

## Molecular characterization of '*Candidatus Liberibacter*' species/strains causing huanglongbing disease of citrus in Kenya

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**Abbreviations:** HLB: huanglongbing  
LH: lower highlands  
LM: lower midlands  
MA: monoclonal antibodies  
UM: upper midlands

This study was undertaken to characterize the alpha subgroup of the proteobacteria causing the huanglongbing (HLB) disease of citrus from three different ecological zones of Kenya namely the Lower highlands (LH2, LH3, 1800-1900 m above sea level);

Upper midlands (UM3, UM4, 1390-1475m), Lower midlands (LM5, LM4, LM3 of 1290-1340-1390m), by isolation and sequencing DNA encoding the L10 and L12 ribosomal proteins and the intergenic region. A 716-basepair DNA fragment was amplified and

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sequenced and consisted of 536 basepairs of DNA encoding the L10 protein, 44 basepairs of DNA intergenic region and 136 basepairs of DNA that partially encodes the L12 protein. Sequences of *rpL10/L12* protein genes from Kenyan strains were 98% and 81% similar to the South African '*Candidatus Liberibacter africanus* strain Nelspruit' and the Asian '*Candidatus Liberibacter asiaticus*' strains, respectively. The intergenic rDNA sequence of Kenyan strain from UM and LM showed 84% similarity with '*Candidatus L. africanus* strain Nelspruit' and 50% similarity with '*Candidatus L. asiaticus*' strain. However, the LH strain had an 11- basepairs deletion, while the LM4 had a 5-basepair deletion in the intergenic region compared to '*Candidatus L. africanus* strain Nelspruit'. The L10 amino acid sequence was 100% homologous among HLB bacteria obtained from the agro-ecological zones in Kenya and the L10 protein sequence was also homologous to '*Candidatus L. africanus* strain Nelspruit'. Nevertheless, the L10 amino acid sequence of '*Candidatus L. asiaticus*' and the '*Candidatus L. africanus* subsp. *capensis*' differed from the Kenyan strains by 18.36% and 11.82%, respectively. Phylogenetic analysis of both the L10/L12 rDNA sequences and the L10 amino acid sequences clustered the Kenyan strains of the '*Candidatus Liberibacter*' species with members of alpha subdivision of proteobacteria.

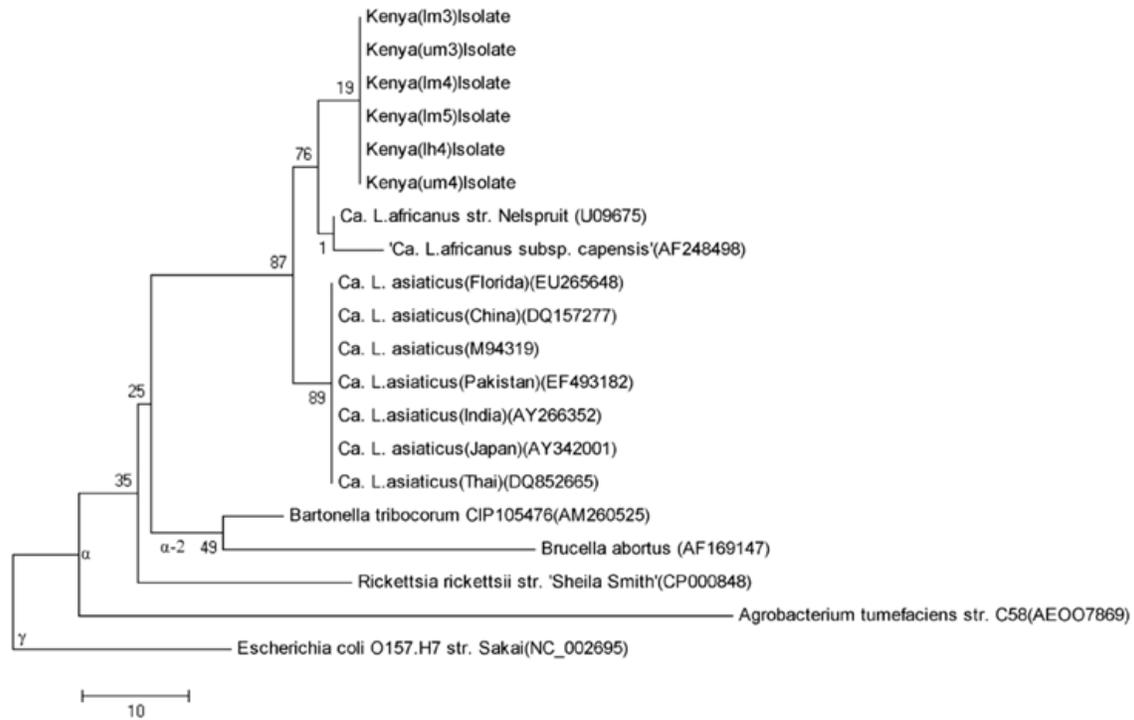
Huanglongbing (HLB) disease of citrus, also called the citrus greening disease was first reported in China in 1919 and in Africa in 1929. It is a severe and widespread disease of citrus in Asia, south-east Asia, south and eastern Africa, the Arabian peninsular (Garnier and Bové, 1993) and the Americas (Coletta-Filho et al. 2005; Teixeira et al. 2005a). In Africa it has been reported in Ethiopia, Kenya, Malagasy, Sudan, Swaziland, Tanzania and Zimbabwe (Bové, 2006). HLB disease is caused by an uncultured obligate, rod shaped, gram-negative phloem restricted bacterium that has recently been characterised (Jagoueix et al. 1994). The '*Candidatus*' generic name *Liberibacter* as defined by Murray and Schleifer (1994), for uncultured organisms has been given to the bacterium. The *Liberibacter* from Asia and Africa belong to two different '*Candidatus*' species, namely *Liberibacter asiaticus* and *Liberibacter africanus* (Villechanoux et al. 1993; Jagoueix et al. 1994; Planet et al. 1995; Hung et al. 2004). Recently, a third species '*Candidatus Liberibacter americanus*' was identified in Brazil (Coletta-Filho et al. 2005; Teixeira et al. 2005a; Teixeira et al. 2005b; Teixeira et al. 2005c).

HLB disease is transmitted among citrus trees by two psyllid vectors, *Trioza erytrae* (Del Guercio) in Africa and *Diaphorina citri* (Kuwayawa) in Asia (French et al. 2001; Boykin et al. 2007; Grafton-Cardwell et al. 2007). In Asia the proteobacteria causing HLB disease seem to be heat-tolerant as symptoms occur even at temperatures well above 30°C. In Africa, the disease is prevalent only in cold areas where temperatures do not exceed 30°C. The

proteobacteria is thought to be heat-sensitive (Bové et al. 1974). For many years the distinction between the heat-tolerant and the heat-sensitive has been the only indication that two different strains of bacterium occurred, until Garnier et al. (1987), developed MA against "Fuzhou" strain from China and "Nelspruit" strain from South Africa. MAs against proteobacteria are highly specific and only react with the homologous MA serotypes of bacteria; however, the MAs are too specific for detection of all or most strains of the proteobacteria (Villechanoux et al. 1992).

Villechanoux et al. (1992) first reported the use of DNA probes to detect Asia HLB causing bacterium strains. Hocquellet et al. (1997) developed radioactive probes and later non-radioactive probes for distinguishing between the Africa and Asiatic bacterium strains. Planet et al. (1995) cloned and sequenced the *rpL10/rpoBc* operon of the African proteobacteria, which was used for characterization of the African strain of HLB causing bacterium. Teixeira et al. (2008) sequenced the  $\beta$  operon of the American *liberibacter*, and compared it with that of the African and Asian *liberibacter*s. Other molecular studies on the proteobacteria have involved PCR detection based on the 16S ribosomal operon followed by digestion of the PCR products to distinguish between the African and Asiatic strain. Later an improvement on this protocol involved PCR-based detection of the ribosomal protein genes of the  $\beta$  operon without concomitant enzymatic digestion (Hocquellet et al. 1999a; Hocquellet et al. 1999b; Fang et al. 2004; Lan et al. 2004; Okuda et al. 2005; Li et al. 2007; Li et al. 2008; Tatineni et al. 2008). Bastianel et al. (2005) described an *omp*-based PCR-RFLP analysis method for detecting and differentiating '*Candidatus Liberibacter asiaticus*' isolates. Jagoueix et al. (1997) cloned and sequenced the intergenic spacer region 16S/23S rDNA of two strains of '*Candidatus L. asiaticus*' (Fuzhou from China), Poona from India and one strain Nelspruit from South Africa belonging to '*Candidatus L. africanus*'. These three strains from the two species belong to different serotypes and had 79.46% similarity in DNA of the intergenic spacers. The Asian strains were further characterized at the 16S and 16S/23S rDNA showing 85.5% to 100% similarity (Subandiyah et al. 2000; Fusayasu et al. 2006; Urasaki et al. 2007; Urasaki et al. 2008).

It is difficult to get accurate assessments of yield losses from HLB disease in a perennial crop such as *Citrus*. However, infected branches produce little or no marketable fruit. Experiments in South Africa and reunion to rejuvenate HLB infected *Citrus*, using tetracycline injection, have reported increases in yields of up to 140%. In Kenya, yield losses of 100% have been reported and it is speculated that HLB disease causing proteobacteria in Kenya emanated from introduction of infected scions imported from South Africa since most citrus propagules were obtained from South Africa and to a limited extent from Israel. HLB disease was first diagnosed in Kenya



**Figure 1. Minimum- evolution phylogenetic tree constructed from alignment of L10/L12 rDNA of '*Candidatus Liberibacter*' species (accession numbers in parenthesis) and other  $\alpha$  Proteobacteria.** The phylogenetic history was inferred using the Neighbor-Joining method. Bootstrap values are shown at the nodes. Phylogenetic analyses were conducted in MEGA4.

using electron microscopy in 1982 and the disease led to the subsequent destruction of all sources of propagules from National Nurseries located in the highland areas. Although HLB disease has been in Kenya for the last 20 years, there is no information of what species/strain of the proteobacteria present. '*Candidatus L. africanus*' has been thought to be the cause of the disease based on the assumption that HLB disease in Kenya originated from South Africa. In a recent study (unpublished) we adopted the PCR diagnostic procedures developed by Hocquellet et al. (1999) to HLB samples from Kenya collected at different altitudes. In this study we report on DNA sequencing of the L10/L12 ribosomal protein genes and their intergenic region in HLB disease causing proteobacteria samples collected from infected citrus at different altitudes from three agro-ecological zones and define phylogenetic relationships among the proteobacteria.

## MATERIALS AND METHODS

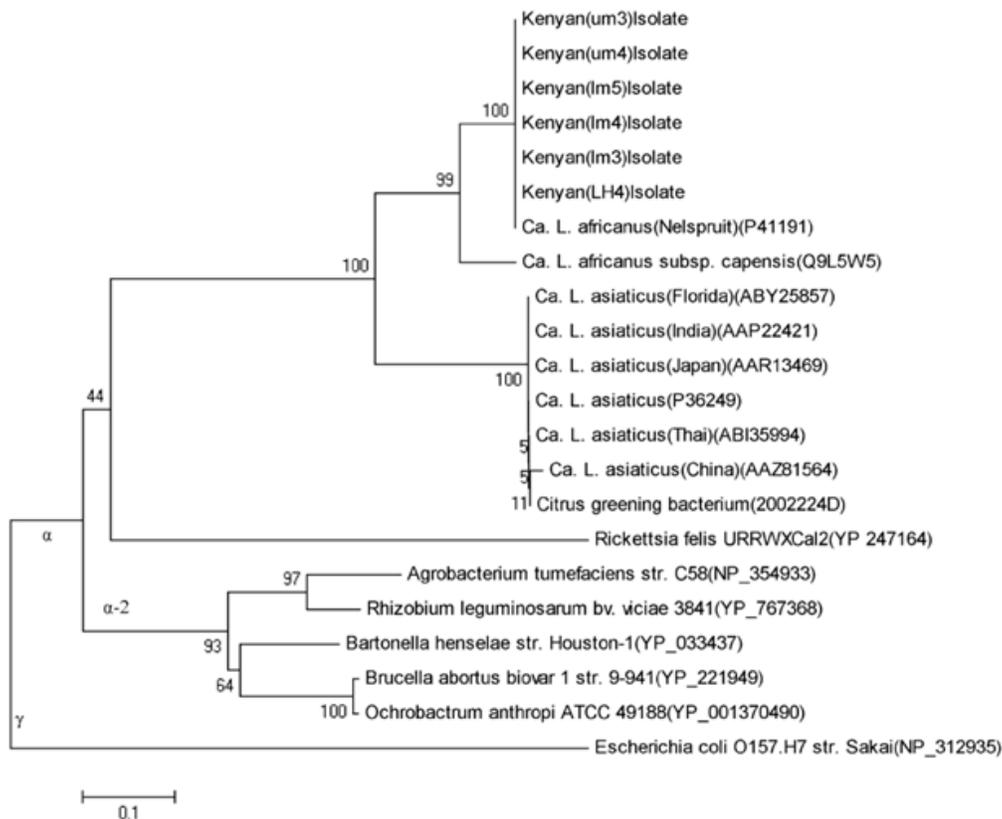
### DNA extraction and partial purification

Leaf midribs (0.1 to 0.3 g) were chopped to a fine mince with a razor blade in a disposable petri-dish in extraction buffer containing 1 mL of TE buffer (10 mM Tris pH 8.0, 400 mM EDTA plus 1% SDS and 0.25 mg of proteinase K) (Jagoueix et al. 1996). The homogenate was transferred to an eppendorf tube and incubated for 2 hrs at 65°C. The suspension was centrifuged for 15 min at 12,000 x g and

the supernatant mixed with 1 mL of Wizard Min Prep DNA purification resin (Promega). The resin was transferred to a mini-column and washed twice with 2 mL of 80% isopropanol. Thereafter, 50  $\mu$ L of hot water (80°C) was added, incubated for 1 min and centrifuged at 16,000 x g for 30 sec in an eppendorf tube. This step was repeated to have a total yield of 100  $\mu$ L of extract. The extract was stored at -20°C.

### Amplification of the L10/L12 ribosomal protein rDNA

Two primers were used to amplify the intergenic region of ribosomal protein L10/L12 rDNA and the L10 rDNA of the bacterium. Forward primer 5' (CATCGGGAGATGAAAGTTGAATA) and reverse primer 5' (TTCCCCTGCCGCAGACGCAACA). Amplification was done by PCR (Hybrid OMN-E Thermal Cycler) with the reaction conditions by Jagoueix et al. (1996). The PCR reaction was performed in 50  $\mu$ L of reaction mixture containing 1  $\mu$ M of each of the primers, Gibco buffer, 200  $\mu$ M of each of the four dNTP, 2 mM MgCl<sub>2</sub>, 0.05% W1 detergent (Gibco BRL), 100  $\mu$ g/mL BSA and 2.5 U of Taq polymerase (Gibco BRL). The reaction mix was amplified for 35 cycles with the thermal cycler at: 92°C for 20 sec (denaturation of template into single strand); 62°C for 20 sec (annealing of primers to each original strand, high temperatures are used due to the



**Figure 2. Minimum-evolution phylogenetic tree constructed from alignment of translated ribosomal protein L10 of ‘*Candidatus Liberibacter*’ species (accession numbers in parenthesis) and other  $\alpha$ Proteobacteria.** The phylogenetic history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values are shown at the nodes.

specificity of the primers); and 72°C for 45 sec (elongation and new strand synthesis). PCR products were analyzed by electrophoresis of 20  $\mu$ L of each sample (Jagoueix et al. 1996).

### Agarose gel electrophoresis and DNA elution from the gel

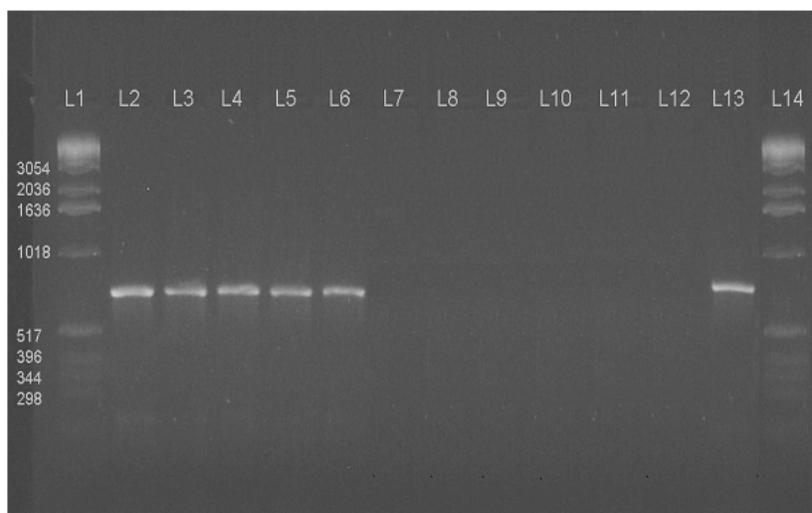
PCR products were electrophoresed on 1% agarose gels and Tris-borate buffer (1 X TBE) and stained in ethidium bromide at 0.5  $\mu$ g/mL. 20  $\mu$ L of the PCR products were mixed with 1  $\mu$ L of gel loading dye and loaded into gel. The gel was run at a voltage of 4 V/cm. After electrophoresis and photography, the DNA was extracted from the gel by use of a Gel Extraction Kit (Life Technologies). A gel slice containing the DNA was cut with a sharp razor under UV Light. The gel slice was weighed and 400 mg of the 1% gel was placed into a 1.5 mL propylene tube. Thereafter, 1.2 mL of gel solubilization buffer (concentrated sodium percholate, sodium acetate, and TBE solubilizer) was added. This was incubated at 50°C for 15 min, during which the solution was mixed every 3 min to ensure gel dissolution. The DNA was recovered through a cartridge based protocol (Life Technologies).

### DNA sequencing

Automated fluorescent DNA sequencing/Sanger-Dideoxy Nucleoside Triphosphate Chain Termination DNA Sequencing method was used. Big Dye chemistry with ABI PRISM<sup>®</sup> BigDye<sup>™</sup> Terminators v 3.0 and AmpliTaq<sup>®</sup> DNA polymerase Dyes attached to the four di-deoxynucleotide terminators were used for sequencing. The cycling reaction was done on a GeneAmp<sup>®</sup> PCR System with a MicroAmp<sup>®</sup> 96-Well Reaction Plate. The PCR fragments obtained were then separated on a denaturing polyacrylamide gel on Perkin Elmer ABI PRISM<sup>®</sup> 377 DNA sequencer. The dyes were detected by fluorescence after excitation. The sequence information was decoded by a 3100-Avant Genetic Analyzer software. The PCR product was sequenced directly without cloning it in vectors.

### Purification of sequencing template

This procedure was performed to remove primers and dNTPs, A G-50 w/spin column was rinsed by adding 300  $\mu$ L H<sub>2</sub>O to spin column and spin in a microfuge. 600  $\mu$ L Sephadex G-50 slurry (1 g / 15 mL in ddH<sub>2</sub>O) was added to the spin column and microfuge at 4900 RPM for 60 sec. 20



**Figure 3. 1% agarose electrophoresis gel of L10/L12 ribosomal protein PCR products. Lanes 7 -11-undiseased; lanes 2-6 - positive 716bp amplification from infected samples; lanes 1 and 14 1kb DNA ladder; lane 12 negative control; lane 13 positive control.** The results show that plants from tissue culture supported nurseries were HLB-disease free, while plants from low altitude nurseries/fields (1000 m above sea level) and high altitude (2000 m) not supported by tissue culture were infected with the '*Candidatus Liberibacter africanus*' giving a PCR fragment of 716 base pairs.

$\mu$ L PCR products was loaded to the center of the Sephadex and microfuged at 4900 RPM for 60 sec and the product was transferred into fresh 1.5 mL eppendorf tube (QIAGEN Guide, 1998; Applied Biosystems, 2002).

#### Extension/termination reactions

A reaction mixture consisting of template DNA 30-90 ng, primer 2.0 -3.0 pmol, buffer 4.0  $\mu$ L, T and terminator Ready Reaction mix 2.0  $\mu$ L was added into a 500  $\mu$ L eppendorf tube and made to final volume of 20  $\mu$ L with sterile H<sub>2</sub>O (Perkin-Elmer Corporation, 1997; Applied Biosystems, 2002). The reaction mixture was briefly mixed by pipetting. It was overlaid with 2 drops of mineral oil and spun down. The reaction tubes were placed into a thermalcycler (ThermoHybaid Omni-E) that had been preheated to 95°C in a start the cycling program. The preheating was to prevent non-specifically annealed primers from being extended (and thus stabilized) by Amplitaq DNA polymerase. Thermalcycler was programmed to run at 95°C for 1 min 30 sec preheating, then 95°C for 30 sec, 59°C for 15 sec (sequence-specific annealing temperature), 60°C for 4 min and 25 cycles. The sample was stored at 4°C at the end of the cycles. The PCR products were purified by running the products through Centri-Sep Columns to remove excess dye terminators (Perkin-Elmer Corporation, 1997; Applied Biosystems, 2002). 1.5  $\mu$ L of the purified PCR products were loaded on 5.0% Long Ranger, 6 M-urea gel.

#### DNA sequencing and homology comparisons

The DNA was sequenced in a 48 cm ABI sequencing gel (5.0% long ranger acrylamide, 6 m urea). The gel consisted of 18.0 g urea, 5.0 mL 10 X TBE, 5.0 mL Long Ranger, 25.0  $\mu$ L TEMED, 250.0  $\mu$ L 10% APS, 27 mL ddH<sub>2</sub>O (up to

50 mL) (Perkin-Elmer Corporation, 1997; Applied Biosystems, 2002). After polymerization for 10-15 min the gel was positioned and warmed for 15 min to 51°C. Samples were heated to 95°C for 2 min and cooled on ice and 1.5  $\mu$ L loaded in each well. Buffer was loaded into any empty wells to the right and left of the samples. The gel was run at a constant 48 watt for 8 hrs and the ABI base-caller results were received in ASCII format and were subjected to homology comparisons with other published sequences in the GenBank, EMBL, DDBJ, PDB Database using the BLAST (basic logical alignment sequencing tool) (Altschul et al. 1997), on the National Center for biotechnology information (NCBI) Genbank website.

#### Phylogeny analysis for the L10/L12 rDNA

Bootstrap test of phylogeny using the neighbourhood joining method and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). The L10/L12 rDNA sequences and the L10/L12 intergenic regions of '*Candidatus Liberibacter*' species from different agro-ecological zones in Kenya and those reported on the National Centre for Biotechnology Information (NCBI) Genbank were used in the analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 99 positions in the final dataset. Seven L10 rDNA, L10/L12 intergenic rDNA and partial L12rDNA sequences from '*Candidatus Liberibacter asiaticus*' species were utilized in the analysis, they included isolate "Thai" (accession no. DQ852665), isolate "Japan" (accession no. AY342001), isolate "India" (accession no. AY266352), isolate

Table 1 Composition of medium in transformation experiment.

Homology Gene	LM4/UM3	LM4/UM4	LM4/LH4	LM4/LM5	LM4/LM3	LM4/LAN	LM4/CLA	LM4/LAC
<i>RPL10/RPL12</i> intergenic gene	100	100	84.09	100	100	83.67	49	65.31
<i>RPL10</i> gene	100	100	100	100	100	99	83	83.18
<i>RPL12</i> partial gene	100	100	100	100	100	99.26	91.9	84.56
L10 amino acid sequence	100	100	100	100	100	100	81.64	88.18
<i>RPL10/RPL12</i> DNA (720BP)	100	100	94.7	100	100	98	81	83

“Pakistan” (accession no. EF493182), isolate “Asia” (accession no. M94319), isolate “China” (accession no. DQ157277), and isolate “Florida” from the USA (accession no. EU265648). Two sequences were from South Africa '*Candidatus* *Liberibacter africanus* subsp. *capensis*' (accession no. AF248498) and '*Candidatus* *Liberibacter africanus* str. Nelspruit' (accession no. U09675). Other *Proteobacteria* belonging to the  $\alpha$  subdivision used included, *Agrobacterium tumefaciens* str. C58 (accession no. AEO07869), *Rickettsia rickettsii* str. 'Sheila Smith' (accession no. CP000848), *Brucella abortus* (accession no. AF169147), and *Bartonella tribocorum* CIP105476 (accession no. AM260525). *Escherichia coli* O157.H7 str. Sakai (accession no. NC\_002695) was used as an outgroup and belongs to the  $\gamma$  subdivision of *Proteobacteria*. Six isolates from Kenya (Kenya (lm3) isolate, Kenya (um3) isolate, Kenya (lm4) isolate, Kenya (lm5) isolate, Kenya (lh4) isolate and Kenya (um4) isolate) were utilized in the analysis (Figure 1).

### Phylogeny analysis for the ribosomal protein L10

The amino acid sequence of the L10 protein of '*Candidatus* *Liberibacter*' species from Kenya and those reported were also used in determining phylogeny within the species. The phylogenetic history was inferred using the Minimum Evolution method with a bootstrap test (500 replicates). The evolutionary distances were computed using the Poisson correction method and the ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000). The Neighbor-joining algorithm was used to generate the initial tree in MEGA4 (Tamura et al. 2007). Six L10 protein amino acid sequences from Kenyan isolates (Kenya (lm3) isolate, Kenya (um3) isolate, Kenya (lm4) isolate, Kenya (lm5) isolate, Kenya (lh4) isolate and Kenya (um4) isolate) were utilized in the analysis with L10 proteins from South African isolates '*Candidatus* *Liberibacter africanus* subsp. *capensis*' (accession no. Q9L5W5) and '*Candidatus* *Liberibacter*

*africanus* str. Nelspruit' (accession no. P41191). The '*Candidatus* *Liberibacter asiaticus*' isolates included isolate “Thai” (accession no. ABI35994), isolate “Japan” (accession no. AAR13469), isolate “India” (accession no. AAP22421), isolate “Asia” (accession no. P36249), isolate “China” (accession no. AAZ81564) and isolate “Florida” from the USA (accession no. ABY25857). Other *Proteobacteria* belonging to the  $\alpha$  subdivision used included, *Agrobacterium tumefaciens* str. C58 (accession no. NP\_354933), *Rhizobium leguminosarum* bv. *viciae* 3841 (accession no. YP\_767368), *Bartonella henselae* str. Houston-1 (accession no. YP\_033437), *Brucella abortus* biovar 1 str. 9-941 (accession no. YP\_221949), *Ochrobactrum anthropi* ATCC 49188 (accession no. YP\_001370490). *Escherichia coli* O157.H7 str. Sakai (accession no. NP\_312935) was used as an outgroup and belongs to the  $\gamma$  subdivision of *Proteobacteria* (Figure 2).

## RESULTS AND DISCUSSION

### DNA sequences of L10 and L12 ribosomal protein genes

A 716-basepair DNA fragment was amplified (Figure 3) and sequenced, producing 536 basepairs of DNA encoding the L10 protein, 44 basepairs of DNA intergenic region and 136 basepairs of DNA that partially encodes the L12 protein. Genes encoding the L10 protein of alpha subgroup *proteobacteria* have been sequenced and characterized in '*Candidatus* *L. africanus* strain Nelspruit' from South Africa (Planet et al. 1995), '*Candidatus* *L. africanus* subsp. *capensis*' (Garnier et al. 2000) in '*Candidatus* *L. asiaticus*' (Asiatic strains) (Villechanoux et al. 1993; Okuda et al. 2005) and in '*Candidatus* *L. americanus*' (Teixeira et al. 2008). The L10 and L12 protein are transcribed from the *rplKAIL-rpoBC* ribosomal protein-RNA polymerase gene cluster. The most abundant transcript is the 2600 nucleotide tetracistronic L11-L1-L10-L12 mRNA initiated at the upstream major PL11 promoter and terminated at the

transcription attenuator in the L12-beta intergenic space (Pettersson, 1979). Comparison DNA sequence of the *rpL10* protein gene showed that the Kenyan isolates from different agro-ecological zones were 100% homologous to one another. In contrast the *rpL10* DNA sequence from the Kenyan strains was 99%, 83% and 83.18% similar to 'Candidatus L. africanus strain Nelspruit', 'Candidatus L. africanus subspecies capensis' and 'Candidatus L. asiaticus' strains, respectively (Table 1). Comparisons of the partial 136 base sequence of the *rpL12* gene showed 100% homology among isolates from the Kenya's agro-ecological zones, but 99.26%, 91.9% and 84.56% similarity to the 'Candidatus L. africanus strain Nelspruit', 'Candidatus L. asiaticus' and 'Candidatus L. africanus subsp. capensis' strains, respectively (Table 1). The low similarity between the Kenyan isolates and 'Candidatus L. africanus subsp. capensis' as compared to 'Candidatus L. asiaticus' may be attributed to the incomplete sequence of the *rpL12* gene. However, the Kenyan strains of the alpha subgroup of HLB disease causing proteobacteria were different from the strains characterized from South African strains and the Asiatic strains, but more closely related to the former than

the later based on the *rpL10* and the *rpL12* protein gene.

**Translation and comparison of L10 ribosomal protein**

In the current study, DNA sequence of L10 gene was translated into a 169 amino acid protein sequence, with a molecular weight of 18637.6 and an atomic formula: C<sub>829</sub>H<sub>1388</sub>N<sub>232</sub>O<sub>248</sub>S<sub>2</sub>. It had an estimated half-life of 30 hrs and an instability index of 32.85, which classifies the protein as stable. It had a start codon of TTG at the seventeenth base while the L12 protein has its start codon at the 580<sup>th</sup> base in Kenyan isolate LM4 (Figure 4). In prokaryotes the ribosomal protein L10 is one of the proteins from the large ribosomal subunit that belongs to the L10 protein family of ribosomal proteins. L10 proteins range in size from 135 amino acids in proteobacteria *Brucella abortus* (Oliveira et al. 1994) to 199 amino acids in both aquificae *Aquifex aeolicus* (Deckert et al. 1998) and *A. pyrophilus* (Klent et al. 1999) and they have only been found so far in eubacteria. The ribosomal protein L8 is a

LM4		Ribosomal protein L10 Machakos district lower midland 4.	
LH4		Ribosomal protein L10 from lower highland 4 Altitude 1650 M ASL	
LAN	Gi507767	ribosomal protein L10 [ <i>Candidatus Liberibacter africanus</i> ]Nelspruit	
CLA	Gi551768	ribosomal protein L10 [ <i>Candidatus Liberibacter asiaticus</i> ]	
LAC	Gi7716606	RplJ [ <i>Candidatus Liberibacter africanus</i> subsp. capensis]	
LM4:	1	MNRQEKSVSELSKIFSSSGSVVVAHYKGISVAQIKDLRKKVREAGGVKVAKNRLVKI	60
LH4:	1	*****	60
LAN:	1	*****	60
LAC:	1	*****L*****RTSKEGX*****	60
CLA:	1	****G*****I*****M*****	60
LM4:	61	AVSDTSLKGVSDLFVQGSLIVYSVDPVIVAPKISVSFANDNKQFVVLGGILEKDILDQDSI	120
LH4:	61	*****	120
LAN:	61	*****	120
LAC:	61	**R*****E*****N*****V***V***C**	120
CLA:	61	*IR**IR*I*****DS*VI*****S***NE*R***VV**GV*N***	120
LM4:	121	KRIASLPNIDGIRSMIISAIQFNSTRVNLNAPQTKIVRAISAFVDKN	169
LH4:	121	*****R**	169
LAN:	121	*****	169
LAC:	121	*Q*****AI*****A*K*LR**S***AQV***L*****	169
CLA:	121	*Q*****DLE***AG*****S*A***R**GT***QV*****	169

The composition of amino acids in the L10 protein is as follows

Ala (A) 12 7.0%;	Lys (K) 16 9	Glx (Z) 0 0.0%;
Arg (R) 8 4.7%;	Met (M) 2 1.2%;	Xaa (X) 0 0.0%
Asn (N) 9 5.2%;	Phe (F) 6 3.5%;	
Asp (D) 10 5.8%;	Pro (P) 4 2.3%;	
Cys (C) 0 0.0%;	Ser (S) 22 12.8%;	
Gln (Q) 9 5.2%;	Thr (T) 3 1.7%;	
Glu (E) 5 2.9%;	Trp (W) 0 0.0%;	
Gly (G) 10 5.8%;	Tyr (Y) 2 1.2%;	
His (H) 1 0.6%;	Val (V) 21 12.2%;	
Ile (I) 19 11.0%;		
Leu (L) 13 7.6%;		

Figure 4. Alignment of the translated ribosomal protein L10 sequences showing amino acid variations between the Kenyan, South African and Asian strains of the bacteria.

LM4	Sample from Machakos district lower midland 4. Altitude 1317 M ASL
UM3	Sample from Machakos district upper midland 3. Altitude 1461 M ASL
UM4	Sample from Machakos district upper midland 4. Altitude 1377.7 M ASL
LH4	Sample from Kabete Lower Highland 4. Altitude 1650 -1900 M ASL
LM5	Sample from Machakos district lower midland 5. Altitude 1309.8 M ASL
LM3	Sample from Machakos district lower midland 3. Altitude 1455.6 M ASL
LAN	gi 507766 <i>Candidatus Liberibacter africanus</i> Welspruit ribosomal protein L10 (rplJ) and ribosomal protein L12 (rplL) genes, complete cds, 1673 bps
CLA	gi 144454 <i>Candidatus Liberibacter asiaticus</i> ribosomal protein and RNA polymerase (rplKAJL-rpoB) gene cluster. 3197 bps
LAC	gi 7716604 <i>Candidatus Liberibacter africanus</i> subsp. capensis RplA (rplA) gene, partial cds; RplJ (rplJ) and RplL (rplL) genes, complete cds; and RpoB (rpoB) gene, partial cds. 1658 bps
LM4:	1 catcgggagatgaaagtgaatagcgaagaagaagagtgtnmaaatctcgaattaagtaa 60 gattttttcttcttctggatcagttgttctgctgcacataaaggaattgtgagcca 120
UM3:	1 *****n*****nm*****nm***** 60 *****n*****nm*****nm***** 120
UM4:	1 *****n*****n*****n*****n***** 60 *****n*****n*****n*****n***** 120
LH4:	1 nnnnnnnnn*****tct***** 60 *****n*****n*****n*****n***** 120
LM5:	1 *****c*****nm***** 60 *****n*****n*****n*****n***** 120
LM3:	1 *****n*****n*****n*****n***** 60 *****n*****n*****n*****n***** 120
LAN:	171 *****gg***** 230 *****n*****n*****n*****n***** 290
CLA:1589	*****t*****g*****c*****ag***** 1648 *****a*****a*****t*****t***** 301
1708	
LAC:	182 *****n*****ag*****a*****a*****a*****g*****g*****g***** 241 *****g*****g*****c*****a*****t*****a***** 301
LM4:	121 gatataaagatcttcgaagaagaagctgcgagaagctggtggaggtgtaaaaggttcgcaaaaa 180 tctctctgttaagattgccgtagctagcttgaaggggtgttcagatctttttgt 240
UM3:	121 *****n*****nm*****nm***** 180 *****n*****nm*****nm***** 240
UM4:	121 *****n*****n*****n*****n***** 180 *****n*****n*****n*****n***** 240
LH4:	121 *****n*****n*****n*****n***** 180 *****n*****n*****n*****n***** 240
LM5:	121 *****n*****n*****n*****n***** 180 *****n*****n*****n*****n***** 240
LM3:	121 *****n*****n*****n*****n***** 180 *****n*****n*****n*****n***** 240
LAN:	291 *****gg***** 350 *****n*****n*****n*****n***** 410
CLA:1709	*****g*****a*****g*****g*****a*****c***** 1768 *****c*****c*****ta*****t*****a*****ga*****a*****g*****a***** 421
1828	
LAC:	302 *****c*****ga*****nm*****g*****nmnn***** 361 *****c*****c*****t*****cg*****a*****t*****c*****gt 421
LM4:	241 tggccaatcattgattgtttatctcgttgaccctattgttgc tnmnaagattctctgtgag 300 ctctgcgaatgataataaacagtttctggtctctggtgtattttggagaagatattct 360
UM3:	241 *****cct***** 300 *****n*****nm*****nm***** 360
UM4:	241 *****cct***** 300 *****n*****n*****n*****n***** 360
LH4:	241 *****nct***** 300 *****n*****n*****n*****n***** 360
LM5:	241 *****cct***** 300 *****n*****n*****n*****n***** 360
LM3:	241 *****cct***** 300 *****n*****n*****n*****n***** 360
LAN:	411 *****cct***** 470 *****n*****n*****n*****n***** 530
CLA:1829	*****g*****t*****c*****a*****ag*****g*****a*****cct*****a*****g*****t***** 1888 *****c*****c*****t*****a*****aga*****gg*****g*****g*****g*****g***** 530
1948	
LAC:	422 *****c*****t*****cct*****a*****a***** 481 *****c*****a*****c*****g*****a*****a*****ag*****a*****a*****t***** 541
LM4:	361 tgaccaagattctatcaaacgattgctctcgttgccctaatattgatnntattcgttctat 420 gatcattagtc tattcaatttaattcgaactagattgtaaaactctttaaagcactca 480
UM3:	361 *****n*****nm*****g***** 420 *****n*****n*****n*****n*****n*****n*****n***** 480
UM4:	361 *****g***** 420 *****n*****n*****n*****n*****n*****n*****n***** 480
LH4:	361 *****gg***** 420 *****n*****n*****n*****n*****n*****n*****n***** 480
LM5:	361 *****gg***** 420 *****n*****n*****n*****n*****n*****n*****n***** 480
LM3:	361 *****gg***** 420 *****n*****n*****n*****n*****n*****n*****n***** 480
LAN:	531 *****gg***** 590 *****n*****n*****n*****n*****n*****n*****n***** 650
CLA:1949	*a*t*****g*aa*****t*a*c*g*t*c*****gg*****ag*gg 2008 *****a*****c*****c*****g*****t*****ga*****gg*a*g*a***** 2068
LAC:	542 *****t*****g*****a*****aa*****t*****a*****g*****c*****g*****g***** 661
LM4:	481 gactaaaaattgctcgtctattctcgtctttgtagataaaaaaacaacaaagtttagactcc 540 aagttatt----aataccaacaagaataaggaataatggtgatatgtccaatattgaat 595
UM3:	481 *****n*****nm*****g***** 540 *****n*****n*****n*****n*****n*****n*****n***** 595
UM4:	481 *****g***** 540 *****n*****n*****n*****n*****n*****n*****n***** 595
LH4:	481 *****g***** 540 *****n*****n*****n*****n*****n*****n*****n***** 595
LM5:	481 *****g***** 540 *****n*****n*****n*****n*****n*****n*****n***** 595
LM3:	481 *****g***** 540 *****n*****n*****n*****n*****n*****n*****n***** 595
LAN:	651 *****gg***** 710 *****n*****n*****n*****n*****n*****n*****n***** 770
CLA:2069	*****t*****g*****g*****g*****g*****tctctg 2128 tca*c*g*t*aaaa*c*t*ag*t*a*a*c*a*****a***** 770
2187	
LAC:	662 *g*ac**g*****c*****ca*****cct*t 721 *****aaaaa**c*taag**c*a**c**g*****g*****g***** 781
LM4:	596 caattgttgaataattatcgtctcttctcgtctcttcaagcggcagaactcttcaaaagat 655 tagaagaagaatggggagttctctgctgctcctgctagcttctgtgctgctgctgag 715
UM3:	596 *****n*****nm*****nm***** 655 *****n*****n*****n*****n*****n*****n*****n***** 715
UM4:	596 *****n*****n*****n*****n***** 655 *****n*****n*****n*****n*****n*****n*****n***** 715
LH4:	596 *****n*****n*****n*****n***** 649 *****n*****n*****n*****n*****n*****n*****n***** 709
LM5:	596 *****n*****n*****n*****n***** 655 *****n*****n*****n*****n*****n*****n*****n***** 715
LM3:	596 *****n*****n*****n*****n***** 655 *****n*****n*****n*****n*****n*****n*****n***** 715
LAN:	771 *****gg***** 830 *****n*****n*****n*****n*****n*****n*****n***** 890
CLA:2188	*****g*****c*****t*****ta*****g*****c*****g*****a***** 2247 *****g*****t*****t*****c*****c*****c*****t*****t***** 890
2407	
LAC:	782 *****g*****t*****ta*****c*****c*****c*****c*****c*****c*****c***** 893
LM4:	716 gggaa 720
UM3:	716 ***** 720
UM4:	716 ***** 720
LH4:	710 ***** 714
LM5:	716 ***** 720
LM3:	716 ***** 720
LAN:	891 ***** 895
CLA:	2408 ca**g 241.3
LAC:	894 ***** 899

Figure 5. Alignment of sequenced L10/L12 ribosomal protein DNA; ribosomal protein L10 DNA (bases 1-535), L10/ L12 intergenic DNA (bases 536-584) and L12 DNA (bases 585-720) showing nucleic acid variations between the Kenyan, South African and Asian strains of the bacteria. Showing a 5 base deletion in L10/ L12 intergenic DNA of LM4, UM3, UM4, LM5, LM3 and an eleven base deletion in LH4.

\*signifies similarity of the base with the reference sequence, - refers to a deletion, while n refers to a missing base. BLAST 2 version BLASTN 2.2.6 (<http://www.ncbi.nlm.nih.gov/blast/>)

complex L7/L12 and L10 (Pettersson et al. 1976). The acidic stalk protein complex, L10.L7/L12 in prokaryotes, binds to a region around residues 1030-1127 in domain II of 23S rRNA, termed the GTPase-associated domain (Beauclerk et al. 1984), and constitutes a part of the functional center, termed the GTPase center or factor-binding center (Ban et al. 1999; Ban et al. 2000). The four copies of L7/L12 bind to L10 and form the pentameric complex (Liljas and Gudkov, 1987); the L10 moiety of the complex appears to bind directly to the rRNA domain (Pettersson, 1979). In the current study, the L10 amino acid sequence of *Proteobacteria* isolates from LM4 and LH4 agro-ecological zones were identical to each other and to '*Candidatus L. africanus* Nelspruit', but had 88.18% homology to the isolate '*Candidatus L. africanus* subsp. *capensis*' and 81.64% similarity to '*Candidatus L. asiaticus*' (Table 1). Previous comparisons of the species on the 16S/23S intergenic regions and the tRNAs<sup>Ala</sup> sequence identified in the 16S/23S intergenic regions of '*Candidatus L. africanus*' and '*Candidatus L. asiaticus*' region were found to be 79.46% and 87.8% respectively (Jagoueix et al. 1997). Furthermore, the outer membrane protein (omp) of '*Candidatus L. africanus*' (isolate 'South Africa-Nelspruit') and '*Candidatus L. asiaticus*' nucleotide sequences exhibited 72.2% identity, whereas the encoded polypeptides had 58% identical and 86.5% similar amino acids (Bastianel et al. 2005). Comparison of the  $\beta$  operon of liberibacters shows that '*Ca. L. africanus*' and '*Ca. L. asiaticus*' shared 81.2% sequence identity, the percentage for '*Ca. L. americanus*' and '*Ca. L. africanus*' was only 72.2%, and identity for '*Ca. L. americanus*' and '*Ca. L. asiaticus*' was only 71.4% (Teixeira et al. 2008). Beyond showing variation on the L10 protein sequence in the species, these results further indicate that the Kenyan strains of the proteobacterium were more closely related to '*Candidatus L. africanus*' than the '*Candidatus L. asiaticus*' in the amino acid sequences of the L10 and L12 ribosomal proteins.

### Intergenic region of L10/L12 ribosomal protein genes

A 44-DNA base sequence comprising of the intergenic region of '*Candidatus L. africanus*' strains from Kenya was obtained from UM3, UM4, and LM5, LM3 agro-ecological zones which were five bases shorter than the sequences obtained from '*Candidatus L. asiaticus*' and '*Candidatus L. africanus* subsp. *capensis*' and '*Candidatus L. africanus* strain Nelspruit' (Figure 5). However, intergenic DNA sequences of Kenyan strain LH4 had an 11-basepair deletion compared to '*Candidatus L. asiaticus*' and '*Candidatus L. africanus*' strains. The intergenic regions from the LM4 isolates were 100%, 100%, 84.09%, 100%, 100%, 83.67%, 49% and 65.31% similar to isolates from LM3, LM5, LH4, UM3, UM4, '*Candidatus L. africanus* strain Nelspruit', '*Candidatus L. asiaticus*' and '*Candidatus L. africanus* subsp. *capensis*' strains, respectively (Table 1). The intergenic regions of '*Candidatus L. africanus*' strain from Kenya isolated from agro-ecological zones LM3,

LM5, UM3 and UM4 were 100% homologous to one another. Similarly, the 595 basepair intergenic region of the 16S/23S of the '*Candidatus L. asiaticus*' strains from India and the People's Republic of China were 100% homologous to one another, although they were isolated distant geographically regions (Jagoueix et al. 1997). However, the level of homology of the entire 16S/23S intergenic regions of '*Candidatus L. africanus*' and '*Candidatus L. asiaticus*' was 87.46% and was only 498 base pairs long (Jagoueix et al. 1997). Because of the high homology between the '*Candidatus L. asiaticus*' strains from India and the People's Republic of China even though the two strains belonged two different serotypes (Jagoueix et al. 1997), concluded that probably in '*Candidatus Liberibacter*' the 16S/23S intergenic region does not vary much within a species. Hence monoclonal antibodies remain the only reagents that allow identification of '*Candidatus Liberibacter*' strains within a given species. However in the current study, the variability in the sequences of '*Candidatus L. africanus* strain Nelspruit' were high, 49%, 65.31%, and 83.67% homology respectively, to '*Candidatus L. asiaticus*', '*Candidatus L. africanus* subsp. *capensis*' and '*Candidatus L. africanus* strain from Kenya isolated from LM and UM. Results from the current study show that variability within the L10/L12 intergenic regions was high and may be used as a means of distinguishing between strains in the alpha subgroup of HLB disease causing proteobacteria. In addition the results from comparison of DNA sequences from the L10/L12 intergenic region further corroborate that the Kenyan strains of the proteobacteria are different from the South African strains and the Asiatic strains but more closely related to the former than the later.

### Phylogeny of the '*Candidatus Liberibacter*' species

The ribosomal DNA sequence and the intergenic rDNA sequences have been previously used to study phylogeny in *Proteobacteria* (Hugenholtz, 2002), among the 21 Phloem-restricted, uncultured walled bacteria two proteobacteria have been phylogenetically characterized; the citrus huanglongbing (greening) (Jagoueix et al. 1994) and the strawberry chlorosis bacterium (Zreik et al. 1998). Both have been phylogenetically characterized based on the polymorphism within the rDNA sequences. The L10/L12 intergenic rDNA sequences were used to cluster the '*Candidatus Liberibacter*' species isolated in Kenya as compared to those posted in the genebank (Figure 1). The Kenyan strains of the '*Candidatus Liberibacter*' species clustered within the alpha ( $\alpha$ ) subdivision of proteobacteria. The Kenyan strains clustered away from the other African strains but with low bootstrap values of 19 (Figure 1), showing that the difference between the African strains is not wide. The Kenyan strains formed a cluster with the South African isolates '*Capensis*' (Garnier et al. 2000) and '*Nelspruit*' (Planet et al. 1995) with bootstrap values of 76 (Figure 1). This showed that the Kenyan strains are more closely related to the South African strains than the Asian strains. However the Asian isolates ('*Thai*', '*India*', '*China*',

'Japan', 'Florida', 'Asia' (Villechanoux et al. 1993), and 'Pakistan' (Chohan et al. 2007) clustered within the  $\alpha$  subdivision. Other members of the  $\alpha$  subdivision including; *Agrobacterium tumefaciens* str. C58 (Wood et al. 2001), *Rickettsia rickettsii* str. 'Sheila Smith', *Brucella abortus* (Campos et al. 1998), and *Bartonella tribocorum* CIP105476 (Saenz et al. 2007), clustered with all  $\alpha$ -2 proteobacteria away from *Escherichia coli* O157.H7 str. Sakai (Makino et al. 1999; Yokoyama et al. 2000; Hayashi et al. 2001) that represented the gamma ( $\gamma$ ) subdivision of proteobacteria.

Ribosomal protein sequences have been utilized for phylogenetic analysis in eubacteria, due to their evolutionary important structural and functional properties and the existence of conserved domains (Müller and Wittmann-Liebold, 1997). The *nusG-rplKAL-rpoBC* gene cluster has been previously used to study phylogenetic relationships in Liberibacters (Villechanoux et al. 1993). The L10 amino acid sequence phylogeny shows the Kenyan isolates (UM3, UM4, LM5, LM4, LM3, and LH4) clustering with other  $\alpha$  proteobacteria. The Kenyan strains and the South African strain 'Nelspruit' (Planet et al. 1995), form a distinct cluster with high bootstrap values away from the South African isolate 'Capensis' (Garnier et al. 2000) (Figure 2). However, the Kenyan and the South African isolates share a common origin more recent than the one they share with the Asian isolates ('Thai', 'Japan', 'India', 'Asia', 'China' and 'Florida') denoting the close relationship between the Kenyan and South African isolates (Figure 2). Other members of the  $\alpha$  subdivision of *Proteobacteria* *Agrobacterium tumefaciens* str. C58 (Goodner et al. 2001; Wood et al. 2001), *Rhizobium leguminosarum* bv. viciae 3841 (Young et al. 2006), *Bartonella henselae* str. Houston-1 (Alsmark et al. 2004), *Brucella abortus* biovar 1 str. 9-941 (Halling et al. 2005), *Ochrobactrum anthropi* ATCC 49188, *Escherichia coli* O157.H7 str. Sakai (Yokoyama et al. 2000; Hayashi et al. 2001), diverged from the Liberibacters earlier and forms a cluster with a bootstrap value of 93.

The L10/L12 rDNA and the L10 protein phylogenetic clusters show that the Kenyan isolates and other Liberibacters are members of the  $\alpha$  subdivision of proteobacteria. This is consistent with findings using the *omp* gene (Bastianel et al. 2005), the 16S rRNA (Jagoueix et al. 1994, Teixeira et al. 2005b), the 16S rDNA (Garnier et al. 2000) and the 16S/23S rDNA (Jagoueix et al. 1997). Therefore, the L10/L12 intergenic rDNA and the L10 protein serves as a good tool for phylogeny analysis among 'Candidatus Liberibacter' species, however, the region cannot be used to cluster within a species as was reported for the 16S rDNA and 16/23S rDNA. (Jagoueix et al. 1997; Teixeira et al. 2005b). These results suggest that, the 'Candidatus Liberibacters' active in the country maybe South African in origin.

## CONCLUDING REMARKS

The isolated HLB causing proteobacteria in Kenya was more closely related to the 'Candidatus L. africanus' strains from South Africa than the 'Candidatus L. asiaticus' strains isolates from Asia with respect to the L10 and L12 rDNA and amino acid sequences and the L10/L12 intergenic DNA sequences. There was high homology in L10 and L12 rDNA and amino acid sequences and the L10/L12 intergenic DNA sequences of alpha proteobacteria isolated from different agro-ecological zones in Kenya. Phylogeny analysis on the L10/L12 rDNA and the L10 amino acid sequence shows significant clusters within the 'Candidatus Liberibacter' species with the Kenyan strains being closely related to the South African strains. The HLB disease causing bacteria in Kenya is distinct from the South African but they have a common origin more recent than the one they share with the Asian HLB causing bacteria.

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