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

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The GenBank accession number for the 16S rRNA gene, *rplJ* gene and *omp* gene sequences of 1) '*Candidatus* Liberibacter africanus subsp. clausenae' is KJ152131, KJ189106 and KJ189104 respectively; 2) '*Candidatus* Liberibacter africanus subsp. vepridis' is KJ152134, KJ189105, and KJ189103 respectively, and of 3) '*Candidatus* Liberibacter africanus subsp. zanthoxyli' is KJ152137, KJ197227, and KJ197228 respectively.

Summary

Greening disease of citrus in South Africa is associated with '*Candidatus* Liberibacter africanus' (Laf), a phloem-limited bacterium vectored by *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 *Clausena anisata*, 289 *Vepris lanceolata* and 231 *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 *Clausena*, 17 *Vepris*, and 10 *Zanthoxylum* tested positive for liberibacter. None of the samples contained typical citrus infecting Laf sequences. Phylogenetic analysis of 16S rRNA sequences indicated that liberibacters obtained from *Vepris* and *Clausena* were identical to 16S rRNA sequences for '*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 per tree species. We propose the following names for these new 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 these are not alternative sources to citrus orchards, of Laf *per se*.

Keywords Horsewood, White ironwood, Knobwood, Liberibacter, Greening, Trioza erytreae

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 (*S. 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* that was isolated from the sap of a papaya (family *Caricaceae*) hybrid (Davis *et al.*, 2008; Fagen *et al.*, 2014), all of the known members of this genus are fastidious and apparently cannot be cultivated (Garnier and Bové, 1983). These bacterial taxa have accordingly been assigned the provisional status '*Candidatus*'(Murray and 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), as well as '*Candidatus* Liberibacter solanacearum' (Lso) associated with potato and tomato (Liefting *et al.*, 2009; Secor *et al.*, 2009).

The pathogens associated with Greening and HLB cause symptoms suggestive of nutrient limitations, e.g., yellowing of the leaves, vigor decline and reduced fruit quality (Kapur *et al*., 1978), which ultimately leads to dieback, stunted growth and plant death (McClean and Oberholzer, 1965; Lopes and Frare, 2008). Laf, which has thus far only been identified from citrus orchards in Africa and the Mascarene islands (Garnier and Bové, 1996; Garnier *et al.*, 1996), is primarily spread through the feeding and flight activities of the triozid, *Trioza erytreae* Del Guercio (order *Hemiptera*, family *Triozidae*) (McClean and Oberholzer, 1965). Lam has been reported from citrus in Brazil (Teixeira *et al.*, 2005a), while Las has been identified from citrus in Asia (Garnier and Bové, 1996), the Americas (Coletta-Filho *et al.*, 2004; Halbert, 2005) and recently Ethiopia (Saporani *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 efficiently transmit both Las and Laf (Masonie *et al.*, 1967; Aubert, 1987).

Whilst being present in most citrus producing regions in South Africa (Pretorius and van Vuuren, 2006), Greening incidence 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 and 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 subspecies capensis' (LafC) has been described from the native ornamental, Cape Chestnut (*Calodendrum capense*, family *Rutaceae*) (Garnier *et al.,* 2000). Despite being widely associated with *Ca. 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 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 *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 and 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'-GGC AGG CCT AAC ACA TGC-3') to target a region of the 16S ribosomal RNA (16S rRNA) gene that is conserved amongst known liberibacter species, thus enabling the detection of various liberibacter species within a single assay (Pietersen, *unpublished*). For these reactions, 1µl of DNA template

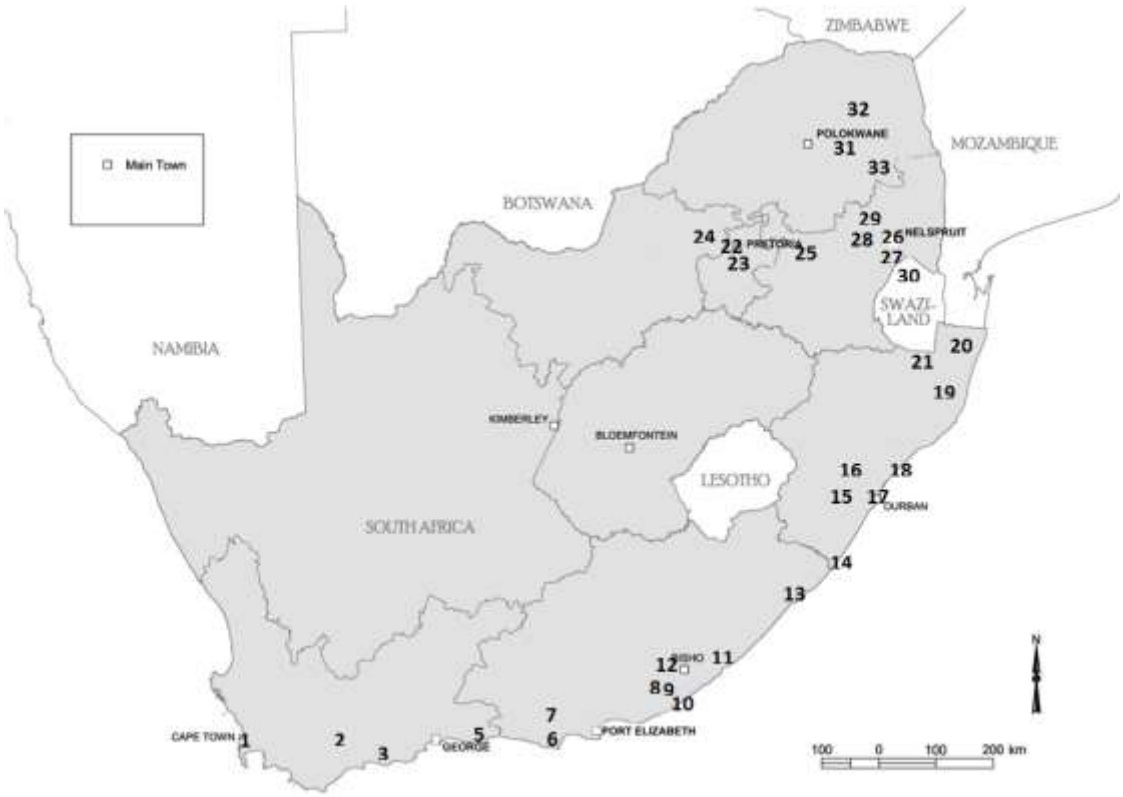

Fig. 1 Sampling sites of indigenous Rutaceous trees across South Africa. Site numbers (1-33) correspond to those listed in Table 1

was added to a final reaction volume of 10µl containing 5.0µl 2X Taqman® universal Master Mix II (ABI, Foster City, CA, USA), 500nM forward primer LibUF, 500nM reverse primer HLBr, 150nM probe HLBp (Li *et al.,* 2006) 2ng/ml BSA and 3.4µl dH_2O. The reaction was performed using a LightCycler® 1.5 (Roche Diagnostics, Manheim, Germany) capillary-based thermocycler. 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, Foster City, CA, USA), with initial denaturation of 10 min at 95˚C, 45 cycles 95˚C for 10s, 62˚C for 50s and 72˚C for 5s, followed by final cooling of 30s at 40˚C. Fluorescence was measured and crossing threshold (Ct) values were determined using LightCycler® 1.4 software (Roche Diagnostics, Mannheim, Germany). A positive/negative of Ct<35 was used as samples with Ct>35 no longer yielded amplicons with conventional PCR reactions.

In order to identify the liberibacters present in the samples yielding Ct values within the threshold limit (Ct<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 0.5µl of DNA template was added to a final reaction volume of 50µl consisting of 0.1% of 2% Triton X-100, 5µl 10X NH_4 reaction buffer (Bioline, Boston, USA), 0.2mM deoxynucleotide triphosphate mix (dNTP), 0.5µM of each primer A2/J5, 0.05M $MgCl_2$, 2000µg/ml BSA, 5u/µl of 2.5 units (U) Biotaq® (Bioline, Boston, USA) and made up to a final reaction volume with molecular grade H_2O (Sigma-Aldrich, St. Louis, MO, USA). PCR cycling reaction was performed on a T100™ Thermal Cycler (Bio-Rad, CA, USA). Cycling conditions were set up as follows; initial denaturation at 92˚C for 3 min, followed by 35 cycles of denaturation at 92˚C for 20s, annealing at 62˚C for 20s and elongation at 72˚C for 45s, with a final elongation step at 72˚C for 5 min.

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 HPIinv/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 30s, annealing at 55˚C for 30s 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 30s, annealing at 62˚C for 30s and elongation at 72˚C for 90s, with a final elongation step at 72˚C for 10 min.

The *rplJ*, *omp* and 16S rRNA PCR products were purified using Exonuclease I (Fermentas, Maryland, USA) and FastAP® (Fermentas, Maryland, USA) according to the manufacturer's instructions. Purified amplicons were sequenced in both directions using the original PCR primers and the Big Dye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, USA) as per manufacturer's instructions. Sequences of amplicon products were determined using an ABI 3500xL automated sequencer (Applied Biosystems, Foster City, CA).

DNA sequences were compiled into different data sets 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 dataset, representatives from related genera for outgroup purposes. These known sequences were obtained from GenBank® (Benson *et al.*, 2008). Alignments were visualized using BioEdit version 7.0.9.0 (Hall, 1999). This software was also used to trim the respective 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 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 (i.e. *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 primer rbcLa F (Levin *et al*. 2003) and primer rbcLa R (Kress and 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 20s, annealing at 55˚C for 20s and elongation at 72˚C for 90s, 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 *Clausena*, 289 *Vepris* and 231 *Zanthoxylum* specimens were sampled from across South Africa (Fig. 1) in both Greening and Greening-free areas. Of these, 33 *Clausena*, 16 *Vepris*, and 10 *Zanthoxylum* samples tested positive for the presence of liberibacter following real-time PCR (Ct<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’ and healthy control samples included per reaction yielded any amplicons. Amplification of *rplJ* and 16S rRNA genes was successful for all of the liberibacter-positive samples. However, for *omp* liberibacter sequences from only 29 of 33 *Clausena*, 14 of 16 *Vepris* and 8 of 10 *Zanthoxylum* samples were successfully amplified and sequenced. Various attempts were made to lower stringency of the *omp* PCR in order to amplify the recalcitrant liberibacter-infected samples but failed. This could possibly be attributed to the primer binding site being too variable.

Table 1 Sampling site information, the number of trees sampled per site and the number of liberibacter-positive* samples per site

Province	District	Site number†	Number of liberibacter positive samples / Number sampled at site		
			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	1/2
	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

*Liberibacter' positive samples were detected using a liberibacter-generic real-time PCR method (Li et al.2006; Pietersen *unpublished*). A positive/negative of crossing threshold (Ct) <35 was used, i.e. samples with a Ct >35 were considered positive for a liberibacter

†The site numbers refer to those depicted in Figure 1.

Phylogenetic analysis of the 16S rRNA 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

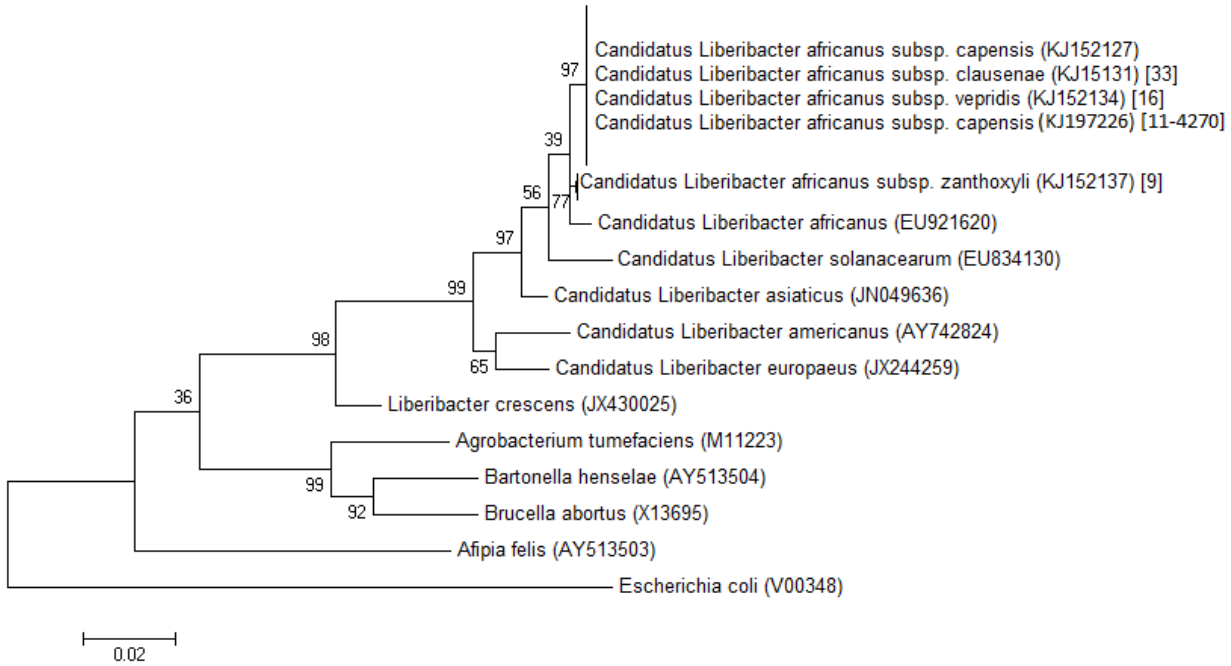

Fig. 2 Maximum likelihood phylogeny of the genus Liberibacter based on the 16S rRNA sequences obtained from the *Clausena*, *Vepris* and *Zanthoxylum* samples examined in this study, as well as for all known liberibacters and related Proteobacteria. The phylogeny was inferred using the Kimura-2-parameter model (Kimura 1980) with gamma correction to account for among site rate variation. Bootstrap support values based on 1000 replicates are indicated at the branches, and terminal branches receiving less that 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 square brackets. *Escherichia coli* (V0038) was used as the outgroup.

sequences of LafC from *Ca. capense* and Laf from citrus are also 99.0% similar. This is greater than previously reported (97.4%) (Garnier *et al.*, 2000) due to having now resolved some ambiguous nucleotide identities from the initial 16S rDNA sequence (GenBank acc. AF137368). These findings indicate that 16S rRNA gene sequences are not sufficiently variable to allow for the differentiation of the subspecies of liberibacters.

Phylogenetic analyses of the *rplJ* and *omp* data sets revealed that none of the *Clausena*, *Vepris* and *Zanthoxylum* samples examined contained sequences typical of Laf or LafC (Figs. 3 and 4). The only exception was a single *Zanthoxylum* sample (denoted 11-4270), which contained sequences of *rplJ* and *omp* (as well as 16S rRNA) 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

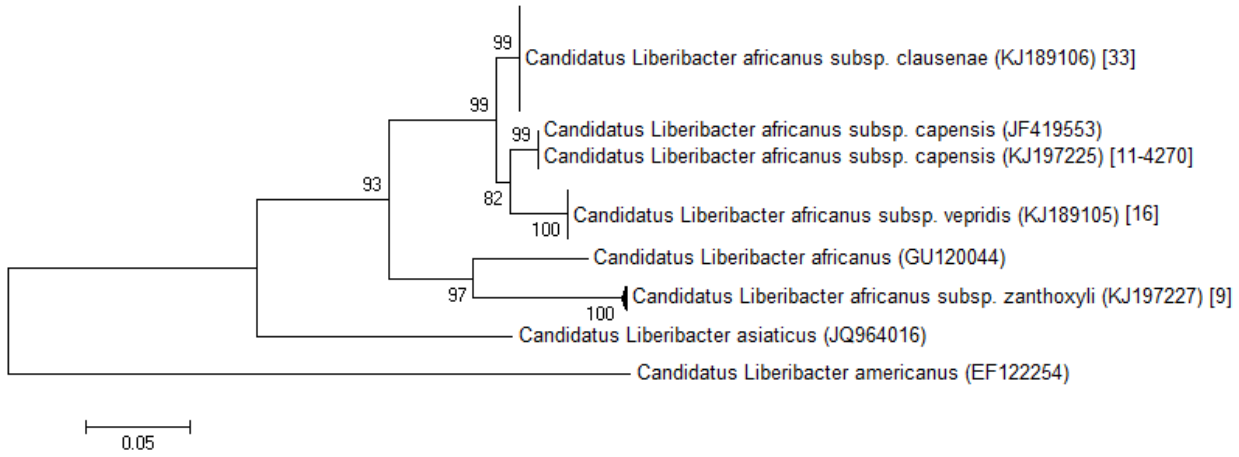

Fig. 3 Maximum likelihood phylogeny of liberibacter based on the *rplJ* sequences obtained from the *Clausena*, *Vepris* and *Zanthoxylum* samples examined in this study, as well as for Laf, Las and Lam. The phylogeny was inferred using the Tamura – Nei model (Tamura & Nei 1993) with gamma correction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

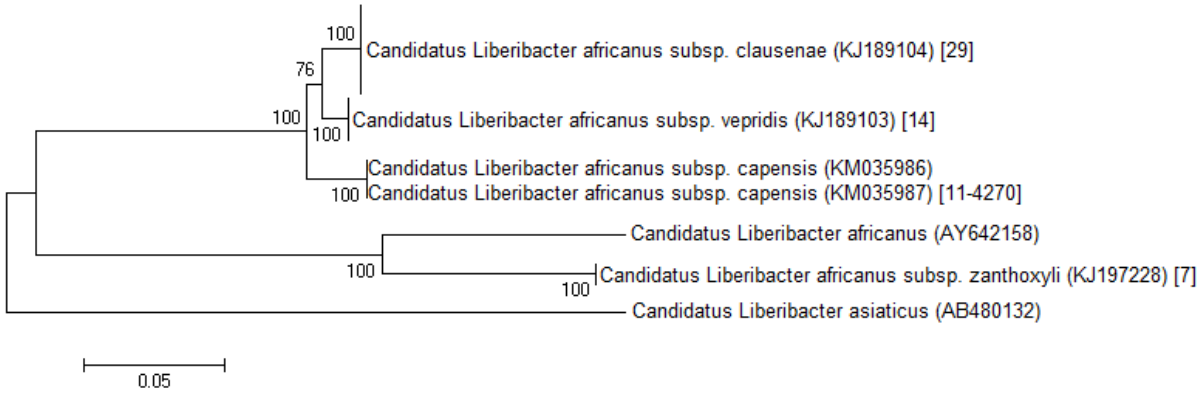

Fig. 4 Maximum Likelihood phylogeny of Liberibacter based on the *omp* sequences obtained from the *Clausena*, *Vepris* and *Zanthoxylum* samples examined in this study, as well as for Laf, Las and Lam. The phylogeny was inferred using the Tamura –Nei model (Tamura and Nei 1993) with gamma correction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

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 ex *Clausena* samples were more similar to that of LafC (i.e. 97.0% and 95.6% identity, respectively) than to the Laf sequences from citrus (i.e. 86.4% and 77.9% identity, respectively). Similarly, the liberibacter ex *Vepris* sequences were more similar to the LafC sequences (i.e. 96.1% and 95.7% identity, respectively) than to those for Laf (i.e., 85.3% and 77.9% identity, respectively). For these genes, however, the nine liberibacter ex *Zanthoxylum* sequences were more similar to those for Laf (i.e., 89.0% and 87% identity) than LafC (i.e. 84.0% and 76.9% identity).

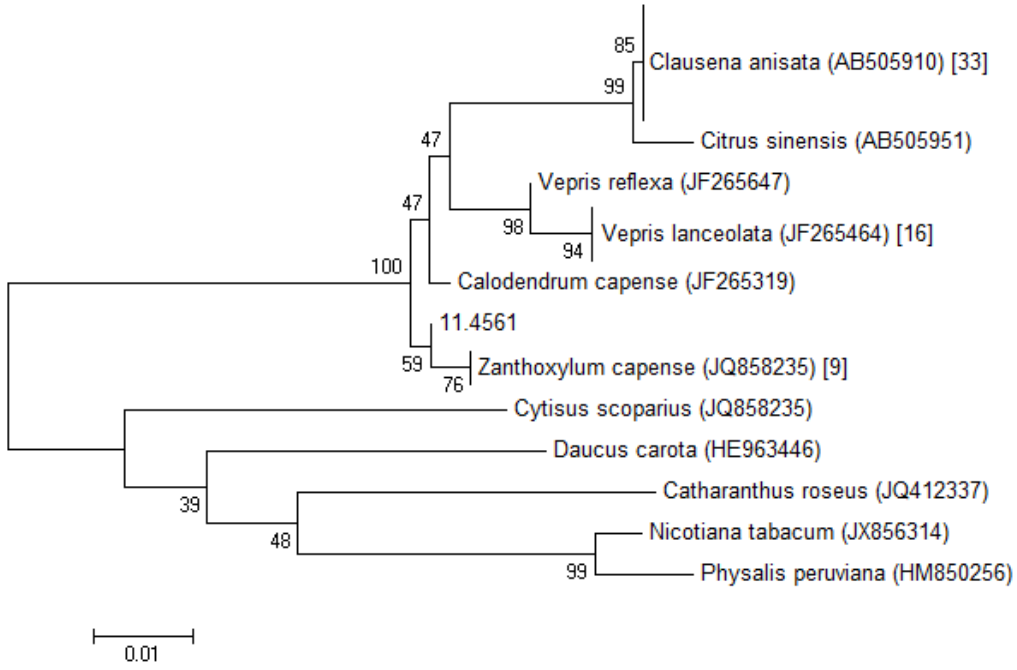

Fig. 5 Maximum likelihood phylogeny of tree host species based on the *rbcL* sequences obtained from the *Clausena, Vepris* and *Zanthoxylum* examined in this study. The phylogeny was inferred using the Kimura-2-parameter model (Kimura 1980) with gamma correction to account for among site rate variation. Bootstrap support values based on 1000 replicates are indicated at the branches, and for the terminal taxa branches receiving less than 70% bootstrap support, branches were collapsed. GenBank® accession numbers are shown on tree for sequences included in analyses. The number of sequenced liberibacter-positive samples per tree host is indicated in square brackets.

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 con-specific with *V. lanceolata*. The same is also true for the *Cl. anisata* samples examined. Of the ten *Zanthoxylum* samples examined, only nine represented *Z. capense* and included the sample (11-4270) containing LafC-like sequences. The remaining *Zanthoxylum* sample (denoted 11-4561) was likely con-specific with *Z. davyi* of which no sequence information is available from Genbank.

Within the current taxonomic framework for the genus liberibacter, the bacteria infecting South African *Cl. anisata*, *V. lanceolata* and *Z. capense* and *Z. davyi* clearly represent members of the Laf species as is evident from the high level of 16S rRNA identity 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 *Ca. capense* is different from Laf associated with Greening on citrus. We therefore propose that the three new 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 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 determine whether natural transmission of the LafCl, LafV and LafZ to commercial citrus can occur.

It has previously been suggested that LafC co-evolved with its only known rutaceous host, *Ca. capense* (Phahladira *et al.,* 2012). Similarly, an apparently 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 geographic regions that have remained undisturbed by urban encroachment. Furthermore, Laf in Africa has been suggested to be due to a host-jumping event of a native liberibacter such as LafC on *Ca. capense* to citrus (Phahladira *et al.,* 2012). The existence of various liberibacter subspecies from South Africa indicates that the evolution of Laf from an indigenous *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 *Zanthoxylum* specimens 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 liberibacter identified here are capable of being acquired by *T. erytreae* and multiplying within commercial citrus. Further assessment of the existence of liberibacter from additional *Rutaceae* species from Africa are 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 *Rutaceae* 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 *Rutaceae* trees 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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