. capensis* | 39 | - |
| | *A. ovata* | 1 | - |
| Kranshoek | *A. ovata* | 5 | - |
| ***Cape floristic region*** | | | |
| Kirstenbosch | *A. apiculata* | 2 | - |
| | *A. capensis* | 1 | - |
| | *A. crenulata* | 5 | - |
| | *A. gonaquensis* | 1 | - |
| | *A. mucronulata* | 3 | - |
| | *A. nova* | 1 | - |
| | *A. ovata* | 26 | - |
| | *A.* spp | 1 | - |
| West coast national park | *A. bisulca* | 30 | - |

| | | | |
|---|---|---|---|
| | *A. imbricata* | 70 | 8 |
| Cape point nature reserve | *A. capensis* | 10 | - |
| | *A. ciliaris* | 17 | 1 |
| | *A. imbricata* | 13 | - |
| | *A. lanceolata* | 35 | - |
| Hermanus | *A. crenulata* | 2 | - |
| | *A. imbricata* | 19 | - |
| | *A. martiana* | 1 | - |
| | *A. ovata* | 3 | - |
| | *A. virgata* | 19 | - |
| Franschoek | *A. bifida* | 14 | - |
| | *A. ciliaris* | 18 | - |
| | *A. crenulata* | 8 | - |
| | *A.* spp | 5 | - |
| Harold Porter | *A. apiculata* | 14 | - |
| | *A. crenulata* | 12 | - |
| | *A. lanceolata* | 3 | - |
| | *A. ovata* | 15 | - |
| | *A. serpyllacea* | 12 | - |
| Paarlberg | *A. hispida* | 40 | - |
| Bainskloof | *A. virgata* | 13 | - |
| Rondevlei nature reserve | *A. acutissima* | 1 | - |
| | *A. muirii* | 1 | - |
| | *A. serpyllacea* | 1 | - |
| Lourensford Wine Estate | *A. ciliaris* | 21 | - |
| | *A. crenulata* | 44 | - |
| Vergelegen Wine Estate | *A. ciliaris* | 34 | - |
| | *A. imbricata* | 19 | - |
| Clanwilliam | *A. virgata* | 8 | - |
| Porcupine hills | *A. ovata* | 5 | - |
| ***Cultivated**** | | | |
| Skimmelberg | *A. betulina* | 43 | 4 |
| | | ***826*** | ***20*** |

* Samples obtained from cultivated *A. betulina*, while all other samples were obtained from natural vegetation or botanical gardens.

**Samples with a Ct<32 following Liberibacter universal real-time PCR assay were considered potentially positive for Liberibacters.

From Kaiju analysis, 93-95% of reads from both datasets were unclassified with less than 1% of the total reads being classified as Proteobacteria of which 45-54% of Proteobacteria reads were made up of Alphaproteobacteria. Sample 16/8127 returned more sequence reads (50 million reads) than sample 16/8025 (41 million reads), and this translated to more sequence reads being mapped against Liberibacter 16S rDNA reference sequences than for sample 16/8025. The average sequence coverage from sample 16/8127 was 87% compared to only 55% for sample 16/8025 (Table 4.4) when reads were mapped against 16S rDNA reference sequences. Reads from each sample were also mapped against available Liberibacter *rplJ* and outer-membrane protein (*omp*) sequences, but no reads mapped to any of these reference sequences. From the *de novo* assembled data, none of the contigs mapped against 16S rDNA Liberibacter sequences.

Table 4.4: Read mapping results of Illumina HiSeq reads obtained for samples 16/8025 and 16/8127 mapped against available Liberibacter 16S rDNA sequences. The length of the consensus sequence obtained per mapping per sample are presented as the base pair (bp) length of the consensus sequence.

| **Reference sequence (Genbank Accession)** | **Reference sequence (bp)** | **No. reads mapped to reference (16/8025)** | **Consensus sequence 16/8025 (bp)** | **No. reads mapped to reference (16/8127)** | **Consensus sequence 16/8127 (bp)** |
|---|---|---|---|---|---|
| Laf (EU921619) | 1,432 | 3,835 | 821 | 6,365 | 1,280 |
| LafC (KY000560) | 1,500 | 3,828 | 828 | 6,424 | 1,306 |
| LafCl (KY000562) | 1,500 | 3,828 | 828 | 6,424 | 1,306 |
| LafT (KX990288) | 1,500 | 3,836 | 830 | 6,420 | 1,304 |
| LafV (KY000561) | 1,500 | 3,828 | 827 | 6,424 | 1,306 |
| LafZ (KX990287) | 1,500 | 3,643 | 830 | 6,098 | 1,305 |
| Las (JQ866401) | 1,122 | 3,267 | 625 | 5,695 | 1,023 |
| Lam (EU921624) | 1,417 | 4,422 | 877 | 7,609 | 1,264 |
| Lso (KF776242) | 1,180 | 3,614 | 647 | 6,171 | 1,037 |
| Lcr (NR102476) | 1,482 | 4,601 | 932 | 7,910 | 1,307 |
| Leu (JX244260) | 2,070 | 4.748 | 958 | 8,130 | 1,322 |
| Carribeanus (KP012551) | 1,125 | 4,088 | 700 | 7,080 | 1,006 |
| Lbr (KY077741) | 1,464 | 3,561 | 920 | 5,976 | 1,271 |

Nucleotide BLAST analyses against NCBI Genbank database were performed on extracted consensus sequences from read mappings for 16/8127 only, due to the greater sequence coverage of these sequences compared to consensus sequences from 16/8025. All of the obtained Liberibacter 16S rDNA consensus sequences from read mappings, with the exception of the Las consensus sequence, shared 90-92% sequence similarity with uncultured bacterial entries on GenBank. The Las 16S rDNA read mapping consensus sequence obtained from sample 16/8127 was the only sequence which shared 89% sequence identity with Las sequences on Genbank. This nucleotide similarity is however not enough to prove that a Liberibacter was present in sample 16/8127, as bacterial 16S rDNA sequences within a genus typically share 97-100% sequence similarity. The BLAST analyses would, however, suggest that the *Agathosma* spp contains unique bacteria, related to the Liberibacter genus.

The maximum-likelihood phylogeny performed on the HTS read mapping derived extracted consensus sequences supports the end-point PCR results, in that Liberibacter sequences were not detected within the *Agathosma* spp submitted for HTS. Phylogenetic analysis revealed that the consensus sequences, obtained from Liberibacter-like 16S rDNA read mappings, represented a distinct clade within the Alphaproteobacteria. It was also demonstrated that each of the two samples submitted for HTS (16/8025 and 16/8127), obtained from two different *Agathosma* spp (*A. betulina* and *A. apiculata*) harbours closely related bacterial species within a potentially novel genus. Further analysis of these sequences and comparisons of bacterial communities from other *Agathosma* species will, however, be required to ascertain the above hypothesis, which was not within the scope of this study.

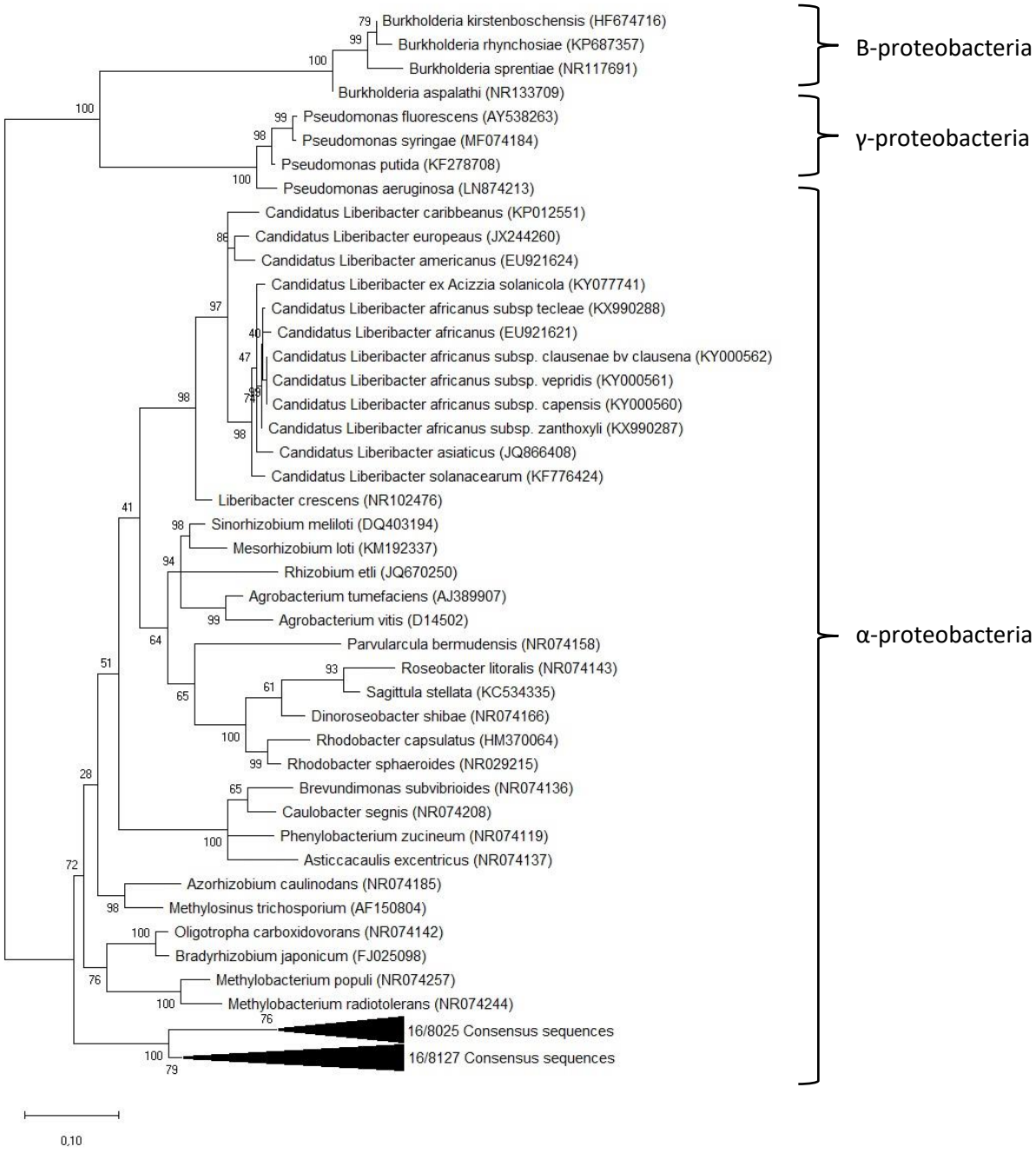


Fig 4.1: Maximum-likelihood phylogeny based on 16S rDNA sequences for members within the genus Liberibacter as well as representative sequences for phytobacteria of the Alpha-, Beta-, and Gammaproteobacteria. Branches with >70% support for consensus sequences obtained from 16/8025 and 16/8127 read mappings were collapsed. The phylogeny was inferred based on the Kimura-2-parameter model (Kimura 1980). Bootstrap support values based on 1000 replicates are indicated at the branches and the Genbank accession numbers for reference sequences are indicated in brackets. *Bar* 0.10 substitutions per nucleotide position.

*4.4.2 Agathosma barcoding*

A total of 30 *Agathosma* samples were collected for submission as voucher specimens within the National herbarium at SANBI, Pretoria, South Africa. In addition to these 30 voucher samples, five *Agathosma* spp collected from Kirstenbosch national botanical gardens and two *A. ovata* samples from the Southern Cape were included in DNA barcoding.

Barcoding of the *psb-trnH* failed for the majority of the voucher specimens with less than 10% of samples being amplified successfully. This barcode was thus omitted from further analysis. For *rbcL* and ITS2 barcodes, samples "Victor and Roberts 16" and "Victor and Roberts 18" failed to amplify with a third voucher specimen, "Victor and Roberts 24" also failing to amplify *rbcL*. Two voucher samples, "Victor and Roberts 20" and "Victor and Roberts 21", generated mixed sequences for *rbcL* barcode which could not be included in further analysis. All *matK* barcodes were however successfully amplified and sequenced.

Table 4.5: List of *Agathosma* spp collected as voucher specimens for deposition within the National Herbarium at SANBI, Pretoria.

| **Voucher** | ***Agathosma* spp**[*] | **Locality** |
|---|---|---|
| Victor and Roberts 1 | *A. apiculata* | Sedgefield |
| Victor and Roberts 2 | *A. muirii* | Knysna |
| Victor and Roberts 3 | *A. capensis* | Wilderness West Coast National Park |
| Victor and Roberts 4 | *A. imbricata* | Wilderness West Coast National Park |
| Victor and Roberts 5 | *A. imbricata* | Wilderness West Coast National Park |
| Victor and Roberts 6 | *A. bisulca* | Cape Point Nature Reserve |
| Victor and Roberts 7 | *A. capensis* | Cape Point Nature Reserve |
| Victor and Roberts 8 | *A. ciliaris* | Cape Point Nature Reserve |
| Victor and Roberts 9 | *A. ciliaris* | Cape Point Nature Reserve |
| Victor and Roberts 10 | *A. crenulata* | Lourensford Wine Estate |
| Victor and Roberts 11 | *A. crenulata* | Lourensford Wine Estate |
| Victor and Roberts 12 | *A. ciliaris* | Lourensford Wine Estate |
| Victor and Roberts 13 | *A. ciliaris* | Vergelegen Wine Estate |
| Victor and Roberts 14 | *A. ciliaris* | Hermanus |
| Victor and Roberts 15 | *A. martiana* | Hermanus |
| Victor and Roberts 16 | *A. ciliaris* | Hermanus |
| Victor and Roberts 17 | *A. imbricata* | Hermanus |
| Victor and Roberts 18 | *A. apiculata* | Harold Porter Botanical Garden |

| | | |
|---|---|---|
| Victor and Roberts 19 | *A. lanceolata* | Harold Porter Botanical Garden |
| Victor and Roberts 20 | *A. lanceolata* | Harold Porter Botanical Garden |
| Victor and Roberts 21 | *A. lanceolata* | Harold Porter Botanical Garden |
| Victor and Roberts 22 | *A. bifida* | Franschoek |
| Victor and Roberts 23 | *A. hisipda* | Paarl |
| Victor and Roberts 24 | *A. virgata* | Bainskloof |
| Victor and Roberts 25 | *A.* spp | Wilderness |
| Victor and Roberts 26 | *A. acutissima* | Wilderness |
| Victor and Roberts 27 | *A.* spp | Sedgefield |
| Victor and Roberts 28 | *A.* spp | Sedgefield |
| Victor and Roberts 29 | *A. capensis* | Sedgefield |
| Victor and Roberts 30 | *A. muirii* | Sedgefield |

*Species identification based on the morphological characterisation

Of the individual barcode sequences assessed for differentiation of *Agathosma* spp, ITS2 (Fig 4.3) showed the highest interspecies resolution, with *rbcL* (Fig 4.2) being the least discriminatory. Phylogeny based on concatenated sequences did, however, give the greatest interspecies discrimination with the highest support based on bootstrap values, for clades obtained (Fig. 4.5). From the phylogenetic analysis performed, *A. betulina, A. crenulata, A. gonaquensis, A. muirii, A. ovata* and *A.* spp Kirstenbosch formed distinct clades for each of these species, irrespective of the barcoding gene assessed. *A. mucronulata* and *A. martiana* grouped as distinct, albeit closely related species, for *rbcL* and ITS2 barcodes but as a single clade for *matK* (Fig 4.4). However, phylogeny based on the concatenated sequences for these two species once again represents these two as distinct species (Fig 4.5).

Based on concatenated phylogenies, vouchers *A. apiculata* (Victor and Roberts 1) and *A. spp* (Victor and Roberts 27) are closely related, which is supported by phylogenies based on *rbcL* and *matK* sequences. Barcodes "Victor and Roberts 3, 25 and 26" (*A. capensis, A. spp,* and *A. acutissima,* respectively), grouped within the same clade for concatenated, *matK* and ITS2 phylogenies suggesting that these three specimens represent the same species. Vouchers, morphologically identified as *A. imbricata* (Victor and Roberts 4, 5 and 17), and grouped as a single species for barcode sequence ITS2 and only for Voucher "Victor and Roberts 4 and 5". *A. bisulca* (Victor and Roberts 6), *A. capensis* (Victor and Roberts 7) and *A. ciliaris* (Victor and Roberts 14), formed closely related clades for each of the phylogenies tested suggesting that these vouchers are also closely related genetically, albeit distinct. The phylogenies for

vouchers “Victor and Roberts 8, 9, 12, 13, 19, 22, 23, 28, 29” and *A. nova* were unclear and may be better resolved with the inclusion of additional barcodes from morphologically described specimens.

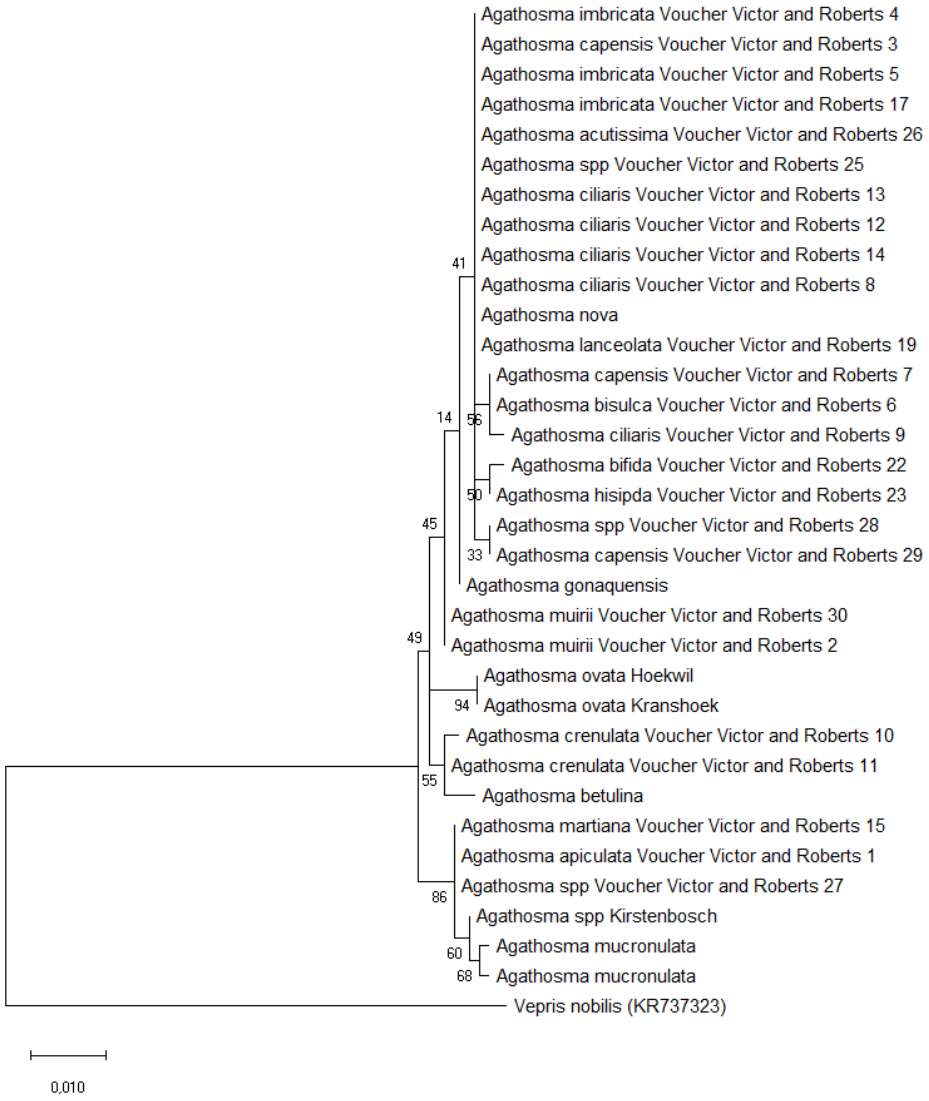


Fig 4.2: Maximum-likelihood phylogeny of *Agathosma* spp based on *rbcL* barcoding sequences. *Vepris nobilis rbcL* sequence obtained from Genbank is included as an outgroup. The Genbank accession for this sequence is presented in brackets. The phylogeny was inferred using the Jukes-Cantor model (Jukes and Cantor, 1969) with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,01 substitutions per nucleotide position.

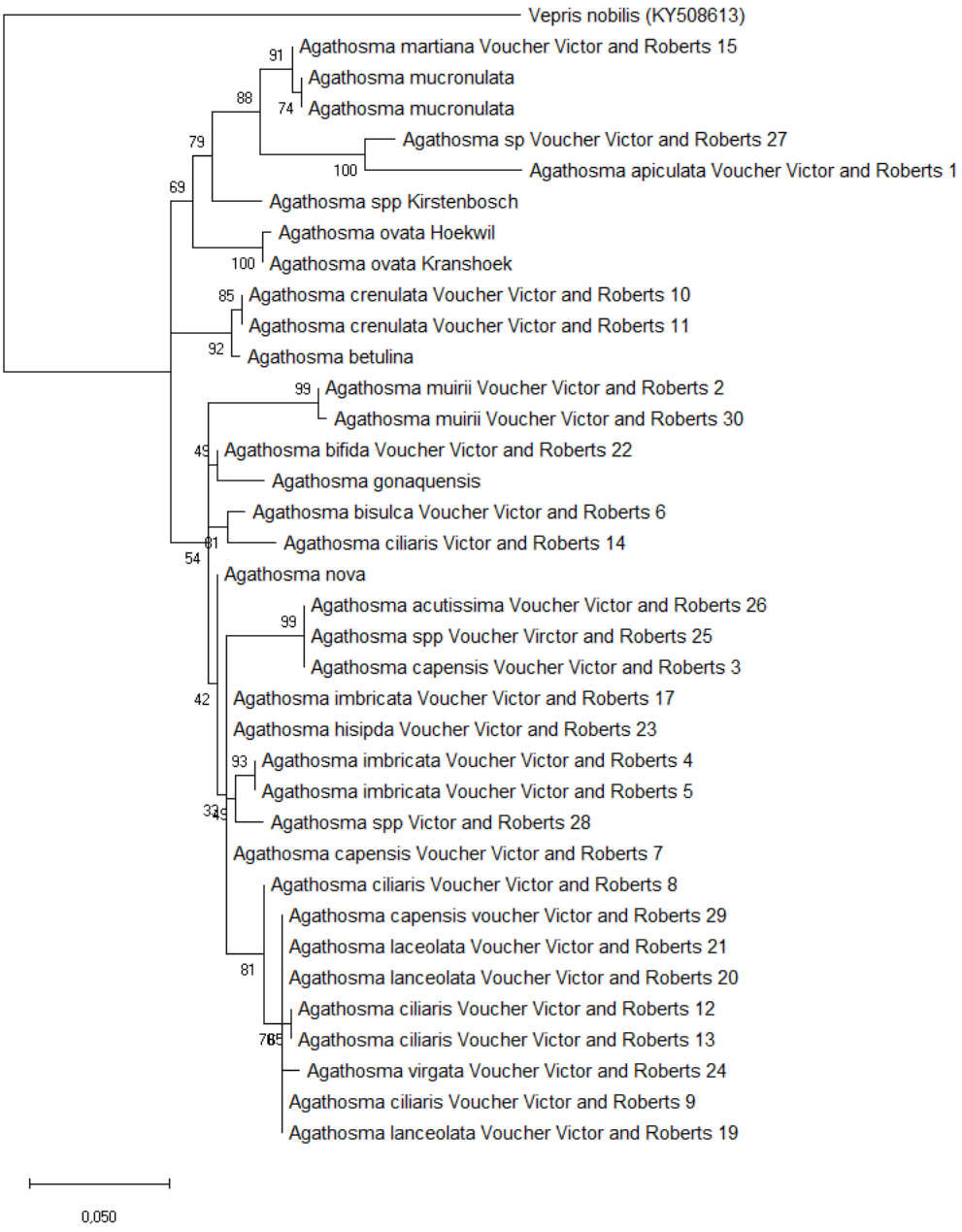


Fig 4.3: Maximum-likelihood phylogeny of *Agathosma* spp based on ITS2 barcoding sequences. *Vepris nobilis* ITS2 sequence obtained from Genbank is included as an outgroup. The Genbank accession for this sequence is presented in brackets. The phylogeny was inferred using the Tamura-Nei (Tamura and Nei 1993) model with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,01 substitutions per nucleotide position.

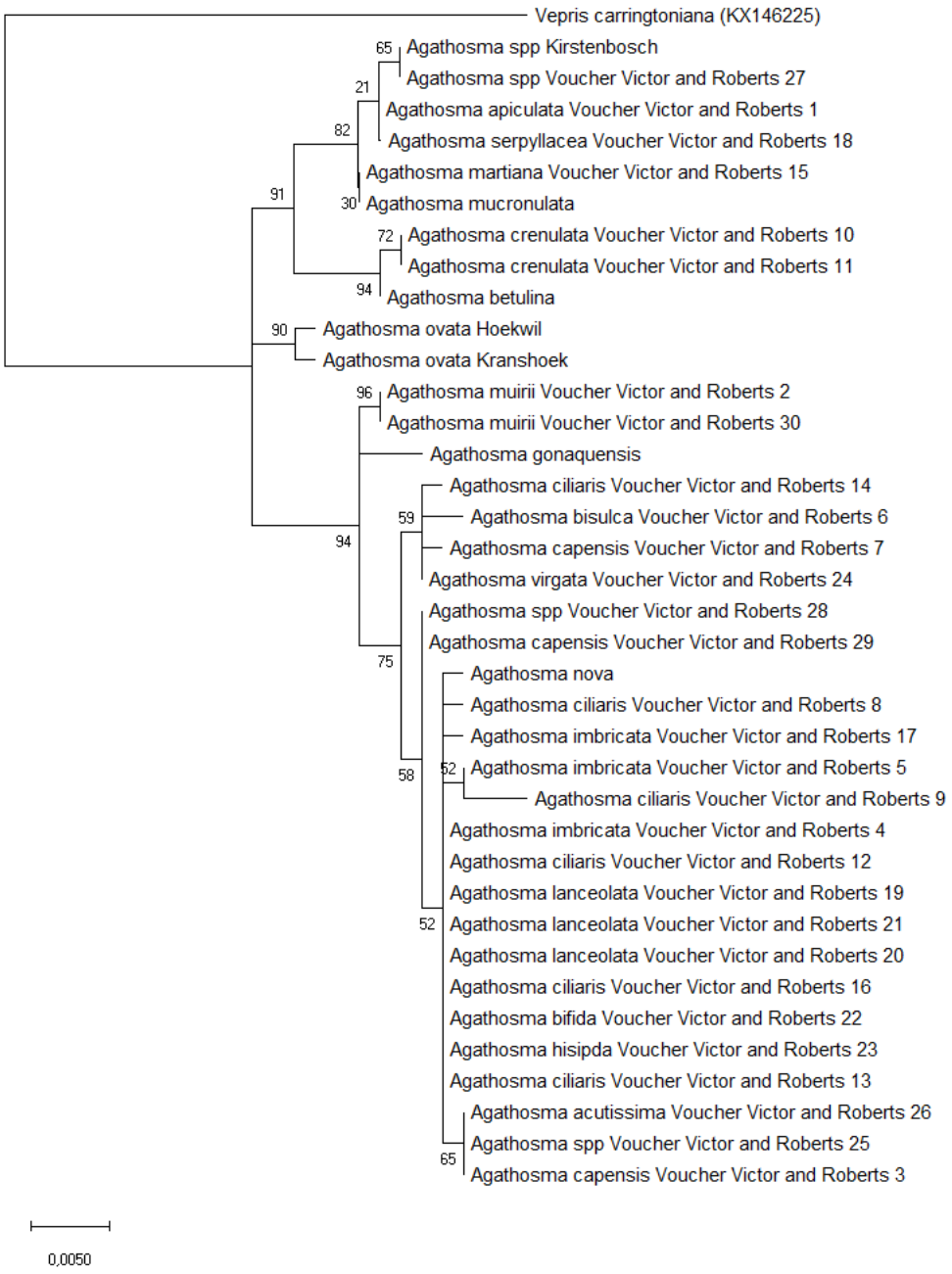


Fig 4.4: Maximum-likelihood phylogeny of *Agathosma* spp based on *matK* barcoding sequences. *Vepris carringtoniana matK* sequence obtained from Genbank is included as an outgroup. The Genbank accession for this sequence is presented in brackets. The phylogeny was inferred using the Hasegawa-Kishino-Yano (Hasegawa et al. 1985) model with gamma corrections. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,005 substitutions per nucleotide position.


Fig 4.5: Maximum-likelihood phylogeny of *Agathosma* spp based on concatenated *rbcL*, ITS2, and *matK* sequences. *Vepris nobilis* sequence obtained from Genbank is included as an outgroup. The Genbank accession for this sequence is presented in brackets. The phylogeny was inferred using the Tamura-Nei model (Tamura and Nei 1993) with gamma corrections and invariable sites. Bootstrap values based on 1000 replicates are indicated at branch nodes. *Bar* 0,01 substitutions per nucleotide position.

## 4.5 Discussion

Based on the current study, no evidence could be obtained for the presence of Liberibacters being associated with *Agathosma* from South Africa, despite samples testing positive following a Liberibacter universal real-time PCR assay. Shin and van Bruggen (2018) demonstrated that the real-time PCR primers published by Li et al. 2006 for the detection of Las, amplified *Bradyrhizobium* populations present in the roots of citrus trees. This non-specific amplification of bacterial populations, especially species within the Proteobacteria, possibly resulted in the initial false-positive amplification of non-Liberibacter sequences from *Agathosma* samples, as it was shown that samples submitted for HTS contained sequences from a potentially novel, non-Liberibacter, bacterial genera within the Alphaproteobacteria. This non-target amplification of the 16S rDNA primer and probe set used for initial screening of *Agathosma* samples for Liberibacters will also explain why no amplification was achieved for end-point PCR reactions which were performed on samples with a Ct<32. These results were further validated with HTS data from *A. betulina* and *A. apiculata* as none of the sequence reads mapped against Liberibacter *rbcL* and *omp* sequences, despite high coverage being obtained for read mappings against Liberibacter 16S rDNA sequences. It is therefore important that the Liberibacter universal real-time PCR assay, which utilises the reverse primer and probe published by Li et al. 2006, used within this study be assessed to prevent future non-target amplification, despite having previously proven successful in the detection of Laf-subspecies from Rutaceae hosts (Roberts et al. 2015, Roberts and Pietersen 2017).

Typical symptoms associated with Liberibacter infection of commercial Rutaceae hosts, such as yellowing and mottling, were observed on a few *Agathosma* specimens sampled, in particular, those from Kirstenbosch Botanical Garden (Appendix B). These symptoms, however, can be due to the presence of other biological agents such as viruses and phytoplasmas, or caused by physiological stresses brought on by environmental conditions, as these samples were generally collected from natural settings. The latter is possible as the majority of sampling was done during 2016-2017, when the Western Cape was experiencing the most severe drought in recorded history. It should be noted, however, that a number of Liberibacters do not appear to cause disease symptoms on the hosts they infect (Raddadi et al. 2010, Roberts et al. 2015), and the absence of symptoms does not necessarily constitute the absence of Liberibacters.

Insect vectors within the Psyllidae play an important role in the transmission of Liberibacters (McClean and Oberholzer 1965, Capoor et al. 1967). Within the current study, no psyllid-like insects were observed on *Agathosma* samples collected. Even though the absence of psyllids may be due to the extremely dry season, or the inexperience of collectors to visually identify psyllids in the field, no information is available on the occurrence of members within the Psyllidae on *Agathosma* (Ian Millar, per comm). The possibility, therefore, exists that *Agathosma* may not be a suitable host for Psyllidae species, which could also explain the absence of Liberibacters from this genus. However, further sampling of different *Agathosma* species and specific focus being given to cultivated *Agathosma* species may change this perspective.

Through HTS and subsequent phylogenetic analyses, it was shown that the *A. betulina* and *A. apiculata* samples assessed, each harboured similar bacterial lineage within the Alphaproteobacteria. Research focused on the diversity of bacterial communities of plants within the fynbos biome has shown that novel members within the Alphaproteobacteria are associated with fynbos of the CFR (Steenkamp et al. 2016, Miyambo et al. 2016). It is therefore not surprising that the current study indicated that *Agathosma* spp also contains unique Alphaproteobacterial sequences. It was also demonstrated that each *Agathosma* spp contained a unique, albeit closely related to Liberibacter, bacterial sequence. The Alphaproteobacteria contains a number of endophytic species which have been shown to promote plant growth (Pini et al. 2011) and it is believed that plants within the CFR harbour a plethora of unique Proteobacteria to assist these species to grow in nutrient-poor soils associated with the CFR (Brink et al. 2017, Miymbo et al. 2017). The results obtained herein further support this notion, however, future characterisation of the Alphaproteobacterial communities associated with *Agathosma* species will be needed to determine whether or not these bacteria act as endosymbionts and exerts any biological benefits within these hosts.

The current taxonomy of *Agathosma* is based purely on morphological characteristics of species within this genus (Pilans 1950) and little information on the molecular characterisation of DNA barcodes for *Agathosma* spp are available to support these morphological speciations (Trinder-Smith et al. 2007). Based on barcoding results obtained herein, only six of the putative 19 *Agathosma* spp included in barcoding could be resolved as distinct species for all of the barcode regions assessed (i.e *A. betulina, A. ovata, A. gonaquensis, A. crenulata, A. muirii, A.* spp Kirstenbosch), with two species, *A. martiana* and *A. mucronulata,* being resolved as separate species for all barcodes except *matK*. This leaves a total of eleven unresolved

morphological species being included in this study. These unresolved phylogenies may indicate that a high level of species hybridizations may occur amongst *Agathosma* species, which would also explain why a high diversity of morphotypes are observed for defined *Agathosma* species such as *A. ovata*. Alternatively, the low level of interspecies separation can be attributed to the barcoding regions used. *Psb-trnH* has a higher evolutionary rate compared to the other barcodes tested. Unfortunately, the amplification success for this barcoding region within *Agathosma* spp sampled was poor, possibly due to mutations within the primer binding sites.

Of the individual barcodes assessed, ITS2 gave the highest interspecies resolution, with *rbcL* barcodes being a poor candidate for interspecies differentiation of *Agathosma* spp. The ITS2 sequence has previously been shown to be a preferred DNA marker for the differentiation of Rutaceae species (Luo et al. 2010). It is however advised that multiple barcodes are used, and assessed as a single concatenated sequence, to determine the interspecies divergence for *Agathosma* spp, based on the results obtained in the current study. It is additionally proposed that a greater number of clearly defined *Agathosma* specimens, per species, be included in future phylogenetic studies pertaining to *Agathosma* taxonomy as this may give better support for the formation of distinct clades following phylogenetic analysis.

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# Chapter 5

# Detection and differentiation of '*Candidatus* Liberibacter africanus subspecies' from commercial citrus orchards in South Africa.

## 5.1 Abstract

‘*Candidatus* Liberibacter africanus subsp. clausenae’ (LafCl) was shown to be associated with citrus trees displaying mottling symptoms, characteristic of Citrus Greening disease (CG), in East Africa. Prior to this, it was accepted that ‘*Ca.* L. africanus’ (Laf) is the sole agent causing CG in the majority of African countries, and that the five known subspecies of Laf i.e. ‘*Ca.* L. africanus subsp. capensis’ (LafC), *‘Ca.* L. africanus subsp. tecleae’(LafT), *‘Ca.* L. africanus subsp. vepridis’ (LafV) and *‘Ca.* L. africanus subsp. zanthoxyli’ (LafZ) in addition to LafCl, were unlikely to play a role in the epidemiology of this disease. To determine whether any of these subspecies infect commercial citrus crops in South Africa, primer sets specific to the available outer-membrane protein (*omp*) sequences of each of these Laf-subspecies were designed. These primer sets were also assessed in various combinations in multiplex PCR reactions. Each primer set was shown to specifically amplify its intended target with no amplification of non-target sequences. All primer sets, except those of LafC, could be multiplexed in different combinations. Using these primer sets, 243 mainly archived DNA extracts, sampled from commercial citrus farms across South Africa were screened for the presence of Laf-subspecies. The results from this study indicated that Laf is the only known Liberibacter species associated with citrus in South Africa, suggesting that the transmission of the Laf-subspecies to citrus, if it happens, is not a common occurrence and may be reliant on various external factors.

## 5.2 Introduction

Citrus Greening disease (CG) in South Africa is associated with the heat sensitive (Schwarz and Green 1972), insect transmissible (McClean and Oberholzer 1965), fastidious bacterium ‘*Candidatus* Liberibacter africanus’ (Laf) (Garnier and Bové 1983, Jagoueix et al. 1994). Previous studies have shown Laf to be the sole bacterium associated with mottling symptoms typical of CG on commercial citrus in this country, and that *‘Ca.* L. asiaticus’ (Las) and ‘*Ca.* L. americanus’ (Jagoueix et al. 1994, Teixeira et al. 2005), both known to be associated with Huanglongbing (HLB) disease of citrus, are not present (Garnier and Bové 1996, Garnier et al. 2000a, Korsten et al. 1993, Korsten et al. 1996, Pietersen et al. 2010). Additionally, Phahladira et al. (2012) found that ‘*Ca.* L. africanus subsp. capensis’ (LafC), first described from indigenous *Calodendrum capense* (L. F.) Thunb (Cape Chestnut) (Garnier et al. 2000b), is unlikely to play a role in the epidemiology of this disease (Phahladira et al. 2012) despite being

widespread in South Africa and identified from *C. capense* specimens sampled in close proximity to citrus orchards.

Subsequent to the discovery of LafC, four subspecies of Laf have been described from indigenous Rutaceae hosts in South Africa, namely; '*Ca.* L. africanus subspecies clausenae' (LafCl), '*Ca.* L. africanus subsp. vepridis' (LafV), *'Ca.* L. africanus subsp. zanthoxyli' (LafZ) (Roberts et al. 2015) and '*Ca*. L. africanus subsp. tecleae' (LafT) (Roberts and Pietersen, 2017). LafCl, LafV and LafZ were characterised from the known native hosts of *Trioza erytreae* del Guercio (Hemiptera: Triozidae) i.e. *Clausena anisata, Vepris lanceolata* and *Zanthoxylum capense*, the triozid vector of Laf (McClean and Oberholzer 1965, Moll and Marin 1973).

The significance of these Laf-subspecies to citriculture was demonstrated when Roberts et al. (2017) established that citrus trees from Uganda and Tanzania, initially reported being infected with Las (Kaylebi et al. 2015, Shimwela et al. 2016), in fact contained a biovar (bv.) of LafCl (LafCl bv. citrus) and not Las. As with Laf, LafCl bv. citrus appears to be heat sensitive (Garnier and Bové 1983, Roberts et al. 2017) as diseased citrus were mainly detected at higher altitudes in the east African countries surveyed. While verifying the nature of the Liberibacter present in samples collected, two different research groups had contradicting results regarding which Liberibacter was present within single samples. To resolve the identity of the Liberibacter present, specific primers spanning portions within the outer-membrane protein gene (*omp*) of both Laf and LafCl were designed and it was shown that these citrus samples were co-infected with both Laf and LafCl bv citrus (Roberts et al. 2017).

The misdiagnosis of Las from Uganda and Tanzania was attributed to the non-targeted amplification of LafCl bv. citrus by the real-time PCR assay used for detection which utilises the primer and probes described by Li et al. (2006) (Roberts et al. 2017). These primers target the 16S rDNA region of Liberibacters and distinguish between Las and Laf based on the forward primer used (Li et al. 2006). It has however been demonstrated that this test does not sufficiently differentiate between Las and Laf, and even amplifies bacterial sequences outside of the Liberibacter genus (Shin and van Bruggen 2018). Roberts et al. (2017) additionally showed that the Li et al. (2006) assay non-specifically amplified Laf-subspecies, irrespective of the primer combination used. This is due to the high sequence homology observed between the various citrus infecting Liberibacters, which shares between 97-100% sequence identities (Roberts et al. 2015).

In South Africa, the real-time PCR assay of Li et al. (2006) is commonly used for the detection of Laf from citrus, the results of which are generally confirmed by end-point PCR using primers A2/J5 against ribosomal protein J sequences (*rplJ*) (Hocquellet et al. 1999), OA1/OI2c amplifying 16S rDNA sequences (Jagoueix et al. 1996) and OMP8inv/HPIinv directed against Liberibacter *omp* sequences (Bastianel et al. 2005). The correct identification of the Liberibacter present is however reliant on the sequencing of the amplification products obtained from end-point PCR using these primers as all of these primer sets amplify Laf and its subspecies (Roberts et al. 2015, Roberts et al. 2017). This makes the process of identifying Liberibacters from citrus samples both laborious and costly.

Within the current study, additional primer sets based on the *omp* sequences of Laf and its subspecies were designed for the specific detection of the Laf-subspecies described from South Africa. These primers were assessed for their ability to be multiplexed and used, in simplex, to determine whether these subspecies are present in archived DNA extracts obtained from South African citrus orchards.

## 5.3 Method and Materials

### *5.3.1 Primer design*

Primers against each of the known Laf-subspecies described from South Africa were designed based on available Laf-subspecies *omp* sequences using the primer blast online tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The amplification product of each primer set was designed to yield bands of different sizes, specific to each of the intended Liberibacter targets (Table 5.1). The specificity of the primers were assessed *in silico* by performing a BLAST analysis of the primers as well as by aligning the primers with available Liberibacter *omp* sequences obtained from Genbank using MAFFT online tool (Katoh et al. 2002).

Table 5.1: Primers sequences, expected product size and binding site within target *omp* sequence for detection of Laf and its subspecies.

| **Liberibacter Target and primer name** | **Primer Sequence (5'-3')** | **Primer binding site on reference sequence (bp)** | **PCR Product Size (bp)** | **Genbank reference of target gene** |
|---|---|---|---|---|
| Laf_F[a] | TCTCCGACGCGTATCAATCT | 1398-1417 | 250 | AY642158 |
| Laf_R | CGCGATGACACCTTAACTGC | 1629-1648 | | |
| LafC_F | TCACGGATCAAGTCCATCTG | 317-336 | 310 | KM035986 |
| LafC_R | TGCAAAAGAAGACTGCGAACG | 606-626 | | |

| | | | | |
|---|---|---|---|---|
| LafCl_F[a] | CGGTAGTCCTCACTCTTTCGTA | 131-152 | 199 | KJ189104 |
| LafCl_R | ATGAATCACCGAAACAGCGG | 310-320 | | |
| LafT_F | ACGCTATTGACGAGGGTGTT | 506-522 | 158 | KU561668 |
| LafT_R | ACGGACTCGTTCTCTACTGTAA | 639-660 | | |
| LafV_F | CCGCATTGAAATTCGCGGT | 353-371 | 401 | KJ197229 |
| LafV_R | TAAGCATCGTCGGCGAAACA | 734-753 | | |
| LafZ_F | GCGCAGAAGTTGTTAGAGCG | 233-252 | 543 | KJ197228 |
| LafZ_R | AACACCCTCGTCAATCGCAT | 756-775 | | |

[a]Primer originally published by Roberts et al. (2017).

Primers were assessed in both simplex and multiplex PCR reactions against a synthetic pooled extract of the known Laf-subspecies, extracts of each single Liberibacter subspecies described from South Africa, a healthy citrus control and a buffer control, to determine the specificity of the primers designed. DNA extractions which previously tested positive for each of the Liberibacters, i.e Laf, LafC, LafCl, LafT, LafV, and LafZ by PCR amplification and sequencing of ribosomal protein J (*rplJ*) (Hocquellet et al. 1999) were used for the synthetically pooled control included in end-point PCR reactions. These amplicons were confirmed by sequencing as described by Roberts et al. (2015) and were standardised to 250ng/µl, and pooled in a 1:1:1:1:1 ratio. Samples containing single Liberibacters included in the analysis were also standardised to 250ng/µl.

For simplex assessment of the specificity of the African Liberibacter specific primers, end-point PCR reactions consisted of 0.2mM dNTP (Bioline, London, UK), 0.2mM of each forward and reverse primer assessed, 2mM MgCl, 5µl Green GoTaq® Flexi Buffer (Promega Corp, Madison, WI, USA), 0.13µl GoTaq® G2 Flexi DNA Polymerase (Promega Corp, Madison, WI, USA), made up to a final volume of 25µl with nuclease-free water. Cycling was performed at initial denaturation of 95˚C for 5 min followed by 35 cycles of 95˚C for 20s, 65˚C for 20s and 72˚C for 30s, and final extension at 72˚C for 5min. The amplification products of each reaction were assessed following gel electrophoresis on a 2% agarose gel. A 100bp ladder (Promega Corp, Madison, WI, USA) was included in gel electrophoresis to ensure that products of the correct size were obtained for each of the primer sets assessed.

Multiplex PCR reactions were prepared as described above with primers being included in a 1:1 ratio, and the amount of nuclease-free water appropriately adjusted depending on whether the primers were multiplexed in duplicate and triplicate as listed in Table 5.2.

### *5.3.2 Primer validation*

To verify that the amplification products obtained for each primer set corresponded to its intended target, these products were subjected to Sanger sequencing. To achieve this, the amplification product of each primer set was enzymatically purified with FastAP (Thermo Fisher Scientific, Waltham, MA, USA) and Exonuclease I (Thermo Fisher Scientific, Waltham, MA, USA) following the protocol of Werle et al. (1994). These purified products were then subjected to a unidirectional sequencing PCR reaction using BigDye v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the forward primer for each primer set designed herein. Sanger sequencing was conducted at the Sequencing facility of the University of Pretoria. Resulting sequences were assessed for quality in BioEdit version 7.2.5 (Hall 1999) and were subsequently compiled into separate datasets for each Liberibacter assessed. Each dataset was aligned with available South African–derived Liberibacter *omp* sequences from Genbank using MAFFT online tool (Katoh et al. 2002). The aligned sequences were trimmed using BioEdit to ensure that cognate regions within the derived sequences were assessed. Using MEGA software version 6.06 (Tamura et al. 2013), the evolutionary, as well as maximum-likelihood phylogenies, were inferred on each of the aligned datasets.

Table 5.2: Primer combinations assessed for multiplex reactions in duplicate and triplicate. Primers were pooled in added to PCR reactions is a 1:1 ratio. Each primer combination was assessed against a pooled extract of Liberibacters, a single extract of each Liberibacter represented, a healthy control and a buffer control.

| | | | | |
|---|---|---|---|---|
| ***Duplicate primer combinations*** | | | | |
| Laf/LafC | Laf/LafCl | Laf/LafT | Laf/LafV | Laf/LafZ |
| LafC/LafCl | LafC/LafT | LafC/LafV | LafC/LafZ | LafCl/LaT |
| LafCl/LafT | LafCl/LafV | LafCl/LafZ | LafT/LafV | LafT/LafZ |
| LafV/LafZ | | | | |
| ***Triplicate primer combinations*** | | | | |
| Laf/LafC/LafCl | Laf/LafC/LafT | Laf/LafC/LafV | Laf/LafC/LafV | Laf/LafC/LafZ |
| Laf/LafCl/LafT | Laf/LafCl/LafV | Laf/LafCl/LafV | Laf/LafCl/LafZ | Laf/LafT/LafV |
| Laf/LafT/LafZ | Laf/LafV/LafZ | | | |

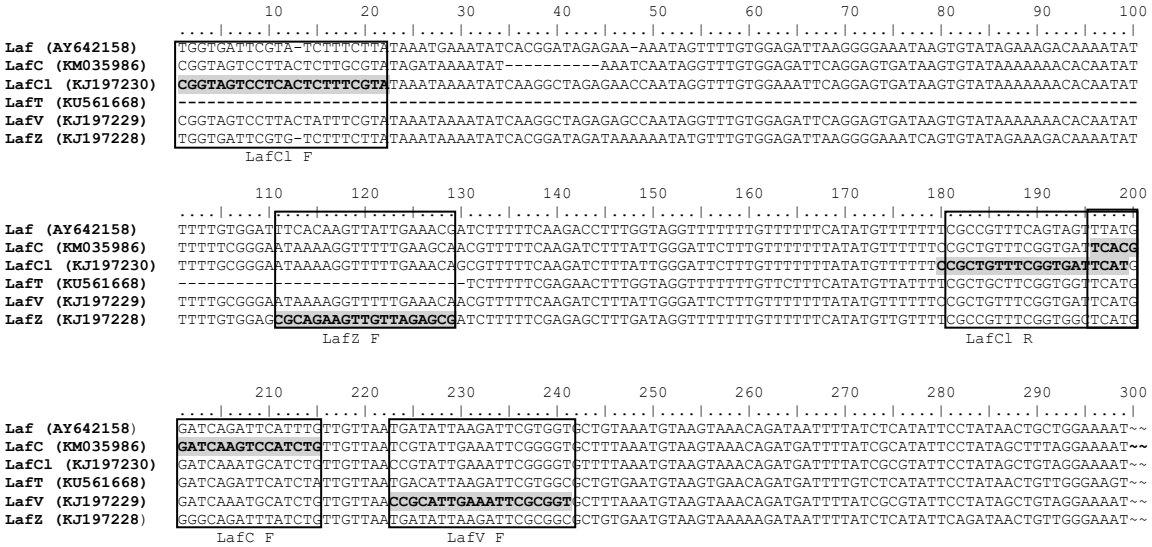
### *5.3.3 Detection of Laf-subspecies from citrus*

243 Total DNA extracts, obtained according to the CTAB protocol described by Doyle and Doyle 1990, from citrus samples collected over an 11 year period (2006-2017) were subjected

to PCR using the primers listed in Table 5.1 in simplex reactions. A positive, healthy and buffer control was included in each of these reactions which were constituted as previously described. The majority of the samples (209/243) were collected during 2006 when 57 greening-affected orchards from the major citrus production areas in South Africa were surveyed, the results of which were published by Pietersen et al. (2010). All of the samples included in the analysis were previously tested for the presence of Laf using the real-time PCR protocol of Li et al. (2006) and PCR using A2/J5 primer set of Hocquellet et al. (1999). Of these 209 samples, 169 again tested positive for the presence of Laf. In addition, 19 samples were obtained from Citrus Research International (CRI), all of which tested positive for Laf following the real-time PCR assay of Li et al. (2006). The remaining 15 samples originated from a survey conducted in the Eastern Cape in 2009, which were also tested with the real-time PCR assay of Li et al. (2006). Sample and distribution data are presented in Table 5.3. The full list of samples tested can be viewed in Appendix C.

## 5.4 Results

Following the design of the Laf-subspecies specific primer, BLAST analysis showed each primer to be specific to its intended Laf-subspecies target. Alignments further revealed that these primers aligned to the intended position within the sequences assessed and were unique to their intended targets. An exception to this, however, was the primer binding site of primer LafC_R which shared 100% nucleotide identity to *omp* sequences of LafV and LafCl. A suitable replacement for this primer could not be found (Fig. 5.1).



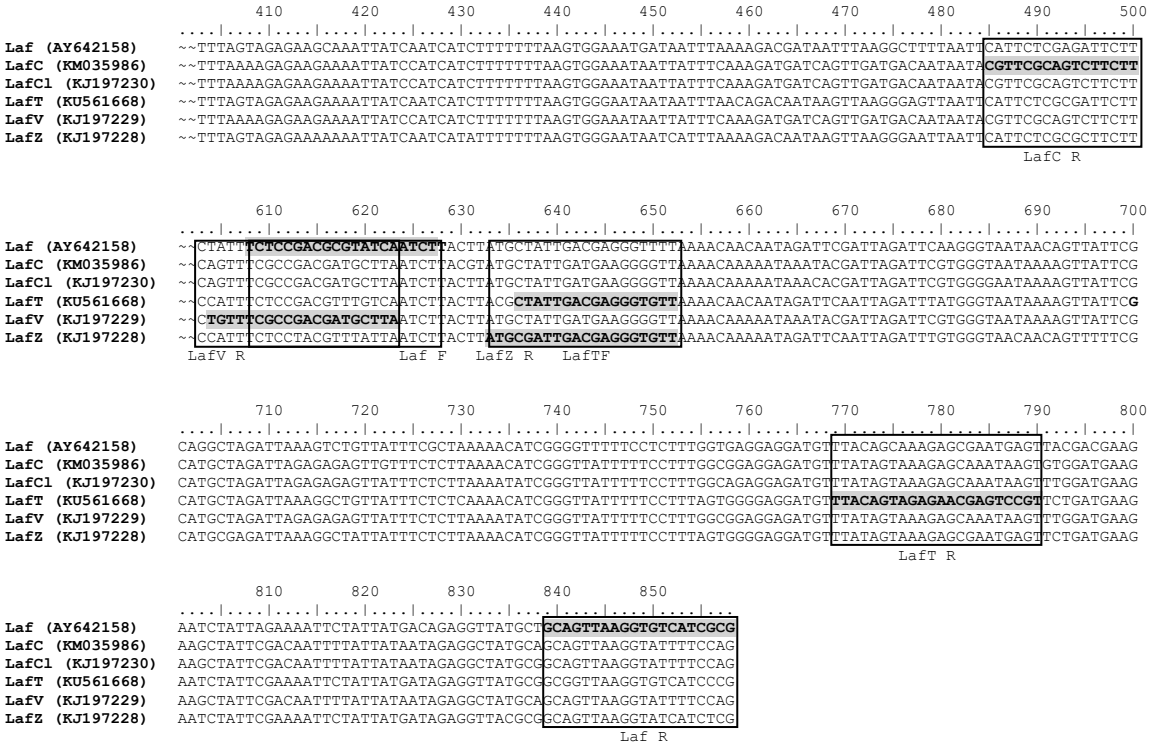



Fig 5.1: *In silico* alignment of the *omp* sequences of Laf and its subspecies. Primers designed for each of these Liberibacters are indicated on the figure.

When assessed against a mixture of Liberibacters, each primer set was able to correctly amplify their intended positive control target (i.e. Laf, LafC, LafCl, LafT, LafV and LafZ) in simplex primer reactions, yielding single bands of the expected sizes. No amplification was obtained in healthy controls, indicating that the primers do not amplify plant DNA. Buffer controls also remained free from amplification bands. The ability of these primers to differentiate between Laf-subspecies, including primer set LafC where the reverse primer binds with both LafCl and LafV *in silico*, is attributed to the stringent conditions used for PCR. Sequencing and phylogenetic analyses performed on the amplification products, further validated the specificity of the primers assessed as each sequence obtained by the amplification of the Liberibacter specific primer clustered with the intended Liberibacter target (Fig 5.2).

Primer combinations were tested in PCR against a mixed pool of Liberibacter templates as well as single Laf-subspecies. This was done to ensure that primers do not preferentially bind to a single template, masking other Laf-subspecies which may be present in a single sample. When assessed in duplicate, the following primer pair combinations yielded two electrophoresis bands of the expected sizes; Laf /LafT, Laf /LafV, Laf /LafZ, LafCl/LafZ, LafT/LafV, LafT/LafZ and LafV/LafZ. Assessed in triplicate, primer combinations Laf/LafT/LafZ and Laf/LafV/LafZ successfully yielded three electrophoresis bands of the correct sizes following

amplification (Fig 5.3). No electrophoresis band could be obtained for LafT and only a very faint electrophoresis band was obtained for Laf in a multiplex of Laf/LafV/LafT/LafZ primers. Regardless of the primer mixtures used in multiplex reactions, LafC could not be amplified from a mixed pool of Liberibacters. All the other Liberibacter targets yielded amplicons in this combination. When tested against an extract containing only LafC, the duplex primer mixtures yielded an electrophoresis band of the correct size for LafC.

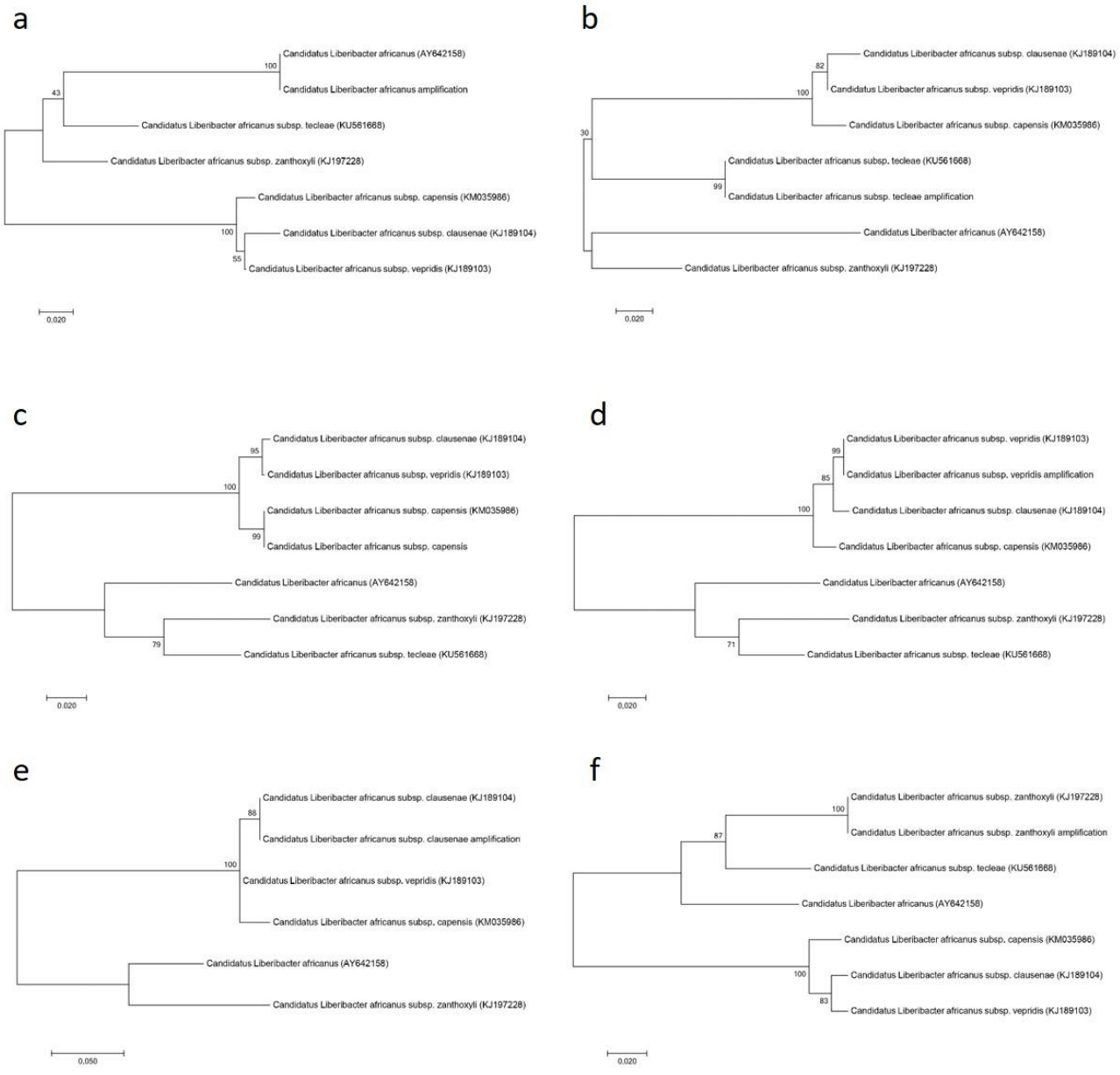



Fig 5.2: Maximum-likelihood phylogeny of African Liberibacter *omp* sequences based on sequences obtained from end-point PCR amplification with primer sets a) Laf, b) LafC, c) LafCl, d) LafT, e) LafV and f) LafZ, in simplex. Each sequence obtained is shown to group with sequences from its intended target thus showing the specificity of the primer sets designed. The maximum-likelihood phylogeny was inferred using the Tamura 3-parameter model (Tamura 1992) with 1000 bootstrap replicates. These values are presented at the nodes of

branches. The Genbank accessions of reference sequences included in alignments are indicated in brackets.

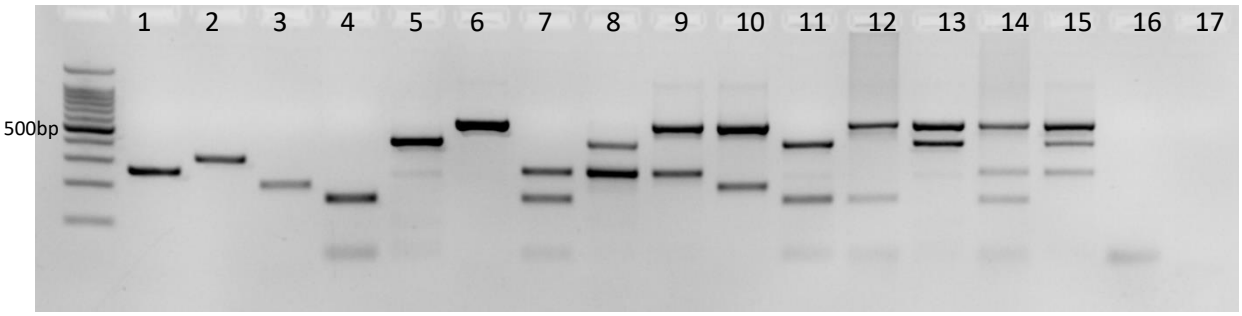



Fig 5.3: Amplification products obtained following end-Point PCR reactions in simplex for primers Laf, LafC, LafCl, LafT, LafV and LafZ (Lanes 1-6); duplex primer reactions for Laf/LafT, Laf/LafV, Laf/LafZ, LafCl/LafZ, LafT/LafV, LafT/LafZ and LafV/LafZ (Lanes 7-13), triplicate primers sets Laf/LafT/LafZ and Laf/LafV/LafZ (Lanes 14-15), healthy control and buffer control (Lanes 16 and 17). All multiplex reactions were subjected against a pool of Liberibacters included in this analysis thus demonstrating the specificity of the primers designed to detect their intended targets.

Using the primers described above, it was found that 192 of the samples tested positive for Laf while none of the extracts screened were positive for any of the Laf-subspecies (Table 5.3). Samples positive for Laf were obtained for all represented citrus types and provinces.

Table 5.3: Nature and locality of historic DNA extract tested within study. The number of samples testing positive for Laf, from this study, are indicated.

| | | **Liberibacter** ***positive samples/Number of samples tested per citrus type*** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Navel** | **Valencia** | **Lemon** | **Minneola** | **Clementine** | **Mandarin** |
| ***Provinces represented*** | **North-West** | 22/25 | 27/37 | 2/7 | 6/6 | 1/1 | 2/2 |
| | **Gauteng** | - | - | 1/1 | - | - | - |
| | **Mpumalanga** | - | 34/35 | 6/6 | - | - | 6/6 |
| | **Limpopo** | 10/14 | 26/28 | - | - | - | - |
| | **Western Cape** | 9/9 | - | 17/27 | - | 4/5 | 9/13 |
| | **Swaziland** | - | 5/5 | - | - | - | - |
| | **Eastern Cape** | 4/15 | - | 1/1 | - | - | - |
| | | **45/63** | **92/105** | **27/42** | **6/6** | **5/6** | **17/21** |

## 5.5 Discussion

Primers specific to the various Laf-subspecies described from South Africa were designed and successfully used to differentiate between these Liberibacters within this study. It was also found that these primers, with the exception of LafC, could be multiplexed. Using these primers, it was further demonstrated that Laf is the sole known Liberibacter species associated with CG in South Africa.

This supports previous findings by Pietersen et al. (2010) who had found only Laf in citrus in South Africa and had discounted the presence of Las and Lam in these samples. Amongst the Laf-subspecies, LafC appears not to play a role in the epidemiology of CG despite being found on *C. capense* specimens planted in close proximity to citrus orchards (Garnier et al. 2000b; Phahladira et al. 2012). However, LafCl has been found to infect citrus in eastern Africa (Roberts et al. 2017) and we had hypothesized that *C. anisata,* a known native host of *T. erytreae,* is potentially the original host of LafCl, with *T. erytreae* contributing to the host jump to citrus. It is also possible that LafV and LafZ could infect citrus as these Laf-subspecies share indigenous host plants of *T. erytreae* (Moran 1968; Roberts et al. 2015). While LafZ was routinely found in *Z. capense* (Roberts et al. 2015) a single *Z. capense* tree contained LafC, and not the expected LafZ. This illustrates that Laf-subspecies have the potential to be transmitted to other hosts. Furthermore, LafT shares a high sequence homology in the 16S rDNA and β-operon Laf which would suggest a recent separation between these Liberibacters (Roberts and Pietersen 2017), and therefore citrus samples were also tested for LafC and LafT in this study.

The lack of Laf-subspecies from South African citrus samples is in contrast to that of eastern Africa, where LafCl bv. citrus was found to be the dominant Liberibacter associated with CG symptoms (Roberts et al. 2017). This may be due to sampling in eastern Africa, which was focused on citrus trees growing in subsistence farming scenarios, while in the current study, commercial trees were sampled. Commercial farmers in South Africa generally follow stringent integrated control strategies to ensure that inoculum sources of CG are reduced within orchards and vector populations are suppressed, potentially preventing Laf-subspecies, other than Laf *senso stricto* from infecting citrus. The commercial citrus farms sampled in South Africa were generally proximal to natural indigenous vegetation containing Rutaceae hosts which are in contrast to eastern Africa where few Rutaceae hosts vegetation occurred in the

proximity of the citrus orchards sampled (T. Grout, pers. comm). This may have placed selective pressure on LafCl to adapt to a new host.

It is also possible that an unknown vector, which is present in eastern Africa, is absent in South Africa. It is of course also possible that citrus is unable to serve as a host to most of the subspecies of Laf. Studies in which the various Laf-subspecies are transmitted to citrus through both grafting and insect transmission approaches could resolve whether citrus is a host for these Liberibacters. Past studies have however indicated that both grafting and insect transmissions of citrus infecting Liberibacter spp. occur at relatively low rates (<20%) (Lopes and Frare 2008; Lopes et al. 2009). It will, therefore, be important that such experiments be conducted using large numbers of repetitions to obtain meaningful results.

PCR is the preferred diagnostic tool for detection and differentiation of Laf from other citrus infecting Liberibacters and the method of Laf-subspecies detection and differentiation used in this study will be very useful for further surveys of citrus at subsistence farmers or abandoned orchards in South Africa and in further surveys in Africa. The two gene regions traditionally used for primer design, the β-operon (*rplJ*) and 16S rDNA, respectively shared sequence homologies of >85% and >99% amongst Laf and its various subspecies (Garnier et al. 2000b; Roberts et al. 2015; Roberts and Pietersen, 2017). In comparison, the *omp* gene sequences from which the primers used in this study were derived, are less homologous (>70%) amongst the Liberibacters studied here (Roberts et al. 2015; Roberts and Pietersen, 2017). Primers derived from the *omp* gene sequences successfully differentiated between the various Laf-subspecies when assessed in simplex reactions within this study. Laf, LafCl, LafT, LafV and LafZ could successfully be multiplexed in duplex reactions and Laf, LafT, LafV and LafZ primers could be multiplexed in triplicate (Laf/LafT/LafZ; Laf/LafV/LafZ). The LafC primer set was, however, unable to successfully amplify LafC from a mixed pool of Liberibacter primers, possibly due to preferential binding of the LafC reverse primers to non-target sequences through PCR selection (Elnifro et al. 2000, Wagner et al. 1994) given that this primer is homologous to LafCl and LafV sequences. The reverse primer of LafC has a low GC content (47%) compared to all the other primers described (50-55%) with the exception of the LafT reverse primer (GC content of 45%). However, as the latter subspecies was successfully multiplexed, the failure of LafC primers to successfully amplify LafC within a mixture of Liberibacters is unlikely due to the low GC content of the primers themselves. The overall GC content of the amplified region of LafC is lower (29%) than all other Liberibacter targets (31-38%) assessed. Hence, the dissociation of the LafC target is probably more efficient than that

of the other Liberibacter targets present and it is expected that LafC would be preferentially amplified by its corresponding primer set. It is, therefore, possible that the lack of amplification of LafC in a mixed pool is a result of the secondary structure of the amplification target (Suzuki and Giovannoni, 1996).

In conclusion, the current study demonstrated that Laf *senso sitricto* appears to be the sole agent causing CG in South Africa and that the Laf-subspecies described from this country does not play a role in the epidemiology of this disease. The primers described herein can be used in subsequent studies on the Laf-subspecies from South Africa.

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# Chapter 6

# Population structure of '*Candidatus* Liberibacter africanus' in South Africa based on microsatellite markers.

## 6.1 Abstract

Citrus Greening disease (CG) in South Africa is associated with the fastidious bacterium '*Candidatus* Liberibacter africanus' (Laf). It has been observed that Laf isolates obtained from different geographic localities in SA differed in the rate of transmission during grafting experiments leading to the hypothesis that multiple populations of Laf exist in this country. To determine this, 167 Laf isolates obtained from Limpopo, North West, Mpumalanga and the Western Cape were subjected to microsatellite analyses, using four polymorphic markers. From UPGMA and STRUCTURE analysis, it was shown that two major genetic groups of Laf exist in the country which comprises of 25 distinct haplotypes. Four samples included within this study did not group with these two mayor groups, suggesting a potential third and fourth genetic group of Laf being present, which can only be validated by further sampling. Results further indicate that Laf populations in SA are formed by geographic locality. The high genetic diversity observed for Laf within this study supports the hypothesis that Laf originated on the African continent, warranting further analysis of Laf populations from Africa. This is also the first study on the genetic diversity of Laf.

## 6.2 Introduction

Citrus greening disease (CG), which is characterised by leaf mottling, the production of lopsided bitter fruit and early fruit drop, has afflicted citrus species of commercial value in South Africa since 1928 (Oberholzer et al. 1963). It was initially speculated that this disease was due to mineral deficiencies as the leaf symptoms observed were similar to that of a zinc shortage. It was however not until the 1960's that it was demonstrated that this disease was biological in nature and could be transmitted both mechanically and by the triozid vector, *Trioza erytreae* Del Guercio (Hemiptera: Tiozidae) (McClean and Oberholzer 1965a, McClean and Oberholzer 1965b). It has since been determined that CG in Africa is associated with the fastidious bacterium, *'Candidatus* Liberibacter africanus' (Laf) (Garnier and Bové 1983, Jaqoueix et al. 1994). A more invasive and serious emerging disease worldwide, Huanglongbing (HLB), causes foliar and fruit symptoms on citrus identical to CG, but is associated with the close relatives of Laf, '*Ca.* L. asiaticus' (Las) (Jaqoueix et al. 1994) and '*Ca.* L. americanus' (Lam) (Teixeira et al. 2005a), both of which are vectored by the liviid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Capoor and Singh 1967, Teixeira et al. 2005b).

Various levels of tolerance exist amongst citrus cultivars against Laf, with sweet orange, tangelo and mandarin being severely affected by CG, grapefruit, lemon and sour orange having moderate tolerance and lime, pomelo and trifoliate orange being tolerant against this disease (Manicom and van Vuuren 1990). These different levels of susceptibility of citrus to Laf were based on the severity of symptoms observed on different cultivars in South Africa (Manicom and van Vuuren 1990). The possibility, however, exists that the level of symptom severity being expressed may be attributed to the presence of different genetic populations of Laf (da Graça 1991). The only study on the genetic diversity of Laf, associated with CG in South Africa was conducted in the late 1960s when it was widely accepted that CG in this country was due to a viral infection (McClean and Oberholzer 1965a). Schwarz (1972) proposed that different strains of the 'greening virus' were present in the country. These assumptions were based on transmission studies in which CG affected material collected from different geographical localities were graft inoculated onto citrus seedlings and monitored over time. This study found that the rate of transmission, as well as the disease progression, differed depending on the geographical origin of the grafting material, suggesting the presence of different geographical strains of the organism associated with CG.

In contrast to Laf, the genetic diversity of Las populations, infecting citrus globally, have been extensively studied. Las populations have been determined using sequence-specific approaches including PCR-restriction fragment length polymorphisms (PCR-RFLP) profiles of *omp* sequences (Bastianel et al. 2005, Hu et al. 2011), identification of single nucleotide polymorphisms (SNP) between 16S rDNA sequences (Adkar-Purushothama et al. 2009; Tomimura et al. 2009; Gupta et al. 2011,Ghosh et al. 2013; Moreno-Enríquez et al. 2014) and ribosomal protein gene clusters (Adkar-Purushothama et al. 2009; Furuja et al. 2009) as well as the comparison of prophage sequences (Liu et al. 2011, Zheng et al. 2017).

The use of microsatellite markers (synonymous with Variable Number Tandem Repeats – VNTR and simple sequence repeats – SSR) to determine the genetic diversity of Las populations have proven popular due to the ability of these markers to resolve genetic populations across limited isolates (Chen et al. 2010, Katoh et al. 2011, de Paula et al. 2019). Using microsatellite markers, researchers were able to determine that Las originated in India from where it spread to the rest of Asia (Islam et al. 2012). Based on results obtained from microsatellite analyses it was also demonstrated that the occurrence of Las in the Americas were due to a single introduction of Las from Southeast Asia to Brazil, and two separate

introduction events of Las into Florida from China and either Southeast Asia or Brazil. (Chen et al. 2010, Islam et al. 2012, Matos et al. 2013).

The 16S rDNA sequence of Laf from South Africa is known to be homologous across different geographical isolates as well as sharing 99% sequence identity with Laf-subspecies described from this country (Roberts et al. 2015, Roberts and Pietersen 2017). This is also true for *omp* and other ribosomal protein sequences of Laf (Roberts et al. 2015). Upon obtaining the complete genome of Laf, it has been revealed that this Liberibacter contains two prophage sequences (Lin et al. 2015). It, however, remains uncertain whether these sequences are shared amongst different Laf populations in South Africa, making these unsuitable markers for population studies of Laf in this country. With the complete genome of Laf now being available, the current study aimed to determine the genetic composition of Laf populations from South Africa using microsatellite markers.

## 6.3 Method and Materials

### *6.3.1 Samples assessed*

A total of 144 DNA extracts obtained from a country-wide survey conducted in 2006 were used to perform the population study. These samples were from citrus trees showing CG symptoms and were collected from different geographical regions within South Africa as well as from different citrus cultivars. An additional 23 DNA extracts were received from Citrus Research International's (CRI) DNA collection and were included in fragment analysis. All 167 samples had previously tested positive for Laf. To ensure that the samples only contained Laf populations and that known Laf-subspecies were not present, primers described previously were utilised (Chapter 5).

### *6.3.2 Microsatellite primer design*

A genome-wide search for microsatellite sequences from the complete Laf genome (Genbank accession CP004021) was performed and primers flanking such regions were obtained using msatcommander software (Faircloth 2008). Initial screening of primer sets for polymorphisms was carried out using a subset of Laf-positive samples originating from different geographical localities as well as from different citrus cultivars. Reactions were set up using GoTaq® G2 Flexi DNA polymerase (Promega, USA) as follow; 5µl of 5X Green GoTaq® Flexi buffer, 2µl 25mM $MgCl_2$, 0,13 µl GoTaq® G2 Flexi DNA polymerase (5U/µl), 200nM per primer, 200nM dNTP mix, 0,5µl target DNA and made up to a final volume of 25µl with nuclease-free water.

PCR cycling was performed using the following parameters; initial denaturation of 5 min at 94˚ followed by 35 cycles of 94˚C for 30s, 58˚C for 30s and 72˚C for 40s. Final extension was performed for 10min at 72˚C. Amplification products were viewed under UV following gel electrophoresis on 3% agarose gel.

The amplification products for primer sets targeting polymorphic loci were subsequently purified enzymatically with exonuclease I (Thermo Fisher Scientific, USA) and FastAP (Werle et al. 1994). The purified products per polymorphic primer set were sequenced unidirectionally with their respective forward primer using Big Dye Terminator v3.1 cycling sequencing kit (ABI, USA) according to the manufacturer's instructions. Sanger sequencing was conducted on an ABI 3500xL automated sequencer at the University of Pretoria, South Africa. The sequences were then inspected with Chromas v2.6 to ensure the presence of the repeat motifs targeted.

### *6.3.3 Capillary electrophoresis*

The forward primer of each of the polymorphic primer sets identified were labelled with the fluorescent dyes FAM, NED and VIC respectively (Table 6.1). PCR amplification using these fluorescently labelled primers were performed in simplex on all 167 Laf-positive citrus samples using GoTaq® G2 Flexi DNA polymerase (Promega, USA) system, as previously described. Following visualization of amplification products with electrophoresis on a 2% agarose gel, the amplification products from each primer set were pooled in a 1:1:1:1 ratio per sample. The pooled samples were then further diluted in a 1:100 ratio with nuclease-free water. Prior to capillary electrophoresis, 1µl of each pooled-diluted sample was added to 10,7µl of Hi-Di Formamide and 0,3µl GeneScan Liz500® (Thermo Fisher Scientific, USA). The reaction cocktail was then heated to 95˚C for 3min and cooled on ice before being loaded onto a 3500 Genetic Analyzer (Applied Biosystems, USA) for fragment analysis. Analysis of the data obtained was conducted using Geneious® 11.1.5 software.

### *6.3.4 Genetic diversity analyses*

From the multilocus allelic data obtained following fragment analysis, different genotypes were identified and compiled into a clonally corrected dataset. This dataset was used to determine the number of alleles per locus (Na), number of effective alleles (Ne) and haploid genetic diversity (H) using GenAIEx 6.503 (Peakall and Smouse, 2006) for each loci.

### *6.3.5 Genetic structure analyses*

Using Nei's genetic distance, a UPGMA dendrogram, derived from a genetic tri-square matrix obtained in GenAIEx, was constructed using MEGA X (Kumar et al. 2018). This was performed to obtain a visual representation of the genetic relationship amongst Laf isolates included in this study. A principal coordinate analysis ($PC_oA$) analysis was performed in GenAIEx to further determine the genetic diversity and genetic distance of Laf populations from South Africa. To validate the results obtained from UPGMA and $PC_oA$ analysis, a Bayesian modelling analysis using STRUCTURE 2.3.1 (Pritchard et al. 2000) was performed in which the number of clusters (K) was determined by running 100 independent runs of K=1-10. A burn-in period of 25,000 and a run length of 50,000 was set for each run. The results from STRUCTURE were assessed in STRUCTURE harvester (http://taylor0.biology.ucla.edu/structureHarvester/)

## 6.4 Results

None of the 167 samples appeared to contain any of the known Laf-subspecies, only testing positive for Laf *senso stricto,* following end-point PCR using the *omp* primers designed in Chapter 5. In total, 50 primer sets for the amplification of microsatellite loci were designed and tested for polymorphisms (Appendix D). Of these, only five detected polymorphisms, of which four were able to amplify their intended target for all Laf-positive samples included in initial screening. The sequences of these polymorphic primer sets are listed in Table 6.1.

For the four loci assessed by fragment analysis, the number of alleles ranged from 2 to 8 and the haploid genetic diversity from 0.053 to 0.802 (Table 6.1). When the genetic diversity per province was determined, the highest genetic diversity was observed in the Western Cape (0.442), whereas the lowest diversity was observed in Limpopo (0.399) (Table 6.2).

Table 6.1: List of primer sequences used for fragment analysis with repeat motives per locus and allelic range of each locus tested.

| **Primer Name** | **Primer Sequence** | **Repeat Motif** | **Fluorescent Label** | **Allelic Range** | **Number of alleles (N)** | **Haploid genetic diversity (h)** |
|---|---|---|---|---|---|---|
| Laf_P1_F | CTTGGGATTTGGAGCTTCAGG | $(TA)_6$ | VIC | 205-243 | 8 | 0,496 |
| Laf_P1_R | GAGTGGTACGCACGTATACTATAC | | | | | |
| Laf_P6_F | GGGTGACTATAGCCCACAAG | $(TATGAG)_4$ | FAM | 160-202 | 8 | 0,802 |
| Laf_P6_R | CTGTTTGGTCTCCCGGTTTG | | | | | |
| Laf_P9_F | AACCAGAACACAATGATATAATACC | $(GAG)_5$ | FAM | 398-458 | 6 | 0,570 |
| Laf_P9_R | CCACCCACAGTATCTACAGG | | | | | |
| Laf_P14_F | GCCTCCGTTTGGAGTATTGG | $(GAATAA)_6$ | NED | 317-323 | 2 | 0,053 |
| Laf_P14_R | AGTCTGCCAGGTGATATTGAAG | | | | | |

Table 6.2: The number of alleles (Na), number of effective alleles (Ne) and haploid genetic diversity (H) of Laf isolates observed per province represented using a clonally corrected dataset.

| Province | Total number of isolates included in fragment analysis | Total samples included in clonal corrected data | Na | Ne | H |
|---|---|---|---|---|---|
| North West | 59 | 21 | 4.3 | 2.3 | 0.421 |
| Limpopo | 32 | 12 | 3.3 | 1.8 | 0.399 |
| Mpumalanga | 47 | 22 | 3.3 | 2.2 | 0.436 |
| Western Cape | 29 | 19 | 3.8 | 2.4 | 0.442 |
| | 167 | 74 | 6 | 2.6 | 0.480 |

UPGMA analyses revealed the presence of 29 distinct Laf haplotypes being present in South Africa (Fig 6.1). Sixteen of these observed haplotypes contain multiple Laf isolates found in different provinces and citrus types and 13 haplotypes consist of only a single Laf isolate. This analysis further demonstrated the presence of two major genetic Laf populations (denoted as Group I and Group II), with a collective of 163 Laf isolates included in this study being represented within these two groups. The four remaining isolates (4/167) grouped outside of these two clusters in a potential third and fourth genetic population. However, the data from this analysis is insufficient to support the existence of a third and fourth genetic population and further sampling will be required to justify the existence of these clusters.

STRUCTURE analysis indicated that the number of genetic groups are 2 (K=2) (Fig 6.2), further demonstrating the need for future sampling to include samples from areas not represented within this study such as KwaZulu-Natal, the Southern Cape (i.e Knysna) and Gauteng.

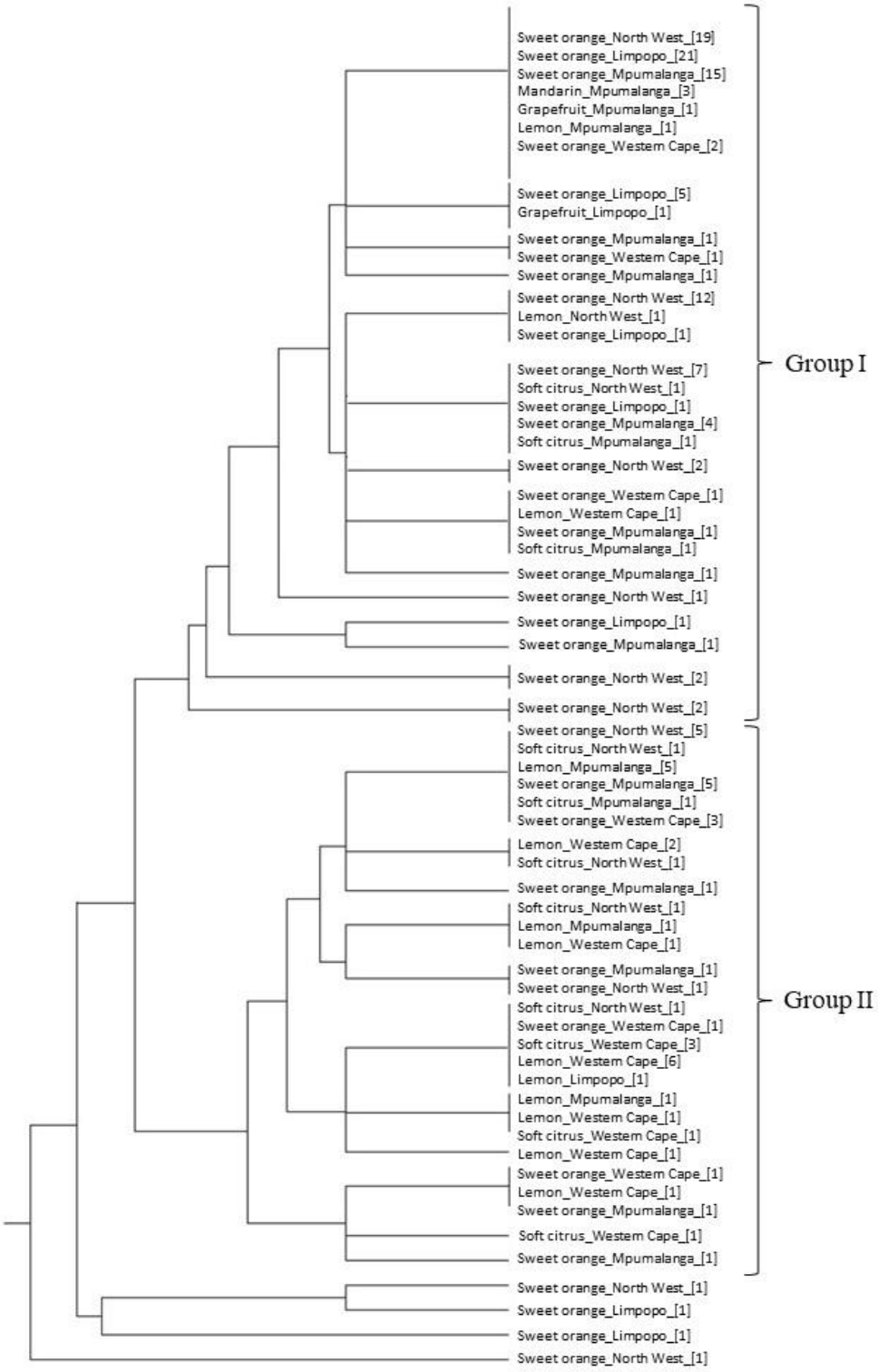


Fig 6.1: UPGMA dendrogram showing the genetic relationship of all 167 Laf isolates included in this study. The province, citrus type and number of Laf isolates per branch are indicated. The four remaining haplotypes which does not assign to Group 1 and Group 2 are indicated as unassigned.

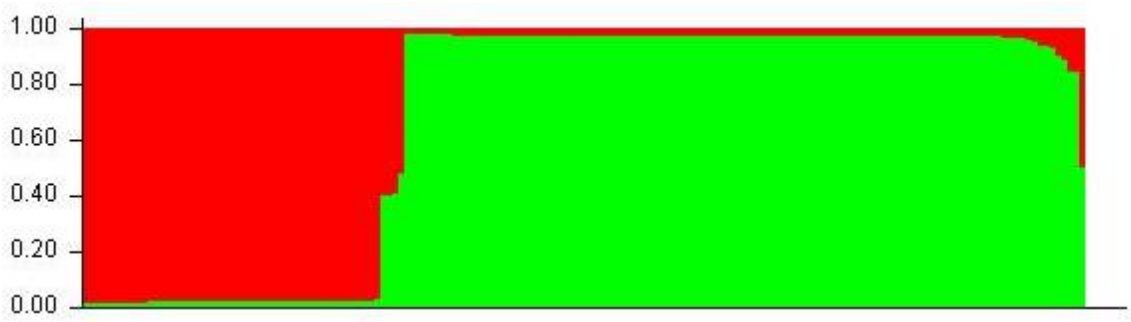


Fig 6.2: Genetic structuring of Laf populations across four provinces in South Africa as determined by STRUCTURE analysis, in which K=2. Red presents Laf isolates, mostly obtained from the Western Cape within genetic group II and green represents Laf isolates from group I, which mainly contains isolates from the Northern provinces.

Within the two major Laf genetic populations (i.e. Group I and Group II) Group I is primarily composed of Laf isolates found in the northern provinces, i.e. North West, Limpopo and Mpumalanga. Group II, however, contains most of the Laf isolates from the Western Cape (22/27) included in this study, isolates from Mpumalanga and North West and only a single isolate from Limpopo (Fig. 6.3). When assessed by citrus type, Laf isolated from Lemon mainly fell within Group II (20 of 23 lemon isolates included), and Laf isolated from sweet orange mainly clustered in Group I (100 of 120 isolated). Of the soft citrus Laf isolates, 10/16 isolates clustered with Group II haplotypes whereas all three Laf isolates from grapefruit clustered in Group I. These two citrus types are however underrepresented within the current study, and therefore further studies are needed to conclusively determine whether distinct Laf populations are formed based on these citrus types.

PCoA analysis supported the presence of the 29 distinct Laf haplotypes in South Africa. Isolates from Limpopo, North West and Mpumalanga (orange squares, blue diamonds and yellow crosses on Fig 6.4), were shown to be made up of a number of single haplotypes, mainly obtained from sweet orange, which are not shared by other provinces. Interestingly, a number of isolates from Mpumalanga and the Western Cape (Yellow crosses and grey triangles on Fig 6.4) grouped together, suggesting that these haplotypes are either shared amongst these two provinces or closely related.

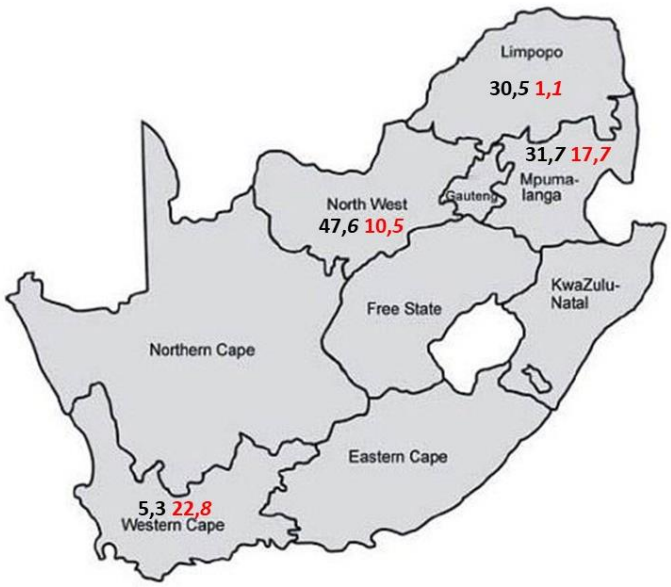


Fig 6.3: Distribution of Laf populations in South Africa. The number of samples contained in group I for each province is represented in black, whereas the number of samples for group II is represented in red. The number of haplotypes for each genetic group per province is indicated in italics.

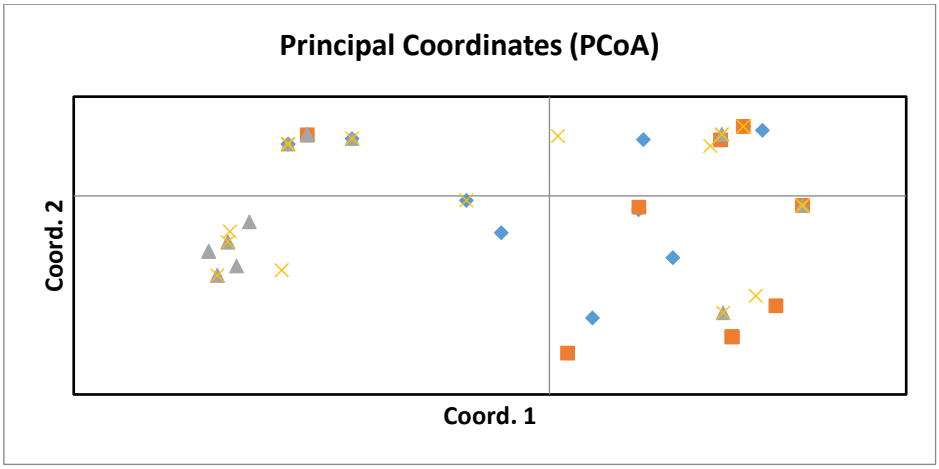


Fig 6.4: Principle coordinate analysis (PCoA) performed on clonal corrected dataset, representing the genetic distance and diversity of Laf populations in South Africa across different provinces and citrus types. Blue: North West isolates, Orange: Limpopo isolates, Yellow: Mpumalanga isolates, Grey: Western Cape isolates

## 6.5 Discussion

The haploid genetic diversity for Laf populations in South Africa was shown to be 0.480, which is comparatively high, considering that Las populations characterised from China and India have haploid genetic diversities of 0.342 and 0.360, respectively (Islam et al. 2012). Additionally, two distinct genetic groups containing a collective 25 haplotypes could be distinguished, with an additional 4 haplotypes being assigned to a potential third and fourth genetic group.

Microsatellite repeats are known to be influenced by environmental conditions (Zhou et al. 2014) and therefore it is not surprising that the two major genetic groups of Laf as described in this study were dominantly formed based on the geographical locality of the isolates studied. Group I is dominated by Laf isolates from the Northern provinces (i.e Limpopo, North West and Mpumalanga) whereas group II mainly contained Laf isolates from the Western Cape. Of these four provinces represented, the Western Cape is separated from the three aforementioned Northern provinces by both physical barriers and climatic conditions. The Western Cape comprises of a mountainous landscape, isolated from other citrus producing regions represented in this study, to the North by the arid landscape of the Northern Cape. Laf has not yet been identified to the east of the Western Cape, in the Eastern Cape, which potentially forms a bridge for the movement of Laf populations by its triozid vector to the eastern coastal production areas, therefore further isolating Laf in the Western Cape. Additionally, this province has a Mediterranean climate with a winter rainfall season, whereas Limpopo, Mpumalanga and the North West are all temperate climatic zones and receive rain during the summer months.

When considering the history of CG in SA, this disease was first observed in Rustenburg, North West during the late 1920's from where is spread to Mpumalanga, Limpopo and KwaZulu-Natal (Pretorius and van Vuuren 2006). It was not until 1994 that the first CG-like symptoms were observed on citrus in the Western Cape with another 4 years to confirm the presence of Laf from affected samples (Garnier et al. 2000). This timeline would suggest that Laf was introduced into the Western Cape from the Northern provinces. The PCoA analyses revealed that Laf populations from Mpumalanga and the Western Cape are closely related, indicating the likelihood that Laf was introduced into the Western Cape from Mpumalanga. Since the introduction of Laf into the Western Cape, numerous haplotypes of Laf in this province has been formed, aided by the environmental conditions as previously explained.

The almost 100 years of CG being known to infect commercial citrus in South Africa has contributed to the shaping of diverse genetic population across different provinces. This geographical separation of genetic Liberibacter populations has been observed for populations of Las (Chen et al. 2010, Islam et al. 2012, Katoh et al. 2011, Katoh et al. 2012) as well as '*Ca.* L. solanacearum' (Lso) (Lin et al. 2011), suggesting that the host has little influence on the genetic make-up of Liberibacter populations. Katoh et al. 2015, suggested that the number of alleles within polymorphic loci are influenced by psyllid transmission. The presence of Laf in South Africa for a century has allowed ample time for multiple passages through psyllid vectors and hosts allowing for genetically unique populations to form, explaining the high genetic diversity observed. The high genetic diversity of Laf populations in South Africa supports the hypothesis that Laf originated on the African continent. It would, however, be valuable to conduct a comparative population study on Laf isolates across Africa, using the microsatellite markers described herein. Such a study will give valuable insights into the origin and evolutionary processes that help shape Laf populations.

With this said, the current study was biased towards Laf isolates from sweet orange, with 124 of the 167 isolates included in this study being obtained from sweet oranges. This is not surprising as the production of sweet orange in the four provinces represented (30,764 ha), far exceeds that of soft citrus (8,915 ha), grapefruit (6,054 ha) and lemons (5,693 ha) (CGA Annual report, 2018). The majority of Laf isolates from sweet orange fell within genetic Group I, and was mainly represented by isolates from the Northern provinces (i.e. Limpopo, North West and Mpumalanga). As for Laf isolated from lemon, these were mainly confined to Genetic group II, with 20 of the 23 lemon isolates included in the current study falling in this group. However, 13 of the 23 Laf isolates from lemons included in this study originated from the Western Cape, further indicating that Laf isolates are predominantly shaped by geographic locality rather than citrus type.

In addition to the two major genetic groups of Laf as revealed in this study, four Laf isolates fell outside of these groupings, suggesting the existence of a potential third and fourth genetic group of Laf being present in the country. Of the 8 commercial citrus production provinces in the country, only four were represented. This may contribute to the low support from this study for the existence of other genetic Laf populations. Additionally, only four polymorphic markers were assessed in this study, which is comparatively few considering that population studies of Las typically comprises of 7 to 8 polymorphic primers (Islam et al. 2012; de Paula et al. 2019). By including Laf isolates from other geographical localities, additional polymorphic loci could

potentially be obtained from the primers described herein (Appendix D). Using these additional markers and including Laf isolates from other provinces not represented in the current study, greater support for the existence of a third and fourth genetic group could potentially be obtained.

The microsatellite markers described herein can be utilised for future studies in the population structure of Laf isolates from across Africa. This is the first study on the genetic variability of Laf in South Africa, and the results obtained herein is the first validation of an African origin of Laf.

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# Chapter 7

# Concluding Remarks

## 7.1 General conclusions

The research presented within this study gave further insights into the association, and existence of Laf-subspecies from indigenous rutaceous hosts in South Africa, with a fifth subspecies being described, this time from *Teclea gerrardii.* It was however anticipated that more subspecies would have been identified as a large number of *Agathosma* species were collected (826 samples across 19 morphotypes). These samples were collected from different geographical regions which should, theoretically, increase the chance of identifying Liberibacters, as different environments may be more suited to supporting Liberibacters. From the population study conducted within Chapter 6, it was demonstrated that Laf populations are formed based on geographic locality, rather than citrus type, further supporting the idea that it is more likely to identify Liberibacters within a larger area sampled. It is also known that the climatic conditions of the Western Cape support multiplication of Laf, as GC occurs in the Western Cape. Therefore, it is plausible that the lack of evidence of *Agathosma* spp containing Liberibacter sequences is attributed by factors such as the inability of *Agathosma* spp to serve as host for Liberibacters, or even as a suitable host for potential psyllid vectors of Liberibacters. This study further demonstrated that the detection of Liberibacters from novel hosts must be approached holistically, as a suitable host, vector and climate must all be present for Liberibacters to thrive.

The existence of diverse subspecies of Laf in South Africa does pose a unique challenge to South African citriculture. With the current South-eastern movement of *Diaphorina* citri along the eastern shores of Africa, it is anticipated that this vector will soon arrive in South Africa. Any of the indigenous hosts which harbours Laf-subspecies may act as alternative hosts supporting the development and feeding of *D. citri.* Should *D. citri* be able to acquire and transmit these subspecies, it remains to be seen whether these subspecies will be able to infect commercial citrus species. The study has shown that these Laf-subspecies are as yet absent from commercial citrus species in South Africa. *D. citri* does not have the same temperature restrictions as *Trioza erytreae* and therefore, it is possible that the association of Laf-subspecies with a potential new vector could place sufficient pressure on these subspecies to adapt to new hosts. *D. citri* may survive a wider climatic range than the speculated vectors of these subspecies or have a different plant host range. The ability of *D. citri* to transmit Laf to citrus also possess a unique threat to South Africa. This study has now shown that different populations of Laf exist in South Africa which are shaped by geographic locality. Thus, the potential exists that some of these Laf populations may be able to infect wider geographical

ranges, assisted by spread through *D. citri.* Such an expansion in the range of CG in South Africa can lead to new epidemics of this disease.

Finally, when considering the research conducted within this dissertation as a whole, more informed hypotheses can be made as to how Laf evolved in South Africa. The existence of suitable woody Rutaceae hosts in South Africa supports the idea that Laf evolved from an indigenous host present in Africa prior to the introduction of commercial citrus species. The high sequence similarities for the core genes studied between Laf and its subspecies confirms that Laf is more related to these subspecies than Las, suggesting a more recent divergence of Laf from its subspecies compared to Las. Laf was the sole agent identified from commercial citrus in South Africa. As previously mentioned, '*Ca.* L. africanus subsp. clausenae' bv citrus was found to be the dominant Liberibacter present in eastern Africa. This is in contrast to what is being observed in South Africa, even in the presence of orchards planted next to indigenous vegetation containing Laf-subspecies, Laf was still the only Liberibacter found in commercial citrus.

Laf has not enjoyed the same attention as Las as the disease associated with Laf, i.e. CG, is easily controlled through integrated management practices and is also a disease limited to the African continent and Mascarene Islands. However, from the work presented herein, it is clear that we have limited knowledge of the genus, Liberibacter, in Africa and conducting research on this genus may potentially lead to the discovery of novel ways to control HLB disease globally.

# Appendix A

Table A1: List of samples collected in Oribi Gorge and Umtamvunu Nature reserve for detection of Liberibacters from Rutaceous hosts within the *Teclea*, and *Oricia* genera.

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Evidence of psyllid infestation | Ct Value |
|---|---|---|---|---|---|---|---|
| 13-2102 | 2013/11/04 | *Teclea spp* | UKZN | Durban Botanical Gardens | Blotchy mottle | Depression Marks | |
| 13-2107 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge | - | - | |
| 13-2108 | 2013/11/05 | *Teclea spp* | UKZN | Oribi Gorge | Blotchy mottle | - | - |
| 13-2109 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge | Blotchy mottle | - | - |
| 13-2110 | 2013/11/05 | *Teclea spp* | UKZN | Oribi Gorge | Blotchy mottle | - | - |
| 13-2111 | 2013/11/05 | *Teclea spp* | UKZN | Oribi Gorge | Blotchy mottle | - | - |
| 13-2112 | 2013/11/05 | *Teclea spp* | UKZN | Oribi Gorge | - | - | - |
| 13-2113 | 2013/11/05 | *Unknown spp* | UKZN | Oribi Gorge | - | - | - |
| 13-2114 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge | - | - | - |
| 13-2115 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge | Blotchy mottle | - | - |
| 13-2116 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge, Samango Falls Trail | - | Depression Marks | - |
| 13-2117 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge, Samango Falls Trail | - | - | - |
| 13-2118 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge, Samango Falls Trail | - | - | - |
| 13-2119 | 2013/11/05 | *Unknown spp* | UKZN | Oribi Gorge, Samango Falls Trail | - | - | - |
| 13-2120 | 2013/11/05 | *Teclea natalensis* | UKZN | Oribi Gorge | Blotchy mottle | - | - |
| 13-2121 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge | - | - | - |
| 13-2122 | 2013/11/05 | *Teclea spp* | UKZN | Oribi Grge | - | - | - |
| 13-2123 | 2013/11/05 | *Teclea natalensis* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2124 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2125 | 2013/11/05 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2126 | 2013/11/05 | *Teclea natalensis* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2127 | 2013/11/05 | *Teclea natalensis* | UKZN | Oribi Gorge, Hoopoe Falls | - | Depression Marks | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Evidence of psyllid infestation | Ct Value |
|---|---|---|---|---|---|---|---|
| 13-2128 | 2013/11/05 | *Teclea natalensis* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2129 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | Depression Marks | - |
| 13-2130 | 2013/11/05 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | Depression Marks | - |
| 13-2131 | 2013/11/06 | *Teclea spp* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2132 | 2013/11/06 | *Teclea spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | Depression Marks | - |
| 13-2133 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | Depression Marks | - |
| 13-2134 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2135 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2136 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | Depression Marks | - |
| 13-2137 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2138 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2139 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2140 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | Depression Marks | - |
| 13-2141 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2142 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | Blotchy mottle | Depression Marks | - |
| 13-2143 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2144 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | Depression Marks | - |
| 13-2145 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2146 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2147 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2148 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2149 | 2013/11/06 | *Oricia bachmanii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2150 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2151 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Evidence of psyllid infestation | Ct Value |
|---|---|---|---|---|---|---|---|
| 13-2152 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2153 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2154 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2155 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2156 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2157 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Loerie Trial | - | - | - |
| 13-2158 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2159 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2160 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2161 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | Depression Marks | - |
| 13-2162 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2163 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2164 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2165 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2166 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2167 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2168 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2169 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2170 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2171 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2172 | 2013/11/06 | *Oricia bachmannii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2173 | 2013/11/06 | *Teclea gerrardii* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2174 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | Depression Marks | - |
| 13-2175 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Evidence of psyllid infestation | Ct Value |
|---|---|---|---|---|---|---|---|
| 13-2176 | 2013/11/06 | *Unknown spp* | UKZN | Port Edward, Umtamvunu, Fish Eagle | - | - | - |
| 13-2180 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2181 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2182 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2183 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2184 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2185 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | Depression Marks | - |
| 13-2186 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | Blotchy mottle | - | - |
| 13-2187 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | Depression Marks | - |
| 13-2188 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2189 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | 25.11 |
| 13-2191 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2192 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2193 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2194 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2195 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2196 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2197 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2198 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2199 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2200 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2201 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2202 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2203 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Evidence of psyllid infestation | Ct Value |
|---|---|---|---|---|---|---|---|
| 13-2204 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2205 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2206 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2207 | 2013/11/07 | *Teclea natalensis* | UKZN | Oribi Gorge, Hoopoe Falls | Blotchy mottle | - | - |
| 13-2208 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2209 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2210 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2211 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2212 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2213 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2214 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2215 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2216 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2217 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2218 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2219 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2220 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2221 | 2013/11/07 | *Teclea gerrardii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2222 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2223 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2224 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2225 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2226 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2227 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Evidence of psyllid infestation | Ct Value |
|---|---|---|---|---|---|---|---|
| 13-2228 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2229 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2230 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2231 | 2013/11/07 | *Unknown spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2232 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2233 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Hoopoe Falls | - | - | - |
| 13-2234 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Nkonka | - | Depression Marks | - |
| 13-2235 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Nkonka | - | - | - |
| 13-2236 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Nkonka | - | - | - |
| 13-2237 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | Depression Marks | - |
| 13-2238 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2239 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2240 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2241 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2242 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2243 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2244 | 2013/11/07 | *Oricia bachmannii* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2245 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Main road | - | - | - |
| 13-2246 | 2013/11/07 | *Teclea spp* | UKZN | Oribi Gorge, Main road | - | - | - |

# Appendix B

Table B2: *Agathosma* samples collected for the detection of Liberibacters. The location, symptoms and Ct values of each sample tested is presented and samples used for vouchering purposes are indicated.

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8000 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 34.28 |
| 16-8001 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 38.04 |
| 16-8002 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8003 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8004 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8005 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 33.82 |
| 16-8006 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 29.82 |
| 16-8007 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8008 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8009 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8010 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8011 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8012 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8013 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8014 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8015 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8016 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8017 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8018 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8019 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8020 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8021 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8022 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8023 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8024 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8025 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 22.49 |
| 16-8026 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 30.63 |
| 16-8027 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8028 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8029 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8030 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8031 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8032 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8033 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8034 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8035 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8036 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8037 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8038 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8039 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8040 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | 30.53 |
| 16-8041 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8042 | 2016/01/26 | *A. betulina* | Western Cape | Skimmelberg, Clanwilliam | - | | - |
| 16-8043 | 17/2/2016 | *A. ovata* | Southern Natal | Port Edward | - | | - |
| 16-8044 | 17/2/2016 | *A. ovata* | Southern Natal | Port Edward | - | | - |
| 16-8045 | 17/2/2016 | *A. ovata* | Southern Natal | Port Edward | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8046 | 17/2/2016 | *A. ovata* | Southern Natal | Port Edward | - | | - |
| 16-8047 | 17/2/2016 | *A. ovata* | Southern Natal | Red Decent | - | | - |
| 16-8048 | 17/2/2016 | *A. ovata* | Southern Natal | Red Decent | - | | - |
| 16-8049 | 17/2/2016 | *A. ovata* | Southern Natal | Red Decent | - | | - |
| 16-8050 | 17/2/2016 | *A. ovata* | Southern Natal | Red Decent | - | | - |
| 16-8051 | 17/2/2016 | *A. ovata* | Southern Natal | Opp Banners Rest | - | | - |
| 16-8052 | 17/2/2016 | *A. ovata* | Southern Natal | Opp Banners Rest | - | | - |
| 16-8053 | 17/2/2016 | *A. ovata* | Southern Natal | Opp Banners Rest | - | | - |
| 16-8054 | 17/2/2016 | *A. ovata* | Southern Natal | Opp Banners Rest | - | | - |
| 16-8055 | 2016/11/03 | *A. capensis* | Western Cape | Kirstenbosch | - | | - |
| 16-8056 | 2016/11/03 | A. nova | Western Cape | Kirstenbosch | - | | - |
| 16-8057 | 2016/11/03 | A. gonaquensis | Western Cape | Kirstenbosch | - | | 36.56 |
| 16-8058 | 2016/11/03 | A. spp | Western Cape | Kirstenbosch | - | | - |
| 16-8059 | 2016/11/03 | A. apiculata | Western Cape | Kirstenbosch | - | | - |
| 16-8060 | 2016/11/03 | A. apiculata | Western Cape | Kirstenbosch | - | | - |
| 16-8061 | 2016/11/03 | A. mucronulata | Western Cape | Kirstenbosch | - | | - |
| 16-8062 | 2016/11/03 | A. mucronulata | Western Cape | Kirstenbosch | - | | - |
| 16-8063 | 2016/11/03 | A. mucronulata | Western Cape | Kirstenbosch | - | | - |
| 16-8064 | 2016/11/03 | A. crenulata | Western Cape | Kirstenbosch | - | | - |
| 16-8065 | 2016/11/03 | A. crenulata | Western Cape | Kirstenbosch | - | | - |
| 16-8066 | 2016/11/03 | A. crenulata | Western Cape | Kirstenbosch | - | | - |
| 16-8067 | 2016/11/03 | A. crenulata | Western Cape | Kirstenbosch | - | | - |
| 16-8068 | 2016/11/03 | A. crenulata | Western Cape | Kirstenbosch | - | | - |
| 16-8069 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8070 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8071 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8072 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | 37.25 |
| 16-8073 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8074 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8075 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8076 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8077 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8078 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8079 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8080 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8081 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8082 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8083 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8084 | 2016/11/03 | A. ovata | Western Cape | Kirstenbosch | - | | - |
| 16-8085 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8086 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8087 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8088 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8089 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8090 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8091 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8092 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8093 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | 35.89 |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8094 | 2016/11/03 | *A. ovata* | Western Cape | Kirstenbosch | - | | - |
| 16-8095 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | - | | - |
| 16-8096 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Yellowing | | - |
| 16-8097 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Yellowing | | - |
| 16-8098 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | - | Victor & Roberts 1 | - |
| 16-8099 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Single dead branch | | - |
| 16-8100 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Partial die-off | | - |
| 16-8101 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Some Yellowing | | - |
| 16-8102 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | - | | - |
| 16-8103 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Yellowing | | - |
| 16-8104 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Yellowing | | - |
| 16-8105 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | - | | - |
| 16-8106 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Yellowing | | - |
| 16-8107 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Yellowing | | - |
| 16-8108 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | Stunted | | - |
| 16-8109 | 14/4/2016 | *A. apiculata* | Western Cape | Sedgefield | - | | - |
| 16-8110 | 14/4/2016 | A. apiculata | Western Cape | Lake Pleasant | Yellowing | | - |
| 16-8111 | 14/04/2016 | A. apiculata | Western Cape | Lake Pleasant | Yellowing | | - |
| 16-8112 | 14/04/2016 | A. apiculata | Western Cape | Lake Pleasant | - | | - |
| 16-8113 | 14/04/2016 | A. apiculata | Western Cape | Lake Pleasant | - | | - |
| 16-8114 | 14/04/2016 | A. apiculata | Western Cape | Lake Pleasant | - | | - |
| 16-8115 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8116 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8117 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | Yellow, Stunted | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8118 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8119 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | Victor & Roberts 2 | - |
| 16-8120 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8121 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8122 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8123 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8124 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8125 | 14/04/2016 | *A. muirii* | Western Cape | Groenvlei Trail | - | | 37.6 |
| 16-8126 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8127 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | 27.52 |
| 16-8128 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8129 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8130 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8131 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | Dying | | - |
| 16-8132 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8133 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8134 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | - | | - |
| 16-8135 | 14/04/2016 | A. apiculata | Western Cape | Groenvlei Trail | Dying | | - |
| 16-8136 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8137 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | Sickly | | - |
| 16-8138 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | 38.94 |
| 16-8139 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8140 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8141 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8142 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8143 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8144 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8145 | 14/04/2016 | A. muirii | Western Cape | Groenvlei Trail | - | | - |
| 16-8146 | 14/04/2016 | A. capensis | Western Cape | Brenton Butterfly Reserve | - | | 37.48 |
| 16-8147 | 14/04/2016 | A. capensis | Western Cape | Brenton Butterfly Reserve | - | | |
| 16-8148 | 14/04/2016 | A. ovata | Western Cape | Brenton Butterfly Reserve | - | | 36.95 |
| 16-8149 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8150 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8151 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | 36.15 |
| 16-8152 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8153 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8154 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Mottling | | - |
| 16-8155 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Mottling | | - |
| 16-8156 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8157 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | - | | - |
| 16-8158 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8159 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8160 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8161 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8162 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8163 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8164 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | 29.59 |
| 16-8165 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8166 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8167 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8168 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | - | | 36.21 |
| 16-8169 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8170 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | - | | - |
| 16-8171 | 15/04/2016 | *A. apiculata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8172 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | Partial yellowing | | - |
| 16-8173 | 15/04/2016 | *A. ovata* | Western Cape | West of Herolds Bay | - | | - |
| 16-8174 | 15/04/2016 | *A. apiculata* | Western Cape | Hoekwil | Partial yellowing | | 35.92 |
| 16-8175 | 15/04/2016 | *A. apiculata* | Western Cape | Hoekwil | Partial yellowing | | 35.02 |
| 16-8176 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | - |
| 16-8177 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | 35.85 |
| 16-8178 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | Very Sickly, leaf loss | | - |
| 16-8179 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | 26.58 |
| 16-8180 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | - |
| 16-8181 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | - |
| 16-8182 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | - |
| 16-8183 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | - |
| 16-8184 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | - |
| 16-8185 | 15/04/2016 | *A. ovata* | Western Cape | Hoekwil | - | | 25.04 |
| 16-8186 | 15/04/2016 | *A. apiculata* | Western Cape | Wilderness | - | | - |
| 16-8187 | 15/04/2016 | *A. apiculata* | Western Cape | Wilderness | - | | - |
| 16-8188 | 15/04/2016 | *A. capensis* | Western Cape | Wilderness | - | Victor & Roberts 3 | - |
| 16-8189 | 15/04/2016 | *A. capensis* | Western Cape | Wilderness | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8190 | 15/04/2016 | *A. capensis* | Western Cape | Wilderness | - | | - |
| 16-8191 | 15/04/2016 | *A. capensis* | Western Cape | Wilderness | - | | - |
| 16-8192 | 15/04/2016 | *A. apiculata* | Western Cape | Wilderness | - | | - |
| 16-8193 | 15/04/2016 | *A. ovata* | Western Cape | Kranshoek | Yellowing | | - |
| 16-8194 | 15/04/2016 | *A. ovata* | Western Cape | Kranshoek | Yellowing | | - |
| 16-8195 | 15/04/2016 | *A. ovata* | Western Cape | Kranshoek | Yellowing | | - |
| 16-8197 | 15/04/2016 | *A. ovata* | Western Cape | Kranshoek | Yellowing | | - |
| 16-8198 | 15/04/2016 | *A. ovata* | Western Cape | Kranshoek | Yellowing | | - |
| 16-8199 | 05/05/2016 | *A. ovata* | Western Cape | Porcupine Hills | - | | - |
| 16-8200 | 05/05/2016 | *A. ovata* | Western Cape | PorcupineHills | - | | - |
| 16-8201 | 05/05/2016 | *A. ovata* | Western Cape | Porcupine Hills | - | | - |
| 16-8202 | 05/05/2016 | *A. ovata* | Western Cape | PorcupineHills | - | | - |
| 16-8203 | 05/05/2016 | *A. ovata* | Western Cape | PorcupineHills | - | | - |
| 16-8204 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | Victor & Roberts 4 | 30.65 |
| 16-8205 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 30.68 |
| 16-8206 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8207 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8208 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8209 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | 34.73 |
| 16-8210 | 03/10/2016 | A. imbricata | Western Cape | West Coast National Park | - | | 27.78 |
| 16-8211 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8212 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | 1 |
| 16-8213 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 33.78 |
| 16-8214 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Browning | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8215 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Browning | | - |
| 16-8216 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | - |
| 16-8217 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8218 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8219 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8220 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8221 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8222 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8223 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Sickley | | - |
| 16-8224 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | - |
| 16-8225 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8226 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | - |
| 16-8227 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | - |
| 16-8228 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | - |
| 16-8229 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8230 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8231 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8232 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8233 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8234 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8235 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8236 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Some die-off | | - |
| 16-8237 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8238 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 35.03 |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8239 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Dying | | - |
| 16-8240 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8241 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 30.91 |
| 16-8242 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8243 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8244 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Reduced vigour | | - |
| 16-8245 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8246 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8247 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | - |
| 16-8248 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8249 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Yellowing | | 32.29 |
| 16-8250 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8251 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 30.76 |
| 16-8252 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 30.65 |
| 16-8253 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | Victor & Roberts 5 | 30.90 |
| 16-8254 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | Victor & Roberts 6 | - |
| 16-8255 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | 35.65 |
| 16-8256 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8257 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8258 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8259 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8260 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8261 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8262 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | 31.46 |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8263 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8264 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8265 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8266 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8267 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8268 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8269 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8270 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8271 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8272 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8273 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8274 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8275 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8276 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8277 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8278 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8279 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8280 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8281 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8282 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8283 | 03/10/2016 | *A. bisulca* | Western Cape | West Coast National Park | - | | - |
| 16-8284 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8285 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8286 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8287 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 33.11 |
| 16-8288 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 37.75 |
| 16-8289 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8290 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8291 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8292 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8293 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8294 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 28.52 |
| 16-8295 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8296 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8297 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 27.61 |
| 16-8298 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8299 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | 30.02 |
| 16-8300 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8301 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8302 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | - | | - |
| 16-8303 | 03/10/2016 | *A. imbricata* | Western Cape | West Coast National Park | Sunburnt flowers | | 29.53 |
| 16-8306 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | 35.85 |
| 16-8307 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | Victor & Roberts 7 | - |
| 16-8308 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8309 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8310 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8311 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8312 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8313 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 31.29 |
| 16-8314 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8315 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8316 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8318 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8319 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 34.99 |
| 16-8320 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8321 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 34.68 |
| 16-8322 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 31.60 |
| 16-8323 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8324 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8325 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 31.04 |
| 16-8326 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8327 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8328 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | Victor & Roberts 8 | - |
| 16-8329 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8330 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 34.90 |
| 16-8331 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8332 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 36.95 |
| 16-8333 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8334 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8335 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8336 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8337 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | 36.08 |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8338 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8339 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8340 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8341 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8342 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8343 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8344 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8345 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8346 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8347 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8348 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8349 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8350 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | Yellowing | | - |
| 16-8351 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8352 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8353 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8354 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8355 | 04/10/2016 | *A. lanceolata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8356 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8357 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8358 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | Victor & Roberts 9 | - |
| 16-8359 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8360 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8361 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8362 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8363 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | 38.81 |
| 16-8364 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | Yellowing | | - |
| 16-8365 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8366 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8367 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8368 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8369 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8370 | 04/10/2016 | *A. imbricata* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8371 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8372 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8373 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8374 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8375 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8376 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8377 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | 28.95 |
| 16-8378 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8379 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8380 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8381 | 04/10/2016 | *A. ciliaris* | Western Cape | Cape Point Nature reserve | - | | - |
| 16-8382 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8383 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | 33.96 |
| 16-8384 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8385 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8386 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8387 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | Victor & Roberts 10 | - |
| 16-8388 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8389 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8390 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8391 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8392 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8393 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8394 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8395 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8396 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Yellowing | | 36.72 |
| 16-8397 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Yellowing | | - |
| 16-8398 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8399 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8400 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8401 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8402 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8403 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8404 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Reduced vigour | | - |
| 16-8405 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8406 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8407 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8408 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8409 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8410 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8411 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8412 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8413 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | 33.68 |
| 16-8414 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8415 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8416 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8417 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8418 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Reduced vigour | | - |
| 16-8419 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8420 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8421 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8422 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8423 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | 37.49 |
| 16-8424 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | Mottling | | - |
| 16-8425 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | Victor & Roberts 11 | - |
| 16-8426 | 05/10/2017 | *A. crenulata* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8427 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8428 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8429 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8430 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8431 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8432 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8433 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8434 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8435 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8436 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | 39.99 |
| 16-8437 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8438 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8439 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8440 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8441 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | 32.19 |
| 16-8442 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8443 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | 33.91 |
| 16-8444 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8445 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | 33.13 |
| 16-8446 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | | - |
| 16-8447 | 05/10/2016 | *A. ciliaris* | Western Cape | Lourensford Wine Estate | - | Victor & Roberts 12 | - |
| 16-8448 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8449 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8450 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8451 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8452 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8453 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8454 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8455 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8456 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8457 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8458 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8459 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8460 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | Victor & Roberts 13 | - |
| 16-8461 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8462 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | 35.45 |
| 16-8463 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8464 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8465 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8466 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8467 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8468 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8469 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8470 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8471 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8472 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8473 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8474 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8475 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | 33.50 |
| 16-8476 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8477 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8478 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8479 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8480 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8481 | 05/10/2016 | *A. ciliaris* | Western Cape | Vergelegen wine estate | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8482 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8483 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8484 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8485 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8486 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8487 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8488 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8489 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8490 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8491 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8492 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8493 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8494 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | 35.77 |
| 16-8495 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8496 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8497 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | 32.10 |
| 16-8498 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8499 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | 30.87 |
| 16-8500 | 05/10/2016 | *A. imbricata* | Western Cape | Vergelegen wine estate | - | | - |
| 16-8501 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | Victor & Roberts 14 | - |
| 16-8502 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8503 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8504 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8505 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8506 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | 32.47 |
| 16-8507 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | 32.34 |
| 16-8508 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | 31.69 |
| 16-8509 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | - |
| 16-8510 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | - |
| 16-8511 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | - |
| 16-8512 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | 33.42 |
| 16-8513 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | - |
| 16-8514 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus | - | | - |
| 16-8515 | 06/10/2016 | *A. crenulata* | Western Cape | Hermanus | - | | - |
| 16-8516 | 06/10/2016 | *A. crenulata* | Western Cape | Hermanus | - | | - |
| 16-8517 | 06/10/2016 | *A. ovata* | Western Cape | Hermanus | - | | - |
| 16-8518 | 06/10/2016 | *A. ovata* | Western Cape | Hermanus | - | | - |
| 16-8519 | 06/10/2016 | *A. ovata* | Western Cape | Hermanus | - | | - |
| 16-8520 | 06/10/2016 | *A.martiana* | Western Cape | Hermanus | - | Victor & Roberts 15 | 33.77 |
| 16-8521 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8522 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8523 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8524 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8525 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8526 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | Infected with dodder | Victor & Roberts 16 | - |
| 16-8527 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8528 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8529 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8530 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8531 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8532 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8533 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8534 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8535 | 06/10/2016 | *A. virgata* | Western Cape | Hermanus | - | | - |
| 16-8536 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Vogel | - | | - |
| 16-8537 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Vogel | - | | - |
| 16-8538 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Vogel | - | | - |
| 16-8539 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Vogel | - | | - |
| 16-8540 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Vogel | - | | - |
| 16-8541 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Rotary Way | - | Victor & Roberts 17 | - |
| 16-8542 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Rotary Way | - | | - |
| 16-8543 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Rotary Way | - | | - |
| 16-8544 | 06/10/2016 | *A. imbricata* | Western Cape | Hermanus/Rotary Way | - | | - |
| 16-8545 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8546 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8547 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8548 | 06/10/2016 | *A. serpyllacea* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8549 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8550 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8551 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8552 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8553 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8554 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8555 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8556 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8557 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8558 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | 36.31 |
| 16-8559 | 06/10/2016 | *A. serpyllacea* | Western Cape | Harold Porter Botanical garden | - | Victor & Roberts 18 | - |
| 16-8560 | 06/10/2016 | *A. serpyllacea* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8561 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8562 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8563 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8564 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8565 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8566 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8567 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | 33.19 |
| 16-8568 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8569 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | 33.65 |
| 16-8570 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | 37.23 |
| 16-8571 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8572 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | Victor & Roberts 19 | - |
| 16-8573 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8574 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | Victor & Roberts 20 | - |
| 16-8575 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8576 | 06/10/2014 | *A. serpyllacea* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8577 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8578 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8579 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8580 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8581 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8582 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8583 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8584 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8585 | 06/10/2016 | *A. crenulata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8586 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8587 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | Victor & Roberts 21 | - |
| 16-8588 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8589 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8590 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8591 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8592 | 06/10/2016 | *A. lanceolata* | Western Cape | Harold Porter Botanical garden | - | | 31.62 |
| 16-8593 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8594 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8595 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8596 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8597 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8598 | 06/10/2016 | *A. ovata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8599 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8600 | 06/10/2016 | *A. apiculata* | Western Cape | Harold Porter Botanical garden | - | | - |
| 16-8601 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8602 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8603 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8604 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8605 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8606 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8607 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8608 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8609 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8610 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8611 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8612 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8613 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8614 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8615 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8616 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | Victor & Roberts 22 | - |
| 16-8617 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8618 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8619 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8620 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8621 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8622 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8623 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8624 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8625 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8626 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8627 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8628 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8629 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8630 | 07/10/2016 | *A. bifida* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8631 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8632 | 07/10/2016 | *A. cilliaris* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8633 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8634 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8635 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8636 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8637 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8638 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8639 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8640 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8641 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8642 | 07/10/2016 | *A. crenulata* | Western Cape | Franschoek Mont Rochelle | - | | - |
| 16-8643 | 07/10/2016 | Unknown | Western Cape | Jan Joubertsgat brug | - | | - |
| 16-8644 | 07/10/2016 | Unknown | Western Cape | Jan Joubertsgat brug | - | | - |
| 16-8645 | 07/10/2016 | Unknown | Western Cape | Jan Joubertsgat brug | - | | - |
| 16-8646 | 07/10/2016 | Unknown | Western Cape | Jan Joubertsgat brug | - | | - |
| 16-8467 | 07/10/2016 | Unknown | Western Cape | Jan Joubertsgat brug | - | | - |
| 16-8648 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8649 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8650 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8651 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8652 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8653 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8654 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8655 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8656 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8657 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8658 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8659 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8660 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8661 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8662 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8663 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8664 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8665 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8666 | 07/10/2016 | Unknown | Western Cape | Paarlberg | - | | - |
| 16-8667 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | Victor & Roberts 23 | - |
| 16-8668 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | 38.89 |
| 16-8669 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8670 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8671 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8672 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8673 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8674 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8675 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8676 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8677 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8678 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8679 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8680 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8681 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8682 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8683 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8684 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8685 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8686 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8687 | 07/10/2016 | *A. hispida* | Western Cape | Paarlberg | - | | - |
| 16-8688 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8689 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8690 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8691 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8692 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8693 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8694 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8695 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8696 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8697 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8698 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8699 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | | - |
| 16-8700 | 07/10/2016 | *A. virgata* | Western Cape | Bainskloof | - | Victor & Roberts 24 | - |
| 16-8701 | 6/12/2016 | *A. muirii* | Western Cape | Rondevlei Nature Reserve | - | | - |
| 16-8702 | 6/12/2016 | *A. apiculata* | Western Cape | Swartvlei | - | | 35.98 |
| 16-8703 | 6/12/2016 | *A. apiculata* | Western Cape | Swartvlei | - | | - |
| 16-8704 | 6/12/2016 | *A. apiculata* | Western Cape | Swartvlei | - | | - |
| 16-8705 | 6/12/2016 | *A. apiculata* | Western Cape | Swartvlei | - | | - |
| 16-8706 | 6/12/2016 | *A. cf acutissima* | Western Cape | Rondevlei Nature Reserve | - | | - |
| 16-8707 | 6/12/2016 | *A. cf serpyllacea* | Western Cape | Rondevlei Nature Reserve | - | | - |
| 16-8709 | 7/12/2016 | *A. ovata* | Western Cape | Ballots Bay | - | | - |
| 16-8710 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8711 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8712 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8713 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8714 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8715 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8716 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8717 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8718 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8719 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8720 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8721 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8722 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8723 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8724 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8725 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8726 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8727 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8728 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8729 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8730 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8731 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8732 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8733 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8734 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8735 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8736 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8737 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8738 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8739 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8740 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8741 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8742 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8743 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8744 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8745 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8746 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8747 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8748 | 7/12/2016 | *A. capensis* | Western Cape | Ballots Bay | - | | - |
| 16-8749 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8750 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8751 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8752 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8753 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8754 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8755 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8756 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8757 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8758 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8759 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8760 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8761 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8762 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8763 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8764 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8765 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8766 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8767 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8768 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8769 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8770 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 16-8771 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8772 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8773 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8774 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8775 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8776 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8777 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8778 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8779 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8780 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8781 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8782 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8783 | 9/12/2016 | *A. apiculata* | Western Cape | Gericke Point, Swartvlei beach | - | | - |
| 16-8784 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8785 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8786 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8187 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8188 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8189 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8190 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 16-8191 | 16/06/2017 | *A. virgata* | Western Cape | Clanwilliam | - | | - |
| 18-8792 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8793 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8794 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 18-8795 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8796 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8797 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8798 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8799 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8800 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | Victor & Roberts 25 | - |
| 18-8801 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8802 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8803 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8804 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8805 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8806 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8807 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8808 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | - | | - |
| 18-8809 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8810 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8811 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | 37.43 |
| 18-8812 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8813 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8814 | 11/1/2018 | *A. acutissima?* | Western Cape | Wilderness | | | - |
| 18-8815 | 11/1/2018 | *A. acutissima?* | Western Cape | Wilderness | | | 28.23 |
| 18-8816 | 11/1/2018 | *A. acutissima?* | Western Cape | Wilderness | | | - |
| 18-8817 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8818 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |

| Accession | Date Collected | Species Collected | Province | Location | Symptoms | Voucher | Ct values |
|---|---|---|---|---|---|---|---|
| 18-8819 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8820 | 11/1/2018 | *A. sp* | Western Cape | Wilderness | | | - |
| 18-8821 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | Victor & Roberts 26 | - |
| 18-8822 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8823 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8824 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8825 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8826 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8827 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8828 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8829 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8830 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8831 | 11/1/2018 | *A. acutissima* | Western Cape | Wilderness | | | - |
| 18-8832 | 12/1/2018 | *A. sp* | Western Cape | Rondevlei, Sedgefield | | Victor & Roberts 27 | 35.33 |
| 18-8833 | 12/1/2018 | *A. sp* | Western Cape | Rondevlei, Sedgefield | | Victor & Roberts 28 | 35.66 |
| 18-8834 | 12/1/2018 | *A. capensis* | Western Cape | Rondevlei, Sedgefield | | Victor & Roberts 29 | - |
| 18-8835 | 12/1/2018 | *A. muirii* | Western Cape | Rondevlei, Sedgefield | | Victor & Roberts 30 | - |

# Appendix C

Table C1: Samples tested for the presence of Laf-subspecies obtained from commercial citrus orchards across South Africa.

| Accession | Date Sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0137 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | | | | Greening |
| 06-0138 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | | | | Greening |
| 06-0139 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.324 | 27 04.548 | 1173 | Greening |
| 06-0140 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.316 | 27 04.542 | 1193 | Greening |
| 06-0141 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.309 | 27 04.531 | 1181 | Greening |
| 06-0142 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.306 | 27 04.530 | 1186 | Greening |
| 06-0144 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.303 | 27 04.528 | 1189 | Greening |
| 06-0145 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.317 | 27 04.519 | 1189 | Greening |
| 06-0146 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.294 | 27 04.521 | 1186 | Greening |
| 06-0147 | 10/23/06 | Palmer Navel | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.290 | 27 04.539 | 1187 | Greening |
| 06-0148 | 10/23/06 | Midnight | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.333 | 27 04.544 | 1184 | Greening |
| 06-0149 | 10/23/06 | Robyn | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.409 | 27 04.549 | 1190 | Greening |
| 06-0150 | 10/23/06 | Robyn | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.415 | 27 04.592 | 1190 | Greening |
| 06-0151 | 10/23/06 | Robyn | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.424 | 27 04.578 | 1191 | Greening |
| 06-0152 | 10/23/06 | Robyn | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.432 | 27 04.567 | 1195 | Greening |
| 06-0153 | 10/23/06 | Robyn | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.434 | 27 04.504 | 1190 | Greening |
| 06-0154 | 10/23/06 | Robyn | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.483 | 27 04.603 | 1189 | Blight? |
| 06-0155 | 10/23/06 | Orange | Unknown | Hunters Rest Hotel | 25 46.599 | 27 15.537 | 1222 | Greening |
| 06-0156 | 10/23/06 | Orange | Unknown | Hunters Rest Hotel | 25 46.600 | 27 15.535 | 1227 | Greening |
| 06-0157 | 10/23/06 | Orange | Unknown | Hunters Rest Hotel | 25 46.599 | 27 15.527 | 1231 | Greening |
| 06-0158 | 10/23/06 | Orange | Unknown | Hunters Rest Hotel | 25 46.592 | 27 15.571 | 1228 | Greening |
| 06-0159 | 10/23/06 | Orange | Unknown | Hunters Rest Hotel | 25 46.589 | 27 15.513 | 1230 | Greening |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0160 | 10/23/06 | Eureka Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.616 | 27 18.774 | 1180 | Greening |
| 06-0161 | 10/23/06 | Eureka Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.618 | 27 18.771 | 1183 | Greening |
| 06-0162 | 10/23/06 | Eureka Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.615 | 27 18.762 | 1183 | Greening |
| 06-0163 | 10/23/06 | Eureka Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.616 | 27 18.754 | 1184 | Greening |
| 06-0164 | 10/23/06 | Eureka Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.608 | 27 18.734 | 1181 | Greening |
| 06-0165 | 10/23/06 | Eureka Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.600 | 27 18.738 | 1184 | Greening |
| 06-0166 | 10/23/06 | Fino Lemon | Kroondal-Rustenberg | Wohlfshule | 25 44.603 | 27 18.718 | 1190 | Greening |
| 06-0167 | 10/23/06 | Delta Valencia | Buffelspoort-Rustenburg | | 25 49.118 | 27 24.541 | 1418 | Greening |
| 06-0168 | 10/23/06 | Delta Valencia | Buffelspoort-Rustenburg | | 25 49.117 | 27 24.524 | 1414 | Greening |
| 06-0169 | 10/23/06 | Delta Valencia | Buffelspoort-Rustenburg | | 25 49.099 | 27 24.570 | 1417 | Greening |
| 06-0170 | 10/23/06 | Delta Valencia | Buffelspoort-Rustenburg | | 25 49.105 | 27 24.582 | 1411 | Greening |
| 06-0171 | 10/23/06 | Delta Valencia | Buffelspoort-Rustenburg | | 25 49.118 | 27 24.591 | 1417 | Yellow vein |
| 06-0173 | 10/23/06 | Minneola | Buffelspoort-Rustenburg | | 25 49.175 | 27 24.633 | 1392 | Greening |
| 06-0174 | 10/23/06 | Minneola | Buffelspoort-Rustenburg | | 25 49.175 | 27 24.630 | 1392 | Greening |
| 06-0175 | 10/23/06 | Minneola | Buffelspoort-Rustenburg | | 25 49.177 | 27 24.626 | 1393 | Greening |
| 06-0176 | 10/23/06 | Minneola | Buffelspoort-Rustenburg | | 25 49.180 | 27 24.624 | 1397 | Greening |
| 06-0177 | 10/23/06 | Minneola | Buffelspoort-Rustenburg | | 25 49.181 | 27 24.622 | 1395 | Greening |
| 06-0178 | 10/23/06 | Valencia (late) | Buffelspoort-Rustenburg | | 25 49.133 | 27 24.558 | 1402 | Greening |
| 06-0179 | 10/23/06 | Valencia (late) | Marikana-Rustenburg | Barnardsvlei | 25 48.131 | 27 28.552 | 1304 | Greening |
| 06-0181 | 10/23/06 | Valencia (late) | Marikana-Rustenburg | Barnardsvlei | 25 48.143 | 27 28.549 | 1304 | Greening |
| 06-0182 | 10/23/06 | Valencia (late) | Marikana-Rustenburg | Barnardsvlei | 25 48.142 | 27 28.529 | 1304 | Greening |
| 06-0183 | 10/23/06 | Valencia (late) | Marikana-Rustenburg | Barnardsvlei | 25 48.161 | 27 28.520 | 1306 | Greening |
| 06-0184 | 10/23/06 | Valencia (late) | Marikana-Rustenburg | Barnardsvlei | 25 48.189 | 27 28.514 | 1304 | Greening |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0185 | 10/23/06 | Clemantine | Marikana-Rustenburg | Barnardsvlei | 25 48.161 | 27 28.512 | 1306 | Greening |
| 06-0186 | 10/23/06 | Valencia (late) | Marikana-Rustenburg | Barnardsvlei | 25 48.141 | 27 28.522 | 1305 | Greening |
| 06-0187 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.064 | 27 36.899 | 1283 | Greening |
| 06-0188 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.060 | 27 36.897 | 1280 | Greening |
| 06-0189 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.058 | 27 36.896 | 1280 | Greening |
| 06-0190 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.057 | 27 36.892 | 1279 | Greening |
| 06-0191 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46. | 27 36.891 | 1278 | Greening |
| 06-0192 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.051 | 27 36.894 | 1279 | Greening |
| 06-0193 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.050 | 27 36.893 | 1279 | Greening |
| 06-0194 | 10/23/06 | Palmer Navel | Mooinooi-Rustenburg | Buffelsfontein | 25 46.080 | 27 36.864 | 1282 | Greening |
| 06-0195 | 10/23/06 | Valencia (late) | Mooinooi-Rustenburg | Buffelsfontein | 25 46.081 | 27 36.870 | 1282 | Greening |
| 06-0196 | 10/23/06 | Valencia (late) | Mooinooi-Rustenburg | Buffelsfontein | 25 46.073 | 27 36.883 | 1283 | Greening |
| 06-0197 | 10/23/06 | Valencia (late) | Mooinooi-Rustenburg | Buffelsfontein | 25 46.263 | 27 36.440 | 1280 | Greening |
| 06-0198 | 10/23/06 | Valencia (late) | Mooinooi-Rustenburg | Buffelsfontein | 25 46.246 | 27 36.435 | 1278 | Greening |
| 06-0199 | 10/23/06 | Valencia (late) | Mooinooi-Rustenburg | Buffelsfontein | 25 46.242 | 27 36.426 | 1279 | Greening |
| 06-0200 | 10/23/06 | Valencia (late) | Mooinooi-Rustenburg | Buffelsfontein | 25 46.248 | 27 36.427 | 1279 | Greening |
| 06-0201 | 10/23/06 | Nova | Wolhuterskop-Brits | Wolhuterskop | 25 43.547 | 27 42.350 | 1244 | Greening |
| 06-0202 | 10/23/06 | Nova | Wolhuterskop-Brits | Wolhuterskop | 25 43.543 | 27 42.348 | 1242 | Greening |
| 06-0203 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.325 | 27 38.370 | 1263 | Greening |
| 06-0204 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.310 | 27 38.370 | 1266 | Greening |
| 06-0205 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.290 | 27 38.384 | 1265 | Greening |
| 06-0206 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.276 | 27 38.390 | 1273 | Greening |
| 06-0207 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.284 | 27 38.377 | 1264 | Greening |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0208 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.262 | 27 38.369 | 1273 | Greening |
| 06-0209 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.258 | 27 38.362 | 1264 | Greening |
| 06-0211 | 10/24/06 | Valencia | Wolhuterskop-Brits | Groenkloof | 25 45.248 | 27 38.373 | 1271 | Greening |
| 06-0212 | 10/24/06 | Valencia | Mooinooi-Rustenburg | Buffelshoek | 25 45.292 | 27 35.294 | 1237 | Greening |
| 06-0213 | 10/24/06 | Valencia | Mooinooi-Rustenburg | Buffelshoek | 25 45.291 | 27 35.294 | 1231 | Greening |
| 06-0214 | 10/24/06 | Valencia | Mooinooi-Rustenburg | Buffelshoek | 25 45.293 | 27 35.297 | 1228 | Greening |
| 06-0215 | 10/24/06 | Valencia | Mooinooi-Rustenburg | Buffelshoek | 25 45.323 | 27 35.302 | 1227 | Greening |
| 06-0216 | 10/24/06 | Valencia | Wolhuterskop-Brits | Bokfontein | 25 43.170 | 27 43.153 | 1226 | Greening |
| 06-0217 | 10/24/06 | Valencia | Wolhuterskop-Brits | Bokfontein | 25 43.162 | 27 43.113 | 1231 | Greening |
| 06-0218 | 10/24/06 | Valencia | Wolhuterskop-Brits | Bokfontein | 25 43.160 | 27 43.116 | 1232 | Greening |
| 06-0221 | 10/25/06 | Robyn | Groblersdal | Moosrivier J14 | 25 00.745 | 29 21.066 | 874 | No mottling |
| 06-0222 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier J1 | 25 01.065 | 29 21.849 | 865 | Greening |
| 06-0223 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier H14 | 25 00.864 | 29 22.856 | 863 | Greening |
| 06-0225 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier H14 | 25 00.884 | 29 22.854 | 873 | Greening |
| 06-0226 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier H14 | 25 00.897 | 29 22.877 | 873 | Greening |
| 06-0228 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier H14 | 25 00.844 | 29 22.848 | 872 | Greening |
| 06-0229 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier H14 | 25 00.841 | 29 22.844 | 874 | Greening |
| 06-0230 | 10/25/06 | Washington Navel | Groblersdal | Moosrivier H10 | 25 01.121 | 29 22.785 | 871 | Greening |
| 06-0232 | 10/25/06 | Palmer Navel | Zebedelia | Zebedelia 3B | 24 19.429 | 29 14.992 | 1112 | Greening |
| 06-0233 | 10/25/06 | Palmer Navel | Zebedelia | Zebedelia 3B | 24 19.424 | 29 14.996 | 1114 | Greening |
| 06-0234 | 10/25/06 | Palmer Navel | Zebedelia | Zebedelia 3B | 24 19.424 | 29 14.998 | 1112 | Greening |
| 06-0235 | 10/25/06 | Palmer Navel | Zebedelia | Zebedelia 3B | 24 19.409 | 29 15.010 | 1111 | Greening |
| 06-0236 | 10/25/06 | Palmer Navel | Zebedelia | Zebedelia 3B | 24 19.408 | 29 15.011 | 1113 | Greening |

| Accession | Date Sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0237 | 10/25/06 | Palmer Navel | Zebedelia | Zebedelia 3B | 24 19.407 | 29 15.016 | 1110 | Greening |
| 06-0238 | 10/25/06 | Delta Valencia | Zebedelia | Zebedelia 3A | 24 19.071 | 29 15.118 | 1110 | Greening |
| 06-0239 | 10/25/06 | Delta Valencia | Zebedelia | Zebedelia 3A | 24 19.069 | 29 15.125 | 1127 | Greening |
| 06-0240 | 10/25/06 | Midnight | Letsitele | Letaba estates B16 | | | 590 | Greening |
| 06-0242 | 10/25/06 | Midnight | Letsitele | Letaba estates B16 | | | 590 | Greening |
| 06-0243 | 10/25/06 | Midnight | Letsitele | Letaba estates B16 | | | 590 | Greening |
| 06-0244 | 10/25/06 | Midnight | Letsitele | Letaba estates B16 | | | 590 | Greening |
| 06-0245 | 10/25/06 | Midnight | Letsitele | Letaba estates B16 | | | 590 | Greening |
| 06-0246 | 10/25/06 | Delta Valencia | Letsitele | Letaba estates A1 | | | 590 | Greening |
| 06-0247 | 10/25/06 | Midnight | Letsitele | Letaba estates A1 | | | 590 | Greening |
| 06-0248 | 10/25/06 | Valencia (late) | Letsitele | Letaba estates A71 | | | 540 | Greening |
| 060249 | 10/25/06 | Valencia (late) | Letsitele | Letaba estates A71 | | | 540 | Greening |
| 06-0250 | 10/25/06 | Valencia (late) | Letsitele | Letaba estates A71 | | | 540 | Greening |
| 06-0251 | 10/25/06 | Valencia | Hoedspruit | Bosveldcitrus | 24 21 42.0" | 30 41 26.9 | 480 | Greening |
| 06-0252 | 10/25/06 | Valencia | Hoedspruit | Bosveldcitrus | 24 21 30.4" | 30 41 33.6" | 450 | Greening |
| 06-0253 | 10/25/06 | du Roi selection | Hoedspruit | Bosveldcitrus | 24 21 37.9" | 30 42 07.3" | | Greening |
| 06-0254 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | | | 590 | Greening |
| 06-0256 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | 24 26 48.9" | 30 49 03.5" | 590 | Greening |
| 06-0257 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | 24 26 48.9" | 30 49 03.5" | 590 | Greening |
| 06-0258 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | 24 26 48.9" | 30 49 03.5" | 590 | Greening |
| 06-0259 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | 24 26 48.9" | 30 49 03.5" | 590 | Greening |
| 06-0260 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | 24 26 48.9" | 30 49 03.5" | 590 | Greening |
| 06-0261 | 10/25/06 | Valencia | Hoedspruit | Moriah Citrus | 24 26 48.9" | 30 49 03.5" | 590 | Greening |

| Accession | Date Sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0263 | 10/25/06 | Delta Valencia | Hoedspruit | Blyderivier Citrus | 24 29 43.5" | 30 50 08.8" | 600 | Greening |
| 06-0264 | 10/25/06 | Delta Valencia | Hoedspruit | Blyderivier Citrus | 24 29 43.5" | 30 50 08.8" | 600 | Greening |
| 06-0265 | 10/25/06 | Delta Valencia | Hoedspruit | Blyderivier Citrus | 24 29 43.5" | 30 50 08.8" | 600 | Greening |
| 06-0266 | 10/25/06 | Delta Valencia | Hoedspruit | Blyderivier Citrus | 24 29 43.5" | 30 50 08.8" | 560 | Greening |
| 06-0267 | 10/25/06 | Delta Valencia | Hoedspruit | Blyderivier Citrus | 24 29 43.5" | 30 50 08.8" | 560 | Greening |
| 06-0268 | 10/25/06 | Delta Valencia | Hoedspruit | Blyderivier Citrus | 24 29 43.5" | 30 50 08.8" | 560 | Greening |
| 06-0270 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0272 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0273 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0274 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0275 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0276 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0277 | 10/26/06 | Valencia | Nelspruit | Crocodile Valley | | | 540 | Greening |
| 06-0279 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0280 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0282 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0283 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0284 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0285 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0286 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0287 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0288 | 10/26/06 | Delta Valencia | Nelspruit | ARC-ITSC C3 | | | 540 | Greening |
| 06-0289 | 10/26/06 | Eureka Lemon | Nelspruit | ARC-ITSC D4 | | | 540 | Greening |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0290 | 10/26/06 | Eureka Lemon | Whiteriver | Fontains | | | 740 | Greening |
| 06-0291 | 10/26/06 | Eureka Lemon | Whiteriver | Fontains | | | 740 | Greening |
| 06-0292 | 10/26/06 | Eureka Lemon | Whiteriver | Fontains | | | 740 | Greening |
| 06-0294 | 10/26/06 | Eureka Lemon | Whiteriver | Fontains | | | 740 | Greening |
| 06-0295 | 10/26/06 | Eureka Lemon | Whiteriver | Fontains | | | 740 | Greening |
| 06-0297 | 10/26/06 | Karino | Larten | | | | 610 | Greening |
| 06-0300 | 10/26/06 | Karino | Larten | | | | 610 | Greening |
| 06-0301 | 10/26/06 | Karino | Larten | | | | 610 | Greening |
| 06-0302 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0303 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0304 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0305 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0306 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0307 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0308 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0309 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0311 | 10/31/06 | Navelina | Swellendam | Thornlands | | | 40 | Greening |
| 06-0312 | 10/31/06 | Afurer | Ashton | Uitkyk | 33 47.373 | 20 00.310 | 280 | Greening |
| 06-0314 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0315 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0316 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0317 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0318 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0319 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0320 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0321 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0322 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0323 | 10/31/06 | Afurer | Ashton | Uitkyk | | | 280 | Greening |
| 06-0324 | 11/01/06 | Nules Clemantine | Caledon | | | | 100 | Greening |
| 06-0325 | 11/01/06 | Nules Clemantine | Caledon | | | | 100 | Greening |
| 06-0326 | 11/01/06 | Nules Clemantine | Caledon | | | | 100 | Greening |
| 06-0327 | 11/01/06 | Nules Clemantine | Caledon | | | | 100 | Greening |
| 06-0328 | 11/01/06 | Nules Clemantine | Caledon | | | | 100 | Greening |
| 06-0329 | 11/01/06 | Eureka lemon | Caledon | | | | 100 | Mottle |
| 06-0330 | 11/01/06 | Eureka lemon | Caledon | | | | 100 | Mottle |
| 06-0332 | 11/01/06 | Eureka lemon | Caledon | | | | 100 | Mottle |
| 06-0333 | 11/01/06 | Eureka lemon | Caledon | | | | 100 | Mottle |
| 06-0334 | 11/01/06 | Eureka lemon | Caledon | | | | 100 | Mottle |
| 06-0335 | 11/01/06 | Eureka lemon | Stellenbosch | Rust & Vrede | | | 150 | |
| 06-0336 | 11/01/06 | Eureka lemon | Stellenbosch | Rust & Vrede | | | 150 | |
| 06-0337 | 11/01/06 | Eureka lemon | Stellenbosch | Rust & Vrede | | | 150 | |
| 06-0339 | 11/01/06 | Eureka lemon | Stellenbosch | Rust & Vrede | | | 150 | |
| 06-0341 | 11/01/06 | Eureka lemon | Stellenbosch | Rust & Vrede | | | 150 | - |
| 06-0342 | 11/01/06 | Eureka lemon | Stellenbosch | Rust & Vrede | | | 150 | |
| 06-0344 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |
| 06-0345 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0347 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |
| 06-0348 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |
| 06-0349 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |
| 06-0350 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |
| 06-0351 | 11/01/06 | Eureka lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | |
| 06-0352 | 11/01/06 | Satsuma | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | Greening |
| 06-0353 | 11/01/06 | Satsuma | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | Greening |
| 06-0356 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0357 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0358 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0359 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0360 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0361 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0362 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0363 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0364 | 11/01/06 | Eureka lemon | Wellington | Olyvenhout | | | 180 | Greening |
| 06-0401 | 10/28/06 | Delta Valentia | Ngonini, Swaziland | Block 28A | | | 360 | |
| 06-0403 | 10/28/06 | Delta Valentia | Ngonini, Swaziland | Block 28A | | | 360 | |
| 06-0404 | 10/28/06 | Delta Valentia | Ngonini, Swaziland | Block 28A | | | 360 | |
| 06-0405 | 10/28/06 | Delta Valentia | Ngonini, Swaziland | Block 28A | | | 360 | |
| 06-0406 | 10/28/06 | Delta Valentia | Ngonini, Swaziland | Block 28A | | | 360 | |
| 06-0407 | 10/28/06 | Valentia | Malelane | | | | 240 | |
| 06-0410 | 10/28/06 | Valentia | Malelane | Next to sugarcane | | | 300 | |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| 06-0411 | 10/28/06 | Valentia | Malelane | Next to sugarcane | | | 300 | |
| 06-0412 | 10/28/06 | Valentia | Malelane | Berg boord | | | 350 | |
| 06-0413 | 10/28/06 | Valentia | Malelane | Magnesite mine | | | 360 | |
| 09-0853 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0854 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0855 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0856 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0857 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0858 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0859 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0860 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0861 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0862 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0863 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0864 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0865 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0866 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| 09-0868 | 01/09/09 | Unknown | Eastern Cape | Unknown | | | | |
| CRI4 | 25/07/14 | Valencia | Nelspruit | Croc Valley | | | | |
| CRI5 | 25/07/14 | Valencia | Nelspruit | Croc Valley | | | | |
| CRI6 | 11/08/14 | Valencia | Nelspruit | Croc Valley | | | | |
| CRI7 | 11/08/14 | Valencia | Nelspruit | Croc Valley | | | | |
| CRI8 | 11/08/14 | Valencia | Nelspruit | Croc Valley | | | | |

| Accession | Date sampled | Citrus Type | Locality | Farm | Latitude | Longitude | Elevation | Symptoms |
|---|---|---|---|---|---|---|---|---|
| CRI9 | 11/08/14 | Mandarin | Nelspruit | Croc Valley | | | | |
| CRI10 | 11/08/14 | Mandarin | Nelspruit | Croc Valley | | | | |
| CRI11 | 11/08/14 | Mandarin | Nelspruit | Croc Valley | | | | |
| CRI12 | 11/08/14 | Mandarin | Nelspruit | Croc Valley | | | | |
| CRI13 | 05/09/14 | Valencia | Nelspruit | CRI | | | | |
| CRI14 | 05/09/14 | Valencia | Nelspruit | CRI | | | | |
| CRI15 | 05/09/14 | Mandarin | Nelspruit | CRI | | | | |
| CRI16 | 05/09/14 | Mandarin | Nelspruit | CRI | | | | |
| CRI18 | 23/08/15 | Valencia | Nelspruit | Croc Valley | | | | |
| CRI19 | 12/08/15 | Valencia | Nelspruit | Croc Valley | | | | |
| CRI21 | 30/03/16 | Lemon | Port Elizabeth | | | | | |
| CRI22 | 18/01/16 | Valencia | Hoedspruit | | | | | |
| CRI23 | 18/04/16 | Valencia | Hoedspruit | | | | | |
| CRI24 | 30/01/14 | Lemon | Pretoria | | | | | |

# Appendix D

Table D1: List of primer sequences used for screening of polymorphic loci during microsatellite analyses. Primers were designed using MSAT commander and annealing temperatures as well as product lengths are indicated.

| Primer Name | Sense Primer | Tm | Primer Name | Anti-sense Primer | Tm | Product Length |
|---|---|---|---|---|---|---|
| Laf-SSR1_F | CTTGGGATTTGGAGCTTCAGG | 56.1 | LaR-SSR1_R | GAGTGGTACGCACGTATACTATAC | 56.3 | 230 |
| Laf-SSR2_F | GTGCCCCTTAAAAGAACAAGC | 55.2 | LaR-SSR2_R | TCCTCCATCAAGAATCTGTGC | 54.9 | 165 |
| Laf-SSR3_F | TCGTCCGAATTCTTGATGATAAAGC | 57.9 | LaR-SSR3_R | AGACATATTACCAACAACCGCAAC | 57.7 | 203 |
| Laf-SSR4_F | CCTGGATCACCCTTAGACC | 52.8 | LaR-SSR4_R | TCATTCTTTCATCTTGAGTGTATTC | 53.5 | 211 |
| Laf-SSR7_F | GGAAACCAACAAAGGAAGAAGG | 55.1 | LaR-SSR7_R | TACAGGCTTACGGCATTGC | 54.9 | 226 |
| Laf-SSR11_F | ATGGGTTTGTTTCCTCATTCTTCAC | 58 | LaR-SSR11_R | GAGAGCAAGGTCGATACTCAACTC | 58.6 | 250 |
| Laf-SSR12_F | TGATTCTTTACTCTCTATCTTTTCCC | 54.7 | LaR-SSR12_R | CATCTTGCATATGGTGTTAGTGG | 55.3 | 209 |
| Laf-SSR13_F | ACGCAATACAGTATCATCATCTAAAC | 56 | LaR-SSR13_R | GCGGCTGTTACATGGTCAC | 56 | 209 |
| Laf-SSR15_F | TCGCATAGACGCAATAATATGAGATC | 57.6 | LaR-SSR15_R | CCAGAAGTGAAGATACCGTTAATGC | 58.1 | 226 |
| Laf-SSR16_F | GATCTCAAGCTACTCGATGGC | 55.6 | LaR-SSR16_R | TTAGATTCAATCCTGCATGATAACC | 55.3 | 157 |
| Laf-SSR17_F | TGGAGCAGATAGAAGCGAACC | 57.1 | LaR-SSR17_R | CGGTGGGAACAGACAGAGG | 56.4 | 196 |
| Laf-SSR18_F | AGCCTAATACCATATTCCTATTCTG | 53.8 | LaR-SSR18_R | TAAGAATGGCATTCGCTACATG | 54.4 | 240 |
| Laf-SSR19_F | TTGTGACCAACCCATTTCCC | 55.2 | LaR-SSR19_R | GTGTATGAATCTCGTCGGCTAG | 56 | 211 |
| Laf-SSR20_F | CTACAGGCACTCCCAATAATTCC | 56.5 | LaR-SSR20_R | TGGTTGTAAAGTAGATTGTTTGTCG | 56.1 | 181 |
| Laf-SSR21_F | ACTCACGCTTACTCATCTTATTCC | 56.3 | LaR-SSR21_R | AGAATCAAGAGTGATGGTGTGG | 55.5 | 215 |
| Laf-SSR22_F | TGAACCGTAGAAATTGTTTATCTCC | 55.5 | LaR-SSR22_R | AATCGTTGATCCATTGTTAGCG | 55 | 155 |
| Laf-SSR23_F | TGAACCGTAGAAATTGTTTATCTCC | 55.5 | LaR-SSR23_R | CGAATCGTGATCCATTGTTAGC | 55.4 | 156 |
| Laf-SSR24_F | CCCTTTATCACCATGCGAACC | 56.6 | LaR-SSR24_R | CGTGTTGGAGCCTTGGATTTAG | 57.1 | 167 |
| Laf-SSR25_F | TTGCTCCACCACCATCGG | 56 | LaR-SSR25_R | GGATTTGTATGGGACGTTGGG | 56.2 | 206 |
| Laf-SSR26_F | ACGATCAACATCATCTCCAACC | 55.9 | LaR-SSR26_R | GCTCTCAATATACACCATGACCTC | 56.7 | 218 |
| Laf-SSR27_F | TATGAGAGAACACTAGCAGAATACC | 55.8 | LaR-SSR27_R | ACCTGTGACCCTTGGAATCG | 56.6 | 216 |
| Laf-SSR28_F | AGACCAAAACCCAGCAAAGC | 56.2 | LaR-SSR28_R | CAATGACTTAGGTGTTTATTTGTGC | 55.5 | 202 |
| Laf-SSR29_F | TGCCGCCGCTGACTTATG | 56.9 | LaR-SSR29_R | AAGTAGGTGTCTTTGAAACTGGTG | 57 | 150 |
| Laf-SSR30_F | GAGCATCTTTCATAGCCATAGC | 54.6 | LaR-SSR30_R | ATTGCACCAGTTCCGTAATTC | 54.1 | 246 |
| Laf-SSR5_F | CTTCATTTCAGACATAGATTAGACC | 53.4 | LaR-SSR5_R | GTATTTTGTGTGATAGTTGTTATCG | 53 | 184 |

| Primer Name | Sense Primer | Tm | Primer Name | Anti-sense Primer | Tm | Product Length |
|---|---|---|---|---|---|---|
| Laf-SSR6_F | CAAATAAAGTATCAGCGAATTGC | 52.8 | LaR-SSR6_R | GTGTATCATAAAAGGCATTAATTTC | 51.5 | 151 |
| Laf-SSR14_F | TTCATCAATACAATATCAATTTCTGG | 52.7 | LaR-SSR14_R | CGCCGTCAGGAATAAATGG | 53.3 | 160 |
| Laf-SSR8_F | AATCCATCTCCTATCTCCTTAACC | 54.9 | LaR-SSR8_R | GTTGTACTTTGGCGATGAAGC | 55.5 | 250 |
| Laf-SSR9_F | AACCAGAACACAATGATATAATACC | 53.3 | LaR-SSR9_R | CCACCCACAGTATCTACAGG | 53.7 | 170 |
| Laf-SSR10_F | TGAGTATGAGTATGAGTATGAGTATG | 53.8 | LaR-SSR10_R | TGTATCATCAGATATTTAAGAGTTAGTG | 53.9 | 137 |
| Saf_1F | ACGCTCCTCACGATTCTGC | 59,8 | Saf_1R | CGGTCATACTATTGCTTCGGC | 59,6 | 344 |
| Saf_2F | GCAGAACTGCGAACCGAC | 58,8 | Saf_2R | TTTGAGCGCGGATACGTTG | 59 | 228 |
| Saf_3F | TTCCGCCACTTCACATTCC | 59,5 | Saf_3R | GTGCTTGTGTTAATGCGCC | 59,9 | 342 |
| Saf_4F | ATTCCACCTTTGCGAACCG | 59,9 | Saf_4R | CGGTCATACTATTGCTTCGGC | 59,7 | 212 |
| Saf_5F | CAGGTGCGGATTGTTTACCC | 58,1 | Saf_5R | CGTATCCCACGTTTGCGAG | 59,7 | 340 |
| Saf_6F | GGGTGACTATAGCCCACAAG | 59,8 | Saf_6R | CTGTTTGGTCTCCCGGTTTG | 58,5 | 414 |
| Saf_7F | GTTGTGTGAGAGCAAGGTCG | 58,9 | Saf_7R | AAGGGCGCTAAGTTCAAGG | 58,4 | 275 |
| Saf_8F | CTTGCTGTGCATATTTCGCC | 59,7 | Saf_8R | ACCAGATTGCTATAAATGATGTGC | 60 | 273 |
| Saf_9F | TGCCACTCCCTTCCCTAAAG | 57,7 | Saf_9R | TGCGCATTCGTTATGGCG | 58,8 | 444 |
| Saf_10F | CAGCTGCCATGCAAATTAAAC | 59,8 | Saf_10R | ACTTGTTCCGCATCCACAG | 59,6 | 289 |
| Saf_11F | GAAGCGGAAGTTGTTGGAGG | 59,9 | Saf_11R | TCGCCAGTCATTTGAAAGCG | 60,2 | 432 |
| Saf_12F | TCCATTGCCTTAGATATTTCGTC | 57,2 | Saf_12R | TGAGCTCGTCCGAATTCTTG | 58,7 | 536 |
| Saf_13F | TGTATCTGGCGTTGGTAGC | 58 | Saf_13R | AGTGAATAGCATCTGTGCATAAAG | 58,2 | 372 |
| Saf_14F | GCCTCCGTTTGGAGTATTGG | 59,4 | Saf_14R | AGTCTGCCAGGTGATATTGAAG | 58,6 | 293 |
| Saf_15F | CACTCACTCGTCCCAATACG | 58,8 | Saf_15R | GGTATTGCCGAGGTCTTGC | 59,3 | 379 |
| Saf_16F | AGTCAGTAAGTTTATTCCACGAAAG | 58,1 | Saf_16R | GAGAAGCGACACGATCTCC | 58,5 | 203 |
| Saf_17F | CTGCCGGCCCTTTCTTAAC | 59,6 | Saf_17R | ACAGGTAGCGTTGATGTAGAAC | 58,9 | 143 |
| Saf_18F | ACCACTGTTTCTGCGCATC | 59,5 | Saf_18R | TCACCTACCGCCTTACGAG | 59,3 | 245 |
| Saf_19F | TTGATTGCGGGCGAGAAGG | 61,5 | Saf_19R | GAGCATTGTCAGGCTGGTG | 59,6 | 213 |
| Saf_20F | CTCTGAAAGCTGGTGAGCG | 59,3 | Saf_20R | TGTTGGCTCTGAATAACATTGAAC | 58,8 | 128 |

Table D2: List of samples included in fragment analysis. Blue represents samples which fell within Laf Genetic Group I whereas, green samples represents Group II. Group III is represented in orange and Group IV is represented in yellow. Different shades of a colour represent distinct haplotypes within a given genetic group.

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0137 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | | | | North West |
| 06-0138 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | | | | North West |
| 06-0139 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.324 | 27 04.548 | 1173 | North West |
| 06-0140 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.316 | 27 04.542 | 1193 | North West |
| 06-0141 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.309 | 27 04.531 | 1181 | North West |
| 06-0142 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.306 | 27 04.530 | 1186 | North West |
| 06-0144 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.303 | 27 04.528 | 1189 | North West |
| 06-0146 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.294 | 27 04.521 | 1186 | North West |
| 06-0147 | 10/23/06 | Palmer Navel | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.290 | 27 04.539 | 1187 | North West |
| 06-0149 | 10/23/06 | Robyn | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.409 | 27 04.549 | 1190 | North West |
| 06-0150 | 10/23/06 | Robyn | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.415 | 27 04.592 | 1190 | North West |
| 06-0151 | 10/23/06 | Robyn | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.424 | 27 04.578 | 1191 | North West |
| 06-0152 | 10/23/06 | Robyn | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.432 | 27 04.567 | 1195 | North West |
| 06-0153 | 10/23/06 | Robyn | Rough Lemon | Boshoek-Rustenburg | Witkrans Nurseries | 25 29.434 | 27 04.504 | 1190 | North West |
| 06-0155 | 10/23/06 | Orange | | Rustenburg | Hunters Rest Hotel | 25 46.599 | 27 15.537 | 1222 | North West |
| 06-0156 | 10/23/06 | Orange | | Rustenburg | Hunters Rest Hotel | 25 46.600 | 27 15.535 | 1227 | North West |
| 06-0157 | 10/23/06 | Orange | | Rustenburg | Hunters Rest Hotel | 25 46.599 | 27 15.527 | 1231 | North West |
| 06-0159 | 10/23/06 | Orange | | Rustenburg | Hunters Rest Hotel | 25 46.589 | 27 15.513 | 1230 | North West |
| 06-0160 | 10/23/06 | Eureka Lemon | Volckermeriana | Kroondal-Rustenberg | Wohlfshule | 25 44.616 | 27 18.774 | 1180 | North West |
| 06-0167 | 10/23/06 | Delta Valencia | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.118 | 27 24.541 | 1418 | North West |
| 06-0168 | 10/23/06 | Delta Valencia | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.117 | 27 24.524 | 1414 | North West |
| 06-0169 | 10/23/06 | Delta Valencia | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.099 | 27 24.570 | 1417 | North West |
| 06-0170 | 10/23/06 | Delta Valencia | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.105 | 27 24.582 | 1411 | North West |
| 06-0171 | 10/23/06 | Delta Valencia | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.118 | 27 24.591 | 1417 | North West |

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0173 | 10/23/06 | Minneola | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.175 | 27 24.633 | 1392 | North West |
| 06-0174 | 10/23/06 | Minneola | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.175 | 27 24.630 | 1392 | North West |
| 06-0175 | 10/23/06 | Minneola | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.177 | 27 24.626 | 1393 | North West |
| 06-0177 | 10/23/06 | Minneola | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.181 | 27 24.622 | 1395 | North West |
| 06-0178 | 10/23/06 | Valencia (late) | Rough Lemon | Buffelspoort-Rustenburg | | 25 49.133 | 27 24.558 | 1402 | North West |
| 06-0179 | 10/23/06 | Valencia (late) | Rough Lemon | Marikana-Rustenburg | Barnardsvlei | 25 48.131 | 27 28.552 | 1304 | North West |
| 06-0181 | 10/23/06 | Valencia (late) | Rough Lemon | Marikana-Rustenburg | Barnardsvlei | 25 48.143 | 27 28.549 | 1304 | North West |
| 06-0182 | 10/23/06 | Valencia (late) | Rough Lemon | Marikana-Rustenburg | Barnardsvlei | 25 48.142 | 27 28.529 | 1304 | North West |
| 06-0183 | 10/23/06 | Valencia (late) | Rough Lemon | Marikana-Rustenburg | Barnardsvlei | 25 48.161 | 27 28.520 | 1306 | North West |
| 06-0184 | 10/23/06 | Valencia (late) | Rough Lemon | Marikana-Rustenburg | Barnardsvlei | 25 48.189 | 27 28.514 | 1304 | North West |
| 06-0187 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.064 | 27 36.899 | 1283 | North West |
| 06-0188 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.060 | 27 36.897 | 1280 | North West |
| 06-0189 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.058 | 27 36.896 | 1280 | North West |
| 06-0190 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.057 | 27 36.892 | 1279 | North West |
| 06-0191 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46. | 27 36.891 | 1278 | North West |
| 06-0192 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.051 | 27 36.894 | 1279 | North West |
| 06-0193 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.050 | 27 36.893 | 1279 | North West |
| 06-0194 | 10/23/06 | Palmer Navel | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.080 | 27 36.864 | 1282 | North West |
| 06-0195 | 10/23/06 | Valencia (late) | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.081 | 27 36.870 | 1282 | North West |
| 06-0196 | 10/23/06 | Valencia (late) | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.073 | 27 36.883 | 1283 | North West |
| 06-0197 | 10/23/06 | Valencia (late) | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.263 | 27 36.440 | 1280 | North West |
| 06-0198 | 10/23/06 | Valencia (late) | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.246 | 27 36.435 | 1278 | North West |
| 06-0199 | 10/23/06 | Valencia (late) | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.242 | 27 36.426 | 1279 | North West |
| 06-0200 | 10/23/06 | Valencia (late) | Rough Lemon | Mooinooi-Rustenburg | Buffelsfontein | 25 46.248 | 27 36.427 | 1279 | North West |
| 06-0203 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Groenkloof | 25 45.325 | 27 38.370 | 1263 | North West |
| 06-0204 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Groenkloof | 25 45.310 | 27 38.370 | 1266 | North West |

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0205 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Groenkloof | 25 45.290 | 27 38.384 | 1265 | North West |
| 06-0208 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Groenkloof | 25 45.262 | 27 38.369 | 1273 | North West |
| 06-0209 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Groenkloof | 25 45.258 | 27 38.362 | 1264 | North West |
| 06-0212 | 10/24/06 | Valencia | Rough Lemon | Mooinooi-Rustenburg | Buffelshoek | 25 45.292 | 27 35.294 | 1237 | North West |
| 06-0213 | 10/24/06 | Valencia | Rough Lemon | Mooinooi-Rustenburg | Buffelshoek | 25 45.291 | 27 35.294 | 1231 | North West |
| 06-0214 | 10/24/06 | Valencia | Rough Lemon | Mooinooi-Rustenburg | Buffelshoek | 25 45.293 | 27 35.297 | 1228 | North West |
| 06-0215 | 10/24/06 | Valencia | Rough Lemon | Mooinooi-Rustenburg | Buffelshoek | 25 45.323 | 27 35.302 | 1227 | North West |
| 06-0217 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Bokfontein | 25 43.162 | 27 43.113 | 1231 | North West |
| 06-0218 | 10/24/06 | Valencia | Rough Lemon | Wolhuterskop-Brits | Bokfontein | 25 43.160 | 27 43.116 | 1232 | North West |
| 06-0224 | 10/25/06 | Washington Navel | Rough Lemon | Groblersdal | Moosrivier H14 | 25 00.863 | 29 22.850 | 870 | Limpopo |
| 06-0231 | 10/25/06 | Palmer Navel | Rough Lemon | Zebedelia | Zebedelia 3B | 24 19.444 | 29 14.994 | 1110 | Limpopo |
| 06-0232 | 10/25/06 | Palmer Navel | Rough Lemon | Zebedelia | Zebedelia 3B | 24 19.429 | 29 14.992 | 1112 | Limpopo |
| 06-0233 | 10/25/06 | Palmer Navel | Rough Lemon | Zebedelia | Zebedelia 3B | 24 19.424 | 29 14.996 | 1114 | Limpopo |
| 06-0235 | 10/25/06 | Palmer Navel | Rough Lemon | Zebedelia | Zebedelia 3B | 24 19.409 | 29 15.010 | 1111 | Limpopo |
| 06-0236 | 10/25/06 | Palmer Navel | Rough Lemon | Zebedelia | Zebedelia 3B | 24 19.408 | 29 15.011 | 1113 | Limpopo |
| 06-0237 | 10/25/06 | Palmer Navel | Rough Lemon | Zebedelia | Zebedelia 3B | 24 19.407 | 29 15.016 | 1110 | Limpopo |
| 06-0240 | 10/25/06 | Midnight | Rough Lemon | Letsitele | Letaba estates B16 | | | 590 | Limpopo |
| 06-0242 | 10/25/06 | Midnight | Rough Lemon | Letsitele | Letaba estates B16 | | | 590 | Limpopo |
| 06-0243 | 10/25/06 | Midnight | Rough Lemon | Letsitele | Letaba estates B16 | | | 590 | Limpopo |
| 06-0245 | 10/25/06 | Midnight | Rough Lemon | Letsitele | Letaba estates B16 | | | 590 | Limpopo |
| 06-0246 | 10/25/06 | Delta Valencia | Swingle | Letsitele | Letaba estates A1 | | | 590 | Limpopo |
| 06-0247 | 10/25/06 | Midnight | Rough Lemon | Letsitele | Letaba estates A1 | | | 590 | Limpopo |
| 06-0248 | 10/25/06 | Valencia (late) | Rough Lemon | Letsitele | Letaba estates A71 | | | 540 | Limpopo |
| 06-0249 | 10/25/06 | Valencia (late) | Rough Lemon | Letsitele | Letaba estates A71 | | | 540 | Limpopo |
| CRI 20 | 18/3/2015 | Star Ruby Grapefruit | | Letsitele | | | | | Limpopo |
| 06-0254 | 10/25/06 | Valencia | Rough Lemon | Hoedspruit | Moriah Citrus Estate | | | 590 | Limpopo |

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0256 | 10/25/06 | Valencia | Rough Lemon | Hoedspruit | Moriah Citrus Estate | 24 26 48.9 | 30 49 03.5" | 590 | Limpopo |
| 06-0257 | 10/25/06 | Valencia | Rough Lemon | Hoedspruit | Moriah Citrus Estate | 24 26 48.9 | 30 49 03.5" | 590 | Limpopo |
| 06-0258 | 10/25/06 | Valencia | Rough Lemon | Hoedspruit | Moriah Citrus Estate | 24 26 48.9 | 30 49 03.5" | 590 | Limpopo |
| 06-0260 | 10/25/06 | Valencia | Rough Lemon | Hoedspruit | Moriah Citrus Estate | 24 26 48." | 30 49 03.5" | 590 | Limpopo |
| 06-0261 | 10/25/06 | Valencia | Rough Lemon | Hoedspruit | Moriah Citrus Estate | 24 26 48.9 | 30 49 03.5" | 590 | Limpopo |
| 06-0262 | 10/25/06 | Marsh | Carrizo citrange? | Hoedspruit | Moriah Citrus Estate | 24 26 15.9 | 30 49 27.8" | 550 | Limpopo |
| 06-0263 | 10/25/06 | Delta Valencia | Carrizo Citrange | Hoedspruit | Blyderivier Citrus | 24 29 43.5 | 30 50 08.8" | 600 | Limpopo |
| 06-0264 | 10/25/06 | Delta Valencia | Carrizo Citrange | Hoedspruit | Blyderivier Citrus | 24 29 43.5 | 30 50 08.8" | 600 | Limpopo |
| 06-0265 | 10/25/06 | Delta Valencia | Carrizo Citrange | Hoedspruit | Blyderivier Citrus | 24 29 43.5 | 30 50 08.8" | 600 | Limpopo |
| 06-0266 | 10/25/06 | Delta Valencia | Carrizo Citrange | Hoedspruit | Blyderivier Citrus | 24 29 43.5 | 30 50 08.8" | 560 | Limpopo |
| 06-0267 | 10/25/06 | Delta Valencia | Carrizo Citrange | Hoedspruit | Blyderivier Citrus | 24 29 43.5 | 30 50 08.8" | 560 | Limpopo |
| 06-0268 | 10/25/06 | Delta Valencia | Carrizo Citrange | Hoedspruit | Blyderivier Citrus | 24 29 43.5 | 30 50 08.8" | 560 | Limpopo |
| CRI22 | 18/04/16 | Valencia | | Hoedspruit | | | | | Limpopo |
| CRI23 | 18/04/16 | Valencia | | Hoedspruit | | | | | Limpopo |
| 06-0270 | 10/26/06 | Valencia | Cleo Mandarin | Nelspruit | Crocodile Valley | | | 540 | Mpumalanga |
| 06-0273 | 10/26/06 | Valencia | Cleo Mandarin | Nelspruit | Crocodile Valley | | | 540 | Mpumalanga |
| 06-0274 | 10/26/06 | Valencia | Cleo Mandarin | Nelspruit | Crocodile Valley | | | 540 | Mpumalanga |
| 06-0275 | 10/26/06 | Valencia | Cleo Mandarin | Nelspruit | Crocodile Valley | | | 540 | Mpumalanga |
| 06-0276 | 10/26/06 | Valencia | Cleo Mandarin | Nelspruit | Crocodile Valley | | | 540 | Mpumalanga |
| 06-0278 | 10/26/06 | Valencia | Cleo Mandarin | Nelspruit | Crocodile Valley | | | 540 | Mpumalanga |
| 06-0279 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0282 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0283 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0284 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0285 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0286 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0287 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0288 | 10/26/06 | Delta Valencia | Troyer Citrange | Nelspruit | ARC-ITSC C3 | | | 540 | Mpumalanga |
| 06-0289 | 10/26/06 | Eureka Lemon | | Nelspruit | ARC-ITSC D4 | | | 540 | Mpumalanga |
| CRI1 | 30/4/2014 | Eureka Lemon | | Nelspruit | | | | | Mpumalanga |
| CRI2 | 30/4/2018 | Eureka Lemon | | Nelspruit | | | | | Mpumalanga |
| CRI3 | 3/4/2014 | Eureka Lemon | | Nelspruit | Unknown | | | 540 | Mpumalanga |
| CRI4 | 25/7/2014 | Eureka Lemon | | Nelspruit | | | | | Mpumalanga |
| CRI5 | 25/7/2014 | Valencia | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI6 | 11/08/14 | Valencia | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI7 | 11/08/14 | Valencia | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI8 | 11/08/14 | Valencia | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI9 | 11/08/14 | Mandarin | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI10 | 11/08/14 | Mandarin | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI11 | 11/08/16 | Mandarin | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI12 | 11/08/16 | Mandarin | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI13 | 05/09/14 | Valencia | | Nelspruit | CRI | | | | Mpumalanga |
| CRI14 | 05/09/14 | Valencia | | Nelspruit | CRI | | | | Mpumalanga |
| CRI15 | 05/09/14 | Mandarin | | Nelspruit | CRI | | | | Mpumalanga |
| CRI16 | 05/09/14 | Mandarin | | Nelspruit | CRI | | | | Mpumalanga |
| CRI18 | 12/08/15 | Valencia | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI19 | 12/08/15 | Valencia | | Nelspruit | Croc Valley | | | | Mpumalanga |
| CRI24 | 30/01/17 | Lemon | | Nelspruit | | | | | Mpumalanga |
| 06-0401 | 10/28/06 | Delta Valentia | Citrange | Ngonini, Swaziland | Block 28A | | | 360 | Mpumalanga |
| 06-0402 | 28/10/06 | Delta Valencia | Citrange | Ngoni, Swaziland | Block 28A | | | 2019 | Mpumalanga |
| 06-0405 | 28/10/06 | Delta Valencia | Citrange | Ngoni, Swaziland | Block 28A | | | 2020 | Mpumalanga |
| 06-0406 | 28/10/06 | Delta Valencia | Citrange | Ngoni, Swaziland | Block 28A | | | 2022 | Mpumalanga |

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0407 | 28/10/06 | Valencia | Rough Lemon | Malelane | Kellerman | | | 2024 | Mpumalanga |
| 06-0410 | 28/10/06 | Valencia | Rough Lemon | Malelane | Radley | | | 2027 | Mpumalanga |
| 06-0411 | 10/28/06 | Valentia | Rough lemon | Malelane | Next to sugarcane | | | 300 | Mpumalanga |
| 06-0412 | 10/28/06 | Valencia | Swingle | Malelane | Berg Boord | | | 350 | Mpumalanga |
| 06-0413 | 10/28/06 | Valentia | Citrange | Malelane | Magnesite mine | | | 360 | Mpumalanga |
| 06-0290 | 10/26/06 | Eureka Lemon | | Whiteriver | Fontains | | | 740 | Mpumalanga |
| 06-0291 | 10/26/06 | Eureka Lemon | | Whiteriver | Fontains | | | 740 | Mpumalanga |
| 06-0292 | 10/26/06 | Eureka Lemon | | Whiteriver | Fontains | | | 740 | Mpumalanga |
| 06-0294 | 10/26/06 | Eureka Lemon | | Whiteriver | Fontains | | | 740 | Mpumalanga |
| 06-0297 | 10/26/06 | Midnight Valencia | | Karino | Larten | | | 610 | Mpumalanga |
| 06-0300 | 10/26/06 | Midnight Valencia | | Karino | Larten | | | 610 | Mpumalanga |
| CRI21 | 30/3/2016 | Lemon | | Western Cape | | | | | Western Cape |
| 06-0302 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0303 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0304 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0306 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0308 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0309 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0310 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0311 | 10/31/06 | Navelina | Carrizo citrange | Swellendam | Thornlands | | | 40 | Western Cape |
| 06-0312 | 10/31/06 | Afurer | Carrizo citrange | Ashton | Uitkyk | 33 47.373 | 20 00.310 | 280 | Western Cape |
| 06-0315 | 10/31/06 | Afurer | Carrizo citrange | Ashton | Uitkyk | | | 280 | Western Cape |
| 06-0316 | 10/31/06 | Afurer | Carrizo citrange | Ashton | Uitkyk | | | 280 | Western Cape |
| 06-0320 | 10/31/06 | Afurer | Carrizo citrange | Ashton | Uitkyk | | | 280 | Western Cape |
| 06-0324 | 11/01/06 | Nules Clemantine | Troyer citrange | Caledon | | | | 100 | Western Cape |
| 06-0328 | 11/01/06 | Nules Clemantine | Troyer citrange | Caledon | | | | 100 | Western Cape |

| Accession | Date Collected | Citrus Type | Rootstock | Area | Farm | Latitude | Longitude | Elevation | Province |
|---|---|---|---|---|---|---|---|---|---|
| 06-0329 | 11/01/06 | Eureka lemon | Rough lemon | Caledon | | | | 100 | Western Cape |
| 06-0332 | 11/01/06 | Eureka lemon | Rough lemon | Caledon | | | | 100 | Western Cape |
| 06-0333 | 11/01/06 | Eureka lemon | Rough lemon | Caledon | | | | 100 | Western Cape |
| 06-0335 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede | | | 150 | Western Cape |
| 06-0336 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede | | | 150 | Western Cape |
| 06-0337 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede | | | 150 | Western Cape |
| 06-0338 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede | | | 150 | Western Cape |
| 06-0339 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede | | | 150 | Western Cape |
| 06-0341 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede | | | 150 | Western Cape |
| 06-0342 | 11/01/06 | Eureka lemon | Rough lemon | Stellenbosch | Rust & Vrede Row1/Plant1 | | | 150 | Western Cape |
| 06-0348 | 11/01/06 | Eureka lemon | Rough lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | Western Cape |
| 06-0350 | 11/01/06 | Eureka lemon | Rough lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | Western Cape |
| 06-0351 | 11/01/06 | Eureka lemon | Rough lemon | Groot Drakenstein | Wilde Paarde Jagt | | | 320 | Western Cape |