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Infection Density Dynamics of the Citrus Greening Bacterium "*Candidatus* Liberibacter asiaticus" in Field Populations of the Psyllid *Diaphorina citri* and Its Relevance to the Efficiency of Pathogen Transmission to Citrus Plants

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Huanglongbing, or citrus greening, is a devastating disease of citrus plants recently spreading worldwide, which is caused by an uncultivable bacterial pathogen, "*Candidatus* Liberibacter asiaticus," and vectored by a phloem-sucking insect, *Diaphorina citri*. We investigated the infection density dynamics of "*Ca.* Liberibacter asiaticus" in field populations of *D. citri* with experiments using field-collected insects to address how "*Ca.* Liberibacter asiaticus" infection density in the vector insect is relevant to pathogen transmission to citrus plants. Of 500 insects continuously collected from "*Ca.* Liberibacter asiaticus"-infected citrus trees with pathological symptoms in the spring and autumn of 2009, 497 (99.4%) were "*Ca.* Liberibacter asiaticus" positive. The infections were systemic across head-thorax and abdomen, ranging from 10^3 to 10^7 bacteria per insect. In spring, the infection densities were low in March, at ~10^3 bacteria per insect, increasing up to 10^6 to 10^7 bacteria per insect in April and May, and decreasing to 10^5 to 10^6 bacteria per insect in late May, whereas the infection densities were constantly ~10^6 to 10^7 bacteria per insect in autumn. Statistical analysis suggested that several factors, such as insect sex, host trees, and collection dates, may be correlated with "*Ca.* Liberibacter asiaticus" infection densities in field *D. citri* populations. Inoculation experiments with citrus seedlings using field-collected "*Ca.* Liberibacter asiaticus"-infected insects suggested that (i) "*Ca.* Liberibacter asiaticus"-transmitting insects tend to exhibit higher infection densities than do nontransmitting insects, (ii) a threshold level (~10^6 bacteria per insect) of "*Ca.* Liberibacter asiaticus" density in *D. citri* is required for successful transmission to citrus plants, and (iii) *D. citri* attaining the threshold infection level transmits "*Ca.* Liberibacter asiaticus" to citrus plants in a stochastic manner. These findings provide valuable insights into understanding, predicting, and controlling this notorious citrus pathogen.

Huanglongbing (HLB), or citrus greening, is one of the most serious diseases of citrus plants in many countries across Asia, Africa, and North and South America (1–5). This disease is caused by the phloem-inhabiting, noncultivable, and insect-vectored bacterial pathogens "*Candidatus* Liberibacter asiaticus" (Asia, North America, and Brazil), "*Ca.* Liberibacter africanus" (Africa), and "*Ca.* Liberibacter americanus" (Brazil) (6–8).

The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is the principal vector of "*Ca.* Liberibacter asiaticus" in Asia, North America, and Brazil (1–5). On the grounds that "*Ca.* Liberibacter asiaticus"-infected *D. citri* insects are primarily responsible for infection and spread of HLB among citrus plants, monitoring and investigation of the infection dynamics of "*Ca.* Liberibacter asiaticus" in field populations of *D. citri* are of pivotal importance (9–12). Furthermore, infection densities of "*Ca.* Liberibacter asiaticus" within vector insects, which are likely relevant to the *in vivo* localization and proliferation of the pathogen (13, 14), may influence various host phenotypes, such as growth, survival, fecundity, physiology, and behavior (15), thereby potentially modulating the vectoring capacity of *D. citri* via, for example, affecting the efficiency of transmission of "*Ca.* Liberibacter asiaticus" to infested citrus plants (16–18).

Since "*Ca.* Liberibacter asiaticus" is noncultivable (6), most recent studies have adopted culture-independent approaches using conventional PCR and/or quantitative PCR techniques for the detection and quantification of "*Ca.* Liberibacter asiaticus" not only in citrus plants (15, 18–25) but also in *D. citri* (9, 13, 15–18). Meanwhile, the biological relevance and consequence of the infection density of "*Ca.* Liberibacter asiaticus" in *D. citri* have been poorly investigated, except for a recent experimental study demonstrating a positive correlation between the infection density of "*Ca.* Liberibacter asiaticus" in *D. citri* and its efficiency of transmission to excised citrus leaf preparations (18). Despite the labo-

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ratory and experimental studies cited above, there has been no report on the infection density dynamics of "*Ca.* Liberibacter asiaticus" in field populations of *D. citri*.

In Japan, while *D. citri* has been endemic in the subtropical Ryukyu islands of Okinawa Prefecture and also in many southern islands of Kagoshima Prefecture, HLB was first detected in 1988 on Iriomote island, one of the southernmost Ryukyu islands (26), and has currently spread across almost all Ryukyu islands toward Kikai island of Kagoshima Prefecture (27–29). The flat lemon *Citrus depressa* Hataya, known as "Shiikuwasha" in Japanese, is the most popular citrus cultivar in commercial plantations and private gardens of the Okinawa region, where *D. citri* infestation as well as HLB symptoms are frequently found on *C. depressa* trees, particularly in private gardens without systematic insecticide spraying (30).

In the annual life cycle of *D. citri* in Okinawa, the insect population exhibits a primary peak in spring (April and May), when citrus trees shoot actively, and a secondary peak in autumn (November), when citrus shooting also occurs (Y. Sadoyama and T. Takushi, unpublished data). This pattern is reminiscent of the population dynamics of *D. citri* in Florida (9).

In this study, we continuously sampled strictly staged (0- to 7-day-old postemergence) adult insects of *D. citri* from "*Ca.* Liberibacter asiaticus"-infected *C. depressa* garden trees in spring and autumn, experimentally investigated their capability of transmitting "*Ca.* Liberibacter asiaticus" to seedlings of *C. depressa*, and quantified the infection density of "*Ca.* Liberibacter asiaticus" in these *D. citri* individuals, thereby attempting to gain insights into the infection density dynamics of "*Ca.* Liberibacter asiaticus" observed in the *D. citri* populations and how the infection density of "*Ca.* Liberibacter asiaticus" is relevant to the transmission capability of the vector insects.

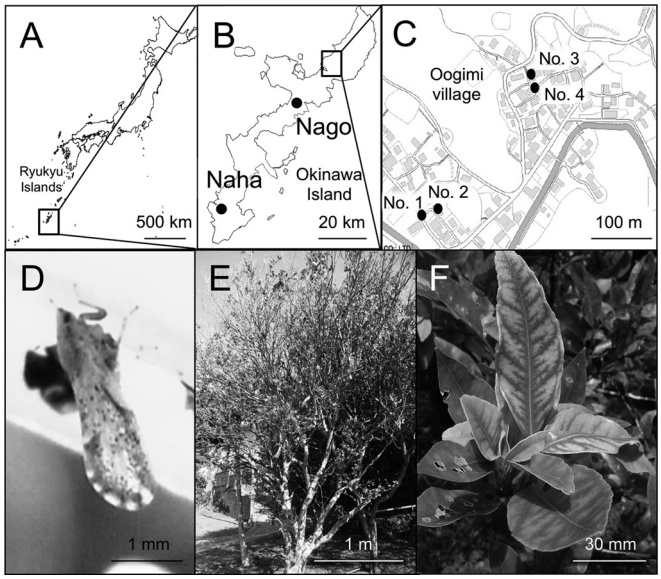

FIG 1 Study field. (A) The Japanese Archipelago, in which Okinawa Island is indicated by a rectangle. (B) Okinawa Island, in which Oogimi village is indicated by a rectangle. (Panels A and B created by using Blank Map, which is freely available at http://www.vector.co.jp/soft/win95/writing/se192183.html [in Japanese].) (C) Enlarged map of Oogimi village on which the locations of *C. depressa* trees 1 to 4 are shown. (Panel C created by using the Okinawa Prefecture Map Information System, which is freely available at http://gis.pref.okinawa.jp/pref-okinawa/top/select.asp?dtp=52&pl=3# [in Japanese].) (D) Adult *D. citri* insect. (E) *C. depressa* tree 1. (F) Leaves of *C. depressa* with pathological symptoms.

MATERIALS AND METHODS

Field collection of *D. citri* from "*Ca.* Liberibacter asiaticus"-infected citrus trees. Adult *D. citri* insects were collected from four *C. depressa* trees (trees 1 to 4), which were located in Oogimi village, northern Okinawa Island, Japan (Fig. 1A to C). All the trees were heavily infested by *D. citri* (Fig. 1D), exhibiting severe visual symptoms of HLB (Fig. 1E and F) and suffering from "*Ca.* Liberibacter asiaticus" infection, as confirmed by conventional PCR tests. For insect staging, after disturbing adult insects to fly away, we covered infested citrus shoots with gauze bags (0.8-mm mesh) and harvested emerging adult insects in the bags by aspiration every 7 days, whereby 0- to 7-day-old postemergence adult *D. citri* insects were collected. In spring, from 19 March to 28 May 2009, we continuously sampled 424 newly emerged adult insects from trees 1 to 4. In autumn, from 12 November to 3 December 2009, we similarly sampled 110 newly emerged adult insects only from tree 1, because trees 2 to 4 were dead. The collected insects were brought to our research center and kept on healthy *C. depressa* tree pots for 7 days prior to transmission tests. During pretransmission rearing, 9 and 5 insects died in spring and autumn, respectively. Climatic data were recorded at tree 1 by using a Thermo Recorder Mini instrument (Espec Mic Corp., Japan) (see Fig. S1 in the supplemental material).

"*Ca.* Liberibacter asiaticus" transmission test. Healthy *C. depressa* seedlings, which were 6 to 12 months old after planting and ~10 cm high with several buds, were grown in a greenhouse in a natural environmental setting without active air conditioning but with ventilation, which was intended to follow seminatural light and temperature conditions. Two consecutive transmission tests were conducted within plant pots placed in the greenhouse. In each plant pot, a healthy citrus seedling was planted, on which a single insect was introduced and maintained. In the first transmission test, each 7- to 14-day-old postemergence adult insect was reared on a potted seedling of *C. depressa* for 7 days, which was covered with a plastic bag with air vents. During the test period, 6 and 5 insects died in spring and autumn, respectively. Next, each insect (14- to 21-day-old postemergence adult) was transferred to a new seedling for the second test, where the insect was kept for 7 days in the same way. During the test period, 2 and 2 insects died in spring and autumn, respectively. After that, the remaining 407 insects in spring and 99 insects in autumn (21- to 28-day-old postemergence adults) were preserved in acetone until subsequent DNA extraction and quantitative PCR analysis (31).

***D. citri* DNA preparation.** The head-thorax and abdomen of each acetone-preserved adult insect were separated by using fine forceps under a dissection microscope. Each insect part was placed into a 1.5-ml plastic tube, air dried, crushed by a sterilized toothpick in 100 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5% SDS, 2 mg/ml proteinase K), and incubated at 55°C for 3 h. After being mixed and centrifuged with phenol-chloroform-isoamyl alcohol, 50 μl of the supernatant was mixed with 125 μl of 100% ethanol and 5 μl of 3 M sodium acetate and centrifuged at 12,000 rpm for 5 min. The pellet was washed with 70% ethanol, air dried, and suspended in 50 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), which was used as the template DNA solution for quantitative PCR.

PCR, cloning, and sequencing of "*Ca.* Liberibacter asiaticus" genes. An 828-bp region of the outer membrane protein (*omp*) gene and a 603-bp region of the 50S ribosomal protein L10 (*rplJ*) gene, which are both single copied on the "*Ca.* Liberibacter asiaticus" genome (32), were amplified by PCR using primers LibOMP1227F (5′-CAC GGG TTA TTT TTC TGA AG-3′) and LibOMP2054R (5′-TTA CCT CCA ATC GCA TAT TT-3′) for the *omp* gene and primers LibRPLJoutF (5′-TTC TGG ATC AAT TGT TGT TG-3′) and LibRPLJoutR (5′-CCC CAT TCC TTT TCT AAT CT-3′) for the *rplJ* gene, under the following temperature profile: 94°C for 4 min followed by 35 cycles consisting of 94°C for 30 s, 55°C for

1 min, and 72°C for 1 min. The PCR products were cloned and sequenced as previously described (33).

Quantitative PCR of "*Ca.* Liberibacter asiaticus" genes. On the basis of the "*Ca.* Liberibacter asiaticus" gene sequences, the following primer sets were designed for quantitative PCR: qLibOMP1773F (5′-GCC ACG TAA AGG CAT GTT GA-3′) and qLibOMP1859R (5′-GCT CGA GAT CCA ATC CGA TG-3′), targeting an 87-bp region of the *omp* gene, and qLibRPLJ198F (5′-GGT TGT AGA GAA GGG CGT CCT-3′) and qLibRPLJ278R (5′-CCA GCT CGA ATA CCC TCA AGA-3′), targeting an 81-bp region of the *rplJ* gene. Quantitative PCR was performed by using SYBR green and the Mx3000P QPCR system (Stratagene, La Jolla, CA) with 4 μl of the DNA template in a 25-μl reaction mixture under a temperature profile of 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 30 s. PCR products of the 828-bp *omp* gene region and the 603-bp *rplJ* gene region, which were purified and serially diluted in TE buffer to contain 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 gene copies per 4 μl, were used as standard DNA samples. All PCR mixtures contained TE buffer samples as no-template controls. Two replicate reactions were run for each sample, and the mean value was calculated. We regarded DNA samples that contained $>10^2$ *omp* gene copies as "*Ca.* Liberibacter asiaticus" positive. In total, 407 and 99 insects collected in spring and autumn, respectively, were individually subjected to DNA extraction and quantitative PCR, during which 3 and 3 insects collected in spring and autumn, respectively, were lost due to experimental failure. Hence, we obtained quantitative PCR data for 404 and 96 insects collected in spring and autumn, respectively.

Evaluation and calibration of quantitative PCR for "*Ca.* Liberibacter asiaticus" genes. To evaluate the reliability and dynamic range of the quantitative PCR, we targeted two "*Ca.* Liberibacter asiaticus" genes, *omp* and *rplJ*. We randomly selected 20 field-collected *D. citri* adults, extracted DNA from them individually, and performed quantitative PCR for *omp* gene copies and *rplJ* gene copies in these DNA samples. The estimated "*Ca.* Liberibacter asiaticus" amounts in the same insects exhibited almost perfect agreement between the genes, comprising a beautiful regression line with an extremely high regression coefficient ($y = 0.8881x - 218.17$ and $R^2 = 0.9902$ in standard regressions; $y = 0.9395x + 0.1741$ and $R^2 = 0.9892$ in log-transformed regressions) (see Fig. S2A and S2B in the supplemental material). Quantification of standard DNA samples confirmed the perfect linearity of the standard curve over the range of 10^2 to 10^8 copies for both the *omp* and *rplJ* genes (see Fig. S2C and S2D in the supplemental material). On the grounds that 1/25 (4 μl of sample/100 μl of lysate) of the whole DNA extract from an insect was subjected to quantitative PCR, these results indicated that the quantitative PCR procedures provide reliable estimates over a range of 10^2 to 10^8 "*Ca.* Liberibacter asiaticus" gene copies per DNA sample or a range of 2.5×10^3 to 2.5×10^9 "*Ca.* Liberibacter asiaticus" gene copies per insect. Here we adopted quantitative PCR targeting the *omp* gene.

PCR detection of "*Ca.* Liberibacter asiaticus" from plant. Each seedling subjected to the transmission test was maintained in the greenhouse for 6 months, and its DNA was extracted from a midrib part of a leaf and subjected to PCR detection of "*Ca.* Liberibacter asiaticus" as previously described (34). Since 404 and 96 insects survived the first and second transmission tests in spring and autumn, respectively, 404 pairs of plants in spring and 96 pairs of plants in autumn were maintained for the PCR test. However, since 15 plants died in spring, we finally obtained "*Ca.* Liberibacter asiaticus" infection data for 389 plant sets in spring and 96 plant sets in autumn, respectively.

Statistics. All statistical analyses were performed by using R version 2.12.1 (35). Linear regression was used to indicate the relationship of "*Ca.* Liberibacter asiaticus" densities between the head-thorax and the abdomen of *D. citri*. To analyze the effects of candidate factors on bacterial densities per insect, analysis of variance (ANOVA) was performed. Response variables were log transformed before the analyses. Differences in "*Ca.* Liberibacter asiaticus" amounts in the head-thorax of *D. citri* across sampling dates were evaluated by Tukey's multiple-comparison tests. Differences in "*Ca.* Liberibacter asiaticus" amounts in the head-thorax of *D. citri* among sampling trees were also evaluated with Tukey's multiple-comparison tests. A generalized linear model (GLM) with a quasibinomial error and logit link function was used to analyze the effects of candidate factors on transmission rates. We used the F test to determine the statistical significance of each coefficient in the GLM. Tukey's multiple-comparison tests were used in the GLM (R add-on package "multcomp") to analyze the significant differences in transmission rates among the sampling dates and trees.

Nucleotide sequence accession numbers. The nucleotide sequences of the *omp* gene and *rplJ* gene of "*Ca.* Liberibacter asiaticus" determined in this study have been deposited in the DNA Data Bank of Japan nucleotide sequence databases under accession no. AB741531 and AB741532, respectively.

RESULTS

Quantification of "*Ca.* Liberibacter asiaticus" infection density in *D. citri*. In the spring of 2009 (on 11 dates from 19 March to 28 May), in total, 424 adult *D. citri* insects (0- to 7-day-old postemergence adults) were collected from *C. depressa* trees 1 to 4. In the autumn of 2009 (on 4 dates from 12 November to 3 December), a total of 110 adult insects (0- to 7-day-old postemergence adults) were collected from tree 1. These insects were subjected to two consecutive trials of the transmission test with healthy seedlings of *C. depressa* for 3 weeks, during which 20 insects in spring and 14 insects in autumn were either dead or lost (see Materials and Methods). Consequently, "*Ca.* Liberibacter asiaticus" quantification data were obtained for 404 insects in spring and 96 insects in autumn (see Table S1 in the supplemental material). Their head-thorax and abdomen were separated and individually subjected to DNA extraction and quantitative PCR of the *omp* gene of "*Ca.* Liberibacter asiaticus." Almost all the insects were "*Ca.* Liberibacter asiaticus" positive: 401 of 404 (99.3%) insects collected in spring, 96 of 96 (100%) insects collected in autumn, and 497 of 500 (99.4%) insects inspected in total were positive (see Table S1 in the supplemental material).

Comparison of "*Ca.* Liberibacter asiaticus" infection densities between head-thorax and abdomen of *D. citri*. Figure S3 in the supplemental material shows the relationships between "*Ca.* Liberibacter asiaticus" infection densities in the abdomen and those in the head-thorax of *D. citri*. In both females and males, and also in both spring and autumn, the infection densities in the abdomen were highly and positively correlated with the infection densities in the head-thorax, indicating systemic "*Ca.* Liberibacter asiaticus" infection throughout the body of *D. citri*. In females, the infection densities were higher in autumn than in spring, and in autumn, the infection densities were higher in the abdomen than in the head-thorax (Fig. 2A). In males, in contrast, the infection densities were not different between the tissues and the seasons (Fig. 2B). Considering that the biomass of the abdomen is 0.98 times lower in females and 0.62 times lower in males than the biomass of the head-thorax (see Fig. S4 in the supplemental material), we normalized the infection densities to the number of *omp* copies per microgram dry weight, but the results exhibited essentially the same patterns (Fig. 2C and D).

Seasonal dynamics of "*Ca.* Liberibacter asiaticus" infection density in *D. citri*. Figure 3 shows "*Ca.* Liberibacter asiaticus" infection densities in adult *D. citri* insects collected from field *C. depressa* trees in the spring (March to May) and autumn (November) of 2009. In March, infection densities were $\sim 10^3$ *omp* copies per insect. In April, infection densities increased drastically up to 10^6 to 10^7 *omp* copies per insect. In May, infection densities were

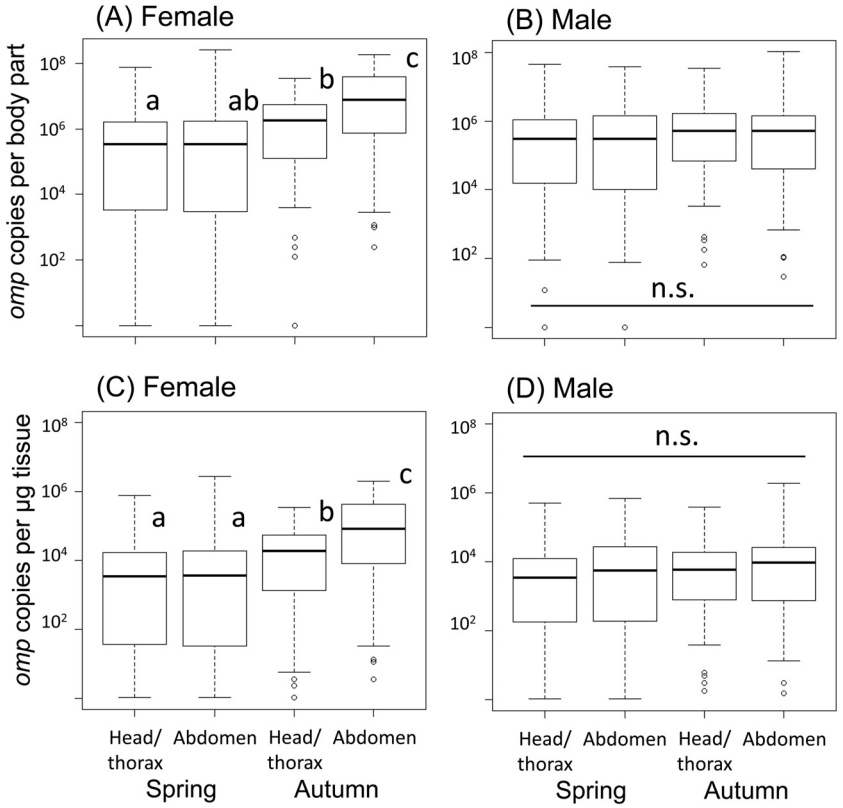

FIG 2 Comparison of "*Ca.* Liberibacter asiaticus" infection densities in *D. citri* insects between head-thorax and abdomen, females and males, and spring and autumn. (A and B) "*Ca.* Liberibacter asiaticus" infection densities per body part in head-thorax and abdomen of adult females (A) and males (B). (C and D) "*Ca.* Liberibacter asiaticus" infection densities per microgram of tissue in head-thorax and abdomen of adult females (C) and males (D). Box-and-whisker plots indicating the median (bold line), the 25th and 75th percentiles (box edges), the range (whiskers), and outliers, which are larger or smaller than 1.5 times the interquartile range from the box edge (open circles). Different lowercase letters indicate statistically significant differences ($P < 0.05$ after Tukey's multiple-comparison tests; n.s., not significant).

constantly as high as 10^6 to 10^7 *omp* copies per insect and declined to 10^5 to 10^6 *omp* copies per insect toward the end of May (Fig. 3A). In November, infection densities were constantly high, at 10^6 to 10^8 *omp* copies per insect (Fig. 3B).

Factors correlated with "*Ca.* Liberibacter asiaticus" infection density in *D. citri*. Table 1 shows ANOVA of candidate factors that may be statistically correlated with "*Ca.* Liberibacter asiaticus" infection densities: in spring, tree and date exhibited statistically significant correlations, as did sex, body site, and date in autumn. Statistical analysis of the respective factors revealed the following patterns: females exhibited significantly higher infection densities than males in autumn (Fig. 4A), the abdomen of females contained more "*Ca.* Liberibacter asiaticus" bacteria than did the head-thorax of females in autumn (Fig. 2A and C), insects collected from tree 3 exhibited significantly lower "*Ca.* Liberibacter asiaticus" densities than did those collected from the other

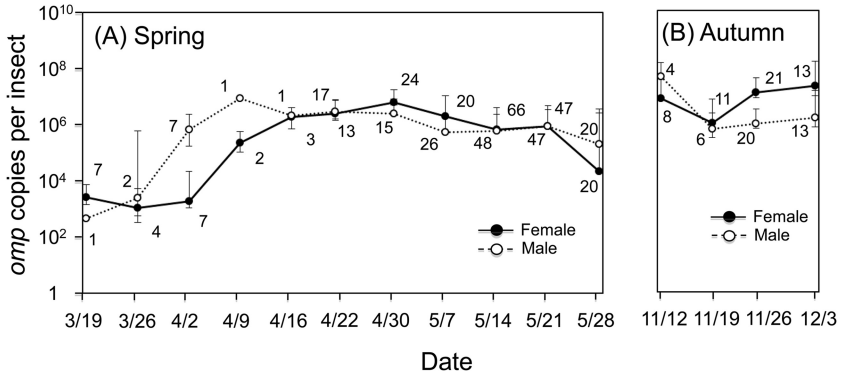

FIG 3 Seasonal dynamics of "*Ca.* Liberibacter asiaticus" infection densities in field-collected *D. citri* insects. (A) Spring of 2009. (B) Autumn of 2009. Means and standard deviations with sample sizes are shown for both adult females and males.

TABLE 1 Analysis of candidate factors correlated with "*Ca*. Liberibacter asiaticus" infection densities in *D. citri* insects collected from "*Ca*. Liberibacter asiaticus"-infected *C. depressa* trees[e]

Season and variable	df[a]	Sum of squares	RSS[b]	F value	*P* value[c]
Spring[d]					
Sex (male/female)	1	3.83	1,889.1	1.589	0.2078
Body (head-thorax/abdomen)	1	1.50	1,886.8	0.623	0.4303
Tree (tree 1/2/3/4)	3	38.07	1,923.3	5.270	0.0013**
Date	10	245.26	2,130.5	10.186	<0.0001***
Temp	1	4.50	1,889.8	1.867	0.1722
Autumn[d]					
Sex (male/female)	1	27.90	431.3	12.863	0.0004***
Body (head-thorax/abdomen)	1	11.47	414.9	5.289	0.023*
Date	3	37.71	441.1	5.796	0.0008***

[a] df, degree of freedom.
[b] RSS, residual sum of squares.
[c] Determined by an F test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[d] The data for spring and the data for autumn were separately analyzed because collective analysis resulted in internal error. The effect of temperature in autumn was unable to be estimated due to internal error, which is probably because of limited temperature differences in autumn.
[e] Bacterial densities per insect were analyzed by ANOVA.

trees (Fig. 4B), and significantly different infection densities were detected across the collection dates in both spring and autumn (Fig. 4C and D).

Rate of "*Ca*. Liberibacter asiaticus" transmission to healthy citrus seedlings by "*Ca*. Liberibacter asiaticus"-infected *D. citri*. Six months after the transmission trials with "*Ca*. Liberibacter asiaticus"-infected *D. citri* adults, the experimental *C. depressa* seedlings were examined by diagnostic PCR for the establishment of "*Ca*. Liberibacter asiaticus" infection. Of 500 pairs of inoculated seedlings, 15 died during maintenance, and thus, 485 plant pairs were analyzed (see Table S2 in the supplemental material). The transmission rates were 8.2% (32/389) in spring, 9.4% (9/96) in autumn, and 8.5% (41/485) in total, which exhibited no apparent seasonal patterns (see Table S2 in the supplemental material).

Relationship between "*Ca*. Liberibacter asiaticus" transmission and "*Ca*. Liberibacter asiaticus" infection density in *D. citri*. In spring, 32 insects transmitted "*Ca*. Liberibacter asiaticus" infection to seedlings, whereas 357 insects did not (see Table S2 in the supplemental material). The "*Ca*. Liberibacter asiaticus"-transmitting insects exhibited significantly higher infection densities than did the nontransmitting insects (Fig. 5A). In autumn, 9 insects transmitted "*Ca*. Liberibacter asiaticus" infection to citrus seedlings, whereas 87 insects did not (see Table S2 in the supplemental material). Between these insect groups, "*Ca*. Liberibacter asiaticus" densities exhibited no significant difference (Fig. 5B). In both spring and autumn, notably, the "*Ca*. Liberibacter asiaticus"-transmitting insects showed remarkably smaller density variances than did the nontransmitting insects (Fig. 5A and B). Of 41 insects that transmitted "*Ca*. Liberibacter asiaticus" infection to citrus seedlings, 26 insects transmitted it at the first trial only, 12 insects transmitted it at the second trial only, and 3 insects transmitted it at both trials. "*Ca*. Liberibacter asiaticus" infection densities exhibited no significant differences between them (Fig. 5C).

Factors correlated with rates of "*Ca*. Liberibacter asiaticus" transmission by *D. citri*. Table 2 shows F test results after GLM of candidate factors that may be statistically correlated with "*Ca*. Liberibacter asiaticus" transmission rates: in spring, sex, tree, and date exhibited statistically significant correlations, as did date in autumn. Statistical analysis of the respective factors revealed the

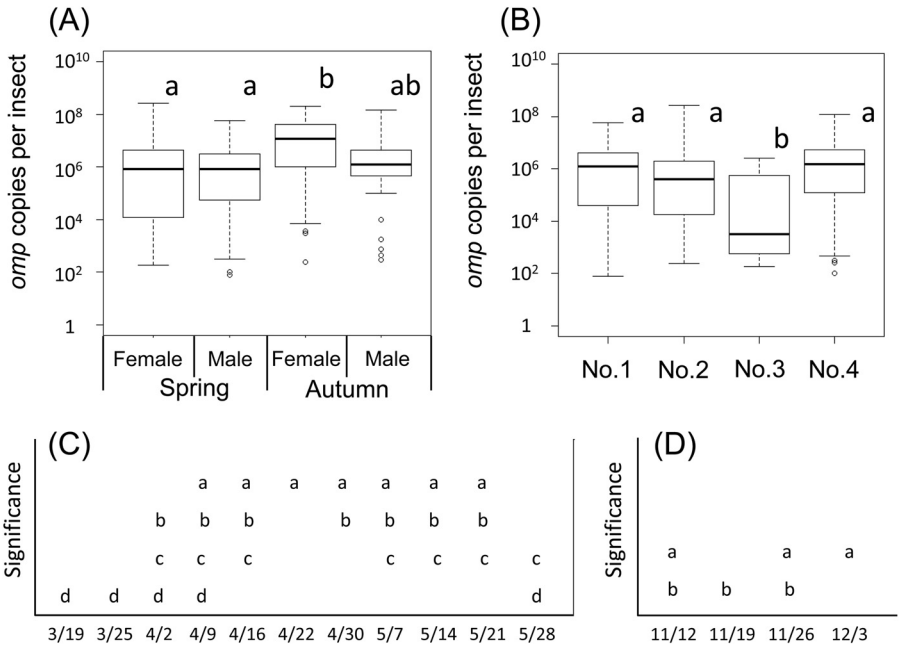

FIG 4 Factors correlated with "*Ca*. Liberibacter asiaticus" infection densities in *D. citri*. (A) Season and sex. (B) Trees. (C) Collection dates in spring. (D) Collection dates in autumn. In panels A and B, box-and-whisker plots are shown, as described in the legend of Fig. 2, where different lowercase letters indicate statistically significant differences ($P < 0.05$ after Tukey's multiple-comparison tests; n.s., no significant difference). In panels C and D, different lowercase letters indicate statistically significant differences ($P < 0.05$ after Tukey's multiple-comparison tests).

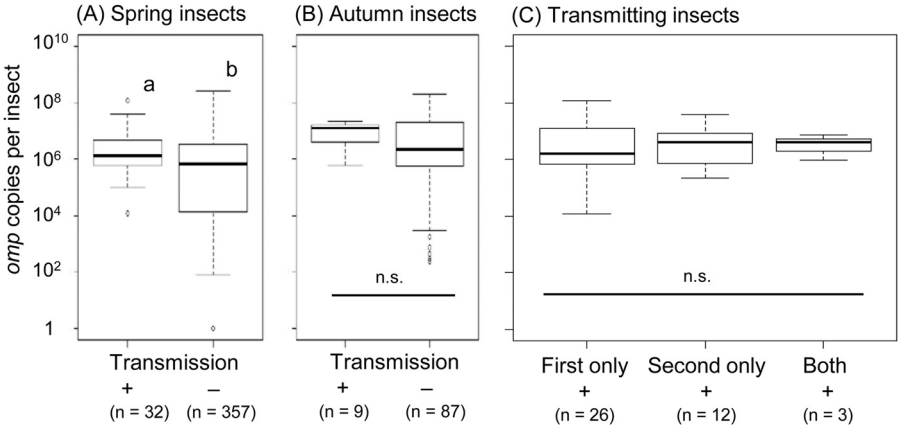

FIG 5 Relationship between "*Ca.* Liberibacter asiaticus" infection densities in *D. citri* and "*Ca.* Liberibacter asiaticus" transmission to *C. depressa* seedlings. (A) "*Ca.* Liberibacter asiaticus"-transmitting insects and nontransmitting insects in spring. (B) "*Ca.* Liberibacter asiaticus"-transmitting insects and nontransmitting insects in autumn. (C) Insects that transmitted "*Ca.* Liberibacter asiaticus" at the first trial only, at the second trial only, and at both trials consecutively. Box-and-whisker plots are shown, as described in the legend of Fig. 2, where different lowercase letters indicate statistically significant differences ($P < 0.05$ after Tukey's multiple-comparison tests; n.s., no significant difference).

following patterns: females exhibited significantly lower transmission rates than did males in spring, whereas, although statistically not significant, females exhibited higher transmission rates than did males in autumn (Fig. 6A); insects collected from tree 3 did not transmit "*Ca.* Liberibacter asiaticus," in contrast to transmission rates of ~10% by insects collected from the other trees, although the difference was not statistically significant (Fig. 6B); significantly different transmission rates were detected across the collection dates in spring (Fig. 6C); and, although statistically not significant, remarkably different transmission rates were observed across the collection dates in autumn (Fig. 6D).

DISCUSSION

Here we conducted a detailed and comprehensive investigation of the infection density dynamics of "*Ca.* Liberibacter asiaticus" in field *D. citri* populations, with special reference to the efficiency of "*Ca.* Liberibacter asiaticus" transmission to citrus plants. In this study, infection frequencies of "*Ca.* Liberibacter asiaticus" in the *D. citri* populations were very high, at 99.3% (401/404) in spring and 100% (96/96) in autumn (see Table S1 in the supplemental material), which is probably because we collected the insects from *C. depressa* trees that were heavily infected with "*Ca.* Liberibacter asiaticus" and had visible pathological symptoms (Fig. 1E and F).

"*Ca.* Liberibacter asiaticus" infection densities varied considerably, ranging from 10^3 to 10^8 *omp* copy equivalents, in the *D. citri* populations (Fig. 2 and 4). Quantitative PCR analysis of body parts revealed systemic "*Ca.* Liberibacter asiaticus" infection within the host body (Fig. 2; see also Fig. S3 in the supplemental material), as shown in previous studies (13, 14) and which is typical of facultative/parasitic bacterial associates of insects in general (36). Statistical analysis suggested several putative factors that may be correlated with "*Ca.* Liberibacter asiaticus" infection densities in *D. citri* populations (Table 1). For example, infection densities were significantly lower in the insects collected from tree 3 than in the insects collected from the other trees (Fig. 4B), which may be due to different "*Ca.* Liberibacter asiaticus" infection densities and/or physiological conditions of the specific host tree. Infection densities in mid- and late April were significantly higher than infection densities in March and late May (Fig. 4C), which may reflect a higher nutritional quality of shooting citrus buds in April and consequent "*Ca.* Liberibacter asiaticus" proliferation in the citrus plant and/or in the host insect. Particularly in females, infection densities tended to be higher in autumn than in spring, and in autumn, infection densities were higher in the abdomen than in the head-thorax (Fig. 2A and C), which may be relevant to the high reproductive activity of adult females in autumn. It should be noted that, although not inspected in this study, other factors may also influence "*Ca.* Liberibacter asiaticus" infection densities in *D. citri*. For example, considering that *D. citri* harbors two bacteriome-associated obligate endosymbionts (*Carsonella* and *Profftella*) and other facultative endosymbionts (*Wolbachia* and *Arsenophonus*, etc.) (37–41), it is conceivable, although speculative, that these coexisting endosymbionts may also affect "*Ca.* Liberibacter asiaticus" infection densities in *D. citri* populations. Host plants may also influence "*Ca.* Liberibacter asiaticus" infection densities in *D. citri*. Previous studies reported that "*Ca.* Liberibacter asiaticus" densities are often remarkably variable not only

TABLE 2 Analysis of candidate factors correlated with "*Ca.* Liberibacter asiaticus" transmission by *D. citri* collected from "*Ca.* Liberibacter asiaticus"-infected *C. depressa* trees[d]

Season and variable	df[a]	Deviance	F value	*P* value[b]
Spring[c]				
Sex (male/female)	1	398.64	15.6775	<0.0001***
Body (head-thorax/abdomen)	1	390.60	0.0000	1.000
Tree (tree 1/2/3/4)	3	397.99	4.8072	0.0025**
Date	10	425.95	6.8963	<0.0001***
Autumn[c]				
Sex (male/female)	1	107.11	1.7814	0.1836
Body (head-thorax/abdomen)	1	106.10	0.0000	1.0000
Date	3	118.39	7.1852	0.0001***

[a] df, degree of freedom.
[b] Determined by an F test after GLM. **, $P < 0.01$; ***, $P < 0.001$.
[c] The data for spring and the data for autumn were separately analyzed because collective analysis resulted in internal error. The effect of temperature was unable to be estimated due to internal error, which is probably because of limited temperature differences.
[d] Transmission rates were analyzed by an F test after GLM.

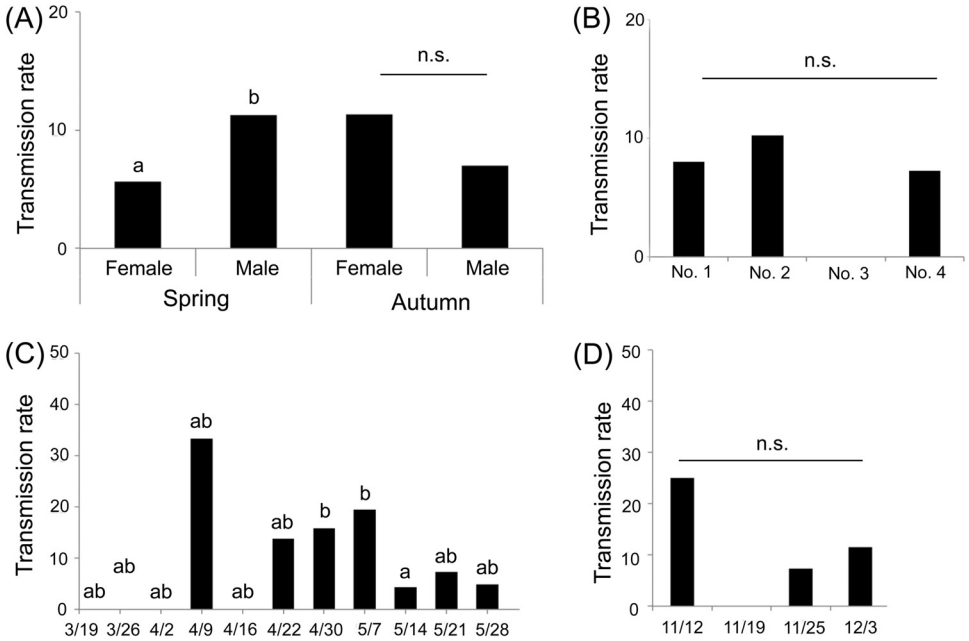

FIG 6 Factors correlated with "*Ca*. Liberibacter asiaticus" transmission rates in *D. citri*. (A) Season and sex. (B) Trees. (C) Collection dates in spring. (D) Collection dates in autumn. Different lowercase letters indicate statistically significant differences ($P < 0.05$ after Tukey's multiple-comparison tests; n.s., no significant difference).

among different citrus trees but also among different plant parts (shoots, leaves, and twigs, etc.) within the same tree (20, 21). Hence, our selection of citrus plants as well as citrus shoots from which the newly emerged adults were collected by a bag-covering method might have affected the "*Ca*. Liberibacter asiaticus" densities determined in this study. Although we detected "*Ca*. Liberibacter asiaticus" from plant samples by conventional PCR in this study, adoption of quantitative PCR for the plant samples could have enabled more sensitive "*Ca*. Liberibacter asiaticus" detection and consequently deeper insights into the relationship between intraplant and intravector pathogen densities. Taken together, these results suggest that a number of biotic and abiotic factors in the environment are relevant to the infection levels of "*Ca*. Liberibacter asiaticus" in field populations of *D. citri*.

In this study, we mainly analyzed the "*Ca*. Liberibacter asiaticus" infection densities in terms of "*Ca*. Liberibacter asiaticus" gene copies per individual, while the "*Ca*. Liberibacter asiaticus" densities normalized to body weight did not affect the observed patterns (Fig. 2C and D; see also Fig. S4 in the supplemental material). Here we did not adopt the "*Ca*. Liberibacter asiaticus" densities normalized to the host insect gene copy number, considering that the insect gene copy number generally exhibits drastic changes, often up to 3-fold, during the adult life span, which is particularly conspicuous between actively reproducing females and pre- or postreproduction females (42, 43).

The efficiency of transmission of "*Ca*. Liberibacter asiaticus" to citrus plants by infected *D. citri* insects is among the critical parameters that facilitate HLB prevalence and spread (3, 10–12). It is expected that *D. citri* insects heavily infected with "*Ca*. Liberibacter asiaticus" may transmit the pathogen to citrus plants more efficiently, which was experimentally confirmed by a previous study using excised citrus leaf preparations in the laboratory (18). Overall, our results also supported this expectation in field *D. citri* populations. In spring, infection densities in "*Ca*. Liberibacter asiaticus"-transmitting insects were significantly higher than those in nontransmitting insects (Fig. 5A). Although infection densities were not significantly different between the "*Ca*. Liberibacter asiaticus"-transmitting insects and the nontransmitting insects in autumn, it is should be noted that in both spring and autumn, infection densities in the "*Ca*. Liberibacter asiaticus"-transmitting insects exhibited remarkably narrower variance than did those in the nontransmitting insects (Fig. 5A and B). These patterns are best explained by assuming that (i) "*Ca*. Liberibacter asiaticus" infection density exhibits a large variance in field *D. citri* populations, (ii) a certain level of "*Ca*. Liberibacter asiaticus" infection density in *D. citri* is required for successful transmission to citrus plants, and (iii) *D. citri* insects attaining the threshold infection level transmit *Liberibacter* to citrus plants not deterministically but stochastically. This "threshold and stochasticity" hypothesis also seems concordant with the observations that infection densities in the insects that consistently transmitted *Liberibacter* to citrus seedlings in two consecutive infection trials were not significantly different from those in the insects that transmitted the pathogen in only one of the two consecutive infection trials (Fig. 5C). Judging from the data shown in Fig. 5, the threshold infection density may be $\sim 10^6$ "*Ca*. Liberibacter asiaticus" gene copies per insect. On account of the variance-narrowing patterns, it seems plausible that not only the insects with low-level infection but also the insects with too much infection transmit "*Ca*. Liberibacter asiaticus" less efficiently, which, although speculative, may be due to deleterious effects of excessive infection on the performance of the host insects. It should be kept in mind that although "*Ca*. Liberibacter asiaticus" infection densities were measured for the whole head-thorax and abdomen in this study (Fig. 2; see also Fig. S3 in the supplemental material), more localized infection densities, such as those in the salivary glands, may be critical for suc-

cessful "*Ca.* Liberibacter asiaticus" transmission to citrus plants (13, 14). Also, it should be noted that the "*Ca.* Liberibacter asiaticus" density measurements by quantitative PCR were conducted 3 to 4 weeks after collection from field citrus trees, and therefore, the "*Ca.* Liberibacter asiaticus" densities determined in this study may reflect, but not strictly agree with, the initial "*Ca.* Liberibacter asiaticus" densities in the field. Plausibly, the density-transmission correlation observed under controlled conditions in the laboratory (18) must be obscured in this study to some extent, because a number of confounding factors inevitably affect the insects collected from field populations. Statistical analysis suggested that several environmental factors may affect "*Ca.* Liberibacter asiaticus" transmission efficiencies in *D. citri* populations (Table 2 and Fig. 6).

In this study, the rate of transmission of "*Ca.* Liberibacter asiaticus" by infected *D. citri* adults to healthy *C. depressa* seedlings was experimentally estimated to be 8.5% (41/485 seedlings tested) under the conditions of a single adult per seedling with an inoculation access period of 2 weeks (see Table S2 in the supplemental material), which is equivalent to "*Ca.* Liberibacter asiaticus" transmission rates reported in previous studies under similar inoculating conditions: 5.0% (12/241 seedlings tested) under conditions of a single adult per *Citrus sinensis* seedling with an inoculation access period of 1 to 24 days (17) and 3.6% (15/422 seedlings tested) under conditions of a single adult per *C. sinensis* seedling with an inoculation access period of a week (18). In the field, however, the low level of "*Ca.* Liberibacter asiaticus" transmission by each *D. citri* individual is presumably summed up on citrus trees that are infested by hundreds of *D. citri* individuals, thereby attaining high "*Ca.* Liberibacter asiaticus" infection rates of both the citrus plants and the vector insects (see Table S1 in the supplemental material). This perspective is concordant with previous reports of high "*Ca.* Liberibacter asiaticus" transmission rates under inoculation conditions of many *D. citri* adults per citrus seedling: 66.7% (4/6 seedlings tested) under conditions of three adults per *Citrus junos* seedling with an inoculation access period of 30 days (16) and 73.3% (11/15 plants tested) under conditions of 200 adults per *C. sinensis* plant with an inoculation access period of 30 days (17).

In conclusion, this study provides a detailed and comprehensive description of the infection density dynamics of the citrus pathogen "*Ca.* Liberibacter asiaticus" in field populations of the Asian citrus psyllid *D. citri*. Experimental studies using field-collected "*Ca.* Liberibacter asiaticus"-infected insects and uninfected citrus seedlings suggest that infection levels in vector insects have substantial relevance to the pathogen transmission efficiency. These findings provide valuable insights into our understanding, prediction, and control of this notorious citrus pathogen that is causing serious agricultural problems worldwide. An understanding of the density-transmission correlation is epidemiologically important not only for "*Ca.* Liberibacter asiaticus" but also for insect-vectored plant pathogens in general, such as "*Ca.* Liberibacter solanacearum" (44), *Spiroplasma citri* (45), *Spiroplasma kunkelii* (46), "*Ca.* Phytoplasma" spp. (47), *Xylella fastidiosa* (48), and many others, which should be pursued in future studies.

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