Low Incidence of ‘*Candidatus Liberibacter asiaticus*’ in *Murraya paniculata* and Associated *Diaphorina citri*

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Abstract

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Huanglongbing (HLB) is one of the most devastating diseases of citrus worldwide. ‘*Candidatus Liberibacter asiaticus*’ is the prevalent species of three HLB-associated *Liberibacter* spp., which is vectored by the psyllid *Diaphorina citri*. The vector and the bacteria have host plants outside the genus *Citrus*, and these plants have the potential to affect disease epidemiology within citrus groves. *Murraya paniculata* could be especially problematic because it is a popular ornamental plant and a host of both psyllid and bacteria. We conducted a year-long survey of eight urban plantings of *M. paniculata* in east-central Florida to char-

acterize ‘*Ca. L. asiaticus*’ infection rates in plants and associated psyllids. Using sensitive quantitative polymerase chain reaction (qPCR) primers targeting two prophage genes of ‘*Ca. L. asiaticus*’, we found infection to be extremely low: less than 1% of psyllids and 1.8% of plants. With qPCR primers targeting ‘*Ca. L. asiaticus*’ 16S rDNA, none of the plants and only one psyllid were ‘*Ca. L. asiaticus*’-positive. Therefore, the titer of ‘*Ca. L. asiaticus*’ is low in *M. paniculata* and associated psyllids. These results suggest that urban plantings of *M. paniculata* may serve as a minor source of ‘*Ca. L. asiaticus*’ inoculum.

Huanglongbing (HLB) is the most destructive disease of cultivated *Citrus* spp. worldwide. It recently invaded North America, with first detection in Florida in 2005 (10). In *Citrus* spp., HLB causes leaf chlorosis; reduced growth; small, asymmetric, or lopsided fruit; and death of the tree in as little as 2 years (3). Although Koch’s postulates have not been completed, the disease is strongly associated with three species of bacteria: ‘*Candidatus Liberibacter asiaticus*’, ‘*Ca. L. americanus*’, and ‘*Ca. L. africanus*’. The bacteria are vectored by one of two species of psyllids: *Diaphorina citri* Kuwayama or *Trioza erytreae* (Del Guercio) (3), the former which was first reported in Florida during 1998 (11). In Florida, HLB is associated with ‘*Ca. L. asiaticus*’ (34) and vectored by *D. citri*.

Both *D. citri* and ‘*Ca. L. asiaticus*’ utilize a number of host plants besides cultivated *Citrus* spp. The psyllid has reported hosts from at least 23 genera within the family Rutaceae (12,24), although germplasm varies in susceptibility to psyllid infestations. *Bergera koenigii*, *Citrus macrophylla*, “commercial citrus”, and *Murraya paniculata* have been reported as preferred host plants of the psyllid (12,37,39). In a study of four host species, the intrinsic rate of increase of the psyllid population was highest on young grapefruit (*C. paradisi*) (32). ‘*Ca. L. asiaticus*’ has been isolated from at least 10 Rutaceae genera (7,9,12), and can also be transmitted to a number of non-Rutaceae plant species, including periwinkle (*Catharanthus roseus*), dodder (*Cuscuta campestris*), tomato (*Solanum lycopersicum*), and tobacco (*Nicotiana tabacum*)

in the lab using dodder (12,41). Host range of the disease may be limited by feeding preferences of the vector rather than by the physiological potential of the bacteria (12). Thus, HLB epidemiology in a citrus grove may be affected by plants in the surrounding landscape that act as host of the vector and the disease.

One alternative host species that has the potential to be especially problematic for *Citrus* production is the ornamental plant *M. paniculata* (3). *M. paniculata* is an excellent host for *D. citri* (12,32,33,37) and can also harbor ‘*Ca. L. asiaticus*’ (8). ‘*Ca. L. asiaticus*’-infected *M. paniculata* often do not show disease symptoms, and may be less affected by the bacterium than *Citrus* spp. (20). There are concerns about the potential for this common plant to serve as an asymptomatic source of inoculum for nearby *Citrus* plantings. *D. citri* regularly disperses from and to citrus throughout the year (13). In Florida, *D. citri* adults collected from *M. paniculata* in garden centers, residential plantings, and nurseries, and nymphs collected from nurseries, have been found to be infected by ‘*Ca. L. asiaticus*’ (22). Because *M. paniculata* may flush frequently or, in the case of regular hedging, nearly continuously, *D. citri* are able to reproduce on this plant at times when they could not reproduce on *Citrus* spp., which may increase the psyllid population in nearby *Citrus* plantings (25,39). Concern about the potential for spread of the vector and disease has led the state of Florida to restrict the conditions for propagation and sale of *M. paniculata* in the state (4) but no programs to manage existing plantings have been implemented.

There are several reasons to question the potential for *M. paniculata* plantings to act as an inoculum reservoir for ‘*Ca. L. asiaticus*’ in Florida. When ‘*Ca. L. asiaticus*’ is detected in *M. paniculata*, only a small amount of polymerase chain reaction (PCR) product is amplified, indicating that fewer bacterial genomes are present or that chemicals extracted from *M. paniculata* during sample preparation interfere with the PCR reaction (8). Under laboratory conditions, the ‘*Ca. L. asiaticus*’ in psyllid-inoculated *M. paniculata* drops to undetectable levels within several months post infection (5). Furthermore, although ‘*Ca. L. asiaticus*’ can be identified in sweet orange inoculated from *M. paniculata*, none of five orange trees back-inoculated with the Florida strain of ‘*Ca. L. asiaticus*’ developed HLB symptoms in a quarantined greenhouse (5). Finally, infection of *M. paniculata* in the field may be rare. In a survey conducted after ‘*Ca. L. asiaticus*’ had become

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dominant over '*Ca. L. americanus*', just over 4% of 661 suspect samples from urban *M. paniculata* plantings in the state of São Paulo, Brazil contained detectable levels of '*Ca. L. asiaticus*' (20).

The objective of this study was to document the incidence of '*Ca. L. asiaticus*' infection in urban *M. paniculata* plantings in an area of Florida with high citrus production and large amount of '*Ca. L. asiaticus*' inoculum as an initial step in determining the importance of *M. paniculata* plantings as a reservoir inoculum source of HLB. The infection status of '*Ca. L. asiaticus*' in *M. paniculata* and *D. citri* that dwell on *M. paniculata* was monitored over the course of 1 year at eight locations. We documented total infection and seasonal trends using two different quantitative (q)PCR detection methods for '*Ca. L. asiaticus*'.

Materials and Methods

Murraya spp. survey locations and dates. Seven sites with substantial plantings of *M. paniculata* were identified in Saint Lucie County, FL, and one site was identified in Palm Beach County, FL. All sites were within the area identified in the initial delimiting surveys of HLB incidence in 2005, and have had high incidence of HLB with noticeable effects of the disease in cultivated citrus since that time (2,11). *D. citri* nymphs and adults are abundant year-round in nearby commercial citrus plantings (14,26), and may migrate out of those plantings at any time (13). Therefore, all sites included in the survey had experienced high inoculum levels for several years prior to the experiment. All sites had mature hedges ranging from 60 cm to over 2 m high, depending on the pruning practices at the site, and were estimated to be at least 5 years old. Frequent pruning occurred at all sites, resulting in a near-constant presence of flush on the plants. In addition, *M. paniculata* leaflets with yellowing symptoms similar to those described by Lopes et al. (20) were observed at all of the study sites.

Each site was visited every 2 months from April 2010 to February 2011. On each sampling date, *D. citri* were sampled by walking down the hedge and removing shoots infested with nymphs. The shoots were returned to the laboratory and placed in water, and the adult *D. citri* that eclosed from the nymphs were collected. A target subsample of 30 adults reared from each site on each sample date was tested for '*Ca. L. asiaticus*' infection. *D. citri* adults were collected within 7 days of eclosion and stored in 70% ethanol at 4°C until DNA was extracted. Collecting and rearing nymphs ensured that assayed adults originated from *M. paniculata* rather than migrating from nearby *Citrus* spp., and rearing the nymphs to adults allowed us to measure '*Ca. L. asiaticus*' prevalence among psyllids with the potential to migrate away from *M. paniculata*. During the same site visits, *M. paniculata* was sampled by removing 8 to 10 leaflets at 30 locations along the hedge at each site. The leaflets composing each sample were selected to be representative of the condition of the plants at that location on the hedge, and included a mixture of young and old growth and leaflets with and without yellowing if yellowing was observed.

DNA extraction methods. All DNA extraction was performed in a sterile laminar flow hood.

Crude extractions of psyllid DNA were made using a modification of the method described by De Barro and Driver (6). Psyllids were stored in 70% ethanol at 4°C from the time of collection until DNA extraction was performed. Individual psyllids were placed in a 2-ml screw-cap tube (USA Scientific, Ocala, FL) containing approximately 12 1.6- to 1.8-mm zirconium silicate beads (Cero-glass, Columbia, TN) and 150 µl of lysis buffer (5% 1 M KCl, 5% 1 M Tris at pH 8.4, 0.45% Tween20, 0.45% NP40, and 89.1% autoclaved deionized water). Tubes were placed in a FastPrep-24 system (MP Biomedicals, Solon, OH) and homogenized for 30 s at 6 m/s. Following homogenization, 100 µl of liquid was transferred to a clean 1.5-ml centrifuge tube (USA Scientific) and placed in a water bath at 95°C for 5 min. Samples were immediately placed on ice for at least 10 min, and then centrifuged at 14,000 rcf for 2 min. The supernatant was removed and stored at -20°C until qPCR was performed.

Leaf midribs were used for DNA extraction from *M. paniculata* because this tissue contains the highest titers of '*Ca. L. asiaticus*' in *Citrus* trees (18). Between collection and midvein extraction (0 to 7 days), plant samples were stored at 4°C. Storage of *Citrus* leaves for up to 10 weeks in this manner did not affect the outcome of qPCR (19). For each sample, the midribs of 10 leaflets collected from the same area of the hedge were removed and finely chopped by hand using a new razor blade. Chopped samples were placed in tubes containing steel beads and stored at -80°C until extraction. Plant samples collected in April were placed in 2-ml screw-cap tubes with two 0.625-cm steel shots (Daisy Outdoor Products, Rogers, AR). All remaining samples were placed in individual tubes cut out of 8-tube strips (Machery-Nagel, Bethlehem, PA) containing approximately 12 1.3-mm chrome steel beads (Biospec Products, Bartlesville, OK).

M. paniculata samples collected in April were extracted using a buffer-based extraction method as follows: 900 µl of extraction buffer (for 1,000 ml of buffer: 12.1 g of Tris-Base, 18.61 g of EDTA [pH of solution adjusted to 8.0 using 5 M NaOH to facilitate EDTA dissolution], 29.22 g of NaCl, 25 g of PVP, and b-mercaptoethanol added to buffer just before use at 0.7 ml/ml) was added to the tubes containing slingshot pellets and plant sample, and the tubes were homogenized in a FastPrep-24 system for 40 s at 6 m/s. Following homogenization, 40 µl of 20% sodium dodecyl sulfate was added and the samples were vortexed. Samples were placed in a 65°C water bath for 30 min and stirred by inverting the tubes every 10 min. Samples were removed from the water bath and centrifuged at 14,000 rcf for 5 min, and 700 µl of supernatant for each sample was transferred to a new 1.5-ml centrifuge tube. A volume of 220 µl of 5 M KoAC was added to the supernatant, and the samples were placed on ice for at least 20 min. After removal from the ice, the samples were centrifuged at 14,000 rcf for 10 min, and 650 µl of the upper aqueous layer was transferred to a new 1.5-ml tube. A volume of 460 µl of cold 70% isopropyl alcohol was added to the samples, and the samples were vortexed and placed on ice for at least 5 min. After removal from the ice, samples were vortexed at 15 000 rcf for 30 min at 4°C, the supernatant was discarded, and the pellet was washed by placing 700 µl of cold 70% ethanol in the tubes for 15 min. The liquid phase was discarded, and the pellet was allowed to dry in a laminar flow hood. Samples were resuspended in 100 µl of nuclease-free water (Qiagen, Valencia, CA).

After April, all *M. paniculata* samples were extracted in a 96-well format using the NucleoSpin 96 Plant II kit (Machery-Nagel) with the following modifications. Lysis buffer PL1 and RNase were added to the tubes containing chopped midvein tissue and beads, and the samples were homogenized in a Mini Bead Beater (BioSpec Products) for 5 min. Following homogenization, the 65°C incubation step was extended to 30 min. The remainder of the protocol followed the recommendation of the manufacturer. Between two and six negative control samples of *Quercus virginiana* were included on each 96-well plate of *M. paniculata* samples from June 2010 to February 2011. If one of the negative control samples consistently amplified in PCR, the entire extraction plate was excluded from further analysis.

Prior to qPCR analysis, the nucleic acid concentration of all plant samples was determined by spectrophotometry at a wavelength of 260 nm using a Nanodrop-1000 detector (NanoDrop Products, Wilmington, DE). Samples were diluted in nuclease-free water so that 300 ng of nucleic acid was loaded in each PCR reaction for the April samples and 100 ng of DNA was loaded per reaction for the rest of the *M. paniculata* samples. Plant samples were stored at -20°C until qPCR was performed.

PCR detection of '*Ca. L. asiaticus*'. All samples were assayed by qPCR with the LJ900 series primers (5'→3' sequences: forward GCCGTTTAAACACAAAAGATGAATATC and reverse ATAAATCAATTTGTTCTAGTTTACGAC) as described by Zhou et al. (40). These primers target the 1-12 tandem repeats of the *hyv_I* and *hyv_{II}* genes of '*Ca. L. asiaticus*' prophages, and were used because they provide a high degree of sensitivity in a one-step qPCR reaction.

Briefly, 2 μ l of the crude psyllid extract or 100 ng of plant DNA (300 ng of nucleic acids for the April samples) were used in a qPCR reaction totaling 15 μ l using the PerfeCTa SYBR Green FastMix 2 $\times$ master mix (Quanta Biosciences, Inc., Gaithersburg, MD), and a reaction concentration of 600 nM forward and 900 nM reverse primer and nuclease-free water. Reactions were run on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) with amplification settings of initial denaturation at 95°C for 5 min, then 50 cycles of 95°C for 3 s, followed by 62°C for 30 s. At the end of each run, a dissociation cycle of 95°C for 15 s, 62°C for 1 min, and a gradual ramp to 97°C for 15 s was performed. All runs included positive and at least four no-template controls. Negative control samples were also included for *M. paniculata* samples after April. All samples were assigned a Cq value based on the cycle when fluorescence exceeded 0.2 Δ Rn. Samples that did not produce the correct melt curve were assigned a value of undetermined. Any sample that amplified (at any cycle) and had the correct melt profile was rerun in triplicate. Samples that amplified and had the correct melt profile in at least two of the three triplicate runs were considered positive detections.

In order to confirm whether the high Cq values we observed using the LJ900 primers represented true positive detections, we prepared a standard curve using negative psyllid and plant samples spiked with varying concentrations of the pLJ153.1 plasmid described by Zhou et al. (40). For psyllid samples, concentrations of 10^1 to 10^8 copies per reaction were run whereas, for plants, the concentration range was 1 to 10^7 copies. The relationship between Cq and log (concentration) was described by linear regression, and efficiency was calculated as $10^{(-1/\text{slope})} - 1$ (ABI 7500 Software v2.0.1; Applied Biosystems).

As a check for our results using LJ900, all psyllid and plant samples that tested positive by the LJ900 primers as well as 50 randomly selected negative samples of each type were run on the HLBaspr primers (5'→3' sequences: forward TCGAGCGCG TATGCAATACG and reverse GCGTTATCCCGTAGAAAAAGG TAG, probe AGACGGGTGAGTAACGCG with 6-carboxy-fluorescein reporter dye on the 5' end and TAMRA quencher on the 3' end; IDT, www.idt.com), which target the 16S rRNA of '*Ca. L. asiaticus*' (17). These primers are among the most common methods for diagnostic detection of '*Ca. L. asiaticus*', and have been used to detect '*Ca. L. asiaticus*' in *D. citri* (22) and *M. paniculata* (5). For HLBaspr, we ran a 20- μ l reaction, including TaqMan Fast Universal PCR Master Mix (Applied Biosystems), a reaction concentration of 0.4 mM each primer, a reaction concentration of 500 nM probe, and nuclease-free water. qPCR reactions were run on the 7500 Fast real-time PCR system with amplification settings of initial denaturation at 95°C for 5 min followed by 50 cycles of 95°C for 3 s and then 60°C for 30 s. All samples were assigned a Cq value based on the cycle when fluorescence exceeded 0.2 Δ Rn.

Data analysis. Psyllid and plant samples were analyzed as separate datasets. Logistic regression of the effect of date, site, and their interaction on the probability that a sample tested positive by the LJ900 primers was performed. For each dataset, one positive detection was added to each of the five or six dates and eight sites in order to eliminate probabilities of zero, which cause inflated error rates in logistic regression. The full model (containing terms for the date, site, and their interaction but no intercept) was reduced by backward selection with an α of 0.05 (PROC LOGISTIC; 28). When a significant effect was detected, pairwise comparisons between observations were performed and evaluated with a Bonferroni-adjusted α of 0.05 across all comparisons. When results are presented as simultaneous 95% confidence intervals, α was adjusted using the Bonferroni correction and confidence intervals were obtained using the exact (Clopper-Pearson) method (PROC FREQ; 28). The number of copies of the tandem repeat present in each sample where amplification was detected by qPCR using the LJ900 series primers was calculated by fitting the Cq value obtained for that sample to the standard curve.

Results

Standard curve of serial dilutions. When purified pLJ153.1 plasmid was added to negative samples of crude psyllid DNA, the efficiency of the reaction was 92.5% (slope = -3.5159, $R^2 > 0.99$). Purified plasmid added to negative *Murraya* samples resulted in an efficiency of 82.5% (slope = -3.8254, $R^2 = 0.9867$) (Fig. 1). High levels of background DNA, and especially purified *Citrus* DNA, have been associated with higher Cq values and lower reaction efficiency with qPCR methods (19). When the Cq values obtained from the experimental samples were fit to the standard curves, all of them yielded a positive estimated number of copies, indicating that our high Cq value detections are legitimate and reflect low titers of '*Ca. L. asiaticus*'.

Prevalence of '*Ca. L. asiaticus*' in psyllid samples. Over the course of the experiment, 669 psyllids were reared from nymphs collected on *Murraya* spp. The number collected for each site and date varied and, for some sites, no psyllids were collected on certain sample dates (Table 1). Only five psyllids tested positive for '*Ca. L. asiaticus*' by the LJ900 method. The Cq values for the psyllids where '*Ca. L. asiaticus*' was detected using the LJ900 primers ranged from 19.19 to 34.64. The psyllid from which we isolated the highest number of copies of *hyvI/hyvII* also had a detectable number of copies of the 16S rRNA gene of '*Ca. L. asiaticus*' (Cq of 28.27). No 16S rRNA '*Ca. L. asiaticus*' DNA was detected from the other four psyllids from which we isolated the *hyvI/hyvII* sequence or from 50 randomly selected negative psyllids. None of the three model terms (date, sample location, and their interaction) were selected for inclusion in our logistic regression model by backward selection (Fig. 2).

Prevalence of '*Ca. L. asiaticus*' in plant samples. In total, 1,046 *Murraya* samples covering all eight sites from the months of April, August, October, and December 2010 and February 2011 were retained in the analysis. Of these 1,046 samples, 19 tested positive for the *hyvI/hyvII* target DNA sequences. These positive samples represented seven of the eight sites and four of the five

Fig. 1. Standard curves generated to calculate *hyvI/hyvII* copy number in A, psyllid samples and B, plant samples by adding known amounts of purified LJ153.1 plasmid to negative samples.

sample dates (Table 1). We were able to amplify 16S rRNA DNA of ‘*Ca. L. asiaticus*’ from 3 of the 19 samples that tested positive for *hyv_I/hyv_{II}* but not from the other 16 positive samples or 50 randomly selected negative samples (Table 2). Whether the 16S rRNA DNA detections would be considered positive for HLB by this method depends on the Cq cutoff threshold employed. All of the Cq values we obtained for *M. paniculata* samples exceed the cutoff threshold suggested for citrus (19) but it is possible that a unique trait of our samples inflated Cq values relative to what was previously observed for *Citrus* samples.

The best model to describe the prevalence of ‘*Ca. L. asiaticus*’ infection using the LJ900 primers was selected with backward selection in logistic regression and included only the effect of month (df = 5, Wald χ^2 = 300.3, *P* < 0.0001). Pairwise comparisons between months revealed that a higher proportion of the *Murraya* samples collected in August relative to October and December contained the *hyv_I/hyv_{II}* tandem repeat sequence (Fig. 3).

Discussion

The prevalence of ‘*Ca. L. asiaticus*’ infection in *M. paniculata* samples using the *hyv_I/hyv_{II}* detection method was extremely low (1.8% of samples infected). The titer of the bacteria also was very low. More importantly, psyllids that were reared from *M. paniculata* were infected at a rate of less than 1%, regardless of fluctuations in plant infection levels. The three plant samples that were amplified using the 16S rRNA primers had very high Cq values, similar to what we have observed running these primers against other species of bacteria (Y. P. Duan, *unpublished observations*). The only sample in the experiment that would be considered positive by 16S rRNA detection methods was the psyllid where amplification was detected using the HLBspr primers. There are conflicting reports about whether transovarial transmission of ‘*Ca. L. asiaticus*’ occurs in *D. citri* (23,38). If transovarial transmission occurs, it is possible that this infection originated from an infected female that migrated to *M. paniculata* from *Citrus* spp.

More plant infections were detected when using primers targeting the *hyv_I/hyv_{II}* tandem repeat sequence than with primers targeting the bacterial 16S rRNA gene. The discrepancies between the two primer sets could occur for several reasons. The most likely is that more infections were detected using the *hyv_I/hyv_{II}* primers because of that method’s higher sensitivity due to higher copy numbers of the *hyv_I/hyv_{II}* sequence relative to the 16S rRNA gene and the use of SYBR green intercalating dye instead of a probe in the qPCR method. If the prophage became lytic, it is also possible that phage particles but not bacteria could be present in the sample. The rate of ‘*Ca. L. asiaticus*’ infection of *D. citri* that developed on *M. paniculata* is low relative to the rates of ‘*Ca. L. asiaticus*’ infection that have been reported for *D. citri* that develop on *Citrus* spp.,

Fig. 2. Proportion of psyllids reared from *Murraya paniculata* from which the target sequence of *hyv_I/hyv_{II}* was detected. Error bars show the upper and lower bounds of 95% simultaneous exact confidence intervals. There was no difference in proportion of psyllids detected between eight sites and six sample dates.

Table 2. Cq values of LJ900 primers and HLBspr primers of positive *Murraya paniculata* samples detected over a 12-month survey of *M. paniculata* plantings

Site	Date	<i>hyv_I/hyv_{II}</i> Cq	16S rRNA Cq
1	April 2010	32.53	Undetermined
2	April 2010	30.80	Undetermined
2	April 2010	34.96	Undetermined
3	April 2010	35.12	Undetermined
5	April 2010	32.77	Undetermined
7	April 2010	33.49	Undetermined
3	August 2010	28.8	Undetermined
3	August 2010	30.01	Undetermined
3	August 2010	31.14	Undetermined
4	August 2010	31.48	Undetermined
5	August 2010	26.55	Undetermined
5	August 2010	35.66	Undetermined
7	August 2010	28.02	Undetermined
3	December 2010	37.50	Undetermined
5	December 2010	27.81	39.07
2	February 2011	36.19	Undetermined
4	February 2011	30.15	Undetermined
6	February 2011	29.62	37.43
6	February 2011	36.03	40.58

^a All LJ900 detections were confirmed in triplicate. Cq values of undetermined denote cases where no amplification took place; *n* = 1,029.

Table 1. Total number of psyllids reared and *Murraya paniculata* samples with number of positive psyllids by the LJ900 primers (in parentheses) for each site and date of a year-long survey of *M. paniculata* in St. Lucie and Palm Beach Counties, FL

	Site ^a								
Date	1	2	3	4	5	6	7	8	Total
Psyllids									
April 2010	0 (0)	19 (1)	31 (0)	19 (0)	2 (0)	6(0)	17 (0)	n/a	94 (1)
June 2010	4 (1)	20 (0)	16 (0)	4 (0)	30 (0)	28 (0)	0 (0)	8 (1)	110 (2)
August 2010	15 (0)	1 (0)	13 (0)	30 (0)	23 (0)	9 (1)	10 (0)	23 (0)	124 (1)
October 2010	0 (0)	0 (0)	30 (0)	9 (0)	14 (0)	9 (0)	7 (0)	17 (0)	86 (0)
December 2010	5 (0)	21 (0)	1 (0)	16 (1)	14 (0)	0 (0)	26 (0)	30 (0)	113 (1)
February 2011	30 (0)	7 (0)	3 (0)	25 (0)	30 (0)	0 (0)	17 (0)	30 (0)	142 (0)
Total	54 (1)	68 (1)	94(0)	103 (1)	113 (0)	52 (1)	77 (0)	108 (1)	669 (5)
<i>M. paniculata</i>									
April 2010	30 (1)	30 (2)	30 (1)	30 (0)	30 (1)	30 (0)	30 (1)	n/a	210 (6)
June 2010	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0
August 2010	12 (0)	10 (0)	11 (3)	12 (1)	17 (2)	15 (0)	13 (1)	9 (0)	99 (7)
October 2010	30 (0)	30 (0)	30 (0)	30 (0)	30 (0)	30 (0)	30 (0)	30 (0)	240 (0)
December 2010	30 (0)	30 (0)	30 (1)	30 (0)	30 (1)	30 (0)	30 (0)	30 (0)	240 (2)
February 2011	30 (0)	30 (1)	30 (0)	30 (1)	30 (0)	30 (2)	30 (0)	30 (0)	240 (4)
Total	132 (1)	130 (3)	131 (5)	132 (2)	137 (4)	135 (2)	133 (2)	99 (0)	1,029 (19)

^a Abbreviation: n/a denotes that samples were not collected for that site and date.

and also lower than infection rates published for the potato-tomato psyllid, *Bactericera cockerelli* and another *Liberibacter* sp., '*Ca. L. solanacearum*'. Using conventional and qPCR, the infection level of *D. citri* collected from infected *Citrus* in the field is 45 to 82% (1,15,29). The infection rate of *B. cockerelli* with '*Ca. L. solanacearum*' was 19 to 20% when the psyllids were collected directly from their host plants (36).

The low rates of infection and bacterial titer in both the plant and psyllid samples raises the possibility that *M. paniculata* may have a unique resistance trait for '*Ca. L. asiaticus*'. Some *Citrus* cultivars do not present severe symptoms when they contract '*Ca. L. asiaticus*' but they have a bacterial load similar to diseased citrus (9) and several orders of magnitude higher than we found in *M. paniculata* (31). The fact that *M. paniculata* has both a low level of infection and a low titer of bacteria in infected plants despite psyllid movement to and from citrus (13) and high levels of HLB incidence in the local citrus population (11) indicates that a trait of the plant may be restricting reproduction or movement of the bacteria. The bacterium appears to remain at a low titer in the psyllids originating on *M. paniculata* despite the fact that '*Ca. L. asiaticus*' has been reported to replicate in *D. citri* (15,16).

The taxonomy of *M. paniculata* is uncertain. Wild *M. paniculata* is distributed across Southeast Asia, including India, Ceylon, Burma, China, Taiwan, the Malay Peninsula, the East Indian Archipelago, the Philippines, Australia, New Caledonia, and the Melanesian Islands (30). The wild plant has been brought into cultivation at least twice (21), once from material likely of Chinese origin and once with material likely from Australia. Wild and domesticated plants in India are genetically distinct (35), although the authors of the study stated that further analysis was necessary before dividing the group into two species. Regardless of origin, the domesticated plant is often referred to as species or cultivar *exotica* (21). Samuel et al. suggested that *M. paniculata* and *M. exotica* should be separated based on differences in noncoding plastid DNA sequences but bootstrap support for this division was low (27). The plants we sampled in Florida were similar morphologically to the *M. exotica* described by Verma et al. (35), although the number of flowers per inflorescence was higher in the Florida plants. Because of uncertainty about whether *M. exotica* from different parts of the world have the same origin, and the area where the domesticated plants in Florida arose, we describe the plants that we sampled in this study as *M. paniculata* sensu lato.

This survey documented very low rates of '*Ca. L. asiaticus*' infection in urban plantings of *M. paniculata* and *D. citri* reared from these plants, much lower than occur in citrus and psyllids from citrus. In the few cases where plant and psyllid samples tested positive, only low titers of the pathogen were present. These results suggest that urban plantings of *M. paniculata* may play only a

minor role as a direct reservoir of '*Ca. L. asiaticus*' for HLB infections in citrus. The influence of urban plantings of *M. paniculata* on disease epidemiology is further questioned due to a lack of evidence that citrus plants inoculated by *D. citri* from infected *M. paniculata* develop HLB (5). Uninfected adult psyllids originating from *M. paniculata* hedges are unlikely to play a role in disease transmission because psyllids that acquire the bacteria as adults may be unable to transmit it to uninfected host plants (16). In order to fully understand the effect of this alternate host on disease epidemiology in citrus, we need to explore factors such as the virulence of the '*Ca. L. asiaticus*' acquired from *M. paniculata* in *Citrus* spp. and the dynamics of psyllid movement between *Murraya* and *Citrus* plantings, particularly flight distance.

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Fig. 3. Proportion of *Murraya paniculata* samples that contained the *hvv1/hvvII* sequence. Error bars show the upper and lower bounds of 95% simultaneous exact confidence intervals. Dates labeled with different letters are statistically significant according to logistic regression with α of 0.05 across all comparisons.

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