Detection of 'Candidatus Liberibacter Asiaticus' in Cacopsylla (Psylla) citrisuga (Hemiptera: Psyllidae)

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DETECTION OF '*CANDIDATUS* LIBERIBACTER ASIATICUS' IN *CACOPSYLLA* (*PSYLLA*) *CITRISUGA* (HEMIPTERA: PSYLLIDAE)

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Abstract

Nymphs of the pomelo psyllid, *Cacopsylla* (*Psylla*) *citrisuga* Yang & Li, were collected from huanglongbing (HLB) symptomatic lemon trees, *Citrus limon* (L.) in Yunnan Province, China. DNA samples extracted from groups of 2-10 pomelo psyllid nymphs of all stages and from leaves of lemon plants were analyzed with nested-PCR in order to detect '*Candidatus* Liberibacter asiaticus' (Las). The results showed that 24 out of the 49 lemon trees were Las-positive. Also psyllid nymphs collected from 10 out of the 24 Las-positive plants were Las-positive. DNA extracted from individual late stage nymphs (3rd to 5th instars) collected from Las-infected trees were then subjected to nested-PCR trials. Twelve out of the 29 nymphs proved to be Las positive. All nymphs collected from Las-negative lemon trees were also Las-negative. Most psyllid samples which showed positive in nested-PCR were also positive in conventional-PCR detection. The amplified fragment of the 16S rRNA gene of '*Ca.* Liberibacter spp.' from positive psyllid samples was 99% similar to those of Las strain psy62 in GenBank. These results demonstrate that *Cacopsylla* (*Psylla*) *citrisuga* is another Las carrier insect. Transmission studies are underway to determine whether Las-positive psyllids can transmit Las to healthy citrus.

Key Words: pomelo psyllid, Huanglongbing, PCR, '*Candidatus* Liberibacter asiaticus', detection

Resumen

Ninfas del psílido del pomelo, *Cacopsylla* (*Psylla*) *citrisuga* Yang & Li, fueron colectadas de árboles de limón, *Citrus limon* (L.), con síntomas de huanglongbing (HLB) en la provincia de Yunnan, China. Muestras de ADN extraídas de grupos de 2-10 ninfas de todos los estadios del psílido, y de hojas de limonero, fueron analizadas mediante PCR- anidada para detectar a '*Candidatus* Liberibacter asiaticus' (Las). Los resultados mostraron que 24 de los 59 árboles fueron Las-positivo. Ninfas provenientes de 10 de los 24 árboles Las-positivos, también fueron Las-positivas. ADN extraído individualmente de ninfas de últimos estadios (3er a 5to instar) colectadas de arboles infectados con Las fueron sujetas a ensayos de PCR anidada. Doce de las veintinueve ninfas demostraron ser Las-positivas. Todas las ninfas colectadas de árboles de limón Las-negativos también fueron Las-negativas. La mayoría de muestras de psílidos que resultaron Las-positivas mediante el PCR anidado también fueron positivas en la detección mediante PCR convencional. El fragmento amplificado del gen 16S rRNA de '*Ca.* Liberibacter spp.' de muestras de psílidos Las-positivos mostró un 99% de similitud con la cepa Las psy62 del GenBank. Estos resultados demuestran que *Cacopsylla* (*Psylla*) *citrisuga* es otro insecto portador de Las. Estudios están en curso para determinar si psílidos que son Las-positivos pueden transmitir Las a plantas de cítricos saludables.

Huanglongbing (HLB) is the most devastating disease of citrus (Sapindales: Rutaceae) in the world. It is associated with 3 species of Liberibacter, i.e., '*Candidatus* Liberibacter africanus' (Laf) - the African form, '*Ca*. L. asiaticus' (Las) - the Asian form, and '*Ca*. L. americanus'

(Lam) - the American form (Planet et al. 1995; Jagoueix et al. 1996; Coletta-Filho et al. 2005; Teixeira et al. 2005). McClean et al. (1965) reported that the African citrus psyllid, *Trioza erytreae* Del Guercio, transmits Laf. Capoor et al. (1967) found that the Asian citrus psyllid, *Diaphorina citri* Kuwayama, vectors Las. *Diaphorina citri* also vectors Lam (Teixeira et al. 2005; Yamamoto et al. 2006). Laboratory studies indicated that *T. erytreae* can also transmit Las (Massonié et al. 1976), while *D. citri* can also transmit Laf (Lallemand et al. 1986). However, field studies and surveys have suggested that *D. citri* is as the sole insect vector of Las, and that *T. erytreae* is the sole insect vector of Laf (Bove 2006).

In addition to *D. citri*, 4 other species of citrus-feeding psyllids have been recorded in Yunnan Province, China, i.e., *Cacopsylla* (*Psylla*) *citrisuga* Yang & Li, *C.* (*P.*) *citricola* Yang & Li, *Trioza citroimpura* Yang & Li (Yang & Li 1984), and *C.* (*P.*) *heterogena* Li (Li 2011). The hosts of these 4 psyllid species include pomelo, *Citrus grandis (L.)* Osbeck, *Citrus reticulata* Blanco, *Citrus medica* L., and other *Citrus* species (Li 2011). However, information about biology and ecology of the psyllid species above is very limited, and no study has been conducted on whether these citrus-feeding psyllids can vector Liberibacter. Among the 4 species, the above authors found that the pomelo psyllid, *C.* (*P.*) *citrisuga* (Fig. 1), is the most frequently encountered in Yunnan Province. The nymphs of *C. (P.) citrisuga* damage the young flush and cause changes in the foliar phenotype (Fig. 2 and Fig. 3). We therefore initiated this investigation to determine whether '*Ca.* L. asiaticus' can be detected in life stages of *C. (P.) citrisuga.*

Materials and Methods

Collection of Psyllids and Host Plant Materials, and Identification of Psyllid Species

Pomelo psyllids were collected from a 6-yr old severely HLB-infected orchard of 'Eureka' lemon, *Citrus limon* (L.) Burm.f. in Bangkong Village, Mengxiu Town (24.0712 N, 97.7999 E, 1200 m asl), Ruili City, Yunnan Province, China. Newly flushed leaves with nymphs were collected from

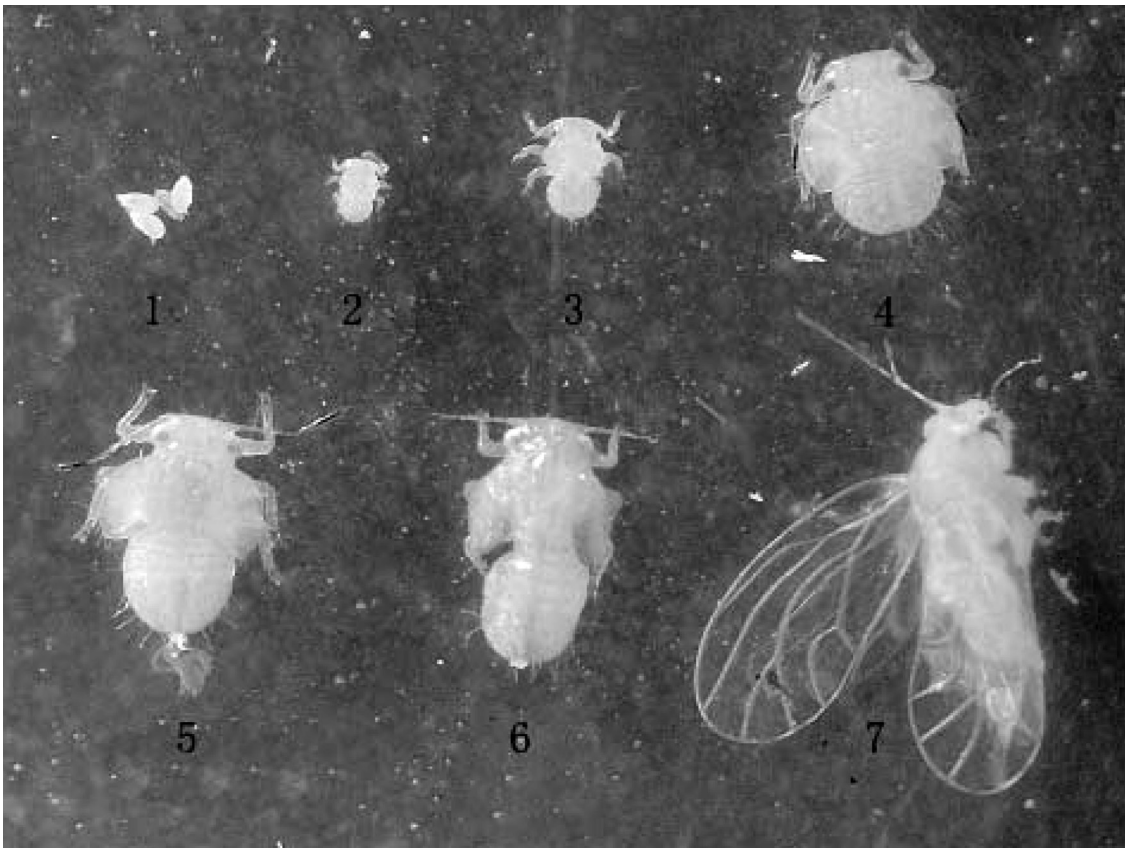

Fig. 1. Pomelo psyllid, *Cacopsylla (Psylla) citrisuga* Yang & Li. 1: egg; 2: 1st instar nymph; 3: 2nd instar nymph; 4: 3rd instar nymph; 5: 4th instar nymph; 6: 5th instar nymph; and 7: adult.

Fig. 2. Damage phenotypes of the pomelo psyllid, *Cacopsylla (Psylla) citrisuga* Yang & Li. The adults lay eggs around the main vein of the upper side of the young leaves. The nymphs feed on the young leaves and the infected leaves become folded.

HLB-symptomatic lemon trees and examined in the laboratory under a microscope. These nymphs were then preserved in 70% ethanol. All nymphs from a single tree were placed together in a bottle and labeled. The mature and HLB-symptomatic leaves from the trees where the nymphs had been collected were also collected and labeled for confirmation of HLB in the laboratory.

Fig. 3. Pomelo psyllid nymphs feeding inside the fold along the main vein.

Psyllid species were identified based on the genitalia of adult psyllid males according to Yang & Li (1984) and Li (2011).

PCR Analyses of DNA of Individual or Groups of Psyllids

The first batch of psyllids was collected on 17 Mar 2011 from 27 HLB-infected lemon trees. The DNA of all 1st to 5th instar nymphs collected from each single tree was extracted in order to determine whether Las DNA could be detected (Table 1). The number of psyllids in each group ranged from 2 to 63 individuals. The second batch was collected on 1 Apr 2011 from each of 22 lemon trees in the same orchard. The DNA of each group of 10 or less late stage-nymphs (3rd to 5th instars) collected from 1 tree was extracted and analyzed by nested-PCR (Table 2). Additionally 29 psyllids from 3 trees from which the above PCR test had been positive for Las in groups of psyllids were subjected to further nested-PCR testing of individual psyllids (Table 2, Tree # 13, 14, and 20). Conventional PCR was then run to detect the psyllid samples that had been shown to be positive in the nested-PCR.

DNA Extraction from Psyllids and from *Citrus limon* Host Plants

The DNA of psyllid nymphs was extracted by TIANamp Genomic DNA Kit (provided by Tiangen Biotech (Beijing) Co., Ltd, Beijing, China). The DNA of lemon leaves was extracted using E.Z.N.A.™ Plant DNA Kit (provided by OMEGA Company, Norcross Georgia, USA).

Nested-PCR and Conventional PCR for Detection of Las DNA

Nested-PCR (Harakava et al. 2000) was used for Las detection in the lemon plants and psyllid nymphs. The primer, 1500R/27F (AAGGAGGTGATCCAGCCGC/ AGAGTTTGATCATGGCTCAG), was used for the first amplification, and OI1 /OI2c (GCGCGTATGCAATACGAGCGGCA/ GCCTCGCGACTTCGCAACCCAT) was used for the second amplification (Jagoueix et al. 1994).

The first amplification system was carried out in a final volume of 25 µL. The mixture contained 17.6 µL of ddH_2O, 2.5 µL of 10 × PCR buffer (Mg^{2+}), 2.5 µL of dNTPs (2.5 mmol/L), 0.5 µL each of primers (10 µmol/L), 0.4 µL of *Taq* enzyme (2.5 U/µL), and 1 µL of Sample DNA. DNA amplification by PCR was performed as follows: reactions were preheated at 94 °C for 5 min; followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 4 min.

The second amplification system was also carried out in a final volume of 25 µL. The mixture contained 17.6 µL of ddH2O, 2.5 µL of 10 × PCR

Table 1. Detection of '*Candidatus* Liberibacter asiaticus' (LAS) by PCR in DNA extracted from groups of Pomelo psyllid nymphs and from host plants, citrus limon - first collection.

Tree No.	Host plant nested-PCR detection result**	Psyllid detection result*		
		Psyllid number	Nested-PCR result**	Conventional-PCR result***
1	—	2	—	
2	+	2	+	—
3		3	—	
4	—	5	—	
5	+	5	—	
6	—	3	—	
7	—	63	—	
8	+	4	—	
9	+	7	—	
10	+	18	—	
11	+	12	—	
12	—	5	—	
13	—	3	—	
14	—	3	—	
15	—	5	—	
16	—	2	—	
17	+	10	—	
18	+	6	—	
19	+	10	—	
20	—	5	—	
21	—	5	—	
22	—	5	—	
23	+	4	—	
24	—	5	—	
25	—	5	—	
26	—	5	—	
27	—	5	—	

* Psyllids used in this table were all stages' nymphs collected from the same tree;
** +: positive; —: negative with respect to '*Ca.* Liberibacter asiaticus' (Las);
***Nested-PCR positive psyllid sample was amplified by the second set of primer of nested-PCR.

buffer (Mg^{2+}), 2.5 µL of dNTPs (2.5 mmol/L), 0.5 µL each of primers (10 µmol/L), 0.4 µL of *Taq* enzyme (2.5 U/µL), and 1 µL of PCR product of the first amplification. DNA amplification by PCR was performed as follows: reactions were preheated at 96 °C for 1 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 4 min.

Conventional PCR was then run to confirm the psyllid samples which had showed positive in nested-PCR. The method was the same as the second amplification of nested-PCR.

DNA Clone and Sequence Analysis

PCR amplification was subjected to electrophoresis on a 1% agarose gel, purified from the gel band according to the Gel Extraction Kit (BioTeke Corporation, Beijing, China) manual instructions, and ligated to the pEASY-T1 cloning vector (*TransGen* Biotech, Beijing, China). Four µL of the ligation mixture was used to transform *E.coli Trans*1-T1 competent cells (*TransGen* Biotech, Beijing, China). Cloned DNA was sent to Invitrogen Biology and Technology Company, Shanghai, China for sequencing. The DNA sequence was compared with the current GenBank database using the BLAST network service available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Results

Among the first batch of 27 lemon tree samples, 10 trees proved to be Las-positive (Table 1). The number of psyllid nymphs collected from each of the lemon trees was also listed in Table 1. The result showed that 1 nymph sample collected from 1 of the 10 Las-infected trees (Tree No. 2) was Las-positive in nested-PCR (Table 1). In this case the psyllid DNA was extracted only from the 2 nymphs in the sample, and the band density was very low.

Among the second batch of 22 samples, 14 lemon trees proved to be Las-positive. Psyllids collected from 9 of these 14 trees also proved to be Las-positive by nested-PCR (Table 2). Psyllid samples collected from the negative trees also showed Las-negative result, the same as the result from the first batch detection.

Twenty-nine psyllids, 2 from tree No. 13, 16 from tree No. 14, and 11 from tree No. 20, were individually tested by nested-PCR after Las-

TABLE 2. DETECTION OF '*CANDIDATUS* LIBERIBACTER ASIATICUS' (LAS) BY PCR IN DNA EXTRACTED FROM INDIVIDUAL AND FROM GROUPS OF POMELO PSYLLID NYMPHS AND FROM HOST PLANTS, CITRUS LIMON - SECOND COLLECTION.

		Las detected in psyllid groups*			Las detected in individual psyllids*		
			Result		Nested-PCR		
Tree No.	Las detected in host plant by means of nested PCR**	Psyllid number	Nested PCR**	Conventional PCR***	Tested individual	Positive individual	Positive individual in conventional PCR***
1	+	10	—				
2	—	10	—				
3	—	8	—				
4	+	8	+	+			
5	—	10	—				
6	—	10	—				
7	—	10	—				
8	+	3	+	+			
9	+	9	+	+			
10	+	3	+	+			
11	—	10	—				
12	+	10	+	+			
13	+	10	+	+	2	2	2
14	+	10	+	+	16	10	8
15	—	10	—				
16	+	10	—				
17	+	10	—				
18	+	10	+	+			
19	+	10	—				
20	+	10	+	—	11	0	
21	+	10	—				
22	—	10	—				

*Psyllids used in this table were late-stage-nymphs collected from the same tree.
**+: positive; —: negative with respect to 'Ca. Liberibacter asiaticus' (Las).
***Nested-PCR positive psyllid samples were amplified by the second set of primer of nested-PCR.

positive results obtained from the group test of the nymphs collected from these 3 trees (Table 2). Twelve of the 29 psyllids (2 from tree No. 13, and 10 from tree No. 14) were Las-positive, but the psyllids from tree No. 20 were not Las-positive (Table 2, Fig. 4). The band densities of all positive samples from the second batch of PCR amplifications were higher than those of the first batch.

Conventional PCR was then run to confirm the 22 psyllid samples (both in groups and individuals) which had been shown to be positive by nested-PCR. The results showed that 18 samples were positive (Tables 1 and 2). The positive sample of the first collection, the sample of tree No. 20 of the second collection, and 2 individual samples of tree No. 14 were negative, which may suggests that nested-PCR is more sensitive than conventional PCR for Las detection.

Sequence analysis showed the DNA sequence of the 16S rRNA gene of '*Ca.* Liberibacter spp.' from positive psyllid samples was 99% similar to that of '*Ca*. L. asiaticus' strain psy62 (Accession NO. CP001677), 97% similar to that of '*Ca*. L. africanus' (Accession NO. L22533), 95% similar to that of '*Ca*. L. africanus subsp. capensis' (Accession NO. AF137368.), and 94% similar to that of '*Ca*. L. americanus' (Accession NO. AY742824) in GenBank. This result demonstrates that pomelo psyllid, *C. (P.) citrisuga*, is a Las carrier.

Discussion

A number of studies have been conducted to investigate potential vectors of '*Ca*. Liberibacter asiaticus', such as the citrus aphid, *Toxoptera citricidus* (Kirkaldy), in South Africa (McClean et al. 1965), *Toxoptera citricidus* (Kirkaldy)*, Toxoptera aurantii* (Boyer de Fonscolombe), citrus red mite *Panonychus citri* (McGregor) and yellow mite *Eotetranychus kankitus* Ehara (Guangxi HLB Research Group 1977), and many other insects with piercing mouthparts in China (Cen et al. 2011, unpublished data). The outcomes of these studies were all negative.

So far psyllids are the only group of insects known to transmit '*Ca.* Liberibacter spp.'. Hansen et al. (2008) reported that in addition to the 3 '*Ca.* Liberibacter' species associated with HLB,

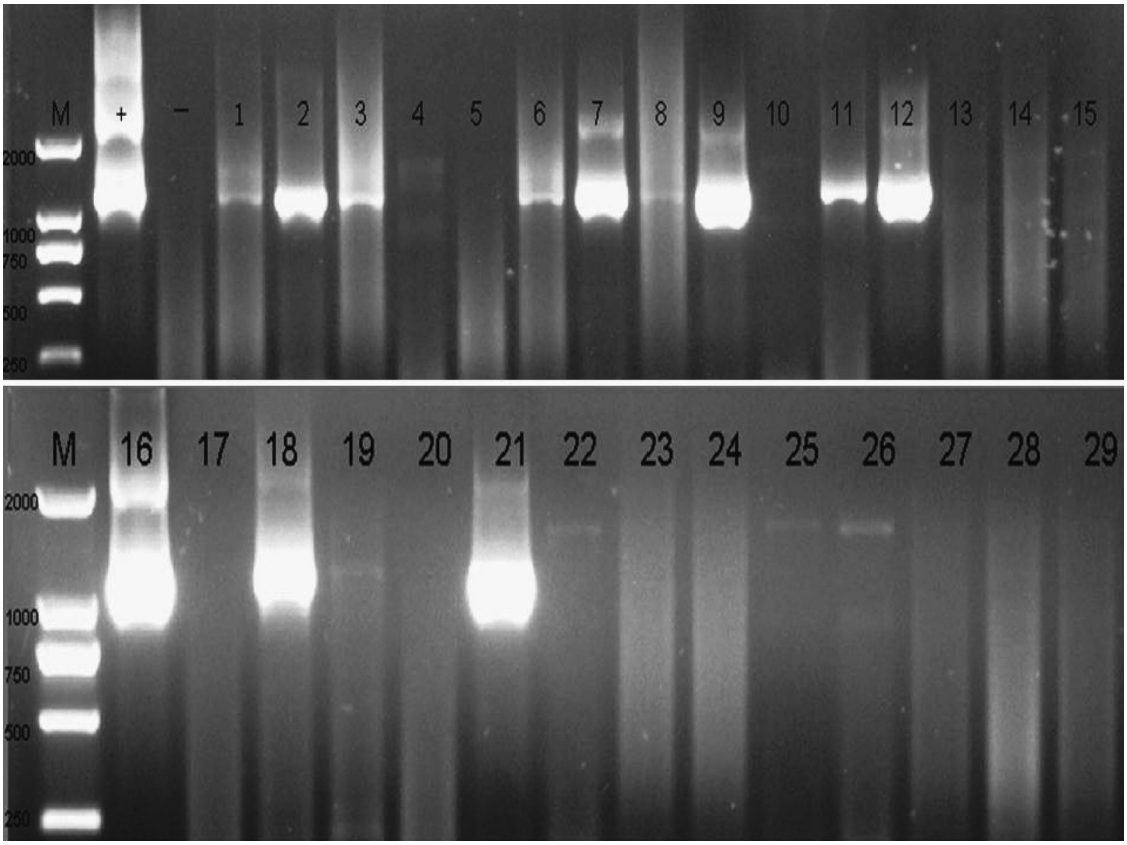

Fig. 4. Detection result of Nested-PCR for Candidatus Liberibacter asiaticus from pomelo psyllid individuals. M: DL2000 DNA marker; +: positive control; —: negative control (ddH20); Lanes 1-29: each lane displays the result of a single psyllid nymph.

'*Ca.* Liberibacter psyllaurous', which infects tomato and potato, is vectored by the potato psyllid, *Bactericera cockerelli* (Sulc). Also '*Ca.* Liberibacter europaeus', which apparently behaves as an endophyte rather than as a pathogen, was found to be transmitted by the pear psylla, *Cacopsylla pyri* (L.) (Raddadi et al. 2010).

According to Halbert & Manjunath (2004), 13 citrus-feeding psyllid species have been reported, and 7 of them belong to the genus of *Diaphorina*. These species are *D. citri* Kuwayama, *D. amoena* Capener, *D. auberti* Hollis, *D. communis* Mather, *D. murrayi* Kandasamy, *D. punctulata* (Pettey 1924), and *D. zebrana* Capener. The other 6 citrus-feeding psyllid species include *Mesohomotoma lutheri* (Enderlein 1918) (= *Udamostigma lutheri* Enderlein), *Psylla citricola* Yang & Li 1984, *Psylla citrisuga* Yang & Li 1984, *Psylla murrayi* Mathur 1975, *Trioza citroimpura* Yang & Li 1984, *Trioza erytreae* (del Guercio 1918) (= *Aleurodes erytreae* del Guercio, = *Trioza citri* Laing, = *Trioza merwei* Pettey, = *Spanioza merwei* (Pettey), = *Spanioza erythreae* (del Guercio) (Hollis 1984)), and *Trioza litseae* Bordage 1898 (= *Trioza eastopi* Orian 1972) (Halbert & Manjunath 2004). But there are uncertainties about the synonymy for some species (Halbert & Manjunath 2004). A new citrus-feeding psyllid species, *C.* (*P.*) *heterogena* Li, was reported in China recently (Li 2011). Among these psyllid species, *D. citri* and *T. erytreae* are the only known insect vectors of HLB (Halbert & Manjunath 2004, Nadarasah & Stavrinides 2011). Donovan et al. (2011) reported Las-positive some *D. communis* individuals. No other citrus-feeding psyllids have been reported to be Liberibacter carriers.

This study is the first to demonstrate that *C.* (*P.*) *citrisuga* carries Las. Transmission studies are underway to determine whether Las-positive *C.* (*P.*) *citrisuga* can transmit Las to citrus.

Acknowledgments

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