A Phytoplasma Related to '*Candidatus* Phytoplasma asteris' Detected in Citrus Showing Huanglongbing (Yellow Shoot Disease) Symptoms in Guangdong, P. R. China

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ABSTRACT

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Citrus huanglongbing (HLB) or yellow shoot disease (i.e., greening disease) is highly destructive to citrus production worldwide. Understanding the etiology of HLB is critical for managing the disease. HLB is currently associated with infection by '*Candidatus* Liberibacter spp.' around the world, including China. However, Koch's postulates have not been fulfilled. In addition, other plant pathogens also may be involved in HLB. In a survey performed in Guangdong Province, P. R. China in 2006 and 2007, 141 citrus samples showing typical symptoms of HLB from 11 different cities were collected. Polymerase chain reaction (PCR) using phytoplasma-specific primer sets fU5/rU3 nested with primer set P1/P7 identified 110 (78.0%) positive samples. A 1,785-bp amplicon was obtained with primer set P1/P7. Analysis showed a 100% identity of this sequence in the region of 16S rDNA and 16S-23S rRNA intergenic tran-

scribed spacer to three strains of '*Candidatus* Phytoplasma asteris' (onion yellows [Japan], aster yellows 'watercress' [Hawaii], and valeriana yellows [Lithuania]). Of the 141 samples, 89 (63.1%) samples were positive for '*Ca. Liberibacter asiaticus*'. When mixed infection was considered, 69 (48.9%) samples were positive for both '*Ca. P. asteris*' and '*Ca. L. asiaticus*'. Transmission electron microscopy (TEM) showed low titers of both walled and wall-less bodies in the phloem sieve tubes of HLB citrus. When transmission from symptomatic citrus to periwinkle (*Catharanthus roseus*) via dodder (*Cuscuta campestris*) was conducted, both phytoplasma and '*Ca. L. asiaticus*' were detected from the affected periwinkle. In addition to yellowing/mottling, the infected periwinkle showed symptoms of virescence and phyllody which are commonly associated with phytoplasma diseases. TEM analysis of affected periwinkle revealed pleomorphic and wall-less organisms, characteristic of phytoplasmas, filling some phloem sieve tubes. In contrast, walled bacteria were at low titer. This study showed that in addition to '*Ca. L. asiaticus*', a phytoplasma related to '*Ca. P. asteris*' could also be detected in citrus showing HLB symptoms in Guangdong.

Huanglongbing (HLB) or yellow shoot disease (i.e., greening disease) is one of the most destructive diseases in citrus production around the world (3). In China, HLB was noted probably in the late 1880s in Guangdong Province (26). The infectious nature of HLB was suggested in 1943 (5) and experimentally demonstrated in 1956 (27). In searching for the etiological agent, Lin (27) suggested the involvement of viruses. Rickettsia-like organisms (RLOs) or bacteria-like organisms (BLOs) and mycoplasma-like organisms (MLOs, now named phytoplasmas) were observed in infected tissue (6,21). Jagoueix et al. (19) reported that HLB was associated with an alpha-proteobacterium, and suggested the trivial name Liberobacter, which was later changed to '*Candidatus* Liberibacter' (15). Despite the lack of fulfillment of Koch's postulates, '*Ca. Liberibacter*' has been considered as the most probable etiological agent of HLB. Currently, three '*Candidatus*' species of Liberibacter, '*Ca. L. asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*', have been proposed (7,19,31,36).

HLB is currently diagnosed by symptomatology and confirmed by polymerase chain reaction (PCR) detection of '*Ca. Liberibacter*'. In China, only '*Ca. L. asiaticus*' has been associated with HLB so far. The sequence of OI1 was used as a signature to define '*Ca. L. asiaticus*' (19,36). Primers OI1 and OI2c are the standard primer set for detection of '*Ca. L. asiaticus*' (19,20). However, we observed that primer set OI1/OI2c may not always yield a positive PCR from citrus samples with typical HLB symptoms (X. Deng, *unpublished data*). The most common explanation is that the titer of '*Ca. L. asiaticus*' cells in the tested plant tissues is below the PCR detection limit. Another possibility is that disease symptoms are due to other causes (e.g., mineral deficiency). However, it is also possible that pathogens other than '*Ca. L. asiaticus*' may be involved in the etiology of HLB.

To facilitate studying non-culturable pathogens of this woody host, Garnier and Bove (14) transmitted HLB-associated bacteria from citrus collected in India and South Africa to periwinkle (*Catharanthus roseus* [L.] G. Don.) via dodder (*Cuscuta campestris* Yunck) and observed a high titer of walled bacteria in the infected periwinkle tissues by transmission electron microscopy (TEM). Subsequently, Tang and Faan (35) in Guangdong successfully performed a reciprocal transmission of HLB-associated bacteria between citrus and periwinkle via dodder. In addition to the yellowing and mottling, they reported that infected periwinkle also showed symptoms of virescence and phyllody and noted the

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presence of pleomorphic organisms in some infected periwinkle and citrus tissues. In this study, our objectives were to (i) verify the occurrence of a phytoplasma in HLB-affected citrus and (ii) characterize the phytoplasma by nucleotide sequence analyses, TEM, and symptomatology in periwinkle after transmission from HLB affected citrus.

MATERIALS AND METHODS

Plant material. Two citrus HLB surveys were carried out in Guangdong (Table 1). In the first survey, 71 HLB samples were collected from six cities in October, 2006. The plant species

surveyed included mandarin (*Citrus reticulata* Blanco), sweet orange (*C. sinensis* [L.] Osbeck) and pummelo (*C. maxima* [Burn.] Merrill) (33). A second survey was performed in September to October 2007 with 70 samples of mandarin, sweet orange, and pummelo collected from five cities. Leaf samples were collected based on typical HLB leaf yellowing/mottling symptoms (Fig. 1) and shipped to the Huanglongbing Research Laboratory at the South China Agricultural University in Guangzhou. Upon arrival, the samples were stored at 4°C and processed within 48 h. Leaves from mandarin trees known to be affected with HLB and free of HLB maintained in the greenhouse on the campus of South China Agricultural University (SCAU) were

TABLE 1. Poloymerase chain reaction (PCR) detection of ‘*Candidatus* Liberibacter asiaticus’ and ‘*Ca.* Phytoplasma asteris’ in leaf tissue samples collected during two citrus Huanglongbing surveys in Guangdong Province, PRC, in 2006 and 2007

				PCR result ^b					
	City	No. of orchards	No. of plants sampled ^a	CLA+	CPA+	CLA + CPA +	CLA – CPA –	CLA + CPA –	CLA – CPA +
2006 Survey									
1	Wengyuan	4	10 (6,1,3)	7	6	5	2	2	1
2	Gaoyao	5	16 (0,0,16)	14	12	11	1	3	1
3	Gaoming	2	3 (1,1,1)	2	3	2	0	0	1
4	Lechang	5	19 (0,2,17)	11	16	10	2	1	6
5	Heshan	4	6 (1,0,5)	6	5	5	0	1	0
6	Fogang	7	17 (0,0,17)	10	14	8	1	2	6
Total		27	71 (8,4,59)	50	56	41	6	9	15
%				70.4	78.9	57.7	8.5	12.7	21.1
2007 Survey									
1	Sihui	1	10 (0,0,10)	4	9	3	0	1	6
2	Changgansan	1	20 (5,6,9)	11	15	7	1	4	8
3	Luoding	1	11 (0,0,11)	9	8	7	1	2	1
4	Huizhou	2	25 (15,0,10)	12	18	8	3	4	10
5	Qinxin	1	4 (0,0,4)	3	4	3	0	0	1
Total		6	70 (20,6,44)	39	54	28	5	11	26
%				55.6	75.6	40	7.1	15.6	37.1
Grand total		33	141 (27,10,103)	89	110	69	11	20	41
%				63.1	78.0	48.9	7.8	14.2	29.1

^a Numbers in parentheses are sweet orange (*Citrus sinensis* [L.] Osbeck), pummelo (*Citrus maxima* [Burm.] Merrill), and mandarin (*Citrus reticulata* Blanco), respectively.

^b CLA = ‘*Candidatus* Liberibacter asiaticus’. The bacterium was identified by nested PCR using primer sets fD1/fD2 (1st round) and OI1/OI2c (2nd round) or by real-time PCR with primer set HLBas/HLBr and TaqMan probe HLNp as detailed in Table 1. CPA = ‘*Candidatus* Phytoplasma asteris’. The bacterium was determined by nested PCR using primer sets P1/P7 (1st round) and fU5/fU3 (2nd round) as listed in Table 2.

Fig. 1. Foliar symptoms of huanglongbing and the results of polymerase chain reaction analyses (+ or –) of these leaves collected in Guangdong, China for ‘*Candiatus* Phytoplasma asteris’ and ‘*Ca.* Liberibacter asiaticus’.

used as sources of DNA for positive and negative controls, respectively.

Experiments with periwinkle were performed in an insect-proof screenhouse on the campus of South China Agricultural University. Each of four 15-cm tall periwinkle plants, grown from seeds, was grafted with one bud from an HLB-affected periwinkle plant previously infected via dodder transmission from an HLB-affected mandarin tree in Sihui City of Guangdong Province and maintained in an insect-proof screenhouse. Symptom development in each grafted periwinkle was recorded weekly. Symptomatic leaves were collected for DNA extraction and bacterial examination. Nongrafted periwinkle plants were used as controls.

DNA extraction. Midribs of plant leaves were excised with a sterile razor blade. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as previously described (9,30). Briefly, 0.5 g of midribs was cut into small pieces with sterilized scissors, frozen with liquid nitrogen then ground into powder with a mortar and pestle. Two milliliters of a 2% CTAB buffer were added, the mixture was transferred into a 1.5-ml microcentrifuge tube and incubated in a water bath at 65°C for 30 min with occasional agitation. After extraction with a phenol/chloroform/iso-amyl alcohol solution (24:24:1), DNA was precipitated by the addition of 1/10 volume of 3M sodium acetate (pH 5.2) and two volumes of alcohol and was collected by centrifugation. The pellets were dissolved in 100 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 0.5 µl of RNaseA (10 mg/ml) and incubated in a water bath at 37°C for 30 min. DNA was stored at -20°C for later use in Guangdong or in California.

PCR. Primer sets used in this study are listed in Table 2. Samples from the first survey were tested in the USDA-ARS laboratory at Parlier, CA (US Lab). Phytoplasma were detected by nested PCR. Primer set P1/P7 (8,34) was used for the first round (10 cycles) and primer set fU5/rU3 (29) was used for the second round (30 cycles) following a previously described procedure (13). PCR reactions (25-µl) were carried out using the TaKaRa taqTM (Hot Start Version) kit (Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan). The reaction mixture contained: 2.5 µl of 10× DNA polymerase buffer, 2.5 µl of dNTPs (2.5 mM of each dNTP), 0.5 µl each of the forward and reverse primers (10 µM), 1 µl of sample DNA, 0.2 µl of Taq DNA polymerase (5 U/µl) and 18.3 µl of H₂O. Amplification was conducted in an MJ Research Tatrada DNA Engine 2 with an initial denaturation at 96°C for 10 min, followed by either 10 or 30 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. ‘*Ca. L. asiaticus*’ was detected by the same nested PCR protocol but using primer set fD1/rD1 (19) in the first round and primer set OI1/OI2c (19) in the second round. Amplified products were evaluated by electrophoresis in 1.5% agarose gels and visualized by staining with ethidium bromide.

Samples from the second survey were tested in the Citrus Huanglongbing Laboratory in South China Agricultural University in Guangzhou, P. R. China (China Lab). Nested PCR was used for phytoplasma detection. PCR was carried out in a final volume of 25 µl with a Perkin Elmer GeneAmp 2400 PCR System. The reaction mixture contained: 2.5 µl of 10× DNA polymerase buffer, 2 µl of dNTPs (2.5 mM of each dNTP), 1 µl

each of the forward and reverse primers (5 µM), 1 µl of sample DNA, 0.2 µl of Taq DNA polymerase (5 U/µl, Shanghai Shenergy Biocolor BioScience and Technology Company, PRC), and 18.3 µl of double distilled H₂O. DNA amplification by PCR was performed as follows: reactions were preheated at 94°C for 5 min, followed by 10 cycles (first round) or 35 cycles (second round) of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. Amplified products were evaluated by electrophoresis in 1% agarose gels after staining with ethidium bromide.

‘*Ca. L. asiaticus*’ was detected by TaqMan real-time PCR following the procedure of Li et al. (25). Briefly, the reaction mixture (20 µl) consisted of PCR master mix, 10 µmol of primers HLBas and HLBBr, 5 µmol TaqMan probe (HLBp) (Table 1), and 1 µl of the extracted sample DNA. A cloned plasmid containing a fragment of 16S rDNA of ‘*Ca. L. asiaticus*’ was used as positive control. Sterilized water was used as negative control, along with DNA from healthy citrus trees as mentioned previously. Amplification, detection, and data analysis were performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal profile consisted of an initial activation step at 95°C for 5 min followed by 40 cycles at 95°C for 30 s and 58°C for 45 s.

DNA sequence analyses. Eighteen selected PCR amplicons from symptomatic leaf samples of mandarin, sweet orange, pummello, and periwinkle obtained with primer set fU5/rU3 were sequenced directly with a 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA). A phytoplasma strain from a mandarin tree in Wengyuan City and a strain from periwinkle were selected for standard PCR amplification with primer set P1/P7 for 30 cycles. P1/P7 flanks the region of 16S rDNA and 16S-23S rRNA intergenic transcribed spacer (ITS). DNA amplicons (≈1,800 bp) were cloned into pGEM-T Easy vector and sequenced. DNA fragments were sequenced three times from both directions to check for accuracy. DNA sequences were compared to the current GenBank database using the BLASTn network service available in the National Center for Biotechnology Information. Multiple sequence alignments were performed using the ‘Query-anchored with identities’ option within the BLASTn tool or using CLUSTAL W (38).

TEM. Bacteria in the sieve tubes of phloem of periwinkle and mandarin showing HLB symptoms were examined by TEM following the protocol of Tang and Faan (35). Briefly, midribs of periwinkle and citrus leaves were collected and cut to 1 mm³ cubes, fixed in 3% glutaraldehyde and then 1% osmium tetroxide, and dehydrated successively in ethanol and embedded in Epon 812 medium. After ultrathin (50 nm) sectioning and staining in uranyl acetate and lead citrate, samples were examined in an FEI-Tecnai 12 transmission electron microscope in South China Agricultural University and the USDA-ARS San Joaquin Valley Agricultural Sciences Center in Parlier, CA.

RESULTS

The presence of phytoplasma was indicated by an expected ≈800-bp DNA amplicon using specific primer set fU5/rU3 nested with primer set P1/P7 (Fig. 2). The phytoplasma is tentatively

TABLE 2. Information about primers used for polymerase chain reaction detection of ‘*Candidatus Liberibacter asiaticus*’ and phytoplasma in this study

Primer set	Sequence (5' to 3')	Amplicon size (bp)	Specificity	Reference
fD1/fD2	agagtttgat cctggctcag/aaggaggatga tccagcc	≈1,500	Bacteria	19
OI1/OI2c	gcgcgtatgc aatacagacg gca/gcctcgcgac ttgcacacc at	1,160	‘ <i>Ca. L. asiaticus</i> ’ and ‘ <i>Ca. L. africanus</i> ’	19
P1/P7	aagagtttga tectgctca ggatt/cgctcttcatt cggtcttt	≈1,800	All phytoplasmas	9,34
fU5/rU3	cgccaatgga ggaact/ttcagctact ctttgaaca	882	All phytoplasmas	29
HLBas/HLBp/HLBr	tcgagcgcgt atgcaatag/FAM-agacgggtga gtaacgcg-BHQ-1/gcgttatccc gtagaaaaag gtag	75	‘ <i>Ca. L. asiaticus</i> ’	25

named as HLB-associated phytoplasma for the convenience of discussion. The presence of ‘*Ca. L. asiaticus*’ was indicated by the expected $\approx 1,100$ -bp DNA amplicon using specific primer set OI1/OI2c nested with primer set fD1/fD2 in the US lab (Fig. 2) and by the cycle threshold (Ct) values smaller than 30 referenced to the values of negative controls in the China lab (data not shown). The results of PCR detection of both HLB-associated phytoplasma and ‘*Ca. L. asiaticus*’ in the two citrus HLB surveys are presented in Table 1. The detection rates of ‘*Ca. L. asiaticus*’ and HLB-associated phytoplasma were slightly higher in the 2006 survey (70.4 and 78.9%, respectively) than those in the 2007 survey (55.6 and 75.6%, respectively). In both years, phytoplasma detection rates were higher than those of ‘*Ca. L. asiaticus*’. Among 141 HLB symptomatic samples, 110 (78.0%) samples were phytoplasma positive. HLB-associated phytoplasma was detected in all three citrus hosts, i.e., sweet orange, pummelo, and mandarin. At the same time, 89 (63.1%) samples were positive for ‘*Ca. L. asiaticus*’. Sixty-nine (48.9%) samples were positive for both HLB-associated phytoplasma and ‘*Ca. L. asiaticus*’, indicating mixed infections.

Nucleotide sequences of 18 amplicons using primer set fU5/rU3 were identical and resulted in high BLASTn matches with phytoplasma DNA sequences in GenBank database. The sequences of the P1/P7 amplicons from mandarin and periwinkle were identical to each other with the length of 1,759 bp. The sequence from mandarin has been deposited in GenBank as accession number EU544303. The sequences showed 100% identity to the sequences of three strains of ‘*Ca. Phytoplasma asteris*’ in the region of 16S rDNA and 16S-23S intergenic spacer. The three phytoplasmas are onion yellows phytoplasma OY-M from Japan (AP006628, locus PAMr01), aster yellows

phytoplasma ‘watercress’ from Hawaii (AY665676), and valeriana yellows phytoplasma clone ValY2 from Lithuania (AY102274). The whole genome of onion yellows phytoplasma OY-M has been sequenced and contains two heterologous ribosomal RNA operons (32). While one operon (PAMr01) shared the identical sequences with EU544303 (i.e., HLB-associated phytoplasma), the sequence of the other operon (PAMr06) showed differences in two SNPs. The comparison of EU544303 to the current version of GenBank database showed that two sequences, AY734453 from barley deformation phytoplasma in Lithuania and AF222063 from aster yellows phytoplasma in United States, showed one single nucleotide polymorphism (SNP); and five sequences, AY566302 from ash witches’-broom phytoplasma in Korea, AY389820 from aster yellows phytoplasma strain AY-BW in Ohio, AY101386 from epilobium phyllody phytoplasma in Estonia, AF322644 from aster yellows phytoplasma strain AY1 clone 14 in United States and AY744070 from *Silene virescens* phytoplasma in United States, showed two SNPs.

All four of the graft-inoculated periwinkle plants showed leaf yellowing symptoms 1 month after grafting. As infected plants began to flower, virescence and phyllody symptoms appeared (Fig. 3A and B). Non-inoculated periwinkle did not show any symptoms (Fig. 3C). TEM revealed that many sieve tubes of infected periwinkle with virescence and phyllody symptoms were filled with pleomorphic cells enclosed by a membrane layer of 6.5 nm ($n = 10$) in thickness (Fig. 3D). A clear unit membrane and distinct pleomorphic morphology (Fig. 3D) were indicative of phytoplasmas (18,23). Cells with thicker border, presumably cell wall, of 27.2 nm ($n = 10$) were also observed. However, they were low in titer, scattered within the sieve tubes, and often observed along with thin-bordered cells (data not shown). Similarly, both

Fig. 2. Agarose gel analysis of polymerase chain reaction amplicons of huanglongbing-associated bacteria: ‘*Candidatus Phytoplasma asteris*’ (top panel) and ‘*Ca. Liberibacter asiaticus*’ (bottom panel) from 14 representative plants in Guangdong, China. GY-10, GY-11, GY-12, GY-13, HS-2-2, HS-2-3, and ZQ-2 were from mandarin; WY-1, WY-2, WY-3, WY-4, HS-6-2, and HS-7-3 were from sweet orange; SH-Peri was from periwinkle; and CK was from healthy mandarin. DNA size markers (bp) on the last lane in both panels.

cells with a thin border (8.0 nm, $n = 10$) and a thick border (20.7 nm, $n = 10$) were observed from HLB citrus samples, both in low titer. Pleomorphic bodies were not found in citrus samples. The thick-bordered cells suggested walled bacteria and the thin bordered cells were indicative of phytoplasma.

DISCUSSION

Currently, it is generally agreed that citrus HLB is associated with '*Ca. Liberibacter*' worldwide (3,19). The presence of '*Ca. L. asiaticus*' in HLB-affected citrus trees in Guangdong has also been confirmed using DNA-based methods (9,10,11,12,13). However, efforts to find '*Ca. L. africanus*' and '*Ca. L. americanus*' in China were not successful (9). In this study, DNA analyses led to the identification of a phytoplasma from HLB citrus. We were unable to identify high titers of the HLB-phytoplasma in citrus sieve tubes by TEM. However, a high titer of phytoplasma was found in periwinkle inoculated with HLB citrus, suggesting that periwinkle could be used for the enrichment of HLB-associated phytoplasma in future studies.

Identification and characterization of '*Ca. L. asiaticus*' began with the Poona strain from HLB-affected sweet orange in India (14,19). Virescence and phyllody symptoms were not observed in periwinkle infected with the Poona strain (14), suggesting that phytoplasmas were not present in the donor citrus tree. In Guangdong, we detected only '*Ca. L. asiaticus*' in 14.2% of the

samples. Whether phytoplasmas are present in HLB citrus in India, as well as in other citrus growing regions, remains to be determined. It is interesting to note that in this study, phytoplasma was associated more with HLB than was '*Ca. L. asiaticus*' (78.0 versus 63.1%, respectively). A paired t test on the infection rates of phytoplasma and '*Ca. L. asiaticus*' returned a P value of 0.057, which could be considered as marginally different at the significance level of 0.05.

A total of 29.1% of the HLB samples were detected with phytoplasma only, evidence of phytoplasma correlated with HLB symptoms. In a separate experiment, we randomly collected and tested 10 asymptomatic mandarin leaves from non-HLB-affected trees in Guangdong. Eight were phytoplasma-negative and two were positive. We also tested 10 leaves of sweet orange collected from California. None was phytoplasma-positive. However, more data, particularly those from controlled experiments, are needed to better establish the association of phytoplasma with HLB. Studies of HLB-associated bacteria from different geographical regions have led to the identification of different putative etiological agents of HLB. DNA sequence analysis on '*Ca. Liberibacter*' strains in South Africa led to the identification of '*Ca. L. africanus*' (14,19), and recent research from Brazil identified '*Ca. L. americanus*' (35).

In China, the highly destructive nature of HLB and the urgent need to control the disease prompted intensive efforts to search for the HLB etiological agents before the recognition of '*Ca. L.*

Fig. 3. Characterization of a citrus huanglongbing (HLB)-associated phytoplasma using periwinkle as a host plant. Top panel: foliar symptoms in periwinkle following inoculation with HLB-associated bacteria ('*Ca. Phytoplasma asteris*' and '*Ca. Liberibacter asiaticus*') through bud grafting. **A**, Virescence symptoms; **B**, leaf yellowing and phyllody; **C**, healthy periwinkle. Bottom panel: Electron micrographs of wall-less bodies in the sieve tubes of a HLB periwinkle sample; **D**, aggregation of phytoplasma in a sieve tube. Inset shows thickness of the phytoplasma membrane; and **E**, pleomorphic phytoplasmas in a sieve tube.

asiaticus' (39). MLOs/phytoplasmas and BLOs were observed in HLB-affected citrus tissue using TEM (6,21). Interestingly, although pleomorphic cells were reported and MLOs/phytoplasma was suspected in HLB-affected citrus (6), the thickness of the cell envelop as reported to be 20 nm, similar to that of the walled bacteria observed in this study. Since the titer level was not mentioned and presumably low, we speculate that the previous confusion could be related to the presence of both walled and wall-less bacteria in HLB-affected citrus.

Based on the current taxonomic scheme (24), the HLB-associated phytoplasma from this study belongs to the Aster yellows group (16SrI-B), and therefore designated as a strain of '*Ca. P. asteris*'. The 16SrI-B group phytoplasmas are associated with more than 100 economically important diseases worldwide and represent the most diverse and widespread phytoplasma group. Analyses of 16S rDNA sequences may not be sufficient to elucidate strain relationships. The biological properties of the '*Ca. P. asteris*' HLB-associated strain, such as pathogenicity, insect vector and plant host range, remain to be determined. The genome of the onion yellows phytoplasma has been sequenced (32). These could provide important information for further genome study of the phytoplasma in HLB-affected citrus.

Meanwhile, phytoplasma in the 16SrI group has never been reported from citrus. Based on our knowledge, only phytoplasmas related to 16Sr II group, '*Ca. P. aurantifolia*', have been identified to be associated with citrus diseases in Ethiopia (1), India (17), Iran (4), Oman (2), and the United Arab Emirates (16). As this manuscript was in submission, a phytoplasma closely related to the pigeon pea witches'-broom phytoplasma (16SrIX) was reported to be associated with citrus HLB symptoms in the State of São Paulo in Brazil (37). The phytoplasma was first identified in a new HLB area where citrus trees showed HLB symptoms but were negative for '*Ca. L. spp.*'. As sample size increased, mixed infection by both phytoplasma and '*Ca. L. spp.*' was also found. The phytoplasmas from China and Brazil are distantly related and could represent two different species. Referenced to the fact that several '*Ca. L. spp.*' were associated with HLB, it is also possible that more HLB-associated phytoplasmas could be found in the future as research on HLB continues.

In summary, we detected both phytoplasma and '*Ca. L. asiaticus*' in HLB-affected citrus in Guangdong. PCR data showed both co-infection (48.9%) and single infection (29.1% for phytoplasma and 14.2% for '*Ca. L. asiaticus*'). We were not able to clearly identify symptoms specifically associated with either bacterium alone or together. Interestingly, by observing two distinct symptomatology, yellow shoot and leaf mottling, in southern China, Lin (28) proposed that HLB could be caused by two different viruses. Nevertheless, the true etiological role(s) of phytoplasma, as well as '*Ca. L. asiaticus*', in HLB remain(s) uncertain until Koch's postulates are fulfilled.

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Erratum

The bacterial name '*Candidatus* Phytoplasma asteri' was corrected to '*Candidatus* Phytoplasma asteris' throughout this article. Changes to this article were made on January 18, 2010.