Three novel lineages of '*Candidatus* Liberibacter africanus' associated with native rutaceous hosts of *Trioza erytreae* in South Africa

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Greening disease of citrus in South Africa is associated with '*Candidatus* Liberibacter africanus' (Laf), a phloem-limited bacterium vectored by the sap-sucking insect *Trioza erytreae* (Triozidae). Despite the implementation of control strategies, this disease remains problematic, suggesting the existence of reservoir hosts to Laf. The current study aimed to identify such hosts. Samples from 234 trees of *Clausena anisata*, 289 trees of *Vepris lanceolata* and 231 trees of *Zanthoxylum capense* were collected throughout the natural distribution of these trees in South Africa. Total DNA was extracted from samples and tested for the presence of liberibacters by a generic Liberibacter TaqMan real-time PCR assay. Liberibacters present in positive samples were characterized by amplifying and sequencing *rplJ*, *omp* and 16S rRNA gene regions. The identity of tree host species from which liberibacter sequences were obtained was verified by sequencing host *rbcL* genes. Of the trees tested, 33 specimens of *Clausena*, 17 specimens of *Vepris* and 10 specimens of *Zanthoxylum* tested positive for liberibacter. None of the samples contained typical citrus-infecting Laf sequences. Phylogenetic analysis of 16S rRNA gene sequences indicated that the liberibacters obtained from *Vepris* and *Clausena* had 16S rRNA gene sequences identical to that of '*Candidatus* Liberibacter africanus subsp. capensis' (LafC), whereas those from *Zanthoxylum* species grouped separately. Phylogenetic analysis of the *rplJ* and *omp* gene regions revealed unique clusters for liberibacters associated with each tree species. We propose the following names for these novel liberibacters: '*Candidatus* Liberibacter africanus subsp. clausenae' (LafCl), '*Candidatus* Liberibacter africanus subsp. vepridis' (LafV) and '*Candidatus* Liberibacter africanus subsp. zanthoxyli' (LafZ). This study did not find any natural hosts of Laf associated with greening of citrus. While native citrus relatives were shown to be infected with Laf-related liberibacters, nucleotide sequence data suggest that these are not alternative sources of Laf to citrus orchards, per se.

The agents associated with diseases such as greening and huanglongbing (HLB) of citrus (family Rutaceae), psyllid yellows of tomato (*Solanum lycopersicum*, family Solanaceae) and zebra chip of potato (*Solanum tuberosum*) are members of the bacterial genus *Liberibacter* (class *Alphaproteobacteria*, family *Rhizobiaceae*) (Jagoueix *et al.*, 1994; Fagen *et al.*, 2014). With the exception of *Liberibacter crescens*, which was isolated from the sap of a papaya (family Caricaceae) hybrid (Davis *et al.*, 2008; Fagen *et al.*, 2014), all known members of this genus are fastidious and apparently cannot be cultivated (Garnier & Bové, 1983). These bacterial taxa have accordingly been assigned the provisional status *Candidatus* (Murray & Stackebrandt, 1995), and currently include the citrus pathogens '*Candidatus* Liberibacter africanus' (Laf), causing greening (Garnier *et al.*, 2000; Pietersen *et al.*, 2010), '*Candidatus* Liberibacter americanus' (Lam) (Teixeira *et al.*, 2005a) and '*Candidatus* Liberibacter asiaticus' (Las), the causal agents of HLB (Jagoueix *et al.*, 1994; Garnier *et al.*, 2000), and '*Candidatus* Liberibacter solanacearum', associated with potato and tomato (Liefting *et al.*, 2009; Secor *et al.*, 2009).

Abbreviations: HLB, huanglongbing; Laf, '*Candidatus* Liberibacter africanus'.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *rplJ* gene and *omp* gene sequences of '*Candidatus* Liberibacter africanus subsp. clausenae', '*Candidatus* Liberibacter africanus subsp. vepridis' and '*Candidatus* Liberibacter africanus subsp. zanthoxyli' are respectively KJ152131, KJ152134 and KJ152137 (16S rRNA gene), KJ189106, KJ189105 and KJ197227 (*rplJ*) and KJ189104, KJ189103 and KJ197228 (*omp*).

The pathogens associated with greening and HLB cause symptoms suggestive of nutrient limitation, e.g. yellowing of the leaves, vigour decline and reduced fruit quality (Kapur *et al.*, 1978), which ultimately leads to dieback, stunted growth and plant death (McClean & Oberholzer, 1965; Lopes & Frare, 2008). Laf, which has so far only been identified from citrus orchards in Africa and the Mascarene islands (Garnier & Bové, 1996; Garnier *et al.*, 1996), is spread primarily through the feeding and flight activities of the triozid *Trioza erytreae* Del Guercio (order Hemiptera, family Triozidae) (McClean & Oberholzer, 1965). Lam has been reported from citrus in Brazil (Teixeira *et al.*, 2005a), while Las has been identified from citrus in Asia (Garnier & Bové, 1996), the Americas (Coletta-Filho *et al.*, 2004; Halbert, 2005) and, more recently, Ethiopia (Saponari *et al.*, 2010). Both Las and Lam are vectored by the liviid *Diaphorina citri* Kuwayama (order Hemiptera, family Liviidae) (Capoor *et al.*, 1967; Teixeira *et al.*, 2005b). *T. erytreae* and *D. citri* have also been shown experimentally to transmit both Las and Laf efficiently (Massonie *et al.*, 1976; Aubert, 1987).

Whilst being present in most citrus-producing regions in South Africa (Pretorius & van Vuuren, 2006), the incidence of greening has been lowered to economically acceptable levels through the implementation of stringent control measures involving the elimination of inoculum sources within orchards (e.g. chemical control of vectors, planting of disease-free material and the removal of infected trees and branches) (Buitendag & von Broembsen, 1993; Belasque *et al.*, 2010; Hung *et al.*, 2000; Shokrollah *et al.*, 2011). However, this strategy may be flawed if other Laf hosts exist nearby. For example, a relative of Laf known as '*Candidatus* Liberibacter africanus subsp. capensis' (LafC) has been described from the native ornamental Cape chestnut (*Calodendrum capense*, family Rutaceae) (Garnier *et al.*, 2000). Despite being widely associated with *Calodendrum capense* (Phahladira *et al.*, 2012), LafC has not been identified from commercial citrus in South Africa (Pietersen *et al.*, 2010), suggesting that this liberibacter does not play a direct role in the epidemiology of greening disease.

The aim of this study was to identify the diversity and distribution of liberibacters associated with *Clausena anisata* (horsewood), *Vepris lanceolata* (white ironwood) and *Zanthoxylum capense* (small forest knobwood) across their natural distribution ranges in South Africa. These plant species were specifically targeted as they represent native members of the family Rutaceae and are known hosts of *T. erytreae* (Moran, 1968a). The identification of native hosts for Laf (or other liberibacters) may help to identify possible reservoirs involved in the spread of greening in South Africa. Additionally, the identification of liberibacters from alternative rutaceous hosts may give insight into the evolution of Laf on the African continent.

Leaf and petiole samples of trees of *Clausena*, *Vepris* and *Zanthoxylum* were collected from across South Africa within their natural distribution ranges (Fig. 1). Trees were located and visually identified with help from both amateur and experienced botanists. GPS coordinates were taken of trees sampled and each tree was tagged with a unique accession number. Trees were sampled at random from both natural and urban settings, irrespective of the presence of symptoms, triozids or proximity to citrus orchards. Total DNA was extracted from petioles and midribs of leafs following a CTAB extraction method as described by Doyle & Doyle (1990).

To identify liberibacter-positive samples, extracted DNA was subject to a generic liberibacter TaqMan real-time PCR assay. This assay was a modification of the real-time PCR protocol described by Li *et al.* (2006) with the forward primer being redesigned (LibUF; 5′-GGCAGGCCTAACACATGC-3′) to target a region of the 16S rRNA gene that is conserved amongst known liberibacter species, thus enabling the detection of various liberibacter species within a single assay (G. Pietersen, unpublished data). For these reactions, 1 µl DNA template was added to a final reaction volume of 10 µl containing 5.0 µl 2× TaqMan universal Master Mix II (ABI), 500 nM forward primer LibUF, 500 nM reverse primer HLBr, 150 nM probe HLBp (Li *et al.*, 2006), 2 ng BSA ml^{-1} and 3.4 µl distilled water. The reaction was performed using a LightCycler 1.5 capillary-based thermocycler (Roche Diagnostics). Reaction conditions were modified from the protocol described by Li *et al.* (2006) to allow optimum detection of unknown liberibacters using TaqMan universal Master Mix II (ABI), with initial denaturation of 10 min at 95 °C, 45 cycles of 95 °C for 10 s, 62 °C for 50 s and 72 °C for 5 s, followed by a final cooling of 30 s at 40 °C. Fluorescence was measured and crossing threshold (C_t) values were determined using LightCycler 1.4 software (Roche Diagnostics). A positive/negative of $C_t<35$ was used, as samples with $C_t>35$ no longer yielded amplicons with conventional PCRs.

In order to identify the liberibacters present in the samples yielding C_t values within the threshold limit ($C_t<35$), the DNAs for these samples were subjected to the conventional PCR to amplify the 16S rRNA gene. We also amplified portions of the *omp* and *rplJ* genes, which respectively encode the outer-membrane protein and the 50S ribosomal protein L10.

A portion of the liberibacter *rplJ* gene was amplified as described previously (Hocquellet *et al.*, 1999), where 0.5 µl DNA template was added to a final reaction volume of 50 µl consisting of 0.1 % 2 % Triton X-100, 5 µl 10× NH_4 reaction buffer (Bioline), 0.2 mM dNTP mixture, 0.5 µM of each primer A2/J5, 0.05 M $MgCl_2$, 2000 µg BSA ml^{-1} and 5 U of 2.5 U Biotaq (Bioline) μl^{-1} and made up to the final reaction volume with molecular-grade water (Sigma-Aldrich). PCR cycling was performed on a T100 Thermal Cycler (Bio-Rad). Cycling conditions were as follows: initial denaturation at 92 °C for 3 min, followed by 35 cycles of denaturation at 92 °C for 20 s, annealing at 62 °C for 20 s and elongation at 72 °C for 45 s, with a final elongation step at 72 °C for 5 min.

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

A portion of the *omp* gene was amplified using the protocol of Bastianel *et al.* (2005). These PCRs were set up as for the *rplJ* amplification, but utilized primer pair HP1inv/OMP8inv (Bastianel *et al.*, 2005). Cycling conditions were as follows: initial denaturation at 92 °C for 3 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 2 min, with a final elongation step at 72 °C for 10 min.

For amplification of the 16S rRNA gene, the protocol described by Jagoueix *et al.* (1996) was used. These reactions were also set up as for the *rplJ* and *omp*, but utilized primers OA1/OI2c (Jagoueix *et al.*, 1996). Amplification was carried out under the following conditions: initial denaturation at 92 °C for 5 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 62 °C for 30 s and elongation at 72 °C for 90 s, with a final elongation step at 72 °C for 10 min.

The *rplJ*, *omp* and 16S rRNA gene PCR products were purified using exonuclease I (Fermentas) and FastAP (Fermentas) according to the manufacturer's instructions. Purified amplicons were sequenced in both directions using the original PCR primers and the Big Dye Terminator version 3.1 cycle sequencing kit (ABI) according to the manufacturer's instructions. Sequences of amplicon products were determined using an ABI 3500xL automated sequencer.

DNA sequences were compiled into different datasets and aligned using the online tool Mafft (Katoh *et al.*, 2002). These alignments included all of the sequences generated in this study, as well as those for the known members of the genus and, in the case of the 16S rRNA gene dataset, representatives from related genera for outgroup purposes. These known sequences were obtained from GenBank (Benson *et al.*, 2013). Alignments were visualized using BioEdit version 7.0.9.0 (Hall, 1999). This software was also used to trim the alignments so that the sequences in each dataset spanned the same region of the gene. These alignments were subjected to maximum-likelihood phylogenetic analyses using MEGA software version 6 (Tamura *et al.*, 2011) and the best-fit substitution models as indicated by jModelTest (Posada, 2008).

The standard DNA barcoding gene for plants (*rbcL*, encoding the large subunit of ribulose-1,5-biphosphate carboxylase) (Chase *et al.*, 2005) was used to verify the

identification of the tree hosts in which liberibacters were detected. For this purpose, PCRs were set up as before, but utilized primers rbcLa F (Levin *et al.*, 2003) and rbcLa R (Kress & Erickson, 2007). The PCR was set up under the following cycling conditions: initial denaturation at 92 °C for 3 min, followed by 35 cycles of denaturation at 92 °C for 20 s, annealing at 55 °C for 20 s and elongation at 72 °C for 90 s, with a final elongation step at 72 °C for 5 min. Products were purified and sequenced as described before, after which DNA sequences were aligned and subjected to maximum-likelihood phylogenetic analysis as described above.

A total of 234 specimens of *Clausena*, 289 specimens of *Vepris* and 231 specimens of *Zanthoxylum* were sampled from across South Africa (Fig. 1) in both greening-affected and greening-free areas. Of these, 33 samples from *Clausena*, 16 samples from *Vepris* and 10 samples from *Zanthoxylum* tested positive for the presence of liberibacter following real-time PCR (C_t<35) (Table 1).

Liberibacter-specific *rplJ*, *omp* and 16S rRNA gene sequences were determined for those samples that had tested positive for the presence of liberibacter. None of the 'no-template' or healthy control samples included in each reaction yielded any amplicons. Amplification of *rplJ* and 16S rRNA genes was successful for all of the liberibacter-positive samples. However, *omp* liberibacter sequences were successfully amplified and sequenced from only 29 of 33 samples from *Clausena*, 14 of 16 samples from *Vepris* and 8 of 10 samples from *Zanthoxylum*. Various attempts were made to lower the stringency of the *omp* PCR in order to amplify the recalcitrant liberibacter-infected samples, but these failed. This could possibly be attributed to the primer-binding site being too variable.

Phylogenetic analysis of the 16S rRNA gene dataset indicated that all of the samples contained sequences related to Laf and LafC (Fig. 2). None of the samples contained sequences related to the other known liberibacters. Within this phylogeny, the sequences recovered from *Clausena* and *Vepris* shared 100 % sequence identity with LafC, and 99.0 % sequence identity to the sequences recovered from *Zanthoxylum*. The high level of conservation among the 16S rRNA gene sequences from the liberibacters examined in this study is consistent with what has been reported before for these bacteria (Ghosh *et al.*, 2013; Garnier *et al.*, 2000). The 16S rRNA gene sequences of LafC from *Calodendrum capense* and Laf from citrus are also 99.0 % similar. This is greater than previously reported (97.4 %; Garnier *et al.*, 2000) because some ambiguous nucleotide identities from the initial 16S rRNA gene sequence (GenBank accession no. AF137368) have now been resolved. These findings indicate that 16S rRNA gene sequences are not sufficiently variable to allow differentiation of the subspecies of liberibacters.

Phylogenetic analyses of the *rplJ* and *omp* datasets revealed that none of the samples from *Clausena*, *Vepris* or *Zanthoxylum* examined contained sequences typical of Laf or LafC (Figs 3 and 4). The only exception was a single sample from *Zanthoxylum* (denoted 11-4270), which contained sequences of *rplJ* and *omp* (as well as the 16S rRNA gene) that were identical to those known for LafC. Nonetheless, analyses of the *rplJ* and *omp* data separated all of the apparently novel liberibacter sequences into three unique clusters. In all cases, these unique clusters correlated with the tree host species from which the liberibacter sequences were obtained (Figs 3 and 4). For both of these gene regions, the respective clusters obtained from *Clausena* and *Vepris* were more closely related to LafC than to citrus-infecting Laf. In these phylogenies, the sequences obtained from *Zanthoxylum* sample 11-4270 grouped with LafC, while the remaining sequences from *Zanthoxylum* formed a cluster that grouped with the Laf sequences from citrus (Figs 3 and 4).

In terms of overall sequence similarities for the *rplJ* and *omp* data, the aligned sequences of liberibacter samples from *Clausena* were more similar to that of LafC (97.0 and 95.6 % identity, respectively) than to the Laf sequences from citrus (86.4 and 77.9 % identity, respectively). Similarly, the liberibacter sequences from *Vepris* were more similar to the LafC sequences (96.1 and 95.7 % identity, respectively) than to those for Laf (85.3 and 77.9 % identity, respectively). For these genes, however, the nine liberibacter sequences from *Zanthoxylum* were more similar to those for Laf (89.0 and 87 % identity) than LafC (84.0 and 76.9 % identity).

To assess the apparent correlation of the liberibacter clusters with plant host species, we confirmed the identities of individual tree samples using *rbcL* sequence data (Fig. 5). Phylogenetic analysis of these data showed that the *Vepris* samples examined were indeed conspecific with *V. lanceolata*. The same was also true for the *C. anisata* samples examined. Of the ten *Zanthoxylum* samples examined, only nine represented *Z. capense*, including the sample (11-4270) that contained LafC-like sequences. The remaining *Zanthoxylum* sample (denoted 11-4561) was probably conspecific with *Zanthoxylum davyi*, for which no sequence information is available in the GenBank database.

Within the current taxonomic framework for the genus *Liberibacter*, the bacteria infecting *C. anisata*, *V. lanceolata*, *Z. capense* and *Z. davyi* from South Africa clearly represent members of the Laf species, as is evident from the high level of 16S rRNA gene sequence identity that they share, as well as their phylogenetic affinity to known Laf isolates based on the DNA sequence information for three gene regions. Garnier *et al.* (2000) introduced the subspecies taxon LafC to recognize that the liberibacter detected from *Calodendrum capense* is different from Laf associated with greening on citrus. We therefore propose that the three novel liberibacters reported here also be given Laf subspecies status, as they are readily distinguishable from LafC and citrus-infecting Laf, as well as from one another by their hosts and phylogenetic affinities based on *rplJ* and *omp* sequences. The proposed names for these newly

Table 1. Sampling site information, numbers of trees sampled per site and numbers of liberibacter-positive samples per site

Liberibacter-positive samples were detected using a generic liberibacter real-time PCR method (Li *et al.*, 2006; G. Pietersen, unpublished results). A positive/negative of crossing threshold (C_t) <35 was used, i.e. samples with a C_t>35 were considered positive for a liberibacter. Site numbers correspond to those depicted in Fig. 1.

District	Site	Liberibacter-positive samples/number sampled		
		Clausena	*Vepris*	*Zanthoxylum*
Western Cape				
Kirstenbosch	1	0	0/1	0
Swellendam	2	0	0/3	0
Heuningbos	3	3/13	0	0/9
George	4	1/1	0/9	0
Knysna	5	4/11	12/70	5/40
Eastern Cape				
St. Francis Bay	6	3/7	0	1/11
Patensie	7	0/2	0/13	0/10
Grahamstown	8	0/14	2/23	0/10
Bathurst	9	0	0/1	0/2
Port Alfred	10	2/4	0/3	0/7
East London	11	0	1/19	0/11
Ngele	12	0/3	0	½
Port St. John	13	0	0/2	0/0
KwaZulu-Natal				
Port Edward	14	0/5	1/15	1/12
Richmond	15	0/5	0/20	0/1
Pietermaritzburg	16	4/8	0/6	0/1
Durban	17	0/1	0/6	0/5
Balito	18	1/1	0	2/7
Hluhlwe	19	0/4	0/29	0/9
Kosi Bay	20	0	0/4	0/1
Pongola	21	0	0/4	0
Gauteng				
Pretoria	22	0	0/24	0/21
Johannesburg	23	0	0	0/2
North West				
Rustenburg	24	0	0/17	0/6
Mpumalanga				
Schoemanskloof	25	0/46	0/15	0/30
Nelspruit	26	0	0	0/4
Baberton	27	4/5	0	0/1
Sabi	28	0/48	0/2	0/3
Mount Sheba	29	0/11	0	0/12
Swaziland				
Unknown	30	0	0	0/2
Limpopo				
Magoebaskloof	31	0/11	0	0/6
Tzaneen	32	0/16	0/1	0/6
Lekgalameetse	33	0/18	0/2	0
Totals		33/234	16/289	10/231

identified liberibacters are '*Candidatus* Liberibacter africanus subsp. clausenae' (LafCl), '*Candidatus* Liberibacter africanus subsp. vepridis' (LafV) and '*Candidatus* Liberibacter africanus subsp. zanthoxyli' (LafZ). As the three novel liberibacters described from this study were identified from native hosts of *T. erytreae*, it is important to ascertain whether these liberibacters are capable of being transmitted to citrus by this vector under controlled conditions. Such transmission studies will help to determine whether natural transmission of LafCl, LafV and LafZ to commercial citrus can occur.

It has been suggested previously that LafC co-evolved with its only known rutaceous host, *Calodendrum capense* (Phahladira *et al.*, 2012). Similarly, an apparently

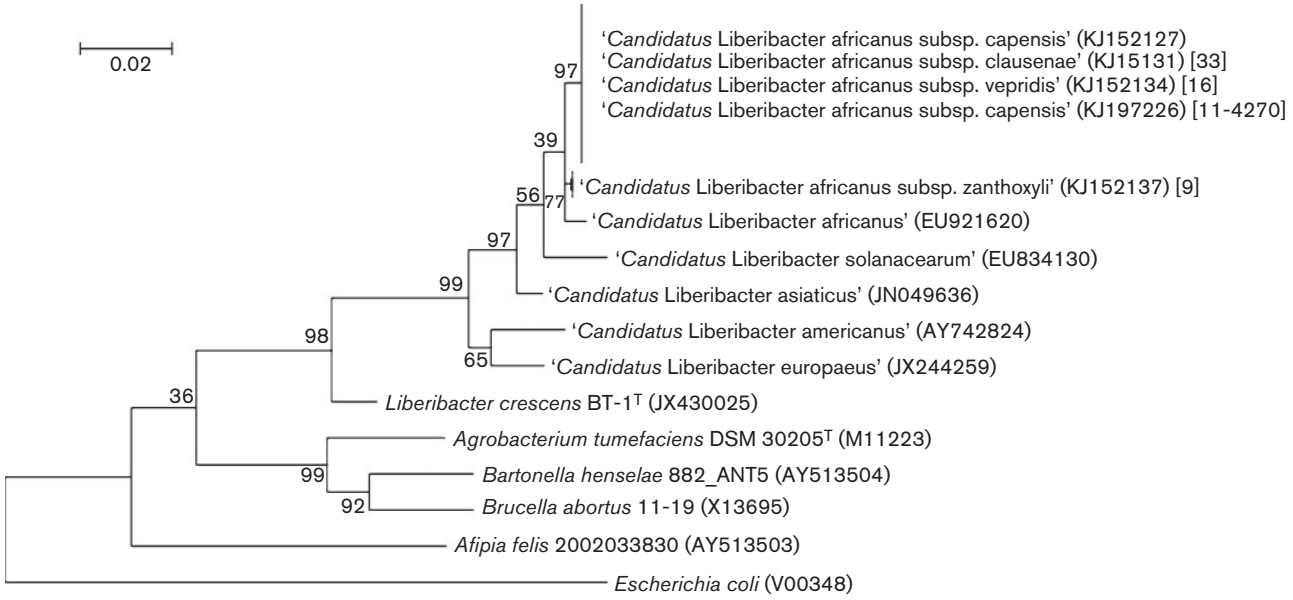

Fig. 2. Maximum-likelihood phylogeny of the genus *Liberibacter* based on 16S rRNA gene sequences obtained from samples from *Clausena*, *Vepris* and *Zanthoxylum* examined in this study, as well as for all known liberibacters and related proteobacteria. The phylogeny was inferred using Kimura's two-parameter model (Kimura, 1980) with gamma correction to account for among-site rate variation. Bootstrap support values based on 1000 replicates are indicated at branches, and terminal branches receiving less than 70 % bootstrap support were collapsed. GenBank accession numbers are shown on the tree for sequences included in analyses. The number of sequenced liberibacter-positive samples per tree host is indicated in brackets. An unknown strain of *Escherichia coli* was used as the outgroup. Bar, 0.02 substitutions per nucleotide position.

long-standing association between LafZ, LafV and LafCl and their respective hosts may also exist, as we frequently detected these liberibacters on trees with no obvious disease symptoms trees in isolated geographical regions that have remained undisturbed by urban encroachment. Furthermore, Laf in Africa has been suggested to be the result of a host-jumping event of a native liberibacter such as LafC on *Calodendrum capense* to citrus (Phahladira *et al.*, 2012). The existence of various liberibacter subspecies from South Africa indicates that the evolution of Laf from an

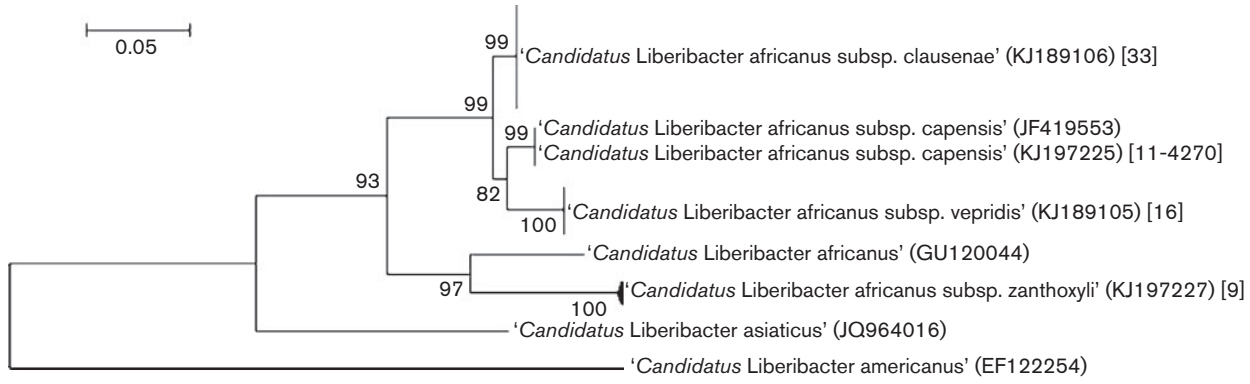

Fig. 3. Maximum-likelihood phylogeny of the genus *Liberibacter* based on *rplJ* sequences obtained from samples from *Clausena*, *Vepris* and *Zanthoxylum* examined in this study, as well as for Laf, Las and Lam. The phylogeny was inferred using the Tamura–Nei model (Tamura & Nei, 1993) with gamma correction to account for among-site rate variation. Bootstrap support values based on 1000 replicates are indicated at branches, and terminal branches receiving less than 70 % bootstrap support were collapsed. GenBank accession numbers are shown on the tree for sequences included in analyses. The number of sequenced liberibacter-positive samples per tree host is indicated in brackets, or, in the case of 11-4270, the sample accession number is given. Bar, 0.05 substitutions per nucleotide position.

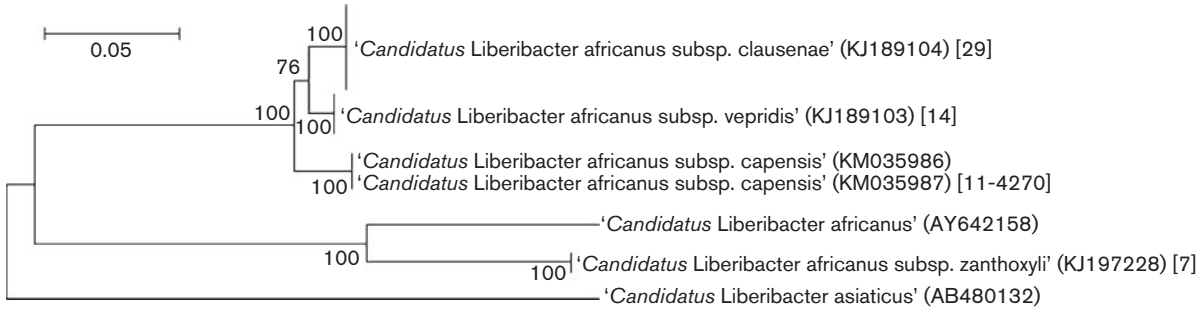

Fig. 4. Maximum-likelihood phylogeny of the genus *Liberibacter* based on *omp* sequences obtained from samples from *Clausena*, *Vepris* and *Zanthoxylum* examined in this study, as well as for Laf, Las and Lam. The phylogeny was inferred using the Tamura–Nei model (Tamura & Nei, 1993) with gamma correction to account for among-site rate variation. Bootstrap support values based on 1000 replicates are indicated at branches, and terminal branches receiving less than 70 % bootstrap support were collapsed. GenBank accession numbers are shown on the tree for sequences included in analyses. The number of sequenced liberibacter-positive samples per tree host is indicated in brackets, or, in the case of 11-4270, the sample accession number is given. Bar, 0.05 substitutions per nucleotide position.

indigenous member of the Rutaceae to commercial citrus may be more complicated than previously speculated. For example, these bacteria could potentially infect multiple host species, as was demonstrated here. Most specimens from *Zanthoxylum* contained LafZ sequences, but a single sample (11-4270) from this host contained LafC sequences across all genes sequenced. The preferential feeding by *T. erytreae* on citrus (Moran, 1968b) and a potentially wider host range of African liberibacters could have assisted in multiple transmissions of any liberibacter from its indigenous host to citrus, indirectly placing selective pressure on the liberibacter to evolve and adapt to a new host. In order to determine whether such transmission of a liberibacter from this study could occur in nature, it would be necessary to perform transmission studies involving both vector and grafting approaches to ascertain whether any of the liberibacters identified here are capable of being acquired by *T. erytreae* and multiplying within commercial citrus. Further assessment of the existence of liberibacters from additional species of the Rutaceae from Africa is

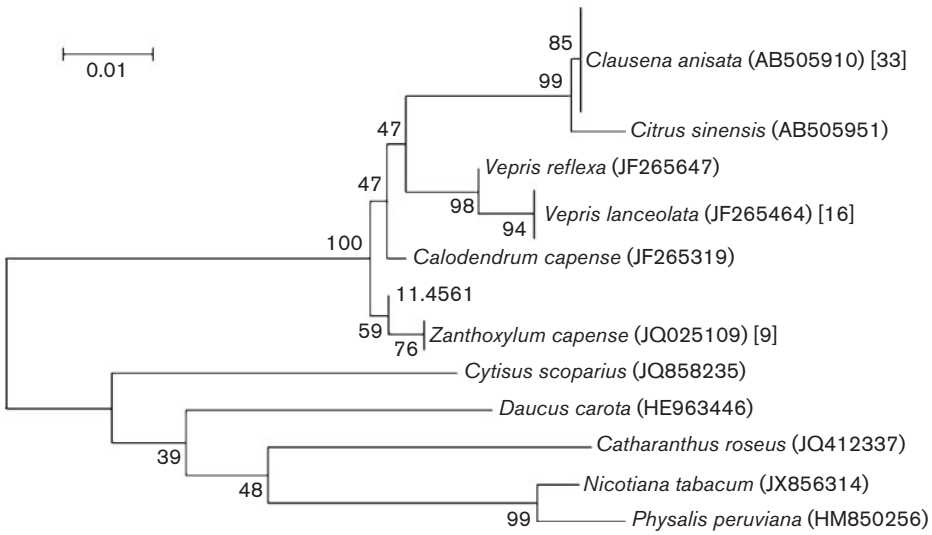

Fig. 5. Maximum-likelihood phylogeny of tree host species based on *rbcL* sequences obtained from samples of *Clausena*, *Vepris* and *Zanthoxylum* examined in this study. The phylogeny was inferred using Kimura's two-parameter model (Kimura, 1980) with gamma correction to account for among-site rate variation. Bootstrap support values based on 1000 replicates are indicated at branches, and branches were collapsed for terminal taxon branches receiving less than 70 % bootstrap support. GenBank accession numbers are shown on the tree for sequences included in analyses. The number of sequenced liberibacter-positive samples per tree host is indicated in brackets. Bar, 0.01 substitutions per nucleotide position.

required before any one liberibacter subspecies can be considered as the most recent ancestor of Laf.

Initially, we set out to determine whether reservoir hosts exist for Laf. However, typical citrus-infecting Laf were not detected in any of the indigenous rutaceous specimens tested; hence, these native rutaceous hosts of *T. erytreae* do not appear to play a role in the epidemiology of Laf on citrus. However, we were able to identify novel liberibacters from all three tree hosts tested. Further studies are needed to determine whether these liberibacters are transmitted to and are capable of causing disease on commercial citrus species. Additionally, the association of various Laf subspecies with native trees belonging to the Rutaceae presents researchers with a unique opportunity to explore the possible evolution of Laf on citrus from a liberibacter source indigenous to the African continent.

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