A Survey for '*Candidatus Liberibacter*' Species in South Africa Confirms the Presence of Only '*Ca. L. africanus*' in Commercial Citrus

G. Pietersen, University of Pretoria, Pretoria, 0002, South Africa; Citrus Research International, P.O. Box 28, Nelspruit 1200 S.A.; and ARC-Plant Protection Research Institute, Private Bag X134, Queenswood 0121, S.A.; **E. Arrebola**, University of Pretoria; **J. H. J. Breytenbach**, Citrus Research International; **L. Korsten**, University of Pretoria; **H. F. le Roux**, Citrus Research International; **H. la Grange**, Capespan, P.O. Box 505, Bellville, 7535 S.A.; **S. A. Lopes**, Fundecitrus, Araraquara, SP, Brazil; **J. B. Meyer** and **M. C. Pretorius**, Citrus Research International; **M. Schwerdtfeger**, University of Pretoria; **S. P. van Vuuren**, Citrus Research International; and **P. Yamamoto**, Fundecitrus

ABSTRACT

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Greening disease of citrus is a serious disease known in South Africa since the late 1920s. In South Africa, it is associated with infection by '*Candidatus Liberibacter africanus*', a heat sensitive, phloem-limited, noncultured alpha-proteobacterium. Huanglongbing (HLB), a similar, but more devastating disease that was described initially from China but which now occurs in several citrus producing countries, is associated with a different *Liberibacter* species, '*Ca. L. asiaticus*'. A '*Ca. L. africanus*' subspecies, '*Ca. L. africanus* subsp. *capensis*', has been found only in South Africa infecting an indigenous Rutaceous species, *Calodendrum capense* (Cape Chestnut), in the Western Cape in 1995. The discovery of a new *Liberibacter* species in Brazil, '*Ca. L. americanus*', and the spread of '*Ca. L. asiaticus*' to a number of additional countries over the last few years prompted us to assess whether only '*Ca. L. africanus*' is present in commercial citrus orchards in South Africa. Samples displaying greening or similar symptoms were collected from 249 citrus trees from 57 orchards distributed throughout the greening affected citrus production areas of South Africa. Multiplex polymerase chain reaction (PCR) was performed on DNA extracts to detect the known citrus *Liberibacter*s. Amplicons were obtained from 197 samples. None of the samples yielded a 1,027-bp amplicon indicative of '*Ca. L. americanus*' infection. The amplicons of 84 samples were sequenced, and all were identical to the cognate '*Ca. L. africanus*' Nelspruit sequence in GenBank. No instance of '*Ca. L. asiaticus*' or '*Ca. L. africanus* subsp. *capensis*' sequence was found. Geographically representative samples that tested negative for *Liberibacter* also tested negative for phytoplasmas based on real-time PCR results. Based on the results of this survey, it is concluded that to date only '*Ca. L. africanus*' is associated with citrus greening in commercial citrus in South Africa.

A serious, debilitating, insect-transmissible citrus disease known in South Africa since the late 1920s as greening disease of citrus (29) is associated locally with '*Candidatus Liberibacter africanus*', a heat sensitive, phloem-limited, noncultured alpha-proteobacterium (17,19). This is in contrast to huanglongbing (HLB) (5) disease, initially described from China and now present in a number of other countries, which is associated with infection by '*Ca. L. asiaticus*'. Greening, therefore, refers to the '*Ca. L. africanus*'-associated disease throughout the rest of this article. Greening is considered heat sensitive, as

the diseased plant appears to recover from symptoms at 32°C but not at 27°C (4). '*Ca. L. africanus*' was shown to be present in South Africa in the Rustenburg, Nelspruit, and Western Cape regions during 1998 (10). In addition, a second *Liberibacter* species, '*Ca. L. africanus* subsp. *capensis*', was detected infecting an indigenous Rutaceous species, *Calodendrum capensis* (Cape Chestnut), in the Western Cape in 1998 (12).

Polymerase chain reaction (PCR) tests on samples from South Africa confirmed the presence of '*Ca. L. africanus*' and the absence of the more destructive '*Ca. L. asiaticus*' (16). However, since 2004, '*Ca. L. asiaticus*' has spread to citrus orchards in, among other countries, Brazil (8), Cuba (26), the Dominican Republic (28), and Florida, USA (13), where it was not previously found; and a new *Liberibacter* species, '*Ca. L. americanus*', was found on citrus in Sao Paulo State, Brazil in 2004 (34). '*Ca. L. americanus*' is similar to '*Ca.*

L. africanus' with regard to its heat sensitivity (25). A new *Liberibacter* species has also recently been discovered on solanaceous hosts in the United States and New Zealand (14,23,24). Recently, *Liberibacter*-negative trees displaying HLB-like symptoms were found in Brazil (35) and in China (6) and were shown to be infected by phytoplasmas. HLB, caused by '*Ca. L. asiaticus*', is a far more debilitating disease than greening, with trees often dying within a few years, whereas '*Ca. L. africanus*' most often will only affect a limited number of branches of a tree for many years (S. P. van Vuuren, *personal communication*). Furthermore, while greening's geographical distribution is limited by its heat-sensitive nature, HLB would affect citrus trees in warmer citrus-production areas in South Africa that are currently unaffected by greening. South Africa's citrus industry is primarily export based, and while ranked as the 14th largest producer in the world by volume, it is the third largest exporter of citrus. A premium is therefore placed on the production of good quality fruit, and the introduction of '*Ca. L. asiaticus*' would severely affect the industry.

As previous surveys in South Africa for greening were done prior to the advent of *Liberibacter* species-specific PCR-based diagnostic tests, this study was conducted to assess whether *Liberibacter* species other than '*Ca. L. africanus*' may have been overlooked in past studies and may already occur in commercial citrus in South Africa.

MATERIALS AND METHODS

Collection of samples. Because of the heat sensitive nature of symptom expression by '*Ca. L. africanus*' and '*Ca. L. americanus*', the survey was conducted during September 2006, as this coincided with the visits of Prof. J. M. Bové from Bordeaux, France and Brazilian co-authors who are well versed in HLB, '*Ca. L. americanus*'-induced disease, and greening. Samples were collected from citrus trees displaying greening, HLB-like, or greening-like symptoms from 57 orchards in 17 magisterial districts representing all

Corresponding author: G. Pietersen
E-mail: gerhard.pietersen@up.ac.za

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the major citrus-production regions of South Africa in which greening disease is known to occur (Fig. 1). Orchards were selected based on having a known history of greening spread. As the incidence of greening is generally low in many of the regions sampled (notable exceptions being Nelspruit and Rustenburg), samples were collected from trees found randomly by the collection team moving through the orchards without a predetermined pattern. Sample numbers at any given site were limited by the low incidence of symptomatic trees found. Where a high enough disease incidence occurred in a given orchard, larger numbers of samples from different symptomatic trees (up to a maximum of 10) were collected. A sample consisted of approximately 20 symptomatic leaves and budwood sticks from an individual tree and was collected from various branches on the tree that displayed symptoms.

Extraction of DNA for PCR. Total DNA was extracted from 0.5 g of petiole or midrib from each field-collected sample using a standard CTAB (hexadecyltrimethylammonium bromide) procedure (9).

Liberibacter PCR amplification. A multiplex PCR for the simultaneous detection of '*Ca. L. asiaticus*', '*Ca. L. afri-*

canus', and '*Ca. L. americanus*' was utilized which combined the previously published A2/J5 (16) and GB1/GB3 (33) primer sets directed at the β -operon of the ribosomal protein genes of '*Ca. L. africanus*' and '*Ca. L. asiaticus*', and the 16S rDNA of '*Ca. L. americanus*', respectively. The PCR was carried out in duplicate on DNA extracted from field-collected samples and was performed in a 25- μ l reaction mixture consisting of 0.5 μ M of each of the four primers, 200 μ M of each of the four dNTPs, 67 mM Tris-HCl, pH 8.8, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Tween 20 (NH_4 reaction buffer, Bioline, London, UK), 10 mM β -mercaptoethanol, 10 μ g Bovine serum albumin, 2 mM MgCl_2 , 0.1% Triton X-100, and 2.5 U *Taq* polymerase (Biotaq, Bioline). A 0.5- μ l volume of DNA extract was used as template in the reaction. Amplification was conducted with an ABI Geneamp 2700 thermocycler (Applied Biosystems, Foster City, CA), and the conditions were 35 cycles of denaturation at 92°C for 20 s, annealing at 63°C for 20 s, and primer extension at 72°C for 45 s. Amplicons were visualized on a UV transilluminator by ethidium bromide staining after electrophoresis at a constant voltage of 150V for 60 min in 2% agarose in sodium borate buffer (5 mM disodium borate decahydrate, adjusted to pH 8.5 with boric

acid) gels. Total DNA extracts from plants known to be infected by '*Ca. L. asiaticus*', '*Ca. L. americanus*', and '*Ca. L. africanus*' were supplied by C. Saillard (INRA, Bordeaux) as positive controls in PCR.

Nucleotide sequencing. To obtain templates for sequencing, samples representative of each production site were amplified using the A2/J5 primer set (16). Reaction conditions were identical to those described for PCR for detection of *Liberibacter* except cycling conditions were conducted at an annealing temperature of 62°C for 20 s. Amplicons were electrophoresed on a 1.0% agarose gel followed by ethidium bromide staining. Desired bands were cut out and purified with a Wizard SV Gel and PCR clean up system (Promega, Madison, WI). 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) according to the manufacturer's specifications. The same forward and reverse primers used for the PCR amplification were used separately in cycle sequencing in order to sequence both sense and anti-sense strands. The extension products were purified by ethanol/sodium acetate precipitation, and then sequenced at the core sequencing facility of the Uni-

Fig. 1. Map of South Africa illustrating sites where samples were collected and showing number of samples collected per site. Main towns within South Africa are indicated by unfilled squares, and filled circles with numbers indicate the number of plant samples collected at the sites named in Table 1.

versity of Pretoria on an ABI Prism 3100/3130 sequencer (Applied Biosystems).

Sequence analysis. Nucleotide sequence was analyzed using the DNAMAN software suite (Lynnon Biosoft, Quebec, Canada). Consensus sequences of the A2/J5 amplicons were prepared based on the sequence in both orientations. Nucleotide similarity searches of GenBank were conducted using the BLAST algorithm (1) of the National Centre for Biotechnology Information. Amplicon sequences were compared to cognate regions of the following GenBank accessions for ‘*Ca. L. africanus*’ (LAU09675), ‘*Ca. L. africanus* subsp. *capensis*’ (AF248498), ‘*Ca. L. americanus*’ (EF122254), ‘*Ca. L. asiaticus*’ (M94319), and a recently discovered liberibacter from tomato, ‘*Ca. L. solanacearum*’ (EU834131) (24). Multiple alignments were prepared in DNAMAN, and alignments were used to prepare phylogenetic trees using a maximum likeli-

hood method with mutation rates estimated from the actual sequences (15,32).

Graft propagation of Liberibacter from field samples. Budwood collected from field collected sources were used to establish the greening samples by side-grafting of two to three buds onto a single plant of sweet orange (*Citrus sinensis* cv. Bahianinha navel) on Carrizo citrange (*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osbeck) rootstock. Inoculated plants could also serve as indicator plants. Plants were maintained in an insect-proof greenhouse under natural light conditions at temperatures between 24 and 28°C. The grafted plants were tested for ‘*Ca. L. africanus*’ after 1 year using real-time PCR (22) using as template 1 µl of the eluant from leaf petioles macerated in an alkaline buffer and spotted onto nitrocellulose membranes (2). 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 ‘*Ca. L. africanus*’-specific primers and probes and conditions described by Li et al. (22).

Detection of phytoplasma with PCR. Of the 52 samples collected from symptomatic trees that yielded negative multiplex PCR results for all three Liberibacter species, 22 samples, one from each site, were tested for the presence of phytoplasmas. PCR was done using the Universal phytoplasma specific Taqman probe and primer sequences previously published (7) using slightly modified conditions. Briefly, each sample was tested in duplicate in 20-µl glass capillaries in a Lightcycler 1.5 apparatus (Roche) in a reagent mixture of 0.5 µM of each of Phytop Forward and PhytoP Reverse primers, 0.25 µM of Phytop Probe in a 1× Taqman Universal PCR master mix (Roche). Cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 92°C for 15 s, 60°C for 60 s, and 72°C for 1 s. Single acquisition fluorescence readings were taken at the conclusion of each cycle, and data processing and threshold cycle (Ct) calculations were done with the Lightcycler instrument software. Positive controls included in all real-time tests were of Bois Noir phytoplasma obtained from Boudon-Padieu (INRA, France).

RESULTS

A total of 249 samples were collected from 57 groves in 17 magisterial districts (Table 1). The number of orchards monitored and the type and number of samples collected per district are shown in Table 1. Samples included leaf material with one or more of the obvious greening symptoms, as agreed upon by most members of the survey team, as well as samples with more obscure symptoms that were collected primarily to ensure the detection of any Liberibacter associated with possible symptom variants of the disease. Graft transmission of Liberibacter to Bahianinha navel sweet orange was very inefficient, with only 12 recipients becoming infected. Real-time ‘*Ca. L. africanus*’-specific PCR performed on extracts from these trees yielded Ct values ranging from 24 to 35 one year postinoculation. This low level of transmission was likely due to the fact that symptomatic branches did not necessarily have budwood of a physiological state adequate for efficient grafting, as poor bud take was experienced, with only 34 buds taking. No instances were obtained where graft inoculated trees displayed disease symptoms or contained ‘*Ca. L. africanus*’ when budded with field collected material that tested negative for ‘*Ca. L. africanus*’ by multiplex PCR.

Of the 249 samples collected and tested in the multiplex PCR, none yielded the 1,027-bp amplicons expected from a ‘*Ca. L. americanus*’-infected plant. Amplicons were obtained from 197 samples. The majority of these were the same size as the amplicon obtained with the ‘*Ca. L. afri-*

Table 1. Results of sampling for ‘*Candidatus Liberibacter*’ species in citrus samples collected from various locations in South Africa

District	Scion	Orchards sampled/ district/scion	Samples/ district/ scion	Infected samples
Brits	<i>Citrus reticulata</i> Blanco cv. Nova	1	2	2
	<i>C. sinensis</i> Osbeck cv. Valencia	2	12	10
Caledon	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	6	6
	<i>C. reticulata</i> Blanco cv. Nules	1	5	4
Groblersdal	<i>C. sinensis</i> Osbeck cv. Robyn Navel	1	1	0
	<i>C. sinensis</i> Osbeck cv. Washington Navel	3	9	4
Hoedspruit	<i>C. paradisi</i> Macfad. cv. Marsh	1	1	1
	<i>C. sinensis</i> Osbeck cv. Delta Valencia	1	6	6
	<i>C. sinensis</i> Osbeck cv. Du Roi Valencia	1	1	0
	<i>C. sinensis</i> Osbeck cv. Valencia	2	9	9
	<i>C. sinensis</i> Osbeck cv. Valencia	5	7	0
Malelane	<i>C. sinensis</i> Osbeck cv. Valencia	2	7	0
	<i>C. sinensis</i> Osbeck cv. Navel	1	1	0
Melmouth	<i>C. sinensis</i> Osbeck cv. Valencia	1	1	0
	<i>C. reticulata</i> Blanco cv. Afurer	1	12	8
Montagu	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	1	1
	<i>C. limon</i> (L.) Burm. f. cv. Limonera	1	2	1
	<i>C. sinensis</i> Osbeck cv. Delta Valencia	1	10	10
	<i>C. sinensis</i> Osbeck cv. Midnight Valencia	1	5	4
	<i>C. sinensis</i> Osbeck cv. Valencia	1	10	9
Nelsons	<i>C. sinensis</i> Osbeck cv. Delta Valencia	1	6	6
	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	8	4
Paarl	<i>C. reticulata</i> Blanco cv. Satsuma	1	2	2
	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	6	6
Rustenburg	<i>C. limon</i> (L.) Burm. f. cv. Fino	1	1	0
	<i>C. reticulata</i> Blanco × <i>C. paradisi</i> Macfad.	1	6	5
	<i>C. reticulata</i> Blanco cv. Clemantine	1	1	0
	<i>C. sinensis</i> Osbeck cv. Delta Valencia	1	5	5
	<i>C. sinensis</i> Osbeck cv. Midnight Valencia	1	2	2
	<i>C. sinensis</i> Osbeck, cv. Not recorded	1	5	2
	<i>C. sinensis</i> Osbeck cv. Palmer Navel	2	20	19
	<i>C. sinensis</i> Osbeck cv. Robyn Navel	1	6	6
	<i>C. sinensis</i> Osbeck cv. Valencia	5	18	18
	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	8	8
	<i>C. sinensis</i> Osbeck cv. Navelina Navel	1	10	9
	<i>C. sinensis</i> Osbeck cv. Delta Valencia	1	1	1
Tzaneen	<i>C. sinensis</i> Osbeck cv. Midnight Valencia	3	10	10
	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	11	4
Wellington	<i>C. limon</i> (L.) Burm. f. cv. Eureka	1	6	6
Whiteriver	<i>C. sinensis</i> Osbeck cv. Delta Valencia	1	2	2
Zebediela	<i>C. sinensis</i> Osbeck cv. Palmer Navel	1	7	7
Totals		57	249	197

canus' control. However, when high yields of amplicons were obtained, electrophoresis conditions could not adequately differentiate the 669-bp band expected of '*Ca. L. africanus*' and the 703-bp band expected of '*Ca. L. asiaticus*' (16). The differing yields of these amplicons, as indicated by staining and comparison to positive controls, suggest possible differences in *Liberibacter* amounts in sample tissues.

Eighty-four amplicons generated by multiplex PCR, selected mainly, but not exclusively, from those where the amplicon size could not be determined accurately due to the thickness of the band, were subjected to cycle sequencing using the A2 and J5 primers to ensure sequence data of both orientations. Sequence analysis confirmed that all sequenced samples were infected with '*Ca. L. africanus*' only. Forty sequences were identical to each other and to the reference '*Ca. L. africanus*' Nelspruit Isolate (LAU09675). The lowest identity among all isolates was 98.7%. The sequence of 13 geographically representative sources were submitted to GenBank. These are Rustenburg 06-0137 (GU120032), Brits 06-0204 (GU120033), Groblersdal 06-0224 (GU120034), Zebedeila 06-0234 (GU120035), Tzaneen 06-0241 (GU120036), Hoedspruit 06-0256 (GU120037), Nelspruit-2 06-0271 (GU120038), Whiteriver 06-0292 (GU120039), Swellendam 06-0307 (GU120040), Caledon 06-0330 (GU120041), Stellenbosch 06-0338 (GU120042), Paarl 06-0351 (GU120043), and Wellington 06-0354 (GU120044). A phylogenetic tree is presented in Figure 2 in which the A2/J5 amplified sequences of 13 samples from infected districts are compared to cognate sequences of known *Liberibacter* species.

None of the 22 samples that had displayed greening-like symptoms but tested negative in the *Liberibacter* multiplex PCR were positive in the phytoplasma real-time PCR test.

DISCUSSION

Only '*Ca. L. africanus*' was detected among the commercial citrus samples during this survey. Therefore, it would appear that the other citrus *Liberibacter*s are still absent from commercial citrus plantings in South Africa. This allays concerns that previous studies done on South African samples may have missed the presence of these other *Liberibacter*s, especially '*Ca. L. americanus*', due to the limitations of techniques available at the time and the relatively low number of samples previously tested for '*Ca. L. asiaticus*' specifically. Symptoms of '*Ca. L. africanus*' and '*Ca. L. americanus*' differ from those of '*Ca. L. asiaticus*' primarily by severity and temperature tolerance (5,25,31). Consequently, it is possible that the symptoms induced by '*Ca. L. africanus*' in South Africa may mask the presence of another *Liberibacter*, especially

'*Ca. L. americanus*', for a significant period. In addition, the presence of a phytoplasma associated with citrus trees displaying greening-like symptoms in Brazil and China (6,35) may indicate that the use of symptoms only to identify the disease may result in erroneous conclusions as to the causal organism. It was therefore essential that a survey be conducted in South Africa with techniques more sensitive and specific for the different *Liberibacter*s.

Specific detection of '*Ca. L. africanus*' was first accomplished in the early 1990s with monoclonal antibodies (11,20), and then later with DNA probes (30). A PCR specific for '*Ca. L. africanus*' (18) was used, along with DNA hybridization which was able to detect '*Ca. L. africanus*' and '*Ca. L. asiaticus*', to confirm the presence of only '*Ca. L. africanus*' in South Africa between 1993 and 1996 (19). During 1998, 82 samples from Nelspruit, Rustenburg, and the Eastern and Western Cape were tested by '*Ca. L. africanus*'-specific PCRs and resulted in the first detection of '*Ca. L. africanus*' in the Western Cape along with detection of '*Ca. L. africanus* subsp. cap-

ensis' infecting *Calodendrum capense* (10,12).

A number of samples collected proved to be negative for '*Ca. L. africanus*' and phytoplasmas. This probably can be ascribed to the collection of samples from trees with greening or HLB-like symptoms that were possibly due to various nutrient deficiencies rather than to *Liberibacter* infection. Although some samples possibly were collected unnecessarily, sample collection based upon visual assessment is preferred to prevent possibly missing an unusual symptom potentially induced by a *Liberibacter* other than '*Ca. L. africanus*'.

Our finding that only '*Ca. L. africanus*' occurs in the commercial citrus samples examined in this study in South Africa is important. The recent spread of '*Ca. L. asiaticus*' to Brazil (8,34), Florida (13), and Cuba (26) and the discovery of a new species, '*Ca. L. americanus*', on citrus in Sao Paulo State, Brazil in 2004 (34), were all preceded by the introduction of the '*Ca. L. asiaticus*' and '*Ca. L. americanus*' natural vector *Diaphorina citri*, an insect not yet reported in South Africa. However,

Fig. 2. A phylogenetic tree of nucleotide sequences amplified by A2/J5 primers from representative South African samples from the indicated districts and the cognate sequences of known *Liberibacter*s. Reference isolates and their GenBank accession numbers are: Laf = '*Candidatus Liberibacter africanus*' (LAU09675); LafC = '*Candidatus Liberibacter africanus* subsp. *capensis*' (AF248498); Lam = '*Candidatus Liberibacter americanus*' (EF122254); Las = '*Candidatus Liberibacter asiaticus*' (M94319); and Lso = '*Candidatus Liberibacter solanacearum*' (EU834131). Geographically representative South African local and GenBank accession numbers (in parentheses) are as follows: Rustenburg 06-0137 (GU120032), Brits 06-0204 (GU120033), Groblersdal 06-0224 (GU120034), Zebedeila 06-0234 (GU120035), Tzaneen 06-0241 (GU120036), Hoedspruit 06-0256 (GU120037), Nelspruit-2 06-0271 (GU120038), Whiteriver 06-0292 (GU120039), Swellendam 06-0307 (GU120040), Caledon 06-0330 (GU120041), Stellenbosch 06-0338 (GU120042), Paarl 06-0351 (GU120043), and Wellington 06-0354 (GU120044). Scale bar refers to branch length.

both *D. citri* and *Trioza erytreae*, the natural vector of '*Ca. L. africanus*' in South Africa, have been shown at least experimentally to transmit both '*Ca. L. africanus*' and '*Ca. L. asiaticus*' (21,27), and the introduction of '*Ca. L. asiaticus*' to South Africa may not require the introduction of *D. citri* to spread. However, the absence of '*Ca. L. africanus* subsp. *capensis*' in any of the citrus samples is interesting, as both occur in at least the Western Cape, where 25% of the samples were collected. It may be that the epidemiology of '*Ca. L. africanus*' and '*Ca. L. africanus* subsp. *capensis*' do not overlap. This is the subject of an additional study.

Very low nucleotide sequence variability in the genome region amplified from the '*Ca. L. africanus*' sources was found, with 40 of the 87 sequenced amplicons yielding identical sequences and the remaining samples diverging by a maximum of only 1.3% of the nucleotides. As the amplified region is part of the 50S ribosomal subunit protein gene, this high level of sequence conservation is expected. It is possible too that some differences among the samples are due to amplification and sequencing errors and that with cloning and sequencing of a number of individual clones the variation may be even less. However, serological variation among South African '*Ca. L. africanus*' populations has been demonstrated in the past, with the 10H8 and MG8 monoclonal antibodies produced against '*Ca. L. africanus*' differentially binding to different '*Ca. L. africanus*' sources (20). Studies to determine the variation that exists among '*Ca. L. africanus*' sources should target a more variable gene, for example the outer membrane protein gene (*omp*), which has been demonstrated to vary with different isolates of '*Ca. L. asiaticus*' (3).

Future studies to ensure that the Liberibacter status quo is being maintained in South Africa and that Liberibacter species reported from other crops (14,23,24) don't occur here (e.g., those from the Solanaceous crops) will require that a multiplex PCR, based on specific primers resulting in amplicons with greater size differences, will need to be developed. In the current study, it was necessary to sequence the amplicons in order to identify the different Liberibacter species.

The aim of the survey was to confirm the validity of previous conclusions from studies using more limited detection techniques that '*Ca. L. africanus*' alone is associated with greening in commercial citrus in South Africa where greening is generally well controlled and the incidence is low. As '*Ca. L. africanus*' remains the only Liberibacter found, it is essential that the South African Phytosanitary authorities and the citrus industry remain vigilant against the unintentional introduction of '*Ca. L. asiaticus*' and '*Ca. L. americanus*'. Finding a newly introduced Liberibacter in

South Africa, where greening symptoms are common, will be much more difficult than in countries where the disease symptoms would be unique. Having absolute certainty that neither '*Ca. L. asiaticus*' or '*Ca. L. americanus*' occurs in South Africa would require that practically all citrus trees showing any HLB or greening-like symptoms would have to be tested, including large numbers of backyard trees. Should '*Ca. L. americanus*', which also induces a heat sensitive disease, be introduced to South Africa, it is likely to remain undetected for a long time as its symptoms would be attributed to '*Ca. L. africanus*' infection. The introduction of '*Ca. L. asiaticus*' possibly may be observed locally as a more severe disease epidemic than greening, or as an expansion of disease to warmer production areas that are currently greening free. Producers are urged to report such an observation through numerous awareness campaigns by the local citrus industry. Should '*Ca. L. asiaticus*' or '*Ca. L. americanus*' be introduced, delineating surveys would probably have to be based on specific laboratory tests rather than on symptoms, as these would be confused with those induced by '*Ca. L. africanus*'. This would make containment and localized eradication extremely difficult if not impossible. Furthermore, due to the successful management of '*Ca. L. africanus*'-induced greening in South Africa and the associated complacency regarding this disease, it would be difficult in the period directly after introduction to convince producers to eradicate trees newly infected with either '*Ca. L. asiaticus*' or '*Ca. L. americanus*', especially the relatively mildly symptomatic '*Ca. L. americanus*'-infected trees. Nevertheless, in view of the crisis that the introduction of HLB would cause in the South African citrus industry, and the need to act as quickly as possible after its introduction, growers are encouraged to submit samples with greening-like diseases for laboratory tests. This is especially critical where such symptoms appear more severe than usual or are from citrus trees in the warmer citrus production areas.

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