Widespread occurrence of "Candidatus *liberibacter africanus subspecies capensis*" *in* Calodendrum capense *in South Africa*

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Widespread occurrence of "*Candidatus* liberibacter africanus subspecies capensis" in *Calodendrum capense* in South Africa

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Abstract Recent studies in citrus orchards confirmed that Citrus Greening, a heat sensitive citrus disease, similar to Huanglongbing (HLB), is associated with the presence of "*Candidatus* Liberibacter africanus" (Laf) in South Africa. Neither "*Candidatus* Liberibacter asiaticus" (Las), associated with HLB, "*Candidatus* Liberibacter americanus", nor "*Candidatus* Liberibacter africanus ssp. capensis" (LafC), previously detected in the Western Cape, South Africa on an indigenous Rutaceous species, *Calodendrum capense* (L. f.) Thunb. (Cape Chestnut), were detected in citrus. The current study aims to determine the potential role of *C. capense* in the epidemiology of Citrus Greening in South Africa and whether LafC poses a risk to citriculture. A total of 278 *C. capense* samples were collected throughout South Africa and tested for Liberibacters using real-time PCR. While LafC was found in 100 samples, distributed from all areas where collected, no evidence of Laf infection in any sample was found . The identity of the LafC present was confirmed by sequencing the amplicon derived from conventional PCR of the ß-operon of the ribosomal protein gene region of the first 17 infected trees found and of a representative sample from each district. The Liberibacter status of 44 *C. capense* and 272 citrus (Midnight Valencia) trees growing in close proximity to each other for over 15 years was determined. Out of 44 *C. capense* specimens, 43 were infected with LafC, but none of the citrus trees were infected with LafC. Based on the results of this it appears that natural spread of LafC to citrus does not occur.

Keywords Cape chestnut · Citrus greening · Huanglongbing · Liberibacter · Trioza erytreae

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Introduction

South Africa appears to be free of Huanglongbing (HLB), a debilitating, insect-transmissible citrus disease, initially described from China, but now spreading in the New World (Bové 2006; da Graça 2008). However a very similar, but heat sensitive (Schwarz and Green 1970) and less severe citrus disease known as greening, has been known in South Africa since the 1920's (Oberholzer et al. 1965).

Unlike HLB, citrus greening diseased plants appear to recover from symptoms at 32 °C but not at 27 °C

(Bové et al. 1974), and the trees seldom die (Van Vuuren, personal communication). Greening disease in South Africa is associated with the presence of "*Candidatus* Liberibacter africanus" (Laf), a phloem-limited, non-culturable, heat-sensitive alpha-Proteobacterium, (Jagoueix et al. 1996; Korsten et al. 1996; Pietersen et al. 2010) that is related to *"Ca.* Liberibacter asiaticus" (Las) which is associated with HLB (Jagoueix et al. 1994).

A further Liberibacter species, "*Ca*. L. africanus ssp. capensis" (LafC) reported only from South Africa was detected by Garnier et al. (1999; 2000) from an indigenous Rutaceous species, *Calodendrum capense* (L. f.) Thunb (Cape Chestnut) in Stellenbosch, Western Cape in 1998.

Recent studies in commercial citrus orchards confirmed earlier reports that Citrus Greening is associated with the presence of Laf in South Africa (Pietersen et al. 2010). The study confirmed the absence of Las and another citrus infecting Liberibacter, "*Ca*. Liberibacter americanus" (Lam) (Teixeira et al. 2005) locally on commercial citrus trees. Also, no instance of LafC infection was found on commercial Citrus during that study, and it is unknown what role *C. capense* or LafC play in the epidemiology of greening in South Africa.

The primary aim of this study was to determine whether *C. capense* is a natural host to Laf;, a secondary aim was to determine the relative incidence and distribution of LafC on *C. capense* in South Africa, and to ascertain whether evidence of natural transmission to Citrus occurs.

Materials and methods

Collection of samples

The location of *C. capense* (Cape Chestnut) trees was obtained by sending a questionnaire to professional and amateur botanists and by surveys during flowering time for specimens in a number of city gardens and parks, and botanical gardens. Samples were collected from *C. capense* trees with no regard for symptoms, although these were noted when observed. Leaf and petiole samples were collected from 278 specimens throughout South Africa (Fig. 1). Initially specimen locations were identified in summer while trees were flowering, with samples being collected only in winter, based on the assumption that LafC may be a heat sensitive bacterium, similar to "*Candidatus* Liberibacter africanus" (Laf), as the natural habitat of *C. capense* is in cool areas. However in the latter phases of the study samples were collected from flowering specimens in summer, due to relatively high titre observed at this time too. Samples consisted of approximately 20 leaves and petioles collected from various branches of an individual tree.

Extraction of DNA for PCR

Total DNA was extracted from 0.5 g of the pooled sample of petioles or midribs from each tree using a standard CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle 1990).

Liberibacter real-time PCR amplification

Total DNA extracts from a known LafC-infected *C. capense* tree was utilized as a positive control in PCR. DNA extracts from *C. capense* specimens were tested for Liberibacters and for LafC specifically using a labelled probe (HLBp) and reverse primer (HLBr) from the real-time PCR of Li et al. (2006) but as the forward primer LibUF, to a conserved region of known Liberibacters (5′-GGCAGGCCTAACA CATGC-3′) ("Generic Liberibacter real-time PCR") or a LafC specific primer, LafC F (5′-ATTGCGCG TATCGAATACGACG-3′) and using as template 1 μl of the DNA extract. The specificity of the LafC specific and generic Liberibacter PCR were tested against total DNA extracts containing Laf, LafC, "*Ca.* Liberibacter asiaticus" (Las); "*Ca.* Liberibacter solanacearum" (Lso) and "*Ca.* Liberibacter americanus" (Lam) (results not shown). Real-time PCR was performed using a Lightcycler® 1.5 (Roche, Mannheim, Germany) capillary-based thermocycler. Lightcycler® Taqman® Master kits were used along with the LibUF, LafC primers and probes and conditions as described (by Li et al. 2006). A positive/negative crossing threshold (Ct) of 30 was used after parallel tests showed that samples with Ct values of 30 in either of the LafC-specific and Generic Liberibacter systems no longer yielded amplicons in conventional PCR.

Nucleotide sequencing

To obtain templates for sequencing, a number of samples were amplified using the A2/J5 primer (Hocquellet

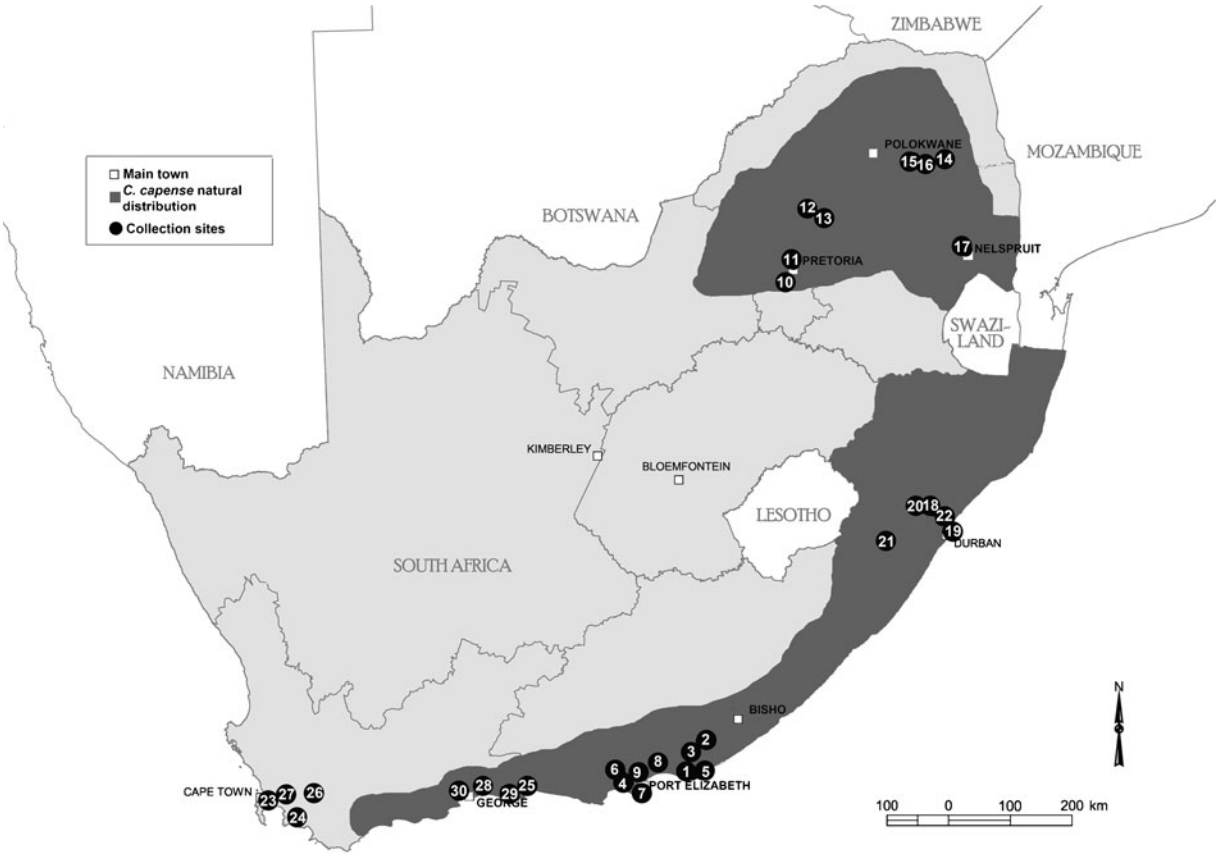

Fig. 1 Map of natural distribution of *Calodendrum capense* (Cape Chestnut) in South Africa (dark grey shaded area) and sites where samples were collected. Site number corresponds with that in Table 1

et al. 1999) targeting the ribosomal protein genes (rplKAJL) portion of the the ß-operon. Reaction conditions were identical to those described for PCR for detection of Laf (Pietersen et al. 2010) except cycling conditions were conducted at an annealing temperature of 62 °C for 20 s. Amplicons were purified by mixing 19 µl of amplicon with 10U Exonuclease I from *Escherischia coli* (Fermentas, Maryland USA) and 2U FASTAP™ Thermosensitive alkaline phosphatase (Fermentas, Maryland USA) at 37 °C for 15 min. The reaction was stopped by incubation at 85 °C for 15 min. The purified PCR products were subjected to cycle sequencing using the Big Dye® Terminator v3.1 Cycle Sequencing kit and Big Dye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. For identification purposes sequencing was only conducted in one direction using the A2 primer (Hocquellet et al. 1999) The extension products were purified by ethanol/sodium-acetate precipitation, and then sequenced at the core sequencing facility of the University of Pretoria on an ABI Prism 3100/3130 sequencer (Applied Biosystems, Foster City, CA).

Sequence analysis

Nucleotide sequence was analyzed using the DNAMAN software suite (Lynnon Biosoft, Quebec, Canada). Sequences of the A2/J5 amplicons were prepared based on the sequence in the single orientation obtained by using the A2 primer. Nucleotide similarity searches of Genbank were conducted using the BLAST algorithm of the National Centre for Biotechnology Information. Amplicon sequences were compared to cognate regions of the following Genbank accessions for Laf (LAU09675); LafC (AF248498), Lam (EF122254), Las (M94319), and a recently discovered Liberibacter from tomato, Lso (EU834131) (Liefting et al. 2009). Multiple alignments were

prepared in DNAMAN and alignments were used to prepare phylogenetic trees using a maximum likelihood method (Hasegawa et al. 1985; Tamura and Nei 1993).

Natural spread of LafC to citrus

This trial was located at a farm near Sunlands, Sundays River Valley, Eastern Cape (33°30.407 S, 25°38.750 E, Elevation 28 m), where two *C. capense* trees had earlier been found to be infected with LafC. These trees were part of a *C. capense* bordered lane of 72 *C. capense* trees of one row on either side. The lane bisected a 15-year old Citrus orchard (Midnight Valencia) with the closest citrus row being 8 m from the C. capense lane. The planting of the *C. capense* trees predated that of the citrus by an unknown period. Leaf and petiole samples were collected from 44 *C. capense* specimens, representing all of those in the lane bisecting the first citrus orchard. Leaf and petiole samples of 273 citrus specimens representing two rows of citrus on either side of the *C. capense*–bordered lane were also collected. All Citrus and *C. capense* nucleic acid extracts were subjected to Liberibacter general real-time PCR tests. This was followed by conventional PCR (Hocquellet et al. 1999) and sequencing as described above on those with Ct values of less than 30 in real-time PCR.

Results

It was difficult to locate naturally occurring *C. capense* specimens, and only 44 of the 278 specimens collected (Table 1) were found in natural indigenous forests. Amongst the trees considered "planted", a number of old, large specimens were found and some of these may have originally occurred naturally, with buildings, roads and gardens subsequently being developed around them. However as it was difficult to determine the history of these they are classified as "planted" for the purpose of this article.

"*Candidatus* Liberibacter africanus" (Laf) was not detected in any of the *C. capense* trees tested. "*Ca.* Liberibacter africanus spp. capensis (LafC) positive samples however were detected throughout the regions sampled including regions considered free of Laf on citrus e.g. Eastern Cape (Fig. 1, Table 1).

No-template and healthy *C. capense* controls and most unknown samples yielded Ct values above 38 in the LibUF primer based Liberibacter real-time PCR designed to detect known Liberibacters ("Generic Liberibacter real-time PCR"), and the test cannot be used to reliably detect Liberibacters at low concentrations, but is useful as an initial screening test when Ct values below 30 are utilized. Thus all samples were tested with a LafC specific real-time PCR tests. A good correlation existed between the two real-time PCR systems with samples below a Ct of 35 in the Generic Liberibacter real-time PCR yielding similar Ct values compared to the LafC specific real-time PCR. When compared to the conventional PCR based on the primers of Hocquellet et al. (1999), samples with Ct values of less than 30 yielded amplicons visible on agarose gels. While this may reflect only the expected lower sensitivity of the conventional PCR system, this value was selected as the positive/negative threshold for LafC in order to avoid any false positives. Based on this positive/negative threshold in the Generic Liberibacter and LafC real-time PCRs, 100 of the 278 samples analyzed were infected with LafC (Table 1).

In order to confirm the identity of the Liberibacter found using the Generic Liberibacter real time PCR and the LafC-specific real-time PCR, the amplicon of the A2/J5 conventional PCR (part of the rplA-rplJ genes) of the first 17 samples (Phahladira 2010) and at least one representative sample from each area was sequenced. All these samples yielded sequences in this part of the genome indicative of LafC (Fig. 2).

While no obvious symptom could be consistently associated with LafC infection of *C. capense*, a number of infected trees displayed a sparseness of foliage. Leaves from such trees tended to be curled backwards along the main vein axis with a marginal chlorosis which gives the whole tree a distinct yellow caste. Various leaf mottles (Fig. 3), generally absent from trees which tested negative for LafC, were also found on individual infected trees but could not be consistently correlated with LafC presence, and may represent a more advanced stage of infection or higher bacterial titres in only a few specimens, or could be due to other biotic or even abiotic disorders.

Forty-three of the 44 *C. capense* trees in the lane bisecting the "Midnight Valencia" orange orchard had Ct values less than 30 (average 21.38; standard deviation 3.89) with only one sample (Accession 10–2250) having a Ct value >30 (Ct=32.75) after being tested using a Generic Liberibacter detection real-time PCR assay. The identity of the Liberibacter present was

Table 1 Number of samples from sites in South Africa indicated in Fig. 1 testing positive for "*Candidatus* Liberibacter africanus spp. capensis" (LafC). Ct values of lower than 30 were considered positive for LafC following real time PCR tests on DNA extracts from naturally occurring and planted specimens of *Calodendrum capense* samples

Province	Site number (Fig. 1.)	District	Planted	Natural	Samples at site	Samples with Ct <30	Sequenced sample
Eastern Cape	1	Boesmansrivermond	7	0	7	0	–
	2	Fort Beauford	9	2	11	0	–
	3	Grahamstown	19	0	19	9	10–0047
	4	Hankey	5	0	5	2	09–0875
	5	Kenton-on-sea	1	0	1	0	–
	6	Patensie	13	10	23	12	10–0029
	7	Port Elizabeth	2	0	2	0	–
	8	Sundaysriver valley	11	2	13	8	10–0093
	9	Uitenhage	6	9	15	1	10–0014
Gauteng	10	Johannesburg	2	0	2	1	–
	11	Pretoria	57	0	57	26	08–1010
KwaZulu-Natal	20	Bulwer	0	3	3	0	–
	19	Durban	3	0	3	1	10–2540
	18	Fort Nottingham	0	2	2	0	–
	21	Ixopo-Kokstad	0	3	3	1	09–0760
	22	Pietermaritzburg	16	0	16	4	09–0754
Limpopo	12, 13	Alma/Nylstroom	0	5	5	5	09–0722
	14	Letsitele	18	0	18	14	09–0738
	15	Magoebaskloof	2	2	4	0	–
	16	Tzaneen	4	0	4	0	–
Mpumulanga	17	Nelspruit	2	0	2	1	07–0608
Western Cape	23	Cape Town	17	0	17	3	09–0824
	30	George	2	0	2	0	–
	24	Kleinmond	1	0	1	1	09–0826
	28, 29	Knysna/Wilderness	19	6	25	11	11–4047
	26	Montagu	5	0	5	0	–
	25	Plettenberg Bay	11	0	11	0	–
	27	Stellenbosch	2	0	2	0	–
Totals			234	44	278	100	

confirmed to be LafC in 36 samples by unidirectional direct sequencing of the entire amplicon from conventional PCR, using primer A2 (Hocquellet et al. 1999) to initiate the reaction (Fig. 4). No amplicons were obtained in conventional PCR using sample 10–2250 as template. Sequence data from the remaining seven samples did not span the entire amplicon length, but in all instances the partial amplicon sequence generated was most closely related to LafC (Genbank Ref. AF248498) when using the BLAST algorithm, and were essentially identical. Three representative sequences amongst the 36 amplicon full length sequences generated were deposited with Genbank with the following accession numbers (JF419553, JF419554, and JF419555). None of the citrus trees tested had Ct values of less than 30, while the majority yielded Ct values between 32 and 35 (average 33.42; standard deviation 1.59) following a Generic Liberibacter detection real-time PCR. Conventional A2/J5 PCR (Hocquellet et al. 1999) on 28 of the citrus samples with the lowest Ct values (higher than 30) did not yield any amplicons and the samples are all considered negative for Liberibacters including LafC. No evidence of *Trioza erytreae* feeding or breeding

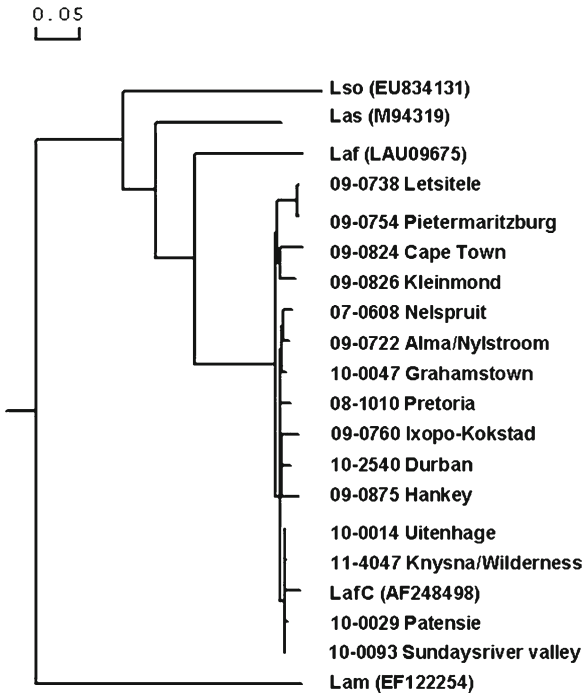

Fig. 2 Comparison of "*Ca.* Liberibacter" sources collected from *Calodendrum capense* specimens from various parts of South Africa following alignments of nucleotide sequences of part of the ribosomal protein genes rplA to rplJ of each source with known "*Ca.* Liberibacter species". Phylogenetic tree obtained using a maximum likelihood distance model and the Hasegawa et al. (1985) nucleotide substitution model. Laf = "Ca. Liberibacter africanus"; LafC = "Ca. Liberibacter africanus spp. capensis"; Lam = "Ca. Liberibacter americanus"; Las = "Ca. Liberibacter asiaticus"; and Lso = "Ca. Liberibacter solanacearum"

was observed during two inspections of the citrus and *C. capense* trees, but the vector is known to occur in this region and over the 15 years of co-existence of the citrus and the *C. capense* trees, numerous outbreaks of *T. erytreae* have probably occurred.

Discussion

During 1995 "*Candidatus* Liberibacter africanus" (Laf) was detected on lemons and clementines in the Western Cape, an area previously free of Greening (Garnier et al. 1999). While investigating the site where this first Laf outbreak occurred, two *C. capense* trees bordering the orchard were found exhibiting leaf mottle. Samples were taken from these trees to ascertain whether they were infected with Laf. PCR, followed by sequencing showed that they were infected with a Liberibacter closely related, but distinct from, Laf. A subspecies status, and the name "*Candidatus* Liberibacter africanus spp. capensis" (LafC) was proposed for the bacteria (Garnier et al. 2000), although the distribution and host range has not been ascertained (Garnier et al. 1999). The biological properties of LafC remain unknown. Furthermore, it has not been established whether *C. capense* can harbour Laf. The recommended control method for greening involves removing infected trees so it is very important to determine whether any of the plants in the vicinity of citrus orchards can harbour Laf. Due to the close

Fig. 3 Detached leaves of *Calodendrum capense* displaying various mottles. Uppermost left leaf from a healthy, greenhouse grown plant

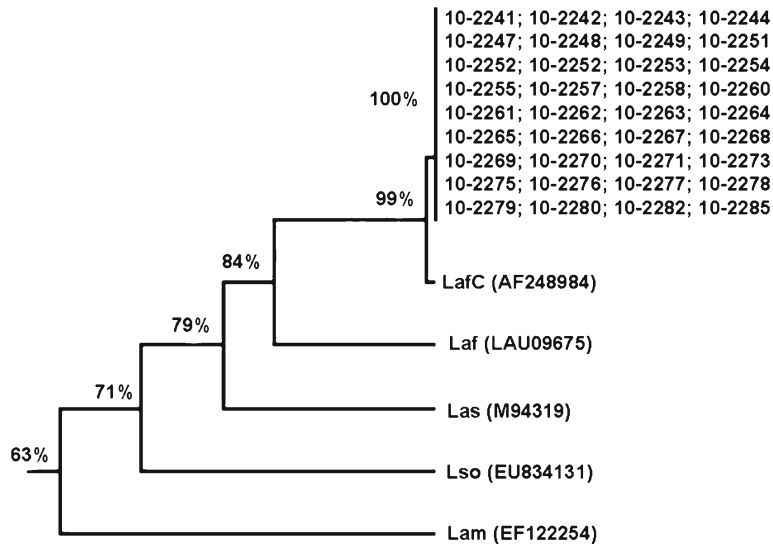

Fig. 4 Dendrogram of observed divergency of partial ribosomal protein genes rplA to rplJ of "*Candidatus* Liberibacter africanus spp. capensis" (LafC) sources from *Calodendrum capense* from a trial site in the Eastern Cape where a lane of *Calodendrum capense* trees bisects a Midnight Valencia orchard (accession numbers between 10 and 2241 and 10–2285). Laf = "*Ca.* Liberibacter africanus"; LafC = "*Ca.* Liberibacter africanus spp. capensis"; Lam = "Ca. Liberibacter americanus"; Las = "*Ca.* Liberibacter asiaticus"; and Lso = "*Ca.* Liberibacter solanacearum". The observed, slight divergence between the reference Laf sequence (AF248984) and the sources obtained in this study are primarily due to a number of unresolved nucleotides occurring in the reference sequence

relatedness of LafC to Laf (Garnier et al. 1999; Garnier et al. 2000), and the frequent proximity of citrus to *C. capense* trees, the potential role of *C. capense* as an alternate host of Laf needs to be ascertained. Furthermore, the presence of only Laf and the complete absence of LafC in citrus samples collected throughout South Africa (Pietersen et al. 2010) suggested that LafC does not contribute to greening disease of citrus here and prompted us to investigate whether the initial discovery of LafC in *C. capense* represented a rare infection, with LafC possibly being confined to a highly localized region in the Western Cape, and hence its absence in commercial citrus in South Africa.

No evidence of Laf infection of *C. capense* trees was found but the survey showed that *C. capense* are often infected with LafC throughout South Africa, including in regions where Laf is not found in citrus. LafC occurred in 38 % of the 278 *C. capense* trees tested, occurring in natural settings and when planted.. The bacterium is not restricted to the Western Cape and the initial discovery of this pathogen in Stellenbosch by Garnier et al. (1999) was more likely due to its relative abundance and wide distribution in *C. capense* rather than being associated with the first outbreak of Laf on citrus in the Western Cape.

The absence of Laf in *C. capense* in citrus greening affected areas, the presence of LafC infected *C. capense* trees in Laf-free citrus production areas, the absence of LafC in citrus samples tested throughout South Africa (Pietersen et al. 2010), and the lack of LafC infected citrus in the orchard adjoining the first *C. capense* from which LafC was reported (Garnier et al. 2000), suggests that transmission of Laf and LafC between citrus and *C. capense* is extremely rare or does not occur. It is important that controlled reciprocal graft transmission experiments between LafC on *C. capense* and Laf on citrus are done to assess whether the two tree species can serve as hosts of both bacteria. Further investigation into the vector of LafC is also needed. No natural spread of LafC to citrus was observed at the site where LafC-infected *C. capense* trees bordered a road bisecting two citrus orchards of Midnight Valencia trees. The vector of Laf, *T. erytreae* occurs naturally in this area and is commonly observed and controlled by systemic insecticides by citrus producers. However, despite two surveys for *T. erytreae* no individuals or tell-tale nymph-induced leaf depressions were observed. Of 44 *C. capense* trees tested, 43 yielded Ct values ≤30. The identity of the Liberibacter present was shown to be LafC in 36 of these trees by performing conventional PCR targeting

the ribosomal protein rplA and rplJ genes of Liberibacters (Hocquellet et al. 1999) and sequencing the resultant full length amplicons. In the remaining seven samples only partial amplicon sequences were obtained but these were most closely related to LafC after performing a BLAST analysis. None of the 273 citrus trees tested positive for any Liberibacters.

We previously demonstrated that LafC is not seed-transmitted at an incidence detectable by PCR tests in two experiments on 34 and 35 seedlings respectively, grown from seed from two LafC infected *C. capense* trees (Pietersen and Viljoen, *unpublished results*). Hence LafC infection of *C. capense* in these areas is most likely due to a vector feeding on *C. capense*. No infection of citrus by LafC was observed close to the infected *C. capense* trees. While *T. erytreae* is a vector of Laf, and therefore a likely candidate as vector for LafC, Moran (1968) observed that *C. capense* leaves attract the adult citrus psyllid for feeding but are not suitable for nymph development. Aubert (1987) also demonstrated that the psyllid feeds on *C. capense* but is not able to complete its life cycle on this host. The fact that it does not complete its life cycle on *C. capense* but merely feeds on this host does not eliminate *T. erytreae* from being a vector for LafC but may make transmission rare or inefficient. The lack of evidence of natural transmission to citrus reported here supports the conclusion that LafC does not readily infect citrus trees and that it does not pose a threat to local citriculture, but needs to be confirmed through controlled transmission experiments.

It has been hypothesized that Laf was present on the African continent before the introduction of citrus, possibly in an indigenous Rutaceous species (da Graça 2008) such as *Vepris sp*. (Beattie et al. 2008), as this is the only indigenous host where the presence of Laf has been confirmed (Korsten et al.1996). While we found no evidence of Laf infection on *C. capense* or of natural spread of LafC to citrus, LafC may represent a "parent lineage" from which Laf emerged through a rare host species "jumping" event followed by selection in citrus. The absence of a clearly associated, severe disease in *C. capense* infected with LafC (Phahladira 2010; this study) suggests a pathogen that has co-evolved with its host. Furthermore, the widespread occurrence of the bacteria in *C. capense* specimens in widely separated and often isolated natural locations in South Africa also suggests a long association with *C. capense* independent of citrus. The occurrence of LafC on *C. capense* in citrus producing parts of South Africa which are free of greening also suggests that LafC on *C. capense* is not due to a transmission and host adaptation event from a Laf infection in citrus to *C. capense*. Much more information is required regarding the Liberibacter status of indigenous Rutaceous plants in Africa, and studies on this aspect are continuing.

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