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'*Candidatus* Liberibacter americanus', associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil

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Symptoms of huanglongbing (HLB) were reported in São Paulo State (SPS), Brazil, in March 2004. In Asia, HLB is caused by '*Candidatus* Liberibacter asiaticus' and in Africa by '*Candidatus* Liberibacter africanus'. Detection of the liberibacters is based on PCR amplification of their 16S rRNA gene with specific primers. Leaves with blotchy mottle symptoms characteristic of HLB were sampled in several farms of SPS and tested for the presence of liberibacters. '*Ca*. L. asiaticus' was detected in a small number of samples but most samples gave negative PCR results. Therefore, a new HLB pathogen was suspected. Evidence for an SPS-HLB bacterium in symptomatic leaves was obtained by PCR amplification with universal primers for prokaryotic 16S rRNA gene sequences. The amplified 16S rRNA gene was cloned and sequenced. Sequence analysis and phylogeny studies showed that the 16S rRNA gene possessed the oligonucleotide signatures and the secondary loop structure characteristic of the α-*Proteobacteria*, including the liberibacters. The 16S rRNA gene sequence phylogenetic tree showed that the SPS-HLB bacterium clustered within the α-*Proteobacteria*, the liberibacters being its closest relatives. For these reasons, the SPS-HLB bacterium is

considered a member of the genus '*Ca*. Liberibacter'. However, while the 16S rRNA gene sequences of '*Ca*. L. asiaticus' and '*Ca*. L. africanus' had 98·4 % similarity, the 16S rRNA gene sequence of the SPS-HLB liberibacter had only 96·0 % similarity with the 16S rRNA gene sequences of '*Ca*. L. asiaticus' or '*Ca*. L. africanus'. This lower similarity was reflected in the phylogenetic tree, where the SPS-HLB liberibacter did not cluster within the '*Ca*. L asiaticus'/'*Ca*. L. africanus group', but as a separate branch. Within the genus '*Candidatus* Liberibacter' and for a given species, the 16S/23S intergenic region does not vary greatly. The intergenic regions of three strains of '*Ca*. L. asiaticus', from India, the People's Republic of China and Japan, were found to have identical or almost identical sequences. In contrast, the intergenic regions of the SPS-HLB liberibacter, '*Ca*. L. asiaticus' and '*Ca*. L. africanus' had quite different sequences, with similarity between 66·0 and 79·5 %. These results confirm that the SPS-HLB liberibacter is a novel species for which the name '*Candidatus* Liberibacter americanus' is proposed. Like the African and the Asian liberibacters, the 'American' liberibacter is restricted to the sieve tubes of the citrus host. The liberibacter could also be detected by PCR amplification of the 16S rRNA gene in *Diaphorina citri*, the psyllid vector of '*Ca*. L. asiaticus', suggesting that this psyllid is also a vector of '*Ca*. L. americanus' in SPS. '*Ca*. L. americanus' was detected in 216 of 218 symptomatic leaf samples from 47 farms in 35 municipalities, while '*Ca*. L. asiaticus' was detected in only 4 of the 218 samples, indicating that '*Ca*. L. americanus' is the major cause of HLB in SPS.

Abbreviations: HLB, huanglongbing; RIR, ribosomal intergenic region; SPS, São Paulo State

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the ribosomal 16S/23S intergenic region gene sequence of '*Candidatus* Liberibacter americanus' isolate SPSA3 are AY742824 and AY859542, respectively.

Comparisons between the gene sequences of the ribosomal 16S/23S intergenic region for liberibacter isolates are available as supplementary data in IJSEM Online.

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In March 2004, symptoms of citrus huanglongbing (HLB), formerly called greening disease, were recognized on sweet orange trees near the city of Araraquara in São Paulo State (SPS), Brazil (Coletta-Filho *et al*., 2004; Teixeira *et al*., 2005a, b). This was the first report of HLB on the American continent. HLB is caused by two sieve-tube-restricted species of the α-*Proteobacteria*, '*Candidatus* Liberibacter africanus' in Africa and '*Candidatus* Liberibacter asiaticus' in Asia (Jagoueix *et al*., 1994). Neither of the two species has been cultured. The HLB liberibacters were among the first bacteria to receive '*Candidatus*' designations according to the rules established for uncultured organisms (Murray & Schleifer, 1994). Phylogenetically, the closest relatives of the liberibacters are members of the alpha-2 subgroup of the *Proteobacteria*. However, the liberibacters do not belong to the alpha-2 subgroup because their 16S rRNA gene sequence has only one of the seven or more oligonucleotide signatures characteristic of the alpha-2 subgroup and also has signatures characteristic of the alpha-1 and alpha-3 subgroups (Jagoueix *et al*., 1994; Woese *et al*., 1984).

The HLB liberibacters are transmitted by two psyllid insects, *Trioza erytreae* (Del Guercio) in Africa and *Diaphorina citri* (Kuwayama) in Asia. The two insects are responsible for the large geographical distribution of HLB in these areas. The Mediterranean region and Australia are free of both HLB and HLB psyllid vectors. However, *Diaphorina citri*, the Asian psyllid vector, is established in South, Central and North America (Florida

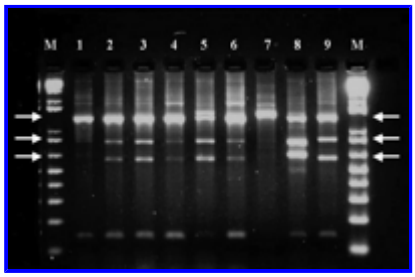
and Texas) and the Caribbean basin. *D. citri* has been present in Brazil for more than 50 years and for less than 10 years in Florida and Texas. Even though symptoms of HLB were recognized in SPS less than a year ago, the disease has been present in some orchards for much longer, probably for 10 years.

Due to their devastating effects on citrus trees and their efficient transmission by the psyllid vectors, '*Ca*. L. africanus' and '*Ca*. L. asiaticus' have been classified by the US Government as 'select agents' with potential for bioterrorism. Working with such agents in the USA requires permission from the US Government. The two liberibacters are quarantine organisms in Europe.

Detection and identification of the liberibacters in citrus leaves showing blotchy mottle, the most characteristic symptom of HLB (McClean & Schwarz, 1970), is based on specific PCR amplification of their 16S rRNA gene sequence with primer pair OA1/OI2c for '*Ca*. L. africanus' and OI1/OI2c for '*Ca*. L. asiaticus' (Jagoueix *et al*., 1996; Teixeira *et al*., 2005b). When, in a given region, the liberibacter involved has not yet been identified, both forward primers are used in the same reaction mixture to favour amplification of either one of the two liberibacters. In all previous cases, amplicons of 1160 bp have been obtained. In order to identify the liberibacter causing HLB in SPS, 43 samples of blotchy mottle-affected leaves were collected in seven citrus farms in the Araraquara region and 16S rRNA gene amplifications with both forward primers OA1 and OI1 and reverse primer OI2c were carried out. Surprisingly, all samples gave negative PCR results under conditions where symptomatic control leaves infected with '*Ca*. L. asiaticus' or '*Ca*. L. africanus' gave positive reactions (Teixeira *et al*., 2005b). These negative results were unexpected because, in the many African and Asian countries previously studied (Jagoueix *et al*., 1996; Garnier & Bové, 1996; Garnier *et al*., 1996; Bové *et al*., 2000), the PCR tests were always positive when symptomatic leaves from HLB-affected trees were used. In SPS, many negative PCR tests were also observed by other investigators using the same PCR method as described above, even though a few samples (2 of 10) tested positive for '*Ca*. L. asiaticus' (Coletta-Filho *et al*., 2004). In view of the many negative PCR tests witnessed by us and others with leaves showing characteristic HLB symptoms, the presence of a new bacterial pathogen in the symptomatic HLB-affected leaves from SPS was suspected.

Evidence for an 'SPS-HLB' bacterium responsible for HLB in SPS was obtained by PCR amplification of its 16S rRNA gene with universal primers fD1/rP1 for prokaryotic 16S rRNA gene sequences (Weisburg *et al*., 1991). The method was essentially that of Jagoueix *et al*. (1994) and is based on the fact that *Bcl*I cuts plant mitochondria 16S rRNA genes, but not bacterial 16S rRNA genes, and *Eco*RI has no effect on chloroplast 16S rRNA genes, but cuts bacterial 16S rRNA genes into two fragments of ~650 and ~850 bp. DNAs from five symptomatic Valencia sweet orange leaf samples that tested negative for '*Ca*. L. africanus' and '*Ca*. L. asiaticus' were digested with *Bcl*I to cut the plant mitochondria 16S rRNA gene and prevent it from being amplified in the ensuing step (Zreik *et al*., 1998). The remaining chloroplast 16S rRNA gene and the putative SPS-HLB 16S rRNA gene were amplified by PCR using universal primers fD1/rP1 and submitted to *Eco*RI treatment. Under these conditions, the bacterial 16S rRNA gene is revealed by the presence of the ~650 and ~850 bp fragments. Fig. 1 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 leaf samples with HLB symptoms from SPS (lanes 2–6). With DNA amplified from healthy leaves (lanes 1 and 7), only the ~1500 bp chloroplast DNA band was seen on the gel. These results gave evidence for the presence of an SPS-HLB bacterium in HLB-affected leaves from SPS, even though such leaves tested negative for '*Ca*. L. asiaticus' and '*Ca*. L. africanus'. Characterization of the novel bacterium required cloning and sequencing of its 16S rRNA gene as follows.

Fig. 1. Electrophoresis on 1 % agarose gel of DNA treated with *Eco*RI after amplification with primers fD1/rP1 from *Bcl*I-treated DNA



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extracted from healthy Valencia sweet orange leaves (lanes 1 and 7), control leaves infected with '*Ca*. L. asiaticus' (lane 9) or '*Ca*. Phytoplasma aurantifolia' (lane 8) and Valencia sweet orange leaves from SPS with blotchy mottle symptoms characteristic of HLB (lanes 2–6). The upper, middle and lower arrows point to DNA bands of approximately 1500, 850 and 650 bp. Lane M, 1 kb Plus DNA ladder.

DNA from Natal sweet orange leaves with HLB symptoms (sample A3 from Luis Antonio municipality) was purified according to Jagoueix *et al*. (1996). It was found to test negative for '*Ca*. L. africanus' and '*Ca*. L. asiaticus' with primers OA1 and OI1/OI2c. The DNA was digested with *Bcl*I and amplified by PCR with universal primers fD1/rP1 as described above. The ~1500 bp amplified DNA (16S rRNA gene from SPS-HLB bacterium and chloroplast 16S rRNA gene) was cloned in *Escherichia coli* using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. In total, 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 rRNA genes. These two clones were selected and their inserts were sequenced. The two sequences were identical. The unique sequence was 1447 bp. Search for homologies in general databases (http://www.ncbi.nlm.nih.gov/blast) were carried out using the BLAST program (Altschul *et al*., 1997). Sequence analysis was also performed using Infobiogen programs (http://www.infobiogen.fr/index.html). Multiple sequence alignments were performed using MULTALIN (Corpet, 1988) (http://www.toulouse.inra.fr/Igc/multalin/multalin.html) and CLUSTAL W (Thompson *et al*., 1994) software.

A BLAST search of the GenBank database applied to the 1447 bp sequence revealed that it was the 16S rRNA gene sequence of a bacterium, with liberibacters as the closest relatives. The sequence was aligned with the 16S rRNA gene sequences of '*Ca*. L. asiaticus' isolate Poona and '*Ca*. L. africanus' isolate Nelspruit, with which it had 96·1 and 95·9 % similarity, respectively.

The following experiments were undertaken to demonstrate that the bacterial 1447 bp 16S rRNA gene sequence did represent the PCR amplification product of the SPS-HLB bacterium involved in HLB in SPS and not that of a contaminating bacterium. Forward primer GB1 and reverse primer GB3 were designed from the bacterial 16S rRNA gene sequence (Teixeira *et al*., 2005b). Two hundred and fourteen leaf samples from SPS with characteristic HLB symptoms and that tested negative for '*Ca*. L. africanus' and '*Ca*. L. asiaticus' were used for PCR amplification with primers GB1/GB3. All 214 samples gave positive PCR results and the amplicons obtained were of the expected size (1027 bp). Healthy leaves, or leaves infected with '*Ca*. L. africanus' or '*Ca*. L. asiaticus', gave negative PCR results (Teixeira *et al*., 2005b). The 1027 bp amplicon, obtained with primers GB1/GB3, specific for 16S rRNA gene amplification, is thus characteristic of HLB leaves from SPS and represents the 16S rRNA gene sequence amplified from the SPS-HLB bacterium present in these leaves. Two amplicons, one from leaf sample A5 (Luis Antonio municipality) and one from AA9 (Boa Esperança do Sul municipality) were cloned and sequenced. The two sequences were identical and they had 100 % similarity with the corresponding sequence of the 1447 bp 16S rRNA gene. Finally, the ~850 *Eco*RI fragment shown in Fig. 1, (lane 5) was also cloned and sequenced. The fragment was 833 bp and its sequence showed 100 % similarity to the corresponding region of the 1447 bp 16S rRNA gene sequence. Therefore, the 833 bp fragment, the 1027 bp 16S rDNA amplicon and the 1447 bp 16S rRNA amplicon, obtained from leaf samples of different geographical origins, were all 16S rRNA gene amplification products from the SPS-HLB bacterium and not from a contaminating bacterium. The 1447 bp 16S

rRNA gene sequence, hereafter referred to as the SPS-HLB 16S rRNA gene sequence, could thus be safely used to characterize the novel SPS-HLB bacterium.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al*., 2001). The bacterial 16S rRNA gene sequences used for comparisons were obtained from the GenBank database and were those of '*Ca*. L. asiaticus' isolate Poona, '*Ca*. L. endosymbiont' from *Diaphorina citri*, '*Ca*. L. asiaticus' isolate Okinawa, '*Ca*. L. asiaticus' isolate *Citrus grandis*, '*Ca*. L. africanus' isolate Nelspruit, '*Ca*. L. africanus subsp. capensis', *Brucella abortus* ATCC 23448^T, *Bartonella henselae* ATCC 49882^T, *Afipia felis* ATCC 53690^T, '*Candidatus* Phlomobacter fragariae' and *E. coli* (MRE 600^T).

The phylogenetic analysis (Fig. 2) indicated that the SPS-HLB bacterium, like the liberibacters, clustered in the α-subclass of the *Proteobacteria* and that the liberibacters were its closest relatives. In addition, the SPS-HLB 16S rRNA gene sequence had oligonucleotide signatures (Woese, 1987; Zeff & Geliebter, 1987) very similar to those of the liberibacters (Jagoueix *et al*., 1997). Also, the secondary loop structure characteristic of the α-*Proteobacteria* (Woese, 1987) was shared by the liberibacters and the SPS-HLB bacterium. For all these reasons, the SPS-HLB bacterium is a member of the genus '*Candidatus* Liberibacter'. The following results indicate that it represents a novel '*Candidatus* Liberibacter' species.

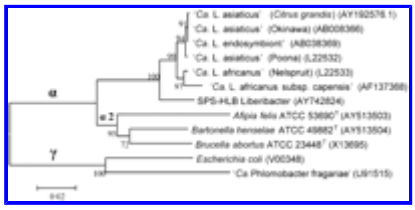

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Fig. 2. Phylogenetic tree constructed from 16S rRNA gene sequences obtained from GenBank (accession nos in parentheses) using MEGA version 2.1.

While the percentage similarity between the 16S rRNA gene sequences of two species of liberibacters as different as '*Ca*. L. africanus' isolate Nelspruit and '*Ca*. L. asiaticus' isolate Poona was 98·4 % (gap-free sequences), the percentage similarity in the case of the SPS-HLB liberibacter and '*Ca*. L. asiaticus' was lower, 95·4 %, indicating that the latter two liberibacters had to represent different species. This was also indicated by the phylogenetic tree (Fig. 2), where 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, the four isolates of '*Ca*. L. asiaticus' clustered together within the liberibacter group. One of these strains, '*Ca*. L. endosymbiont', was described as an endosymbiont of *Diaphorina citri*, the psyllid vector of the Asian liberibacter. Evidence for this liberibacter was obtained by PCR amplification of the 16S rRNA gene sequence from total *D. citri* DNA. Therefore, the *D. citri* liberibacter should not be considered as an endosymbiont, but as the pathogen transmitted by the psyllid, namely '*Ca*. L. asiaticus'. The phylogenetic analysis (Fig. 2) clearly showed that the so-called '*Ca*. L. endosymbiont' clustered with the isolates of '*Ca*. L. asiaticus' and is an isolate of this species. However, the SPS-HLB liberibacter did not cluster in this region and is a novel species. This conclusion was confirmed by analysis of the 16S/23S ribosomal intergenic region (RIR) gene sequence.

The RIR of the SPS-HLB liberibacter was obtained by PCR amplification of DNA extracted from HLB-affected leaf sample AA9 from Boa Esperanca do Sul municipality which was also used for the production, by PCR amplification, of the 1027 bp SPS-HLB 16S rRNA gene sequence (see above). The primers used were forward

primer GB3c, complementary to primer GB3 and located in the 3' half of the SPS-HLB 16S rRNA gene sequence, and reverse primer 23S1, located at the very start of the 23S rRNA gene (Jagoeix *et al*., 1997). The leaf DNA was amplified for 35 cycles with the following program: 92 °C for 40 s, 64 °C for 40 s and 72 °C for 60 s. The amplified DNA was cloned and sequenced and was 1026 bp long. As expected, the RIR was preceded upstream by a 16S rRNA gene sequence, 425 bp, of which the 393 bp sequence at the 5' end was identical to the 3' terminal region of the SPS-HLB 16S rRNA gene. The remaining 32 bp sequence was absent from this 16S rRNA gene sequence because of the position of the reverse universal primer rP1 used for its amplification (see above). Downstream, the RIR was followed by the first 18 bp of the 23S rRNA gene and these 18 bp represented the 23S1 reverse primer used for amplification of the RIR region. The fact that the 16S rRNA gene sequence in the cloned 1026 bp RIR was identical to the equivalent region of the SPS-HLB 16S rRNA gene sequence demonstrated that the cloned 1026 bp RIR was indeed that of the SPS-HLB liberibacter. The bacterial RIR sequences used for comparisons were obtained from the GenBank database and were those of '*Ca*. L. asiaticus' isolates Poona (U61359) and Okinawa (AB019793) and '*Ca*. L. africanus' isolate Nelspruit (U61360). '*Ca*. L. asiaticus' isolate Fuzhou was from Jagoeix *et al*. (1997). The RIR of the SPS-HLB liberibacter was 582 bp long, compared with 595 and 498 bp for the RIRs of '*Ca*. L. asiaticus' isolate Poona and '*Ca*. L. africanus' isolate Nelspruit, respectively (Jagoeix *et al*., 1997). The RIR contained the sequences for two tRNAs: $tRNA^{Ile}$ and $tRNA^{Ala}$. The RIR of '*Ca*. L. asiaticus' contained two similar tRNA sequences, while only the $tRNA^{Ala}$ sequence was present in the '*Ca*. L. africanus' RIR (Jagoeix *et al*., 1997).

RIR sequence similarity comparisons between liberibacter species or isolates are available as Supplementary Table S1 in IJSEM Online. In summary, when '*Ca*. L. asiaticus' isolate Poona was compared with another isolate of the same species, isolate Fuzhou or isolate Okinawa, the RIR sequences were either identical, with 100 % similarity (isolate Fuzhou) or very similar, 99·2 % similarity (isolate Okinawa). Thus, within in a given liberibacter species, the RIR sequence does not vary much, as already described (Jagoeix *et al*., 1997). However, when the RIRs of two different species, such as '*Ca*. L. asiaticus' isolate Poona and '*Ca*. L. africanus' isolate Nelspruit were compared, the similarity value for the total intergenic region was as low as 79·5 %. The figures were even lower when the RIR of the SPS-HLB liberibacter was compared with that of '*Ca*. L. asiaticus', 77·8 % for isolate Poona, 77·7 % for isolate Okinawa, or with '*Ca*. L. africanus', 66·0 %. These results confirm those based on 16S rRNA gene sequence comparisons and indicate that the SPS-HLB liberibacter is a novel species, for which the name '*Candidatus* Liberibacter americanus' is proposed.

Additional properties of the SPS-HLB liberibacter fit those of the other liberibacters. Transmission of the SPS-HLB liberibacter to healthy sweet orange seedlings has been achieved by graft-inoculations under greenhouse conditions in Bordeaux, France, as well as Araraquara, Brazil. The inoculated seedlings showed foliar symptoms of blotchy mottle within 4 months and the SPS-HLB liberibacter could be detected by PCR in the symptomatic leaves. The liberibacters have never been grown in culture and the same is true for the novel species. Liberibacters are restricted to the sieve tubes of the phloem tissue and possess a characteristic double-membrane cell envelope (Garnier *et al*., 1984). Electron microscopy has shown that this is also present in the SPS-HLB liberibacter (Teixeira *et al*., 2005a; Tanaka *et al*., 2004). The natural vector of '*Ca*. L. asiaticus' in Asia is the psyllid *D. citri*, which has been present on citrus in Brazil for more than 50 years. The SPS-HLB liberibacter could be detected in *D. citri* by PCR amplification of 16S rRNA gene sequence with the specific primer pair GB1/GB3, strongly suggesting that the Asian psyllid is also a vector of the SPS-HLB liberibacter in SPS (Teixeira *et al*., 2005b).

In an extensive survey for HLB, 218 samples of leaves showing symptoms of blotchy mottle were collected from 47 citrus farms in 35 municipalities of SPS. Each sample was tested for liberibacters with two pairs of primers; the OA1+OI1/OI2c pair, specific for '*Ca*. L. africanus' and '*Ca*. L. asiaticus' (Jagoeix *et al*., 1996), and the GB1/GB3 pair, specific for the SPS-HLB liberibacter (Teixeira *et al*., 2005b). The SPS-HLB liberibacter could be detected in

214 samples, '*Ca*. L. asiaticus' in two and '*Ca*. L. africanus' in none. Two 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 : 216 or 1·9 %. These figures indicate that the major cause of HLB in SPS is the SPS-HLB liberibacter and not '*Ca*. L. asiaticus', as stated before the characterization of the SPS-HLB liberibacter (Coletta-Filho *et al*., 2004).

It has been suggested previously that '*Ca*. L. africanus' probably originated in Africa and '*Ca*. L. asiaticus' probably originated in Asia (Garnier & Bové, 1996). Similarly, '*Ca*. L. americanus' is likely to be of American origin and will be spread by *D. citri* to further American countries. Therefore, in America, the new liberibacter might be a more serious 'select agent' than '*Ca*. L. africanus' or '*Ca*. L. asiaticus'.

Finally, we propose to eliminate the taxon '*Ca*. L. endosymbiont' and merge it into '*Ca*. L. asiaticus'.

Description of '*Candidatus* Liberibacter americanus'

In view of the genomic properties of the liberibacter associated with citrus huanglongbing in São Paulo State, Brazil, it is proposed that this SPS-HLB bacterium be assigned species status with the following designation according to Murray & Schleifer (1994) and Murray & Stackebrandt (1995):

'*Candidatus* Liberibacter americanus' (a.me.ri.ca'nus. N.L. masc. adj. *americanus* American, referring to the fact that the first detection and occurrence of the organism was on the American continent).

Reference isolate is SPSA3 (from Natal sweet orange leaf sample A3, São José farm, Luis Antonio municipality, SPS, Brazil).

[(α-*Proteobacteria*) NC; G–; F; NAS (GenBank accession no. AY742824 for 16S rRNA gene and AY859542 for ribosomal 16S/23S intergenic region), oligonucleotide sequence complementary to unique region of 16S rRNA 5'-CTATATTTGCCATCATTAAGTTGG-3', S (*Citrus*, phloem; *Diaphorina citri* (Psyllidae), haemolymph, salivary glands); M]. Teixeira *et al*., this study.

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With deep emotion, we dedicate this publication to the memory of Dr Monique Garnier (1949–2003). We would like to thank Dr Pascal Sirand-Pugnet, Université Victor Segalen Bordeaux 2, for fruitful discussions concerning phylogeny.

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