

# Analysis of 16S rDNA Sequences from Citrus Huanglongbing Bacteria Reveal a Different “*Ca. Liberibacter*” Strain Associated with Citrus Disease in São Paulo

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## ABSTRACT

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Citrus huanglongbing (HLB, ex greening) is one of the most serious and destructive citrus diseases in the world. It is caused by a phloem-limited and nonculturable bacterium, “*Candidatus Liberibacter*”. The disease occurs in some Asian and African countries and recently has been reported in the state of São Paulo, Brazil. Analysis of the 16S ribosomal (r)DNA of the HLB bacteria from orchards in São Paulo revealed the presence of two distinct strains of “*Ca. Liberibacter*”. One of them, named LSg1 (*Liberibacter* sequence group 1), was 100% identical to strains from Japan (GenBank accessions AB038369 and AB008366), the Asian forms of the bacteria. The other, LSg2, is genetically distant from the Asian (96.1 to 96.3% identity) and African (95.8 to 96.1% identity) strains. Comparison of the 16S rDNA sequences from the LSg2 and the Asian strain revealed the presence of INDELS and point mutations. Specific primers designed for this Brazilian *Liberibacter* strain revealed that it is more widely distributed throughout the São Paulo orchards compared with the LSg1 strain. The HLB symptoms caused by both strains are almost identical and, interestingly, both strains were found in the same sample, revealing mixed infection in a citrus plant.

Additional keywords: PCR, sweet orange

Huanglongbing (HLB), ex citrus greening, is one of the most serious diseases that occur in citrus. The symptoms include leaf mottling, yellowish shoots, and, frequently, small and lopsided fruit. It is caused by a phloem-limited, gram-negative, nonculturable bacterium designated “*Candidatus Liberibacter*”, a member of the alpha-subdivision of the phylum *Proteobacteria* (11). Two forms of HLB disease are known, African and Asian (1). The African form is caused by a heat-sensitive and *Trioza erytrae*-vectored bacterium designated “*Ca. L. africanus*”, whereas the Asian form is caused by a heat-tolerant and *Diaphorina citri*-vectored bacteria named “*Ca. L. asiaticus*”. Both insect vectors can naturally transmit their respective form of bacteria, which infect all *Citrus* spp. and cultivars. The disease causes severe losses in the production of sweet orange (*Citrus sinensis*), mandarin (*C. re-*

*ticulata*), lemon (*C. limon*), grapefruit (*C. paradisi*), and other economically important citrus species (6). In addition to some Asian countries, where the disease and the vector are endemic, the psylla *D. citri* also is present in the states of São Paulo in Brazil and Florida in the United States, the main orange juice production areas in the world (5,7).

In mid-2004, the Asian form of HLB-bacteria (“*Ca. L. asiaticus*”) was detected by molecular means and characterized from HLB-symptomatic citrus plants in São Paulo (Brazil) (2). More recently, Teixeira et al. (19) detected a different *liberibacter* isolate associated with HLB-diseased plants from São Paulo and designated it as “*Ca. L. americanus*”, which is predominant in the state of São Paulo, being detected in 98% of the samples analyzed compared with 2% of sequences from *Ca. L. asiaticus*. Due to the abundance of the endemic *D. citri* in citrus orchards in the state of São Paulo and the large areas occupied by this culture, the HLB disease has scared citrus researchers and growers in Brazil. An outbreak of an infectious disease often results from an exposure to a common source of a clonally related pathogen. However, in a clonally related pathogen, there is sufficient diversity at the species level that organisms isolated from different hosts and different geographic regions may be differentiated (16). In epidemiology, the knowledge of

genetic variations in the pathogen is important for determining the source of infection and identification of genetic variants that can be associated with the pathogenicity.

Comparison of the 16S ribosomal DNA (16S rDNA) sequences with other microorganisms has increased the knowledge about the biology, diagnosis, and epidemiology of noncultivable or unknown bacteria (14). The 16S rDNA sequences among Asian isolates of “*Ca. Liberibacter*” were quite similar, with 98.8 to 100% identity; whereas, among African isolates, the identity was 97.5% (18). The American isolate shared 93.7 and 93.9% identity with the African and Asian forms, respectively (19). The “*Ca. L. africanus* subsp. *capensis*”, detected by polymerase chain reaction (PCR) in an ornamental rutaceous plant (*Calodendrum capense*), differs from the citrus *liberibacter*s in relation to the 16S rDNA, intergenic 16S/23S rDNA and also serology (4).

The objectives of this study were to detect and compare the HLB bacteria in citrus trees with HLB symptoms in the state of São Paulo, Brazil. For this purpose, the 16S rDNA of HLB *liberibacter*s was amplified, sequenced, compared with Asian and African forms of bacteria, and used to develop primers for specific diagnostic assays. This work confirmed the existence of a third form of HLB *liberibacter*, previously named “*Ca. L. americanus*”, which is the most prevalent form of this bacterium in Brazil.

## MATERIALS AND METHODS

**Plant materials.** Leaves and shoots of citrus plants showing HLB symptoms were collected in different geographic regions of the state of São Paulo, Brazil (Fig. 1). Healthy citrus plants obtained through shoot-tip grafting were used as negative controls. Total DNA from African *liberibacter* was used as a positive control (kindly provided by Dr. Michael Luttig, ARC-Institute for Tropical and Subtropical Crops, Nelspruit, South Africa).

**DNA extraction.** DNA was extracted from leaf midribs and bark of the diseased and healthy plants by the elution procedure as described by Jagoueix et al. (12). Briefly, 300 mg of plant tissue was minced with a razor blade and put in a 1.5-ml tube containing 1 ml of Tris-EDTA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% sodium dodecyl sulfate) to which 0.15 mg

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of proteinase K was added. The tube was incubated at 65°C for 2 h, after which the suspension was centrifuged for 8 min at 12,000 rpm and 600 µl of the supernatant was removed to a new tube. The DNA was further purified using the Wizard Genomic purification kit (Promega Corp., Madison, WI) according to the manufacturer. Of the total DNA obtained, 5 µl were used for PCR assays.

**PCR amplification.** The reactions were performed in 25-µl reaction mixtures (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2 mM each dNTP, 1 unit of *Taq* DNA polymerase (Invitrogen), 5 µl of DNA extract, and 20 ng of each primer. The liberibacter 16S rDNA was amplified specifically with the OI1 and OI2c primers (Table 1; 12). The PCR was carried out in a PTC 100 thermocycler (MJ Research, Woburn, MA) with the following setup: a first step at 94°C for 3 min followed by 36 cycles of a denaturation step at 94°C for 30 s; an annealing step with temperature varying from 55 to 60°C, depending on the primers (Table 1), for 45 s; extension at 72°C for 45 s; and a final extension of 10 min at 72°C. The amplified DNA was analyzed in agarose gel electrophoresis and stained with ethidium bromide. The percentage of agarose in gel was defined according to the molecular weight of the amplicon.

**DNA cloning and sequencing.** The liberibacter 16S rDNA fragment amplified

with the OI1 and OI2c primers from HLB-symptomatic plants were purified from the agarose gels using the GTX PCR kit (Amersham Biosciences, Buckinghamshire, UK). The purified insert was cloned into the pGEM-T vector (Promega Corp.) and sequenced using the BigDye Sequencing kit (PE Applied Biosystems, Foster City, CA) in the ABI 3730 automatic sequencer (Applied Biosystems). The DNA fragments were sequenced in both orientations using the T7 (5' TAATACGACTCACTATAGGG 3') and SP6 (5' ATTTAGGTGACACTATAG 3') primers, and 10 clones of each of the 15 cloned inserts were sequenced.

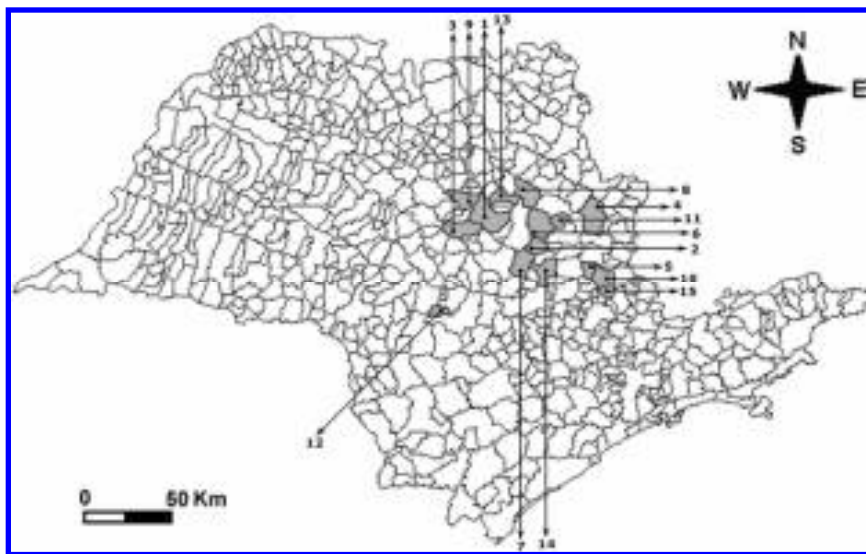
**Data analysis.** The sequences were assembled using the Seqman software of the LaserGene package (DNASTAR, Madison, WI) and multiple alignment were done in Clustal W (20). Phenetic analysis was performed in Phylip (Phylogeny Inference Package, version 3.6; Joseph Felsenstein, Department of Genome Sciences and Department of Biology, University of Washington). The genetic similarity between the samples from the nucleic acid sequences were calculated by the DNADIST program using the Jukes-Cantor model, which assumes that there is independent change at all sites, with equal probability. The phenogram was constructed in the NEIGHBOR software using the unweighted pair group method with arithmetic

means and the consensus tree was established using the CONSENSE tree program. Bootstrapping (1,000 replications) was performed by the SEQBOOT software to estimate the stability and support for the inferred clusters.

## RESULTS

**Detection of HLB bacteria in symptomatic plants.** In the middle of 2004, we detected the Asian form of the liberibacter bacteria in 2 of 10 citrus plants with HLB-like symptoms from two orchards located in Araraquara County in the state of São Paulo, Brazil (2). We then started a survey to verify the distribution of the pathogen in orchards and farms that had plants with HLB-like symptoms. Fifty-three HLB-symptomatic plants from different orchards of the state of São Paulo were analyzed (Table 2). Even though the plants displayed classic HLB symptoms, on the first PCR survey using the OI1 and OI2c primers (12), the bacterium was detected in only 15 of the 53 samples analyzed (28.3% of the plants) in which an amplified fragment of about 1,200 bp of 16S rDNA was generated (Fig. 2; Table 2).

**Sequences of 16S rDNA from HLB bacteria and the phenogram analysis.** Ten clones from each of the 15 16S rDNA fragments amplified using the OI1/OI2c pair of primers together with template DNA obtained from diseased plants were sequenced in both orientations. The sequences were assembled in SeqMan and generated a 1,124-bp contig corresponding to the expected amplicon. The alignment of all the 150 liberibacter sequences allowed the identification of two distinct groups of sequences, which were designated Liberibacter consensus sequence groups 1 (LSg1) and 2 (LSg2). Of the 150 sequences, 23 belonged to the LSg2 group and the other 127 corresponded to sequences of LSg1. These 16S rDNA liberibacter sequences were compared with the 16S rDNA sequences of other liberibacters available in the GenBank database (Table 3; Fig. 3). The LSg1 sequences are identical or very similar to the bacteria of the "*Ca. L. asiaticus*" group (GenBank accession numbers AB008366, AB038369, L22532, and AY192576). The genetic distance from "*Ca. L. africanus*" (GenBank accession numbers L22533 and AF137368) is a little higher. The other 16S rDNA sequence (LSg2) shows an even



**Fig. 1.** Map of São Paulo State, Brazil. Marked regions correspond to counties from which huanglongbing-diseased samples were collected and analyzed for the presence of the LSg1 and LSg2 forms of Liberibacter. Numbers correspond to the names of the counties listed in Table 2.

**Table 1.** Primer sequences used for amplification of different strains of "*Candidatus Liberibacter*" bacteria from huanglongbing-diseased plants

| Primers | Nucleotide sequence (5'-3') | Annealing temperature (°C) | Amplicon size and specificity   |
|---------|-----------------------------|----------------------------|---|
| OI1     | GCGCGTATGCAATACGAGCGGCA     | 60                         | 1,200 bp for both Asian and African forms of <i>Candidatus Liberibacter</i>     |
| OI2c    | GCCTCGCGACTTCGCAACCCAT      |                            |   |
| LSg2f   | TTAAGTTAGAGGTGAAATCC        | 55                         | 545 bp for LSg2 strain of <i>Candidatus Liberibacter</i> from São Paulo, Brazil |
| LSg2r   | CAACTTAATGATGGCAAATA        |                            |   |
| A2      | TATAAAGGTTGACCTTTCGAGTTT    | 60                         | 703 bp for Asian and 669 bp for African forms of <i>Candidatus Liberibacter</i> |
| J5      | ACAAAAGCAGAAATAGCACGAACAA   |                            |   |

higher genetic distance with the known Asian and African liberibacters.

The presence of two sites of deletion in the nucleotide positions 113 to 126 and 131 to 133, 21 transitions, and 10 transversion events plus two point deletions throughout the 1,124 nucleotides were the differences in the 16S rDNA between the HLB-associated organism (LSg2) from São Paulo and LSg1 (Table 4). Despite these differences, the aligned sequences of Liberibacter isolate LSg2 share 1,064 of 1,122 nucleotides. This similarity observed

for LSg2, although not as high as for LSg1 allow us to classify this new associated HLB organism in the “*Ca. Liberibacter*” genus.

The phenetic analysis of HLB strains from São Paulo and the other liberibacter sequences present in the GenBank database delineates two distinct subgroups, one formed by the GenBank Asian sequences AB008366, AY192576, AB038369, and L22532 together with the LSg1 sequence from São Paulo, and the other by the African isolates (GenBank accessions L22533

and AF137368). The LSg2 sequence from São Paulo falls in another subgroup-related liberibacter. The clustering obtained in the phenogram was statistically well supported by 1,000 bootstrappings (Fig. 3).

**Primers designed for PCR amplification of LSg2.** The presence of a genetically different sequence associated with HLB organisms and the absence of a good correlation between HLB symptoms and positive PCR using the already known “*Ca. Liberibacter*” primers OI1 and OI2c led us to design primers for this new HLB-associated organism. The sequence of these primers, named LSg2f and LSg2r, are shown in Table 1. The primer pairs were expected to generate a 545-bp LSg2-specific DNA fragment by PCR.

**Assay for primer specificity.** DNA extracts from diseased and healthy plants were used as templates of PCR amplification. The amplicons were separated in agarose gels (Fig. 4). The HLB-diseased plants from São Paulo, which previously had resulted in negative PCR using the already described pair of primers for liberibacters, were positive for LSg2 DNA sequences in PCRs using the LSg2f and LSg2r primers. The molecular weight of the PCR product was in accordance with the expected size. The African and Asian form of HLB, the samples from healthy citrus, as well as some of the samples that were previously amplified with the OI1/OI2c primers (samples 4, 5, 10, 11, and 15) were all negative when using the LSg2f and LSg2r primers in the same PCR conditions.

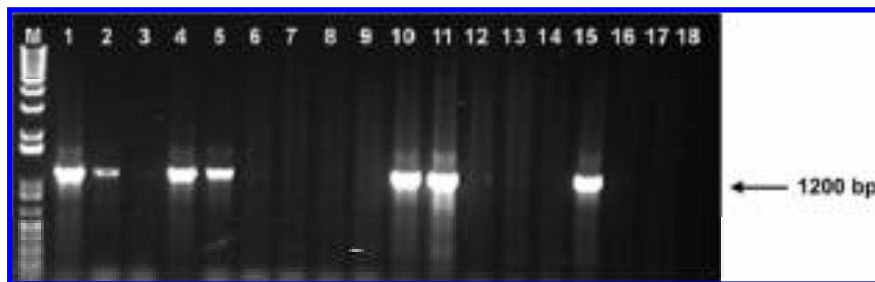
**PCR amplification of HLB-associated organism in diseased plants.** The same 53 samples used for amplification of liberibacter sequences using the pair of primers OI1 and OI2c were used as template of PCR amplifications by the LSg2f and LSg2r primers. The 38 samples that did not previously amplify with the OI1 and OI2c primers were positive with the LSg2f and LSg2r primers. The liberibacter LSg2 isolate also was amplified in 9 of 15 OI1/OI2c positive samples. The Asian “*Ca. Liberibacter*” was found alone in only 6 of the 53 samples tested (Table 2). The liberibacters PCR-positive samples were spread across the 15 municipalities sampled (Fig. 1).

**Table 2.** Polymerase chain reaction (PCR) amplification results using huanglongbing-symptomatic citrus plants from the state of São Paulo (Brazil) for each of the combination of the primers

| Location                             | No. of samples | No. of samples PCR positive |             |
|--------------------------------------|----------------|-----------------------------|-------------|
|                                      |                | OI1/OI2c                    | LSg2r/LSg2f |
| 1. Araraquara <sup>a</sup>           | 5              | 1                           | 4           |
| 2. Analândia                         | 4              | 0                           | 4           |
| 3. Boa Esperança do Sul <sup>a</sup> | 2              | 1                           | 1           |
| 4. Casa Branca <sup>a</sup>          | 2              | 1                           | 1           |
| 5. Conchal                           | 2              | 1                           | 1           |
| 6. Descalvado <sup>a</sup>           | 13             | 1                           | 12          |
| 7. Itirapina <sup>a</sup>            | 3              | 1                           | 2           |
| 8. Luis Antonio <sup>a</sup>         | 5              | 2                           | 3           |
| 9. Matão                             | 1              | 0                           | 1           |
| 10. Mogi Mirim                       | 2              | 1                           | 1           |
| 11. Porto Ferreira                   | 2              | 0                           | 2           |
| 12. Pratânia <sup>a</sup>            | 2              | 1                           | 1           |
| 13. Rincão <sup>a</sup>              | 6              | 2                           | 4           |
| 14. Rio Claro                        | 2              | 2                           | 0           |
| 15. Santo Antonio Posse              | 2              | 1                           | 1           |
| Total                                | 53             | 15 <sup>b</sup>             | 38          |

<sup>a</sup> Location where some samples were positive by both set of primers.

<sup>b</sup> Six samples were amplified only with the OI1/OI2c pair of primers, whereas nine samples were amplified with both OI1/OI2c and LSg2f/LSg2r.



**Fig. 2.** Agarose gel electrophoresis of the amplicons. Total DNA from the citrus trees was amplified with the OI1/OI2c primers and separated in a 1% agarose gel. M, molecular marker, 1-kb ladder (Invitrogen); lane 1, positive control (Asian liberibacter); lane 2, positive control (African liberibacter); lane 3, healthy citrus plant (shot-tip grafted plant); huanglongbing (HLB) symptomatic plants from Rincão (lanes 4 to 9), Luis Antonio (lanes 10 to 14), and Itirapina (lanes 15 to 17); lane 18, HLB symptomless from field.

**Table 3.** Matrix genetic distance for 1,124 nucleotides from 16S rDNA isolates of “*Candidatus Liberibacter*” from São Paulo (LSg1 and LSg2) with the sequences available in the GenBank database<sup>a</sup>

| Huanglongbing organism   | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8    |
|--------------------------|-------|-------|-------|-------|-------|-------|-------|------|
| 1. AB038369 <sup>b</sup> | 0000  | ...   | ...   | ...   | ...   | ...   | ...   | ...  |
| 2. L22532                | 0.004 | 0000  | ...   | ...   | ...   | ...   | ...   | ...  |
| 3. LSg1                  | 0000  | 0.004 | 0000  | ...   | ...   | ...   | ...   | ...  |
| 4. AB008366              | 0000  | 0.001 | 0000  | 0000  | ...   | ...   | ...   | ...  |
| 5. AY192576              | 0.002 | 0.006 | 0.002 | 0.002 | 0000  | ...   | ...   | ...  |
| 6. LSg2                  | 0.037 | 0.039 | 0.037 | 0.037 | 0.039 | 0000  | ...   | ...  |
| 7. L22533                | 0.020 | 0.016 | 0.019 | 0.019 | 0.021 | 0.039 | 0000  | ...  |
| 8. AF137368              | 0.022 | 0.023 | 0.022 | 0.022 | 0.024 | 0.042 | 0.016 | 0000 |

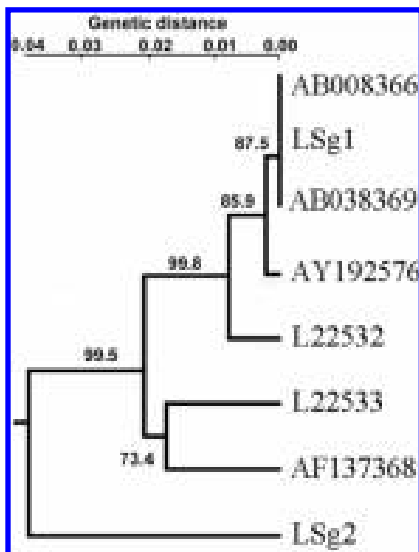
<sup>a</sup> The genetic distances are estimate of the number of nucleotide substitution by site (Jukes and Cantor index).

<sup>b</sup> GenBank accessions number.

## DISCUSSION

In the middle of 2004, the Asian form of “*Ca. Liberibacter*” was detected in citrus plants showing typical HLB symptoms in São Paulo, Brazil (2). Using the set of primers previously described for amplification of the 16S rDNA from “*Ca. Liberibacter*”, we have found a low percentage (28%) of PCR-positive trees displaying classical symptoms of HLB. It has been difficult to detect the HLB bacterium due to its low concentration and uneven distribution in the host (8,10). We observed that the amplification using the OI1 and OI2c primers resulted in this erratic pattern. When using a second set of primers (A2 and J5; Table 1) which amplify a 703-bp DNA fragment from ribosomal protein gene from the Asian liberibacter (9), the results were the same (data not shown). The quality of DNA also was verified by the successful amplification of all samples with the fD1/rD1 universal 16S rDNA primers (21).

The sequences of the 16S rDNA genes are quite conserved among bacteria, but present sufficient variation to design primers for a specific diagnosis of species or strains (12,22). The 16S rDNA sequence of the LSg1 Brazilian “*Ca. Liberibacter*” is identical to sequences of bacteria (GenBank accessions AB038369 and AB008366) identified in Japan from the insect vector and the diseased plants, respectively. The LSg2 Brazilian “*Ca. Liberibacter*” shows few nucleotide sequence differences from the Asian and African strains, but still shows a high percentage of identical nucleotides (signature sequences). The phenogram based on pairwise comparison of all of those se-



**Fig. 3.** Phenogram showing the phylogenetic relationships among strains of “*Candidatus Liberibacter*”. The genetic distances were estimated by pairwise comparisons of the 16S ribosomal DNA sequences using the Jukes-Cantor parameter. Bootstrap values (based on 1,000 replications) are indicated at the nodes.

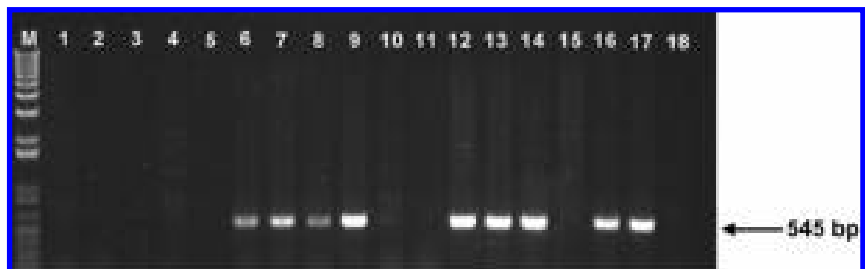
quences define three clusters, Asian + LSg1, African, and LSg2, characterizing the latter as a new liberibacter strain. Genomic characterization by 16S rDNA and serology already had shown differences among the Asiatic and African liberibacters, including the subspecies *capensis* (4,11,13). Genetic variations among the Asian isolates were observed for the different strains, and they also can be sepa-

rated into different groups, those from the Pacific area (Japan, the Philippines, Indonesia, Thailand, and Nepal) and those from India and China (18). Teixeira et al. (19) found a liberibacter variant from HLB-diseased plants in São Paulo, Brazil and, by analysis of the 16S rDNA sequences, they designated it as a new species, “*Ca. L. americanus*”. It is very likely that the LSg2 isolate and this new “*Ca. Liberibacter* sp.”

**Table 4.** Nucleotide comparison of 16S ribosomal (r)DNA sequence between “*Candidatus Liberibacter*” strains LSg1 and LSg2 from São Paulo, Brazil

| Nucleotide position <sup>a</sup> | Liberibacter strain |      |
|----------------------------------|---------------------|------|
|                                  | LSg1                | LSg2 |
| 4                                | A                   | C    |
| 106                              | A                   | T    |
| 113–126                          | ATCTAGGTAAAAA       | –    |
| 127                              | C                   | T    |
| 131–133                          | ACT                 | –    |
| 143                              | C                   | A    |
| 146                              | G                   | T    |
| 226                              | A                   | G    |
| 228                              | A                   | G    |
| 308                              | C                   | T    |
| 326                              | T                   | C    |
| 328–329                          | TA                  | C-   |
| 399                              | T                   | C    |
| 456                              | G                   | A    |
| 503                              | C                   | T    |
| 546                              | C                   | T    |
| 565                              | T                   | A    |
| 581                              | A                   | C    |
| 595                              | A                   | G    |
| 608                              | A                   | G    |
| 621                              | C                   | A    |
| 652                              | C                   | T    |
| 689                              | G                   | A    |
| 778–779                          | AA                  | GT   |
| 962                              | G                   | A    |
| 964                              | A                   | C    |
| 977–978                          | TC                  | CT   |
| 985                              | T                   | C    |
| 1001                             | C                   | T    |
| 1007                             | A                   | G    |
| 1010                             | C                   | T    |
| 1017                             | C                   | T    |
| 1038                             | C                   | T    |
| 1040                             | A                   | T    |
| 1042                             | T                   | –    |
| 1044                             | G                   | A    |
| 1073                             | A                   | G    |
| 1101                             | T                   | C    |
| 1104                             | T                   | C    |

<sup>a</sup> Nucleotide positions are numbered following the sequence of the 16S rDNA of strain LSg1 of “*Candidatus Liberibacter asiaticus*” from São Paulo, Brazil.



**Fig. 4.** Agarose gel electrophoresis of the DNA amplified with the LSg2f/LSg2r pair of primers. M, molecular marker, 1-kb ladder (Invitrogen); positive controls of Asian (lane 1) and African Liberibacter (lane 2); healthy plant (lane 3); huanglongbing (HLB) symptomatic plants from Rincão (lanes 4 to 9), Luis Antonio (lanes 10 to 14), and Itirapina (15 to 17); HLB symptomless (lane 18). Samples of the lanes 4, 5, 10, 11, and 15 previously were positive with the OI1/OI2c primers (see Fig. 2).

are the same *Liberibacter* strain. Although “*Candidatus*” has been used for unculturable bacterium lacking definitive classification (15), the species concept for prokaryotes needs much more information than that provided by comparison of 16S rDNA sequences only (17).

The LSg2 strain of “*Ca. Liberibacter*” was predominant throughout the orchards in the state of São Paulo. Interestingly, in nine samples we were able to amplify both sequences, evidencing a mixed infection. All this work was possible because we were able to amplify the fragment of LSg2 strain of “*Ca. Liberibacter*” using the OI1 and OI2c primers. The frequency of the LSg2 fragment was low compared with LSg1, which could be explained by the variations in the sequence of the region of annealing of primers. Comparing the sequence of LSg2 variant with the OI1 primer, we observed four mismatches and one insertion, whereas the comparison with OI2c reveals the presence of three mismatches, which could explain the low efficiency of amplification observed when using this combination of primers (data not shown). The probable vector for both bacteria is *D. citri*, which has been endemic in Brazilian orchards for at least 40 years (3). There is no correlation between the spatial distribution of the focus of the disease and the variants observed in the plants (Table 1; Fig. 1). The pattern observed in the infected plants is always the same, with a predominance of the LSg2 form over LSg1.

The origin of both forms of liberibacter in Brazil is uncertain. Because of the perfect homology between the sequence of the LSg1 strain and strains from Japan, we believe that it probably was introduced in infected material from this Pacific area. The LSg2 strain has never been detected in other parts of the world but it also was identified in another plant species, *Murraya paniculata*, in Brazil (S. A. Lopes, *personal communication*). This species also was introduced in Brazil from Asia and the bacterium could have come together with this material. Another possibility is that it was introduced from infected citrus material but, because of the variation in the sequence, it has not been identified previously in the center of origin, Asia and Africa, with the previously available primers. Analysis with the LSg2r and LSg2f primers needs to be done with plant material from Asia and Africa to verify whether this form is present in these continents. We cannot exclude the possibility that the bacterium came from native

plants in Brazil and, with the presence of the vector, was transmitted to citrus, where it was shown to be pathogenic.

In fact, in Brazil we have two different strains of “*Ca. Liberibacter*” causing severe and almost identical HLB symptoms in citrus trees. By the distribution of the HLB bacteria across São Paulo State orchards that we verified in this work and by the epidemic situation of its possible vector *D. citri*, an eradication program of HLB-diseased plants needs to be implemented urgently to avoid the rapid spread of the disease.

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