

Genomic characterization of a liberibacter present in an ornamental rutaceous tree, *Calodendrum capense*, in the Western Cape province of South Africa. Proposal of '*Candidatus Liberibacter africanus* subsp. *capensis*'

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In 1994, the uncultured phloem-restricted bacteria of citrus huanglongbing (ex-greening) disease in Asia and Africa were characterized as '*Candidatus Liberobacter asiaticum*' and '*Candidatus Liberobacter africanum*', respectively. Following the rules of the *International Code of Nomenclature of Bacteria*, the two bacterial species have now been renamed '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter africanus*'. A third liberibacter was detected by PCR in an ornamental rutaceous tree, Cape chestnut (*Calodendrum capense*), in South Africa. The new liberibacter was characterized by serology and from the sequences of its 16S rDNA, intergenic 16S/23S rDNA and ribosomal protein genes of the β operon. Phylogenetic analysis showed that the liberibacter present in *C. capense* differed from the two previously described liberibacter species from citrus and that it was more closely related to '*Candidatus Liberibacter africanus*' than to '*Candidatus Liberibacter asiaticus*'. It is proposed that the liberibacter from *C. capense* be assigned a subspecies status, '*Candidatus Liberibacter africanus* subsp. *capensis*'.

Keywords: huanglongbing, phylogeny, citrus, phloem, '*Candidatus Liberibacter africanus* subsp. *capensis*'

INTRODUCTION

Huanglongbing (HLB) (ex-greening) is one of the most destructive diseases of citrus. The uncultured bacterium associated with the disease was characterized in 1994 from its 16S rDNA sequence and shown to be a new genus in the α -Proteobacteria (Jagoueix *et al.*, 1994). The '*Candidatus*' generic name 'Liberobacter', as defined by Murray & Schleifer (1994), was given to the bacterium. Two species, '*Candidatus Liberobacter asiaticum*' and '*Candidatus Liberobacter africanum*', were described on the basis of gene sequences from the

rplKAJL-rpoBC operon (β operon) of African and Asian liberobacters (Jagoueix *et al.*, 1994). Another 16S rDNA sequence of a '*Candidatus Liberobacter* sp.' strain Okinama has recently been deposited in GenBank (accession no. AB008366; T. Iwanami, unpublished results). Following the Rules of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1992; Trüper, 1999), stating that 'bacter' is of masculine gender and that the connecting vowel should be 'i' when the preceding term is of Latin origin, the nomenclature of the two species is now revised, the two species thus becoming '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter africanus*'.

HLB was reported to be present in the eastern and western parts of the former Transvaal province (now

Abbreviations: HLB, huanglongbing; IF, immunofluorescence.

The GenBank accession number for the 16S rDNA sequence of the *Calodendrum capense* bacterium, '*Candidatus Liberibacter africanus* subsp. *capensis*', is AF137368.

North West and Mpumalanga provinces) of South Africa as early as 1929 (Aubert *et al.*, 1988; Da Graça, 1991; Oberholzer *et al.*, 1965; Van de Merwe & Andersen, 1937), where it was shown to be transmitted by the citrus psyllid *Trioza erytreae* (McLean & Oberholzer, 1965). The psyllid was also abundant in other citrus-growing areas of South Africa, but the disease was not present. Thus, strong quarantine measures were applied to prevent movement of citrus from the former Transvaal province to other citrus-growing regions of the country. In Africa, HLB and the psyllid vector are restricted to cool areas, as both *T. erytreae* and '*Candidatus Liberibacter africanus*' are susceptible to temperatures higher than 25/30 °C (Bové *et al.*, 1974). In 1995, symptoms of leaf mottle resembling those of HLB were observed on clementine and lemon trees in the Western Cape province of South Africa (Garnier *et al.*, 1999). When symptomatic leaves were tested by PCR with primers specific for the liberibacters (Hocquellet *et al.*, 1999; Jagoueix *et al.*, 1996) '*Candidatus Liberibacter africanus*' could be detected (Garnier *et al.*, 1999). Bordering the orchard where the disease was first reported, an ornamental rutaceous tree, Cape chestnut (*Calodendrum capense*), also showed leaf mottle symptoms. When tested for the presence of liberibacter, positive PCR reactions were obtained (Garnier *et al.*, 1999). In another location, a second Cape chestnut showed similar symptoms and gave also a positive PCR result. Thus, Cape chestnut was suspected to be responsible for the introduction of the HLB bacterium into the Western Cape area. *Calodendrum* and *Citrus* are two genera in the family *Rutaceae*, but they are phylogenetically distant. However, *T. erytreae*, the citrus psyllid, has been reported to feed on *C. capense*, on which it lays eggs, but nymph development does not take place (Moran, 1968).

As it is important to evaluate the role of *C. capense* in the outbreak of HLB disease in the Western Cape region, the liberibacter-like bacterium present in the *C. capense* tree was phylogenetically characterized by sequencing the 16S rDNA, the intergenic 16S/23S region and part of the *rplKAJL-rpoBC* operon. Comparisons with the equivalent genes from '*Candidatus Liberibacter africanus*' and '*Candidatus Liberibacter asiaticus*', the two liberibacter species that have been found in citrus so far, showed that the symptomatic *C. capense* tree was indeed infected with a liberibacter species, although this species differed from the two previously described ones. The *C. capense* liberibacter was more closely related to '*Candidatus Liberibacter africanus*' than to '*Candidatus Liberibacter asiaticus*'. It is proposed that the liberibacter present in *C. capense* be assigned a '*Candidatus*' subspecies name, '*Candidatus Liberibacter africanus* subsp. *capensis*'. An oligonucleotide defined from the ribosomal protein DNA sequence of this new species has been determined for its specific identification. A preliminary short report of part of these results has been published as a short communication (Garnier *et al.*, 1999).

METHODS

Plant material. Healthy *Citrus* plants or *Citrus* plants infected with '*Candidatus Liberibacter africanus*' were maintained in a glasshouse at 25 °C during the day and at 20 °C at night. *Citrus* plants infected with '*Candidatus Liberibacter asiaticus*' were kept in a greenhouse at 30 °C during the day and 25 °C at night.

Leaves from *Citrus* with or without symptoms of HLB, and leaves of four *C. capense*, with or without mottle were collected in the Western Cape province of South Africa. Two of the symptomatic *C. capense* trees were growing next to an HLB-infected citrus orchard. The symptomless *C. capense* leaves were from a large tree in the botanical garden in Cape Town.

Leaves were kept in plastic bags and tested in South Africa or sent by express mail for testing in the laboratory in Bordeaux, France. Upon arrival, midribs were excised and DNA was extracted for PCR analysis.

Serological reagents. mAbs 14A1 and 10H8 obtained against strain Nelspruit (South Africa) of '*Candidatus Liberibacter africanus*' (Gao *et al.*, 1993), and mAbs 10A6 and 2D12 against strain Poona (India) (Garnier *et al.*, 1987), and mAbs 10F4, 5H10, 12E12 and 11H6 against strain Fuzhou (China) (Gao *et al.*, 1993) of '*Candidatus Liberibacter asiaticus*' were used for immunofluorescence (IF) reactions as described previously (Garnier *et al.*, 1987).

Preparation of leaf extracts and PCR. For DNA extraction, the leaf midribs were worked up according to the Wizard purification procedure of Jagoueix *et al.* (1996). Extracts (2 µl) were used for PCR as described previously with primers OA1, OI1 and OI2c for amplification of 16S rDNA (Jagoueix *et al.*, 1996). PCR amplification of part of the β ribosomal protein operon (Hocquellet *et al.*, 1999; Planet *et al.*, 1995) was carried out with primers A2 (5'-TATAAAGGTTGACCTTTTCGAGTTT-3') and J5 (5'-ACAAAAGCAGAAATAGCACGAACAA-3') with the following programme: 35 cycles each at 92 °C for 20 s, 62 °C for 20 s, 72 °C for 40 s. PCR amplification was also carried out with primers 1897/1898 as described previously (Planet *et al.*, 1995). Following amplification, 8 µl aliquots of each reaction mixture were analysed by electrophoresis on 0.7% agarose gels.

Restriction enzyme analysis of amplified DNA. Twelve microlitres of A2/J5 PCR-amplified DNA were digested with 10 U *ApoI*, *HinfI* or *AciI* restriction enzyme in a final volume of 35 µl according to the manufacturer's instructions. The digested DNA was analysed by electrophoresis on 2.5% (w/v) agarose gels. Digestion of amplified 16S rDNA with *XbaI* was done according to Jagoueix *et al.* (1996).

Cloning and sequencing PCR products. PCR-amplified DNA (80 µl) was electrophoresed on a 0.8% agarose gel. The band at the correct size was cut out of the gel and purified with the Cleanmix kit (Talent) according to the manufacturer's instructions. The DNA was eluted in 20 µl water and 7 µl solution was used for ligation in the pGEM-T easy vector (Promega). Two microlitres of the recombinant plasmid was used to transform competent *E. coli* cells by electroporation.

Plasmids were prepared with the Plasmix kit (Talent) and digested with *EcoRI* to determine the size of the insert. The inserts from three different plasmids were sequenced using the T7 sequencing kit (Pharmacia).

Data analysis. Sequences were compared to those in the NCBI database using the Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences. Multiple sequence alignments were performed using the CLUSTAL software. Phylogenetic studies were done with PHYLIP software. The tree was constructed using the following 16S rDNA sequences (accession numbers are given in parentheses): *Phyllobacterium rubiacearum* strain IAM 13587 (D12790); *Sinorhizobium medicae* strain A321^T (L39882); *Bartonella clarridgeiae* strain ATCC 51734 (89208); *Rochalimaea elizabethae* (L01260); 'Candidatus Liberibacter asiaticus' strain Poona (L22532); 'Candidatus Liberibacter africanus' strain Nelspruit (L22533); and 'Candidatus Liberibacter sp.' strain Okinama (T. Iwanami, unpublished results) (AB008366).

RESULTS

PCR amplification of liberibacter 16S rDNA and ribosomal protein genes from *Citrus* sp. and *C. capense* leaf midribs

Primers OA1/OI2c specific for the amplification of liberibacter 16S rDNA were used with DNA from *Citrus* or *C. capense* in the Western Cape region of South Africa. An amplified product of the expected size (1160 bp) was observed with DNA extracted from HLB-affected citrus leaves as well as from mottled leaves of *C. capense*. No DNA band was obtained from the PCR tubes used as negative controls and containing either water or DNA extracted from healthy citrus and *C. capense* leaves. Similarly, the samples that gave positive PCR results with primers OA1/OI2c also gave positive reactions (680 bp band) when primers A2/J5, specific for the amplification of liberibacter ribosomal protein genes, were used for PCR. No DNA band was observed in the PCR tubes used as negative controls.

Sequence of the 16S rDNA of the liberibacter from *C. capense* and phylogenetic analysis

The sequence of the 16S rDNA of the *C. capense* bacterium was determined (GenBank accession no. AF137368).

The 16S rDNA sequences of the liberibacters, the *C. capense* bacterium and other bacteria present in databases were used for phylogenetic analysis. Fig. 1 presents the phylogenetic tree constructed by using the distance matrix method. It shows that the liberibacters and the *C. capense* bacterium cluster together. The *C. capense* bacterium is more closely related to 'Candidatus Liberibacter africanus' (97.4% homology) than to 'Candidatus Liberibacter asiaticus' (96.9% homology) and, upon digestion of the DNA amplified from symptomatic *C. capense* with *Xba*I (Jagoueix *et al.*, 1996), the restriction profile was that of 'Candidatus Liberibacter africanus' (result not shown). However, the 16S rDNA sequences of the *C. capense* liberibacter and 'Candidatus Liberibacter africanus' were not identical.

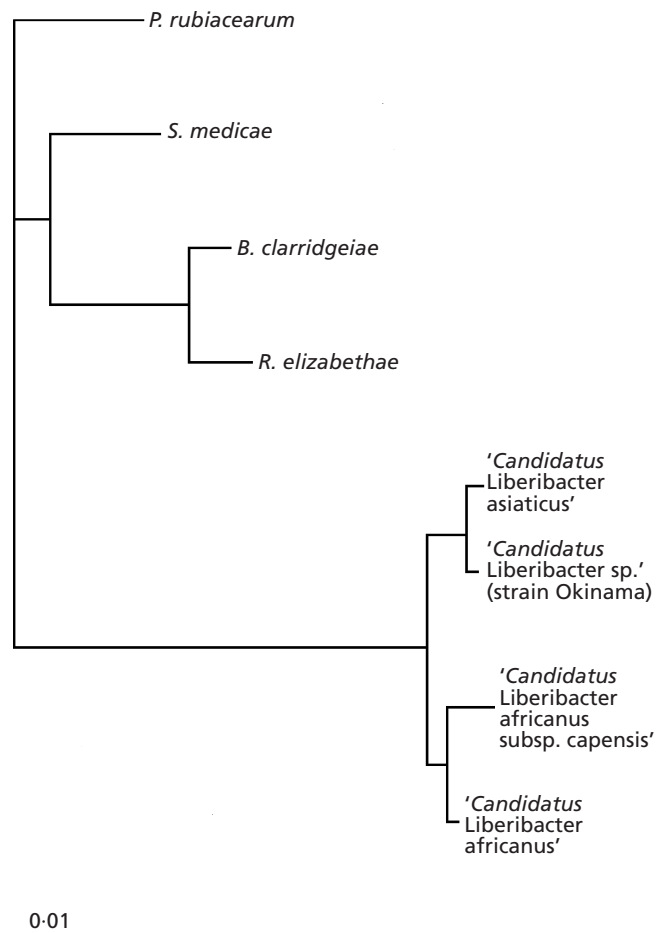


Fig. 1. Phylogenetic tree constructed with 16S rDNA sequences obtained from GenBank (accession numbers in parentheses): *Phyllobacterium rubiacearum* strain IAM 13587 (D12790); *Sinorhizobium medicae* strain A321^T (L39882); *Bartonella clarridgeiae* strain ATCC 51734 (89208); *Rochalimaea elizabethae* (L01260); 'Candidatus Liberibacter asiaticus' strain Poona (L22532); 'Candidatus Liberibacter africanus' strain Nelspruit (L22533); and 'Candidatus Liberibacter sp.' strain Okinama (T. Iwanami, unpublished results) (AB008366). The scale corresponds to 0.01% substitution.

Sequence of the A2/J5 PCR fragment amplified from the *C. capense* liberibacter

The sequence obtained after amplification with primers A2/J5 from the *C. capense* bacterium (GenBank accession no. AF248498) was aligned with the corresponding sequence of 'Candidatus Liberibacter africanus'. This indicated that the sequences were similar and, when the sequence from the *C. capense* bacterium was aligned with those in the NCBI databases, the nearest sequences were those of the liberibacter species. However, the sizes of the amplified products were different: 669 bp for 'Candidatus Liberibacter africanus', 703 bp for 'Candidatus Liberibacter asiaticus' and 688 bp for the liberibacter present in

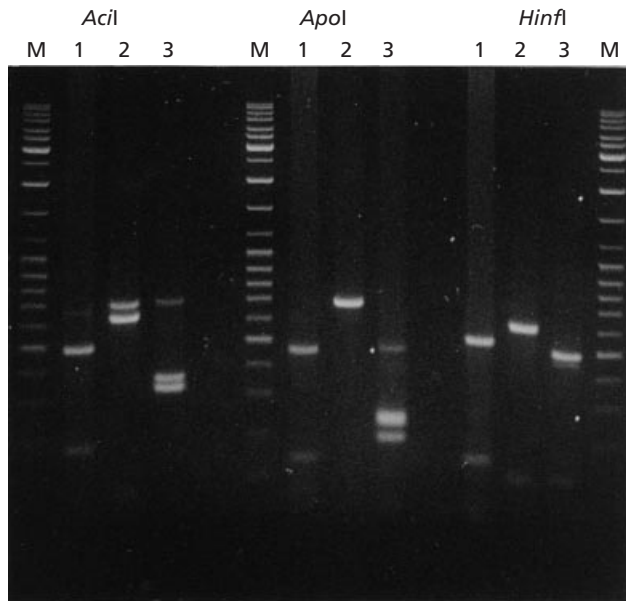


Fig. 2. Restriction enzyme analysis on 2.5% agarose gels of DNAs amplified with primers A2/J5 from symptomatic *C. capense* leaf extracts (lanes 2), and extracts of citrus leaves infected with '*Candidatus Liberibacter africanus*' (lanes 1) and '*Candidatus Liberibacter asiaticus*' (lanes 3). Lane M, 1 kb size marker (MBI, Fermentas).

C. capense. The latter had, in particular, an insertion of 25 bp compared to '*Candidatus Liberibacter africanus*'.

Fig. 2 shows that the three liberibacters (lane 1, '*Candidatus Liberibacter africanus*'; lane 2, *C. capense* liberibacter; lane 3, '*Candidatus Liberibacter asiaticus*') could be distinguished by restriction enzyme digestions of the amplified DNA with *ApoI*, *AciI* or *HinfI*. The fragments obtained were of the sizes expected, according to each sequence.

A 24-mer oligonucleotide, CAL1 (5'-GATTCGTA-GAGGTGTTTTTTGAGG-3') at position 101–124, partially overlapping the 25 bp deletion of '*Candidatus Liberibacter africanus*', was defined on the sequence of the *C. capense* liberibacter. When used as a primer in association with primer J5 in a 35 cycle PCR reaction (92 °C for 20 s, 62 °C for 20 s and 72 °C for 40 s), amplification was obtained with the DNA extracted from infected *C. capense* leaves (Fig. 3, lanes 6 and 7) but not with DNA extracted from citrus leaves infected with '*Candidatus Liberibacter africanus*' or '*Candidatus Liberibacter asiaticus*' (Fig. 3, lanes 3 and 4). No amplification was obtained with water, DNA from healthy citrus or DNA from healthy *C. capense* (Fig. 3, lanes 1, 2 and 5, respectively). The size of the amplified DNA from infected *C. capense* leaves was 588 bp, as expected from the sequence.

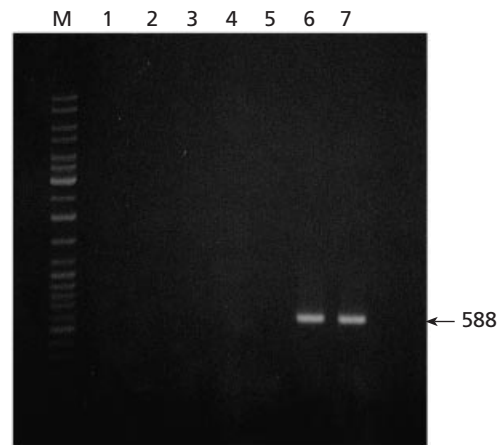


Fig. 3. Agarose gel electrophoresis of DNAs amplified with primers CAL1/J5 from water (lane 1), midrib-extracts from healthy citrus (lane 2) or *C. capense* (lane 5), citrus infected with '*Candidatus Liberibacter africanus*' (lane 3) or '*Candidatus Liberibacter asiaticus*' (lane 4), and symptomatic *C. capense* (lanes 6 and 7).

Comparison of the intergenic 16S–23S rDNA region of the liberibacters

To further compare the *C. capense* liberibacter to the known liberibacter species, the 16S–23S rDNA intergenic region was amplified with primers OI2 and 23S1 as described by Jagoueix *et al.* (1997), cloned and sequenced. When compared to the 16S–23S intergenic region of '*Candidatus Liberibacter africanus*' and '*Candidatus Liberibacter asiaticus*', sequence homology was higher with '*Candidatus Liberibacter asiaticus*' (80.62%) than with '*Candidatus Liberibacter africanus*' (66.2%). This is due to the fact that two tRNAs, those for isoleucine and alanine, were present in the intergenic region of both '*Candidatus Liberibacter asiaticus*' and the *C. capense* liberibacter, whereas only one tRNA (alanine) has been shown in '*Candidatus Liberibacter africanus*' (Jagoueix *et al.*, 1997).

Sequence of the PCR fragment amplified with primers 1897/1898 from the *C. capense* liberibacter

Primers 1897/1898, originally designed on the sequence of the *rplKAJL-rpoBC* operon of '*Candidatus Liberibacter asiaticus*' (Planet *et al.*, 1995, Villechanoux *et al.*, 1993) allowed amplification of a 1.7 kbp fragment of the equivalent operon of '*Candidatus Liberibacter africanus*'. This fragment comprises, at the 5' end, the intergenic region between ribosomal proteins A and J and, at the 3' end, the intergenic region between the ribosomal protein L and the β subunit of RNA polymerase (Villechanoux *et al.*, 1993). When used to amplify DNA extracted

Table 1. Homology (%) between genes in the β operons of the liberibacters

Gene	L. asiaticus/ L. africanus	L. africanus/ <i>C. capense</i> bacterium	L. asiaticus/ <i>C. capense</i> bacterium
3' end of <i>rplA</i>	93.5	96.8	96.8
Intergenic region A/J	55.4	58.9	67.8
<i>rplJ</i>	80.8	82.1	76.8
Intergenic region J/L	61.4	78.1	78.0
<i>rplL</i>	80.1	82.0	82.3
Intergenic region L/B	65.9	80.9	71.4
5' end of <i>rpoB</i>	89.8	90.6	87.8
Total homology	79.3	82.1	79.9

from symptomatic *C. capense* leaves, genes in the *rplKAJL-rpoBC* operon could also be amplified. Table 1 shows the percentage of homologies between the various coding or intergenic regions amplified from the *C. capense* liberibacter and the corresponding sequences from 'Candidatus Liberibacter africanus' and 'Candidatus Liberibacter asiaticus'. The complete coding regions (*rplJ* and *rplL*) have around 80% homology with both 'Candidatus Liberibacter africanus' and 'Candidatus Liberibacter asiaticus'. As expected, less homology was found within the intergenic regions. The overall sequence homologies of the *C. capense* liberibacter DNA were 82.1 and 79.9% with 'Candidatus Liberibacter africanus' and 'Candidatus Liberibacter asiaticus' DNAs, respectively. This is very similar to the degree of homology (79.3%) existing between the same operon from 'Candidatus Liberibacter africanus' and 'Candidatus Liberibacter asiaticus'.

Reaction of the *C. capense* liberibacter with mAbs prepared against several liberibacter strains

To further compare the liberibacter of *C. capense* to those present in citrus, IF reactions with mAbs produced from immunizations with Asian (Poona, India and Fuzhou, China) and African (Nelspruit, South Africa) (Gao *et al.*, 1993; Garnier *et al.*, 1987) strains of the liberibacters were carried out. The only antibodies reacting with infected *C. capense* leaf midrib sections were those specific for 'Candidatus Liberibacter africanus' (results not shown).

DISCUSSION

Using primer pairs defined from ribosomal 16S rDNA or ribosomal protein gene sequences of the two liberibacter species associated with citrus HLB, the corresponding genes of a bacterium inducing HLB-like symptoms in an ornamental rutaceous tree, *Calodendrum capense*, in the Western Cape province of South Africa have been amplified. PCR and phylogenetic analysis demonstrated that this bacterium belongs to the genus 'Candidatus Liberibacter'. From

the 16S rDNA sequence and phylogenetic analysis, as well as from the results of serology, the *C. capense*-liberibacter appears to be more closely related to 'Candidatus Liberibacter africanus' than to 'Candidatus Liberibacter asiaticus'. However, the intergenic 16S–23S region was closer to that of 'Candidatus Liberibacter asiaticus'. Whether this is the result of the preferential amplification of only one of the two rDNA operons present in the liberibacter genomes, as was already discussed in a previous paper (Jagoueix *et al.*, 1997) remains to be established. Indeed, the two liberibacter species contain each at least two ribosomal operons. Up to now, an intergenic 16S–23S region containing only tRNA (alanine) from 'Candidatus Liberibacter africanus' and an intergenic 16S–23S region containing two tRNAs (alanine and isoleucine) from 'Candidatus Liberibacter asiaticus' have been amplified. Thus, it is not known if the two operons of each species have identical 16S–23S intergenic regions.

In the absence of a culture, it is difficult to give a bacterium a species or subspecies status. The classification of the liberibacter from Africa and Asia, into two different species, was based in particular on the genomic differences observed between the conserved β operons of the two bacteria. Indeed, the homology of only 79.3% suggested that the overall homology of their entire genome was likely to be under 70%, the limit which defines species (Wayne *et al.*, 1987). The β operon genes of the *C. capense* liberibacter share a similar percentage of homology (79.9%) with those of 'Candidatus Liberibacter asiaticus' showing that it belongs to a different species. The homology with 'Candidatus Liberibacter africanus' is higher (82.1%) and, interestingly, mAbs specific for 'Candidatus Liberibacter africanus' react with the Cape chestnut liberibacter suggesting that they are closely related. Indeed, within the 'Candidatus Liberibacter asiaticus' species, in which no genomic differences have been observed within the 16S rDNA or β operon sequences, six serogroups have been identified. In India, different liberibacter serotypes can be found in orchards that are separated by only a few kilometres. In South Africa, as of today, only one serotype has been found

and the *C. capense* liberibacter, in spite of the genomic differences with the liberibacter found in *Citrus*, belongs to the same serogroup. The above results indicate that the bacterium infecting Cape chestnut belongs to the species 'Candidatus Liberibacter africanus'. However, it is proposed to give this liberibacter a subspecies status as it can be easily distinguished from the 'Candidatus Liberibacter africanus' species infecting *Citrus* by RFLP analysis of the amplified DNA or using the specific oligonucleotide CAL1.

PCR experiments with primers CAL1/J5 on DNA extracted from several HLB-affected citrus trees grown in the Western Cape area of South Africa and in particular from the orchard adjacent to the infected *C. capense* tree were negative (results not shown). This should be further documented, but suggests that: (i) the *C. capense* liberibacter is not (yet?) present in citrus; (ii) it is closely associated with the ornamental rutaceous tree; and (iii) it does not seem to be involved in the HLB outbreak in the Western Cape. The PCR method with primers CAL1/J5 can now also be used to determine whether the citrus psyllid *T. erytrae* is infected with the *C. capense* liberibacter and whether transmission to citrus is possible.

Because of the genomic and serological properties of the liberibacter from *C. capense*, it is proposed that this bacterium be assigned a subspecies status with the following designation: 'Candidatus Liberibacter africanus subsp. capensis' [(α -Proteobacteria) NC; G-F; NAS (GenBank accession no. AF137368), oligonucleotide sequence complementary to unique region of the ribosomal protein operon 5'-GATTC-GTAGAGGTGTTTTTGGAGG-3', S (*Calodendrum capense*, phloem); M].

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