LOW INCIDENCE OF *CANDIDATUS* LIBERIBACTER ASIATICUS IN *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE) POPULATIONS BETWEEN NOV 2005 AND JAN 2006: RELEVANCE TO MANAGEMENT OF CITRUS GREENING DISEASE IN FLORIDA

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Citrus greening disease or Huanglongbing (HLB) is caused by the gram-negative bacterium *Candidatus* Liberibacter asiaticus (*Ca.* L. asiaticus) (Garnier et al. 2000) and was confirmed in southern Florida in 2005 (Halbert 2005; Bouffard 2006). This disease is vectored by *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), which colonized the citrus-growing regions of Florida after it was discovered in 1998 (Knapp et al. 1998; Halbert 1998; Halbert et al. 2000). *Diaphorina citri* acquires the greening bacterium while feeding on infected phloem (Hung et al. 2004). HLB ultimately is fatal to susceptible citrus trees, so early detection and removal of infected trees is important for disease management. Unfortunately, citrus trees often are asymptomatic for years before the common signs of HLB, including yellowing and mottling of leaf veins and misshapen green-colored fruit, are noticeable (da Graça 1991). Current chemical and biological controls reduce *D. citri* populations (Rae et al. 1997; Hoy et al. 1999; Hoy & Nguyen 2000; Michaud 2004; Browning et al. 2006), but may not be sufficient to eliminate all HLB transmission.

It will be important to understand the epidemiology of HLB to control the spread of this disease. The regions of Florida with citrus showing symptoms of HLB currently are being mapped (http://www.doacs.state.fl.us/pi/chrp/greening/maps/cgsit_map.pdf). However, little currently is known about infection rates and transmission frequency of HLB by the psyllid vector. We surveyed the vector, *D. citri*, for the greening bacterium in 11 citrus-growing counties in Florida (Table 1). In most of the counties sampled, citrus trees did not show signs of HLB infection, so we anticipated a low incidence of the greening bacterium (perhaps <1-2%) in these psyllid populations. However, we hypothesized that if citrus trees had acquired HLB recently and did not show disease symptoms, HLB could still be spread in these regions and detected in vector populations by molecular analyses.

Adult psyllids collected in this survey were killed in 95% ethanol in the field and placed on ice during transit to the Department of Entomology and Nematology at the University of Florida, Gainesville FL. Adult and immature *D. citri* were separated, counted, and stored in fresh 95% ethanol or acetone at -80°C (Fukatsu 2005). Tools used to separate insect specimens were washed with bleach, which degrades DNA, to avoid cross-contamination between samples. A maximum of 10 *D. citri* were pooled for DNA isolation by PUREGENE reagents according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN). DNA pellets were re-suspended in 50 µL of sterile water or TE buffer and stored at -80°C. High-fidelity PCR was used to analyze each sample for the 16S rRNA (Subandiyah et al. 2000) and *nus*G-*rpl*K (Villechanoux et al. 1993; Hoy et al. 2001) gene sequences of *Ca.* L. asiaticus, which would yield DNA bands 0.5 kb and 0.6 kb in length, respectively. The samples also were screened for a 0.6-kb portion of the *wsp* gene of *Wolbachia* (Braig et al. 1998), an endosymbiotic bacterium found in *D. citri* (Subandiyah et al. 2000), to control for DNA quality.

A positive control was obtained from Vernon Damsteegt at the USDA-ARS quarantine facility in Beltsville, MD, where adult *D. citri* fed on citrus trees positive for HLB. A total of 3 DNA extractions from these adult *D. citri*, including 2 extractions from single adults and 1 extraction from 10 pooled adults, was conducted by Micki Kuhlmann with the methods described above. The DNA was shipped from Beltsville, MD to the University of Florida and used in a high-fidelity PCR assay. Amplification products were detected in each of the 3 samples with primers for the *nus*G-*rpl*K gene of *Ca.* L. asiaticus and for the *wsp* gene of *Wolbachia*.

To quantify the sensitivity of our high-fidelity PCR assay, a dilution series of plasmid DNA containing the *nus*G-*rpl*K gene of *Ca.* L. asiaticus, mixed with DNA from adult *D. citri* from a laboratory colony that previously had tested negative *Ca.* L. asiaticus, was amplified with high-fidelity PCR (Fig. 1). As little as 1 fg of the target template could be detected 100% of the time, which is approximately equivalent to 100 copies of the *nus*G-*rpl*K gene sequence (Fig. 1), while as few as 10 copies could be detected 50% of the time (Hoy et al. 2001). Control reactions containing no DNA

TABLE 1. COLLECTION DATA FOR *DIAPHORINA CITRI* AND RESULTS OF THE HIGH-FIDELITY PCR ASSAY FOR *CANDIDATUS* L. ASIATICUS AND THE ENDOSYMBIONT *WOLBACHIA* DURING SEP 2005 AND JAN 2006

County	°N	°W	No. adults tested[1]	No. nymphs tested[1]	Host[2]	Grove[3]	PCR: *Ca.* L. asiaticus	PCR: *Wolbachia*
De Soto	27°13.877'	81°53.990'	56	0	G	C	-	+
Glades	27°06.559'	80°56.427'	30	25	O	A	-	+
Glades	27°00.372'	81°03.003'	10	12	G	D	-	+
Hendry	26°44.256'	81°10.490'	5	0	O	C	-	+
Hendry	26°46.322'	81°12.654'	61	0	G	C	-	+
Hendry	26°33.898'	81°26.202'	0	38	G	D	-	+
Hendry	26°44.423'	81°28.029'	90	0	G	C	-	+
Hendry	26°20.307'	80°54.597'	486	0	O	C	-	+
Highlands	27°24.781'	81°24.714'	48	39	O	C	-	+
Highlands	27°09.201'	81°19.877'	22	27	G	C	-	+
Indian River	27°40.953'	80°27.621'	48	0	G	A	-	+
Lake	28°51.627'	81°38.306'	69	0	O	C	-	+
Lake	28°23.967'	81°41.768'	52	0	O	A	-	+
Lee	26°42.707'	81°36.559'	36	38	O	D	-	+
Marion	28°59.204'	81°55.267'	41	0	G	C	-	+
Pasco	28°19.505'	82°11.240'	116	0	O	C	-	+
Polk	28°03.656'	81°34.937'	140	0	O	C	-	+
Polk	28°02.880'	81°37.035'	91	0	O	C	-	+
Polk	27°52.542'	81°34.654'	70	0	O	C	-	+
Polk	27°47.537'	81°32.044'	79	0	O	C	-	+
Polk	28°06.295'	81°42.895'	197	0	G	R	-	+
St. Lucie	27°32.976'	80°25.852'	40	0	G	N	-	+
St. Lucie	27°23.360'	80°28.376'	6	0	O	C	-	+
		Total	1793	179				

[1]A maximum of 10 specimens were pooled for each DNA extraction.
[2]Host: O = Oranges, G = Grapefruit.
[3]Grove: C = Commercial, A = Abandoned, D = Dooryard, R = Research Plot, N = Non-Commercial.

were negative for the 16S rRNA and *nus*G-*rpl*K genes of *Ca.* L. asiaticus and for the *wsp* gene for *Wolbachia*, as expected.

Altogether, 1,793 adult and 179 immature *D. citri* were collected from 23 sites in 11 counties between Sep 2005 and Jan 2006 (Table 1). All field-collected *D. citri* tested negative for *Ca.* L. asiaticus (<1 in 1,972 psyllids surveyed = <0.05% infection frequency). All samples were positive for *Wolbachia* in the PCR assays, which indicates that the DNA extractions and PCR protocols were working for these samples of microbial DNA mixed with *D. citri* genomic DNA (Table 1). The amount of *Ca.* L. asiaticus DNA in our field-collected *D. citri* was either below the sensitivity of the high-fidelity PCR assay (which reliably detects 100 copies and can detect as few as 10 copies), or the psyllids were truly negative for the HLB-causing bacterium. The failure to obtain any positives for *Ca.* L. asiaticus was surprising, particularly for the site in Hendry County (26°20.307'°N: 80°54.597'°W) where 486 adult *D. citri* were collected from flushing citrus trees with symptoms of HLB. However, the psyllids were aggregating on these trees to mate and oviposit on the tender flush, so they may not have acquired *Ca.* L. asiaticus before they were collected or the greening bacterium had not multiplied sufficiently in the host to facilitate detection.

The results from this study can be used as a benchmark for the infection status of *D. citri* in these sites in Florida during 2005-2006. The absence of *Ca.* L. asiaticus in the psyllid populations surveyed could be due to the recent detection of HLB in Florida and because most psyllids were collected from trees that did not appear to have HLB. However, the lack of any PCR positives in the 486 psyllids collected in Hendry county (Table 1) from trees with HLB symptoms was perplexing; with even a 1% infection frequency we would have expected positives in at least 4 psyllids. In Indonesia, where HLB is more prevalent, up to 45.2% of individual adult *D. citri* tested positive for *Ca.* L. asiaticus in a standard PCR assay (Subandiyah et al. 2000), which is approximately 7-fold less sensitive than the high-fidelity PCR method used here (Hoy et al. 2001). However, in 1992 in India fewer than 1% of the *D. citri* tested were positive for *Ca.* L. asiaticus (Bové et al. 1993). The proportion of *D. citri* carrying *Ca.* L.

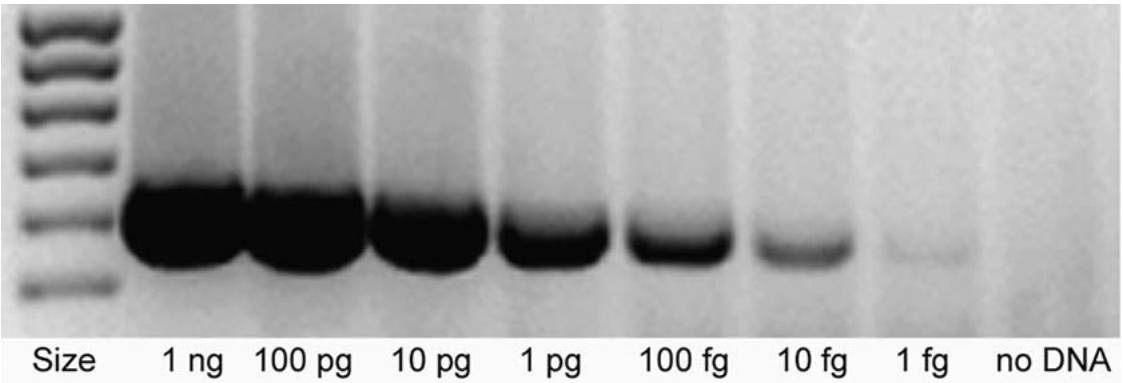

Fig. 1. Sensitivity analysis for high-fidelity PCR-amplification of plasmid DNA containing the *nus*G-*rpl*K gene of *Ca.* L. asiaticus mixed with *D. citri* DNA. PCR-products were obtained with as little as 1 fg (approximately 100 copies) of the 0.6 kb amplification target.

asiaticus in Florida will likely increase if the titer and distribution of the pathogen increases in infected trees and the number of citrus trees with HLB multiplies.

The findings of this study raised a number of important questions concerning the epidemiology and management of HLB in Florida. For instance, how appropriate is it to attempt to kill every psyllid in citrus groves with chemical control if the infection frequency is less than 1%? The cost of widespread chemical control of psyllids and the potential disruption of biological control of other citrus pests needs to be considered. Other questions remain unanswered. How long does it take psyllids to acquire and transmit *Ca.* L. asiaticus when feeding on infected citrus under Florida conditions? How many citrus trees can be infected by a single psyllid hosting *Ca.* L. asiaticus? How does the duration and level of infection in HLB-positive trees affect disease acquisition and transmission by psyllids? Are there seasonal factors that influence HLB transmission? What roles do other host plants, such as *Murraya paniculata* (L.) Jack. play in HLB transmission? Are there other mechanisms that contribute to the spread of HLB, such as mechanical transmission or native vectors? A more extensive effort to survey psyllid populations for *Ca.* L. asiaticus is needed to better understand the epidemiology of HLB. It is thought that destruction of HLB-positive citrus trees, along with suppression of *D. citri* populations in infected citrus groves, will slow the spread of HLB (Stansly & Rogers 2005). However, research is needed to determine which management tactics should have priority in order to minimize the spread of HLB while maintaining existing biological control of other economically-important citrus pests. Possibly, detecting and removing infected trees should be considered a higher priority than attempting to kill all *D. citri* in citrus groves, due to the apparently low proportion of the vector population carrying *Ca.* L asiaticus.

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Summary

Populations of *D. citri* in Florida citrus were surveyed between Sep 2005 and Jan 2006 for *Ca.* L. asiaticus, the causal agent of HLB. No field-collected adults or immatures of the 1,972 *D. citri* tested were positive for the HLB pathogen in these samples, indicating that the proportion of *D. citri* populations hosting *Ca.* L. asiaticus in the regions sampled was very low (<0.05%) during this survey. More extensive surveys for *Ca.* L. asiaticus in *D. citri* are recommended to learn more about the epidemiology of disease transmission in Florida.

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