

INSECT-TRANSMITTED PROCARYOTES

A New *Liberibacter* Species, *Candidatus Liberibacter americanus* sp. nov., is Associated with Citrus Huanglongbing (Greening Disease) in São Paulo State, Brazil*

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ABSTRACT. *Candidatus Liberibacter africanus* and *Ca. L. asiaticus* are α -*Proteobacteria*, responsible for citrus huanglongbing (HLB) in Africa and Asia, respectively. Until 2004, the disease had never been reported on the American continent. However, South, Central, and North America (Argentina, Brazil, Venezuela, the Caribbean basin, Florida, Texas, Yucatan) harbour *Diaphorina citri*, the Asian psyllid vector of *Ca. L. asiaticus*. In early 2004, leaf and fruit symptoms resembling those of HLB were observed in several sweet orange orchards in the Araraquara area of Sao Paulo State (SPS). Shoots with affected leaves were yellowish. Leaves showed characteristic blotchy mottle symptoms. Fruits were small and lopsided, had strong color inversion and contained many aborted seeds. Symptomatic and symptomless sweet orange leaves were analyzed for the presence of the HLB-liberibacters by PCR using the HLB-specific primers OA1+OI1/OI2c. None of the symptomatic leaf samples gave a positive PCR amplification, while liberibacter-infected citrus leaves from the Bordeaux HLB collection, tested positive. These results suggested the occurrence of a new bacterial HLB-agent in SPS. When universal primers fD1/rP1 for the amplification of the bacterial 16S rDNA were used, all symptomatic leaf samples, but none of the symptomless ones, yielded the same 16S rDNA amplification product, indicating the presence of a bacterium in the symptomatic leaves. This 16S rDNA product was cloned and sequenced, and its sequence compared with those of *Ca. L. africanus* and *Ca. L. asiaticus*, and other bacteria. The 16S rDNA sequence of the new bacterium (SPS-HLB bacterium) possessed all the oligonucleotide signatures characteristic of the liberibacters, but on the phylogeny tree, it did not cluster within the *Ca. L. africanus*/*Ca. L. asiaticus* group, and formed instead a separate branch. The 16S/23S ribosomal intergenic region (RIR) of the SPS-HLB was also amplified, cloned and sequenced, and compared to the RIRs of other liberibacters. All tested strains of *Ca. L. asiaticus* had identical or almost identical RIR sequences (99% to 100% sequence identity). However, the SPS-HLB bacterium and *Ca. L. asiaticus* or *Ca. L. africanus* had less than 80% sequence identity. For these reasons, we propose that the SPS-HLB bacterium is a member of the genus *Candidatus Liberibacter*, but sufficiently different from known liberibacter species to warrant a new species designation as *Candidatus Liberibacter americanus* sp. nov.

In 1970, it was shown by electron microscopy that the pathogen associated with citrus greening disease, now officially called huanglongbing (HLB), was not a virus, but a bacterium exclusively located in the phloem sieve tubes (32, 39). Specifically, the bacterium was of the Gram-negative type, as was shown later (18, 22). In spite of many

attempts, the HLB bacterium could never be, and has never been, obtained in culture. Therefore, only when molecular, DNA-based, techniques became available in the early 1990s, could the HLB bacterium be characterized. Analyses of the 16S ribosomal RNA gene (16S rDNA) confirmed that indeed the HLB bacterium was a Gram-negative bacterium, and more precisely, an alpha-proteobacterium to which the genus name "*Candidatus Liberibacter*" was given (27, 29). The prefatory

*We dedicate this work to the memory of Dr. Monique Garnier (1949-2003).

determinant “*Candidatus*” (*Ca.*) indicates that the bacterium is uncultured, and has only been characterized molecularly (36). On the basis of 16S rDNA sequence comparisons, two species within the *Ca* Liberibacter genus could be identified: *Ca. Liberibacter africanus* in Africa, and *Ca. Liberibacter asiaticus* in Asia (27, 29).

Until now, HLB was restricted to two large geographical regions: Africa and Asia. In Africa, the disease is essentially present in the eastern and southern parts, from Ethiopia to South Africa’s Western Cape province, including Somalia, Kenya, Rwanda, Burundi, Malawi, and Zimbabwe, in the west African nation of Cameroon, and on the island of Madagascar in the Indian Ocean (3, 9, 19, 21, 30, 50). In Asia and Southeast Asia, HLB affects Pakistan, India, Sri Lanka, Nepal, Bhutan, Bangladesh, Myanmar, Thailand, Malaysia, Cambodia, Laos, Vietnam, southern China and Taiwan, southern Japan islands including Okinawa, the Philippines, Indonesia including Bali and East Timor, and Papua New Guinea (4, 6, 8, 9, 11, 16, 19, 20, 23, 26, 35, 38, 40, 41, 50). In Africa (African HLB), the pathogen is *Ca. L. africanus*, the vector is the psyllid *Trioza erythrae*, and both are heat sensitive. Therefore, African HLB occurs only in cool areas, as determined by latitude and/or altitude. In Asia (Asian HLB), the pathogen is *Ca. L. asiaticus*, the vector, also a psyllid, is *Diaphorina citri*, and both are heat tolerant. Thus, in nature, there seems to be a good adaptation of the pathogen to its vector. However, experimentally, both vectors are able to transmit both liberibacters (18, 19). In three regions, both African and Asian HLB occur together in the border region between Saudi Arabia and North Yemen (7), on Mauritius island (23), and on Reunion island (23). In these regions, the two psyllid vectors and the two liberibacter species are

encountered in their respective climatic zones. In Mauritius and Reunion islands, some citrus trees could be shown to be infected simultaneously with the two liberibacter species (23).

In March 2004, symptoms of HLB were recognized on sweet orange trees near the city of Araraquara in São Paulo State (SPS), Brazil (1, 12, 43, 44). This was the first reported case of HLB on the American Continent. Leaf symptoms of HLB in SPS were very similar, if not identical, to those in Africa and Asia, with characteristic and pronounced blotchy mottle (34). Fruit symptoms included strong color inversion and seed abortion, and were more similar to those seen in China, than to those observed in South Africa. A survey conducted in September 2004 showed HLB to be present in 46 municipalities of São Paulo State, suggesting that the disease has been present for almost 10 yr, but without being properly diagnosed. The Asian psyllid vector of HLB, *D. citri*, has been established in Brazil for many more years, as it was reported for the first time in 1942 (33), and confirmed in 1970 (10).

At the time this manuscript was written (January 2005), HLB had not been detected in Australia, Central and North America, the Mediterranean region, the Near East, and the Middle East, except the Arabian Peninsula. However, *D. citri* is present in some of these HLB-free regions. The psyllid was seen in 1997 in Iran, 100 km west of the border with Pakistan (5). It is established in Argentina and Venezuela since the late 1990s, entered Guadeloupe and Florida in 1998, the Bahamas in 1999, the Cayman islands in 2000, Cuba and the Dominican Republic in 2001, has been present in Texas since 2001, Puerto Rico since 2002, and in the Yucatan peninsula since 2003 (25).

A PCR method has been previously described for the detection in citrus leaves of the two liberibacters

by amplification of an 1160 bp fragment of their 16S rDNA with forward primers OA1+OI1 and reverse primer OI2c (27, 28). The PCR method has been assayed in many Asian and African countries for the detection of the two HLB liberibacters [see for instance (4, 6, 19, 23)]. Whenever leaves with the classic blotchy mottle symptoms were used, positive PCR reactions were obtained and yielded the characteristic 1160 bp amplicon. Therefore, in April 2004, this PCR technique was used to confirm the presence of HLB in SPS, and identify the liberibacter involved: *Ca. L. asiaticus* and/or *Ca. L. africanus*. Unexpectedly, none of the symptomatic leaf samples from 43 affected trees, of which many had severe fruit symptoms, yielded positive results. Under the same conditions, symptomatic control citrus leaves infected with *Ca. L. asiaticus* or *Ca. L. africanus* from the HLB collection in Bordeaux, gave positive PCR reactions (43, 44). However, at the same time, and using the same PCR technique, *Ca. L. asiaticus* was detected by Coletta-Filho and coworkers in 2 of 10 leaf samples (12). In view of the many negative PCR results obtained, the presence of a new bacterial pathogen in many of the symptomatic, blotchy mottle leaves from SPS was suspected and further investigated. This work has resulted in the discovery of a third Liberibacter species, for which we have proposed the name *Candidatus Liberibacter americanus* (43, 45, 46). For reviews on HLB and its psyllid vectors, see references 14, 15, 18, 24, and 25.

MATERIALS AND METHODS

Plant material. Blotchy mottle leaves from 2-yr-old Hamlin sweet orange seedlings infected with *Ca. L. africanus* or *Ca. L. asiaticus*, as well as Mexican lime leaves infected with *Candidatus Phytoplasma aurantifolia*, were obtained from the HLB collection in Bordeaux, France,

and served as positive controls. Healthy and infected seedlings were maintained under greenhouse conditions (28).

Symptomless citrus leaves and leaves with characteristic blotchy mottle symptoms, hereafter called "HLB-leaves", were collected in April, June and August 2004, on trees from 47 citrus farms within 35 municipalities of SPS. Each leaf sample came from a single tree, and contained 10 to 20 leaves. Leaves were kept in plastic bags at 4°C before they were used for DNA extraction within 48 hr. In total, 218 HLB leaf samples were collected, mainly from sweet orange trees, but some also came from Ponkan and Cravo mandarin trees, and Murcott tangor trees.

The blotchy mottle leaves used for PCR amplification, cloning and sequencing of the 16S rDNA and the 16S/23S ribosomal intergenic region of the new bacterium were checked for absence of *Ca. L. africanus* and *Ca. L. asiaticus* by PCR amplification using 16S rDNA specific primers OA1 +OI1/OI2c as previously described (28).

Extraction of DNA from leaves. Leaf midribs (0.5 g) were finely minced with a razor blade within a disposable Petri dish containing 2 ml of CTAB (cetyl trimethyl ammonium bromide) buffer (37), and the DNA was purified as described (51). The final pellet was resuspended in 100 µl of sterile water. One microliter of the resulting solution was used for PCR.

Amplifying, Cloning and Sequencing of the DNA from the Ribosomal Operon of the Bacterium Associated with HLB in SPS (SPS-HLB Bacterium)

16S rDNA. The method used was essentially as described in Jagoueix et al. (27). This method is based on the fact that mitochondrial 16S rDNA is sensitive to *Bcl* I restriction enzyme digestion, bacterial 16S

rDNA is insensitive to *Bcl* I, but is digested by *Eco* RI into two fragments (~650 bp and ~850 bp in length), while chloroplast 16S rDNA is insensitive to both restriction enzymes (55). In summary, the DNA was extracted from leaves showing blotchy mottle symptoms (Natal sweet orange sample A3 from São José farm in Luis Antonio municipality, that tested negative for the African and Asian liberibacters) and was digested with *Bcl* I to prevent amplification of mitochondrial 16S rDNA in the following step. The bacterial and chloroplast 16S rDNAs were amplified with universal primers fD1 and rP1 (52). The amplification conditions were 35 cycles at 92°C for 60 s, 50°C for 60 s and 72°C for 90 s. The amplified DNA (~1500 bp) was cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clones considered to contain the bacterial 16S rDNA were those with an insert resistant to *Bcl* I digestion, but producing two fragments when digested with *Eco* RI. Of a total of 46 clones assayed, two were obtained with such inserts, and were subsequently sequenced. The two sequences were identical and had a size of 1447 bp.

16S/23S Ribosomal Intergenic Region (RIR). The RIR of the SPS-HLB bacterium was obtained by PCR amplification of DNA extracted from HLB-affected Valencia sweet orange leaf sample AA9 from São João farm in Boa Esperança do Sul municipality. As shown in Figure 3, the primers used were forward primer GB3c, complementary to primer GB3 and located in the 3' half of the 1447 bp 16S rDNA, and reverse primer 23S1, located at the very start of the 23S rDNA (29). The RIR DNA was amplified by PCR for 35 cycles at 92°C for 40 s, 64°C for 40 s, and 72°C for 60 s. The amplified DNA was cloned and sequenced as described above.

Sequence analyses. A search for homologies in GenBank ([\[www.ncbi.nlm.nih.gov/BLAST\]\(http://www.ncbi.nlm.nih.gov/BLAST\)\) was carried out using the BLAST program \(2\). The sequences were also analyzed using the programs proposed by Infobiogen \(<http://www.infobiogen.fr/index.html>\). Multiple sequence alignments were performed using MULTALIN \(13\) \(<http://www.toulouse.inra.fr/Igc/multialin/multialin.html>\) and CLUSTAL W \(49\) software.](http://</p></div><div data-bbox=)

Nucleotide sequence accession number. GenBank accession numbers for 16S rDNA and 16S/23S RIR DNA of the SPS-HLB bacterium were AY742824 and AY859542, respectively.

16S rDNA phylogeny and RIR sequence comparisons. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (31) (<http://evolution.genetics.washington.edu/phyip.html>). The bacterial 16S rDNA sequences used for comparisons were obtained from the GenBank database, and were those of *Ca. L. asiaticus* isolate Poona (L22532), *Ca. L. asiaticus* isolate *D. citri* endosymbiont (AB038369), *Ca. L. asiaticus* isolate Okinawa (AB008366), *Ca. L. asiaticus* isolate *C. grandis* (AY192576), *Ca. L. africanus* isolate Nelspruit (L22533), *Ca. L. africanus* subsp. *capensis* (AF137368), *Brucella abortus* (X13695), *Bartonella henselae* (AY513504), *Afipia felis* (AY513503), *Candidatus* *Phlomobacter fragariae* (PFU91515), and *Escherichia coli* (V00348). The sequences for RIR comparisons by CLUSTALW were those of *Ca. L. asiaticus*, isolate Poona (U61359) and isolate Okinawa (AB019793), and *Ca. L. africanus* isolate Nelspruit (U61360). Information on *Ca. L. asiaticus* isolate Fuzhou was from Jagoueix et al. (29).

PCR detection of *Ca. L. africanus*, *Ca. L. asiaticus*, and *Ca. L. americanus*. For *Ca. L. africanus* and *Ca. L. asiaticus*, the technique was according to Jagoueix et al. (28), and the primers used were OI1+OA1/OI2c (27, 28). PCR detec-

tion of *Ca. L. americanus* was according to Teixeira et al. (44), with forward primer GB1 (5'AAGTCG-AGCGAGTACGCAAGTA3') and reverse primer GB3 (5' CTATAT-TTGCCATCATTAAGTTGG 3').

RESULTS

Evidence for a new bacterial pathogen in HLB-affected leaves from SPS. When 43 leaf samples from SPS with blotchy mottle symptoms characteristic of HLB were tested for the presence of *Ca. L. africanus* and *Ca. L. asiaticus* by PCR amplification of 16S rDNA with specific primers OI1+OA1/OI2c, all PCR reactions were negative under conditions where control leaves, infected with either one of the two liberibacters gave positive reactions. This unexpected result suggested to us that the HLB-leaves from SPS were probably infected with a new HLB bacterium, i.e., a bacterium that was not recognized by the primers specific for *Ca. L. africanus* and *Ca. L. asiaticus*. Evidence for the presence of such a bacterium, hereafter called "SPS-HLB bacterium", in HLB-leaves testing negative for *Ca. L. africanus* and *Ca. L. asiaticus*, was obtained by PCR amplification using universal primers fD1/rP1 for prokaryotic 16S rDNA (52). The DNAs from symptomatic Valencia sweet orange leaf samples 16 to 20 (Chapadão farm, Bueno de Andrada municipality), that tested negative for *Ca. L. africanus* and *Ca. L. asiaticus* as shown on Fig. 1, lanes 5 to 9, were treated with *Bcl* I in order to digest the plant mitochondrial 16S rDNA and prevent it from being amplified in the subsequent step. The remaining chloroplast 16S rDNA and the putative SPS-HLB 16S rDNA were amplified by PCR using universal primers fD1/rP1, and the amplified DNA was subjected to *Eco* RI digestion. Under these conditions, bacterial 16S rDNA is revealed by the presence of two *Eco* RI restriction fragments of ~650 bp



Fig. 1. Agarose gel electrophoresis (1% agarose) of the DNA amplified with primers OA1+OI1/OI2c from water (lane 1), DNA extracted from healthy Valencia sweet orange leaves (lane 3), Hamlin sweet orange control leaves infected with *Ca. L. africanus* (lane 2) or *Ca. L. asiaticus* (lanes 4 and 10), and five samples (16 to 20) from Valencia sweet orange leaves showing the blotchy mottle symptoms characteristic of HLB from Chapadão farm, SPS (lanes 5 to 9). M: Molecular weight marker (1 Kb Plus DNA ladder). The arrows point to the 1160 bp amplicon characteristic of 16S rDNA amplification from *Ca. L. africanus* and *Ca. L. asiaticus* with primers OA1+OI1/OI2c.

and ~850 bp in length. Fig. 2 shows that, indeed, two fragments of the expected size were obtained, not only with control leaves infected with *Ca. L. asiaticus* (lane 9) or *Ca. Phytoplasma aurantifolia* (lane 8), but also with the five samples of HLB-leaves from SPS (lanes 2 to 6). With DNA amplified from healthy leaves (lanes 1 and 7), the ~1500 bp chloroplast 16S rDNA band was seen on the gel, but not the two *Eco* RI fragments. Because PCR is a sensitive technique, the ~650 bp and ~850 bp fragments in lanes 2 to 6 could have been due to exogenous contaminating bacteria present on the HLB-affected leaves. In this case, symptomless leaves from these same trees would be expected to also carry the contaminating bacteria, and yield the two ~650 bp and ~850 bp fragments. This was not the case (data not shown). Therefore, the results of Fig. 2 and the symptoms observed in the leaves suggest the presence of a HLB-like bacterium in HLB-leaves from SPS, even though such leaves tested negative for *Ca. L. asiaticus* and *Ca. L. africanus*. To confirm our hypothesis, the ~850

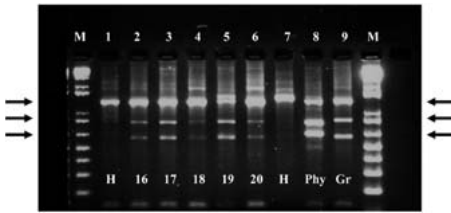


Fig. 2. Agarose gel electrophoresis (1% agarose) of the DNA treated with *Eco* RI after its amplification with the universal primers fD1/rP1 from *Bcl* I-treated DNA extracted from healthy Valencia sweet orange leaves (lanes 1 and 7), Hamlin sweet orange control leaves infected with *Ca. L. asiaticus* (lane 9) or *Ca. Phytoplasma aurantifolia* (lane 8), and five samples (16 to 20) of Valencia sweet orange leaves with blotchy mottle symptoms characteristic of HLB from Chapadão farm, SPS (lanes 2 to 6). M: Molecular weight marker (1 Kb Plus DNA ladder). The upper, middle, and lower arrows point to DNA bands of approximately 1500 bp, 850 bp, and 650 bp, respectively.

Eco RI fragment of sample 19 (Fig. 2, lane 5) was cloned and sequenced. The fragment had a length of 833 bp, and contained, as expected, rP1 as one of the terminal sequences (see Fig. 3).

Cloning and sequencing the 16S rDNA of the SPS-HLB bacterium. To further confirm our hypothesis, DNA from HLB-affected Natal sweet orange leaves (sample A3 from São José farm in Luis Antonio municipality) that tested negative for *Ca. L. asiaticus* and *Ca. L. africanus* was digested with *Bcl* I, and amplified by PCR with universal primers fD1/rP1. The ~1500 bp amplified DNA (16S rDNA from the putative SPS-HLB bacterium, and chloroplast 16S rDNA) was cloned into *Escherichia coli*. A total of 46 clones were obtained, of which two had plasmid inserts resistant to *Bcl* I digestion, but sensitive to *Eco* RI digestion, characteristic of bacterial 16S rDNA. These two clones were selected and their inserts were sequenced. The two sequences were identical. The unique sequence was 1447 bp and was flanked, as expected, by the sequences of primers fD1 and rP1 (Fig. 3). The terminal 833 bp nucleotide sequence at the rP1 end was identical to that of the 833 bp *Eco* RI restriction fragment described in the previous section.

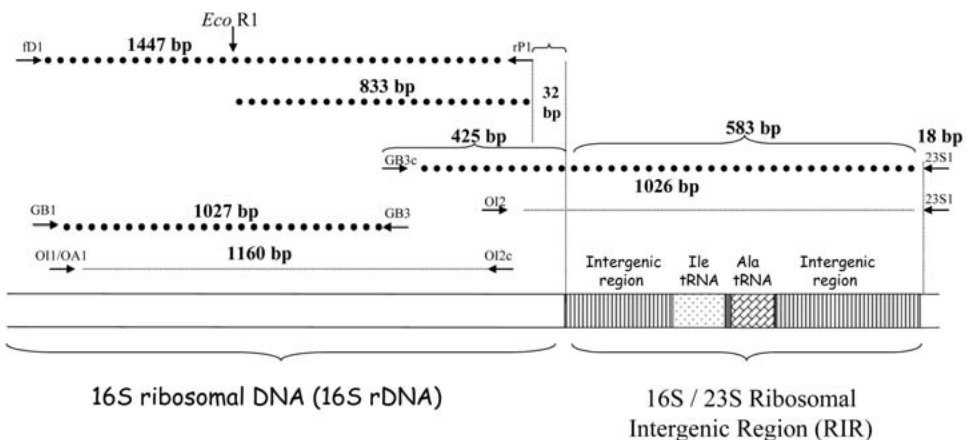


Fig. 3. Schematic representation of the 16S rDNA and the 16S/23S ribosomal intergenic region (RIR) of the SPS-HLB bacterium. PCR amplification products from SPS-HLB bacterium (.....) and *Ca. L. asiaticus* (—) are shown. Primers are indicated by horizontal arrows. The vertical arrow shows the *Eco* RI restriction site. The two tRNAs in the RIR are characteristic of the SPS-HLB bacterium and *Ca. L. asiaticus*. The RIR of *Ca. L. africanus* contains only the Ala tRNA. Amplicon lengths are not to scale.

Detection of the SPS-HLB bacterium by PCR amplification of the 1447 bp 16S rDNA.

Forward primer GB1 and reverse primer GB3 (see Materials and Methods for the sequence of these primers) were designed from the 1447 bp 16S rDNA sequence. Two hundred and fourteen leaf samples from SPS with characteristic HLB symptoms, and testing negative for *Ca. L. africanus* and *Ca. L. asiaticus*, were used for PCR amplifications with primers GB1/GB3 according to Teixeira et al. (44). All 214 samples gave positive PCR reactions, and the amplicons obtained were of the expected size (1027 bp), while healthy leaves or leaves infected with *Ca. L. africanus* or *Ca. L. asiaticus* gave negative PCR reactions (44). Two amplicons, one from Natal sweet orange leaf sample A5 (São José farm in Luis Antonio municipality) and one from Valencia sweet orange leaf sample AA9 (São João farm in Boa Esperança do Sul municipality), were cloned and sequenced. The two sequences were identical. The unique sequence was 1027 bp in size, and had 100% identity with the corresponding sequences of the 1447 bp 16S rDNA and the 833 bp 16S rDNA (Fig. 3). Thus, the 833 bp, 1027 bp, and 1447 bp cloned DNAs obtained from leaf samples of different geographical origins in SPS were all 16S rDNA amplification products from the same SPS-HLB bacterium and were not from contaminating bacteria.

Cloning and sequencing the 16S/23S Ribosomal Intergenic Region (RIR) of the SPS-HLB bacterium. The amplicon obtained by PCR amplification of the DNA from leaf sample AA9 with primers GB3c/23S1 was cloned and sequenced, and found to have a size of 1026 bp (see Fig. 3). The actual RIR was 583 bp. Upstream of RIR were 425 bp corresponding to the 16S rDNA of which 393 bp at the 5' end were identical to the 3' terminal sequences of the 1447 bp and 833 bp

16S rDNA fragments. The remaining 32 bp were missing from those 16S rDNA fragments because of the position of the reverse primer rP1. Downstream of RIR was 18 bp corresponding to the 23S rDNA, and they are the reverse 23S1 primer used for amplification of the RIR region. The fact that the GB3c terminal end (5') of the 1026 bp RIR sequence and the rP1 terminal end (3') of the 1447 bp 16S rDNA had identical sequences over 393 bp indicates that the 1026 bp RIR sequence was that of the SPS-HLB bacterium. In summary, from forward primer fD1 to reverse primer 23S1 (see Fig. 3), we obtained 2080 bp, of which 1479 bp were the 16S rDNA, and 583 bp were the 16S/23S RIR.

The SPS-HLB bacterium is a liberibacter. A BLAST search of the GenBank database was applied to the 1447 bp 16S rDNA sequence and showed that the SPS-HLB bacterium had liberibacters as its closest relatives. The 1447 bp sequence was aligned with the 16S rDNA sequences of *Ca. L. asiaticus* (isolate Poona) and *Ca. L. africanus* (isolate Nelspruit), with which it had identities of 96.1% and 95.9%, respectively. The phylogenetic analysis (Fig. 4) indicated that the SPS-HLB bacterium clustered, like the liberibacters, in the α subdivision of the *Proteobacteria*, close to members of the α 2 subgroup. In addition, as shown in Table 1, the 16S rDNA of the SPS-HLB bacterium had oligonucleotide signatures (53, 54) very similar to those of the liberibacters (27). Also, the secondary loop structure characteristic of the α -*Proteobacteria* (53) was shared by the liberibacters and the SPS-HLB bacterium (Fig. 5). For all these reasons, we propose the SPS-HLB bacterium is a member of the genus *Candidatus Liberibacter*. The following results show that it represents a new species.

The SPS-HLB bacterium is a new *Candidatus Liberibacter* species. Three lines of evidence

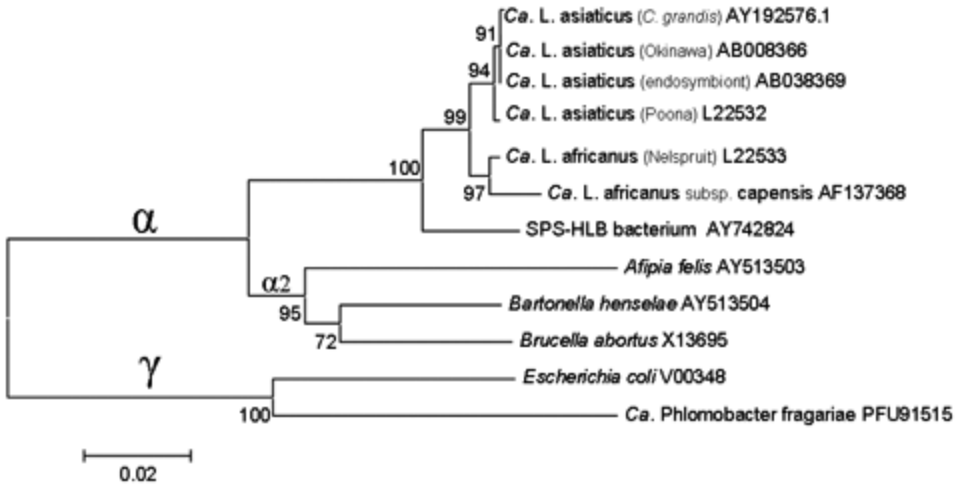


Fig. 4. Phylogenetic tree constructed from the 16S rDNA sequences obtained from the GenBank database using Mega version 2.1. α , γ alpha, gamma subdivision of *Proteobacteria*. $\alpha 2$: subgroup 2 of α *Proteobacteria*. Bootstrap percentages are indicated as numbers on the branches of the tree. Names of the liberibacter isolates are given in parentheses and followed by the accession numbers for their 16S rDNA sequences.

show the SPS-HLB bacterium to be a new liberibacter species.

Evidence from 16S rDNA.

Table 2 indicates the percentage of 16S rDNA sequence identity of one liberibacter species (species A) versus another (species B) for various liberibacter pairs. The comparisons involved only gap-free sequences. The percentages were determined for a sequence size of 1100 bp, the common maximum size available for all liberibacters. As seen in Table 2, for the pair *Ca. L. asiaticus*/*Ca. L. africanus* (line 4), involving two different species, the percent identity was 97.8%. When the comparison involved the same species, *Ca. L. asiaticus*, but different isolates of this species, isolate Poona/isolate Okinawa (line 6) or isolate Poona/isolate *C. grandis* (line 7), the percent of identity was higher than for *Ca. L. asiaticus*/*Ca. L. africanus*, namely 98.9% and 98.8%, respectively. However, when the pair involved the SPS-HLB liberibacter either with *Ca. L. asiaticus* isolate Poona (line 1) or isolate Okinawa (line 2), or *Ca. L. africanus* isolate Nelspruit (line 3), the identities were much lower, averaging 94.6%, indicating that the

SPS-HLB liberibacter was not an isolate of *Ca. L. asiaticus* or *Ca. L. africanus*. This conclusion was also supported by the 16S rDNA phylogeny tree of Fig. 4, showing that the SPS-HLB liberibacter did not cluster within the “*Ca. L. asiaticus*/*Ca. L. africanus*” group, but formed a separate branch with a bootstrap percentage of 100. In contrast, all four isolates of *Ca. L. asiaticus* clustered together within the *Ca. L. africanus*/*Ca. L. asiaticus* group. An isolate of *Ca. L. asiaticus* from SPS also clustered within the “*Ca. L. asiaticus*/*Ca. L. africanus*” group (data not shown). This SPS isolate differed from the “*C. grandis*” isolate from China by only three nucleotides (M. Machado, personal communication). Therefore, the SPS-HLB liberibacter, *Ca. L. asiaticus*, and *Ca. L. africanus* represent different species.

Evidence from the Ribosomal Intergenic Region. The RIR of the SPS-HLB liberibacter was 583 bp long (Fig. 3), compared to 595bp and 498 bp for *Ca. L. asiaticus* isolate Poona and *Ca. L. africanus* isolate Nelspruit, respectively (29). The RIR of the SPS-HLB liberibacter contained the sequences for two

TABLE 1
OLIGONUCLEOTIDE SIGNATURES CHARACTERISTIC OF THE α -PROTEOBACTERIA,
AND THE α -1, α -2, AND α -3 SUBGROUPS.

Oligonucleotide signatures of <i>Proteobacteria</i>	SPS-HLB bacterium (position on 16S rDNA sequence)	<i>Candidatus Liberibacter asiaticus</i>	<i>Candidatus Liberibacter africanus</i>
α subdivision			
AUAAUG	YES ^a (413-418)	YES	YES
AAAUCCAG	AAAUCCCAAG (544-553)	NUAACCG	YES
AAAUUCG	YES (631-637)	YES	YES
AUAUUCG	YES (641-647)	YES	YES
CUCACUG	YES (672-678)	YES	YES
CUAACCG	YES (1378-1384)	YES	YES
α 1 subgroup			
CACUCCG	YES (811-817)	YES	YES
ACAAG	YES (871-875)	CACUCCN	YES
UCACACCAUG	YES (1340-1349)	YES	YES
α 2 subgroup			
CAAUACG	CAAGUACT (68-75)	YES	UUAUACG
α 3 subgroup			
UAAUACCG	YES (145-152)	YES	YES
AUUAAG	YES (529-534)	YES	YES

^a“Yes” indicates that the signature is identical. The figures in parentheses indicate the positions of the 5' and 3' ends of the oligonucleotides on the 16S rDNA sequence (1447 bp) of the SPS-HLB bacterium deposited in GeneBank under accession number AY742824. Positions 1 and 1447 are respectively the 5' and 3' terminal nucleotides of the 16S rDNA sequence.

tRNAs: tRNA^{Ile} and tRNA^{Ala}. The RIR of *Ca. L. asiaticus* contains two similar tRNA sequences, while only the tRNA^{Ala} sequence is present in the *Ca. L. africanus* RIR (29). Table 2 shows the percentages of sequence identity of the RIR of one liberibacter species (species “A”) versus that of another (species “B”). When *Ca. L. asiaticus* isolate Poona was compared to another isolate of the same species, isolate Fuzhou (line 5) or isolate Okinawa (line 6), the partial or total RIR sequences were either identical (100% identity, isolate Fuzhou), or very similar (99.2% identity, isolate Okinawa), indicating that within a given liberibacter species the RIR region does not vary much, as pointed out previously (29). However, when the RIR sequences of two different species such as *Ca. L. asiaticus* isolate Poona and *Ca. L. africanus* isolate Nelspruit (line 4), were compared the percent identity for the total intergenic region was as low as 79.5%. The figures were even lower

when the total RIR sequence of the SPS-HLB liberibacter was compared to that of *Ca. L. asiaticus*, 77.8% for isolate Poona (line 1), 77.7% for isolate Okinawa (line 2), or to that of *Ca. L. africanus* (66.0%). These results confirm those based on 16S rDNA sequence comparisons, indicating that the SPS-HLB liberibacter is a different species.

Evidence from PCR detection of liberibacters, and identification of a unique genomic oligonucleotide. Primer pairs OA1/OI2c, OI1/OI2c, or even OA1+OI1/OI2c, which permit PCR detection of both *Ca. L. asiaticus* and *Ca. L. africanus*, give no 16S rDNA amplification with the SPS-HLB liberibacter, and vice-versa. The primer pair GB1/GB3, designed for the detection of the SPS-HLB liberibacter, gives no amplification with *Ca. L. asiaticus* or *Ca. L. africanus*. The nucleotide sequence of primer GB3 represents a unique oligonucleotide, and is highly characteristic of the SPS-HLB liberibacter. Identification of a unique

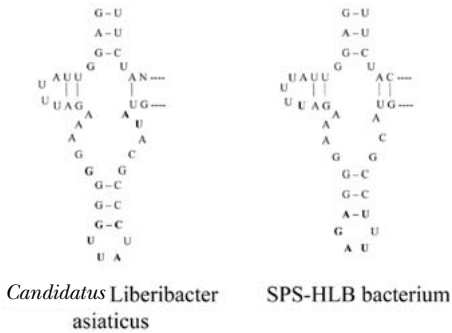


Fig. 5. Comparison of the secondary loop structure of *Ca. L. asiaticus* and the SPS-HLB bacterium.

genomic oligonucleotide is a requirement for designation of a new bacterial species (36).

Additional properties of the SPS-HLB liberibacter. Additional properties of the SPS-HLB liberibacter have been determined and include: (i) Transmission of the SPS-HLB liberibacter to healthy pineapple sweet orange seedlings has been achieved by graft-inoculations under greenhouse conditions in Bordeaux. Leaves of inoculated seedlings developed characteristic blotchy mottle symptoms 4 mo after inoculation at both cool (22°C/24°C) and warm (27°C/32°C) conditions. The SPS-HLB liberibacter was detected in the symptomatic leaves by PCR amplification with primers GB1/GB3. No amplification was obtained with primers (OA1+OI1)/OI2c, indicating that *Ca. L. asiaticus* was absent. Similar results were obtained in Araraquara, Brazil. (ii) The liberibacters have never been obtained in culture, and the same is true for the SPS-HLB liberibacter. (iii) Liberibacters are restricted to the sieve tubes of the phloem tissue, and possess a characteristic double membrane cell envelope (22). Electron microscopy has shown this to be also the case of the SPS-HLB liberibacter (42, 43). (iv) The natural vector of *Ca. L. asiaticus* in Asia is the psyllid *D. citri*, also present on citrus in Brazil for more than 60 yr.

The SPS-HLB liberibacter could be detected in *D. citri* by PCR amplification of 16S rDNA with the specific primer pair GB1/GB3, strongly suggesting that the Asian psyllid is also a vector for the SPS-HLB liberibacter in SPS (44, 45).

The SPS-HLB liberibacter is the major pathogen associated with HLB in SPS. In an extensive survey for HLB, 218 symptomatic leaf samples were collected from 47 citrus farms from 35 municipalities in SPS and each sample was tested for liberibacters with two pairs of primers: (i) the pair OA1+OI1/OI2c, specific for *Ca. L. africanus* and *Ca. L. asiaticus* (24), and (ii) the pair GB1/GB3, specific for the SPS-HLB liberibacter (this article). The SPS-HLB liberibacter could be detected in 214 samples, *Ca. L. asiaticus* in 2, *Ca. L. africanus* in none, and 2 samples were infected with both the SPS-HLB liberibacter and *Ca. L. asiaticus*. The proportion of *Ca. L. asiaticus* to the SPS-HLB liberibacter is thus 4 to 216. These figures indicate that the major cause of HLB in SPS is the SPS-HLB liberibacter and not *Ca. L. asiaticus*, as stated previously at a time when the SPS-HLB liberibacter was not yet characterized (12).

DISCUSSION

In leaves from SPS with characteristic blotchy mottle symptoms of HLB and testing negative for *Ca. L. africanus* and *Ca. L. asiaticus*, evidence for the presence of a new HLB bacterium (SPS-HLB bacterium) was obtained by PCR amplification with universal primers fD1 and rP1 for prokaryotic 16S rDNA. The 16S rDNA of the SPS-HLB bacterium was cloned and sequenced, and the sequence was used to design the specific primer pair GB1/GB3. PCR amplification with these primers made it possible to detect the SPS-HLB bacterium in all HLB-leaf samples testing negative for *Ca. L. africanus* and *Ca. L. asiaticus*. Most leaf

TABLE 2
SEQUENCE IDENTITY (%) OF THE 16S RDNA AND THE 16S/23S RIBOSOMAL INTERGENIC REGION (RIR) AMONG *CANDIDATUS LIBERIBACTER*

	Species A/Species B isolate	16S rDNA (1100 pb)	16 S ←	Intergenic region (partial)	Ile tRNA	Ala tRNA	Intergenic region (partial)	23 S →	Intergenic region (total)
1	SPS-HLB <i>L./L. asiaticus</i> Poona	94.4		72.0	92.5	93.5	76.5		77.8
2	SPS-HLB <i>L./L. asiaticus</i> Okinawa	94.8		72.0	94.8	96.0	74.0		77.7
3	SPS-HLB <i>L./L. africanus</i> Nelspruit	94.5		72.8	Nap	88.2	75.7		66.0
4	<i>L. asiaticus/L. africanus</i> Poona Nelspruit	97.8		71.4	Nap	88.2	86.4		79.5
5	<i>L. asiaticus/L. asiaticus</i> Poona Fuzhou	Nav		100.0	100.0	100.0	100.0		100.0
6	<i>L. asiaticus/L. asiaticus</i> Poona Okinawa	98.9		100.0	97.4	97.4	99.6		99.2
7	<i>L. asiaticus/L. asiaticus</i> Poona <i>C. grandis</i>	96.8		Nav	Nav	Nav	Nav		Nav

For 16S rDNA, the sequence identity was determined over a 1100 bp region, the longest sequence available for all isolates. For the RIR, the sequence identity was calculated for the complete RIR, and selected regions: intergenic region (← 16 S) adjacent to 16S rDNA, Ile tRNA sequence, Ala tRNA sequence, and intergenic region (23 S →) adjacent to 23S rDNA. Nap: not applicable because *C. L. africanus* Ile tRNA is absent from RIR. Nav: not available in the GenBank database.

samples (98%) were infected with the SPS-HLB bacterium, the remaining samples (2%) carried *Ca. L. asiaticus*. Primer GB3c, complementary to GB3, was used in conjunction with reverse primer 23S1 to amplify, clone and sequence the 16S/23S ribosomal intergenic region (RIR). In total, a 1479 bp sequence of 16S rDNA (almost the complete 16S rDNA sequence), followed by the complete 583 bp sequence of the RIR, was available for characterization of the SPS-HLB bacterium by comparison with similar sequences of various isolates of *Ca. L. asiaticus*, and the Nelspruit isolate of *Ca. L. africanus*.

These comparisons clearly showed that the SPS-HLB bacterium was not only a member of the genus *Candidatus Liberibacter*, having all the oligonucleotide signatures of the liberibacters, but also a new species within this genus. In particular, on the 16S rDNA phylogeny tree, all isolates of *Ca. L. asiaticus* clustered together within the *Ca. L. africanus/Ca. L. asiaticus* group, but the SPS-HLB bacterium did not, and formed a separate branch. Also, the RIR sequences of different isolates of *Ca. L. asiaticus* were identical or almost identical (99 to 100% identity). However, the RIR of the SPS-HLB bacterium and that of *Ca. L. asiaticus* had only 78% sequence identity. With *Ca. L. africanus*, the sequence identity was even lower at 66%.

For the above reasons we have proposed to designate the SPS-HLB bacterium as *Candidatus Liberibacter americanus*, sp. nov. (43, 46). This designation refers to the fact that the new liberibacter species was detected for the first time on the American Continent, and that it represents the major *Ca. liberibacter* species associated with HLB in the affected SPS region. The designation is in line with the other *Ca. Liberibacter* names, which also refer to the continents where they occur, Africa for *Ca. L. africanus* and Asia for *Ca. L. asiaticus*.

Until now, *Ca. L. africanus* has only been found in Africa, and *Ca. L. asiaticus*, only in Asia (19). This is probably not due to the fact that the psyllid vector *T. erytrae* is only present in Africa, and the psyllid vector, *D. citri*, only in Asia, because both insects have the ability to transmit both liberibacters, at least under experimental greenhouse conditions. Therefore, it seems as if *Ca. L. africanus* originated in Africa, and *Ca. L. asiaticus* in Asia. After having spread initially within these continents, the disease and its vector subsequently spread to neighboring regions. For instance, in Yemen, on the Arabian Peninsula, African HLB has undoubtedly been introduced from nearby Ethiopia, across the Red Sea, through the narrow Bab al-Mandab strait, and has moved northwards. In Saudi Arabia, Asian HLB has entered the Arabian Peninsula brought probably by pilgrims to Mecca, and has moved southwards. Eventually, African and Asian HLB have met in the Najran region, about half way between Bab al-Mandab and Mecca (7). In Mauritius and Reunion (23), islands of the Indian Ocean with inhabitants originally from Africa and India, African HLB has probably been introduced from eastern Africa or Madagascar and Asian HLB from the Indian subcontinent. In Bhutan (16), where Asian HLB is currently spreading, introduction of the disease has probably been through planting material from India and/or Nepal. In the State of São Paulo in Brazil, the situation is more complex. The Asian psyllid, *D. citri*, has been present, undisturbed, since the 1940s. In these early years, the insect was probably free of the HLB agent, as HLB has been present in SPS only since about 1995. The presence, in SPS, of *Ca. L. asiaticus*, a very minor component of Brazilian HLB, is probably the result of a relatively recent, uncontrolled introduction from Asia.

If, as suggested above, *Ca. L. africanus* and *Ca. L. asiaticus* originated in their respective continents, the

same could apply to *Ca. L. americanus*, thus apparently arising in SPS. However, *Ca. L. americanus* is not a recent "mutation" of *Ca. L. asiaticus*, because the sequence differences in the 16S rDNA and the RIR of the two liberibacters are far too great, and because there is very little sequence variation between isolates of *Ca. L. asiaticus*, including the SPS isolate. The sequence of the 16S rDNA of *Ca. L. americanus* seems to be quite stable too. Indeed, the various 16S rDNAs of Fig. 3, which were amplified from geographically different HLB-leaf samples of different trees (1447 bp DNA from sample A3, 833 bp DNA from sample 19, the two 1027 bp DNAs from sample A5 and AA9, and 1026 bp DNA from sample AA9), have common, overlapping sequences, and these sequences were found to be identical.

The hypothesis proposing that different liberibacter species have evolved in different continents implies that *Ca. L. americanus* is native to SPS, and has not been introduced from known HLB-affected regions. The many PCR-based tests that have been carried out in Africa and Asia for the detection of *Ca. L. asiaticus* and *Ca. L. africanus*, have given no indications for the occurrence of a liberibacter different from *Ca. L. asiaticus* and *Ca. L. africanus*. For instance, in Vietnam, 45 of 47 blotchy mottle leaf samples analyzed by PCR gave positive reactions for *Ca. L. asiaticus* (4). In South Africa, 20 of 21 symptomatic leaf samples were PCR positive for *Ca. L. africanus* (30). In Mauritius, 28 of 30 samples gave positive PCRs, 27 for *Ca. L. asiaticus* and 1 for *Ca. L. africanus* (23). In total, several hundreds of PCR tests have been carried out in Africa and Asia and have always detected either *Ca. L. asiaticus* or *Ca. L. africanus*. However, even though quite numerous, these PCR assays might not have been sufficiently large in number to draw any conclusions, if the proportion of the American liberibacter to the African and Asian liberibacters

is as low as that of *Ca. L. asiaticus* to *Ca. L. americanus* in SPS (2%). Hence, in order to confirm or deny the above hypothesis, search for *Ca. L. americanus*, in Asia in particular, should be continued and intensified, especially since the PCR tools for specific detection of the various liberibacters are available.

The putative mechanism, by which a new liberibacter species might arise, is unknown. As pointed out previously (19), liberibacter species are either insect-borne pathogens, or are acquired by the respective psyllids from indigenous rutaceous plants. Indeed, in Africa, *Toddalia lanceolata*, an indigenous rutaceous species, is a good host of both *T. erythrae* and *Ca. L. africanus* (30). In SPS, *Murraya paniculata*, a widespread ornamental rutaceous species, is the preferred host of *D. citri*, and has been found to be infected with *Ca. L. americanus* (45). Also, it might be relevant to note that the Asian psyllid vector has been present in SPS since the 1940s, while HLB started to spread much later, probably in the 1990s. The psyllids in SPS have never been subjected to control treatments, and have reached, undisturbed, high population numbers on citrus and *M. paniculata* plants. Interestingly, psyllids, including *D. citri*, harbor primary (P) bacterial endosymbionts in specialized cells within the body cavity (47, 48). This association appears to be the consequence of a single infection of a psyllid ancestor with a bacterium. The P-endosymbionts are maternally transmitted to the progeny, and in the psyllids, they are members of the α subdivision of the *Proteobacteria*. However, the liberibacters are members of the γ subdivision, and thus the relationship between the P-endosymbionts and the liberibacters is probably irrelevant. Psyllids also contain secondary (S) endosymbionts (17). The S-endosymbionts are diverse and their association with psyllids may be the result of multiple infections of the psyllids with different precursors of

the S-endosymbionts and/or possible horizontal transmission. It might be worthwhile to examine more closely the S-endosymbionts of *D. citri* and *T. erythrae*, as well as additional rutaceous hosts of these and other citrus psyllids (24).

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