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Nested-quantitative PCR approach with improved sensitivity for the detection of low titer levels of *Candidatus* Liberibacter asiaticus in the Asian citrus psyllid, *Diaphorina citri* (Kuwayama)

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A B S T R A C T

Candidatus Liberibacter asiaticus (*C*Las) is a phloem-limited bacterium transmitted by the Asian citrus psyllid, *Diaphorina citri*. It is the presumptive causal agent of citrus greening disease, also known as huanglongbing (HLB). A critical component of HLB management is the detection of *C*Las within plant and insect samples to avoid the transport of infected material and to monitor the spread of *C*Las through citrus groves and psyllid populations. The current method of surveillance is by a presence/absence qPCR assay which utilizes C_q cutoff values to categorize samples as positive, negative, or inconclusive, using the *C*Las *16S* rDNA gene sequence. Upon reviewing the results from routine monthly assays to monitor infection rates in a *C*Las-positive psyllid colony, it was noted that a large proportion of negative results were being obtained. This observation, coupled with the disjunction between the reported low transmission efficiency of *C*Las by the psyllid vs. how rapidly HLB has spread, led us to suspect that false-negatives were being obtained using the *16S* presence/absence qPCR assay. To investigate this possibility, we developed a nested-qPCR assay with increased sensitivity for *C*Las. Using this method, we found that the false-negative rate of the presence/absence qPCR assay was greater than 50%, confirming our suspicion and demonstrating that psyllids can harbor titers of *C*Las below the detection limit of the assay. To investigate if poor qPCR efficiency could be contributing to the high rate of false-negatives, we conducted studies to determine the amplification efficiency, sensitivity, dynamic range and reproducibility of the qPCR assays for *C*Las *16S* rDNA and *Wingless* (*Wg*), the *D. citri* endogenous gene, using plasmid and psyllid DNA templates. Average efficiencies and sensitivity limits of the plasmid assays were 99.0% and 2.7 copies of template for *Wg*, and 98.5% and 2.2–22.1 copies for *16S*. Variability in efficiency was significantly greater in psyllid samples for both gene targets compared to the corresponding plasmid assays. Efficiencies as low as 76% were obtained for *16S*, suggesting that the sensitivity limit of the assay could be significantly compromised, especially for low-abundant templates. A secondary structure analysis revealed the formation of two stem-loop structures that block the forward and probe binding sites, which could, in part, explain the suboptimal qPCR efficiency of the *16S* assay. In summary, our results suggest that suboptimal qPCR efficiency is not uncommon for the *16S* presence/absence qPCR assay, which combined with low-abundant *C*Las template in some samples, likely contributes significantly to the under-reporting of *C*Las infection in psyllid and plant samples.

1. Introduction

Quantitative PCR is an exquisitely sensitive method that, under optimal conditions, can detect a few molecules of template among a complex background of nucleic acids. This sensitivity combined with the high-throughput and cost-effective nature of qPCR, has made it a standard method for diagnostic screening for the presence of disease using characteristic molecular markers associated with disease states, such as cancer (e.g., Hochhaus et al., 2000). This method is also highly amenable for the detection of disease-causing agents, from viruses to nematodes, and is used regularly to identify these agents in human and plant tissue samples. Examples include the detection of hepatitis C in humans (Santos et al., 2012) and cyst nematodes in potato (Christoforou et al., 2014). Despite the high sensitivity of qPCR, it does have limitations. If the copy number of the template is too low within the sample, or the qPCR reaction is not optimal, adequate amplification may not occur so that the signal from the amplicon is detected above the background fluorescence threshold. In these cases, false-negatives are generated. An approach to increase the sensitivity of qPCR to circumvent these problems, and thus reduce the rate of false-negatives, is to pre-amplify the gene target using conventional PCR, and use the resulting amplicon as template in qPCR. Known as nested-qPCR, this

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method has been used to detect low titers of disease-causing agents in humans and plants, such as the detection of *Mycobacterium tuberculosis* in humans (Takahashi and Nakayama, 2006) and *Phytophthora cinnamoni*, the fungus that causes *Phytophthora* root rot in avocado (Engelbrecht et al., 2013).

Microbial plant pathogens, such as viruses, bacteria and trypanosomes, are often transmitted by Hemipteran insect vectors that feed on plant tissue (reviewed in Mitchell, 2004). *Diaphorina citri* (Kuwayama), the Asian citrus psyllid, transmits *Candidatus* Liberibacter asiaticus (*C*Las),[1] a phloem-limited, gram-negative α-proteobacteria and the presumptive causal agent of citrus greening disease (Huanglongbing, HLB).[2] Huanglongbing is a devastating disease of citrus, causing small, asymmetrical, bitter fruit, aborted seeds, and rapid tree decline (Bové, 2006). At this time, no cure exists, and vector control is a main component of HLB management (Grafton-Cardwell et al., 2013). Rapid screening for *C*Las in psyllid and plant material is imperative in the efforts to control the spread of HLB through inadvertent activities, and qPCR was quickly adopted for the detection of *C*Las in quarantine and field samples. In 2006, Li et al. published a qPCR assay for the detection of the bacterium in plant and insect samples using the *C*Las *16S* rDNA gene sequence, and *Wingless* and *Cytochrome Oxidase I* for the detection of psyllid and citrus DNA, respectively. This protocol was then adapted and implemented by the Animal and Plant Health Inspection Service, US Department of Agriculture (APHIS, USDA) to screen vast numbers of quarantine specimens. APHIS publishes a guideline for conducting the high-throughput, standardized presence/absence qPCR assay using cutoff C_q values to categorize samples as positive, negative or inconclusive (USDA, 2012). Through routine monthly screening to determine infection rates of a *C*Las-positive psyllid colony using the APHIS presence/absence qPCR assay, it was noted that a large proportion of samples were classified as negative or inconclusive, up to 65% and 20% of the total, respectively. Inspection of the amplification plots of the negative samples revealed that some were starting log-phase amplification at the end of the qPCR routine (Fig. A1A). The replicates for these samples were tightly overlaid on one another, suggesting the potential amplification of genuine templates rather than stochastic background noise (Fig. A1B). Other samples crossed the threshold with tight replicates, but did so too late to be considered positive based on APHIS guidelines and were considered negative (Fig. A1C), or inconclusive (Fig. A1D). These observations, coupled with the disjunction between the reported low efficiency by which the psyllid transmits *C*Las (Pelz-Stelinski et al., 2010) vs. the widespread infection rate of citrus with *C*Las in Florida (Gottwald, 2010), led us to suspect that false-negative reporting could be high using the APHIS presence/absence *16S* qPCR assay.

Currently, there are no published methods regarding the low-titer detection of *C*Las in either insect or plant tissue, and the lack of a highly sensitive *C*Las detection method may affect disease containment strategies, as well as hinder research efforts (Pelz-Stelinski et al., 2010). Here we report a method to detect low titers of *C*Las within *D. citri*, using the *16S* rDNA gene target, with a nested-qPCR approach. In this study, we describe the development and validation of the nested-qPCR method, determine the false-negative rate of the presence/absence qPCR assay, report on the efficiency, dynamic range and sensitivity of the qPCR *16S* and *Wingless* qPCR assays using plasmid and *D. citri* DNA templates, and provide evidence that suboptimal qPCR efficiency and secondary structure of template may be contributing to the false-negative reporting of *C*Las.

2. Materials and methods

2.1. *Candidatus Liberibacter asiaticus* — *D. citri* colony

D. citri harboring *Candidatus* Liberibacter asiaticus (*C*Las) were obtained from *C*Las-infected *Citrus aurantium* plants maintained in a secure quarantine facility without exposure to insecticides in a greenhouse at 27–28 °C, 60–65% RH, and L14:D10 photoperiod. Monthly sampling conducted throughout 2013 showed that about 50% of *D. citri* individuals obtained from the colony tested positive for *C*Las according to APHIS guidelines (USDA, 2012). Citrus plants were infected with *C*Las by graft-inoculation of healthy 'Valencia' *C. aurantium* with infected budwood collected from commercial citrus groves in Immokalee, FL (Collier Co.). Infection was verified via qPCR as described in Pelz-Stelinski et al. (2010).

2.2. DNA isolation from *D. citri*

Genomic DNA was isolated from individual psyllids using the QIAGEN DNeasy Blood & Tissue Isolation Kit (69506; Valencia, CA) using the protocol for "Animal Tissues" with modifications. Briefly, per psyllid, 180 μL of ATL buffer and 20 μL of Proteinase K were premixed and 20 μL of the mixture was added to an individual psyllid in a 1.5 mL tube and crushed with a disposable pestle (Kontes, K749520-0090). Another aliquot of 180 μL of the mixture was then added to the psyllid and homogenized with a handheld homogenizer for 5–10 s (Kontes, K749540-0000). Homogenized samples were vortexed for 15 s and placed in a rocking incubator set at 56 °C overnight. The next day, 200 μL of AL buffer was added to each tube and vortexed. Mixtures were then incubated at 70 °C for 10 min. After incubation, 230 μL of 100% molecular grade ethanol was added to each sample and vortexed. The mixture was applied to a Spin Column and centrifuged at 16,000 ×*g* for 1 min. The column was washed with 500 μL AW1 and then 500 μL AW2. An additional centrifugation step at 16,000 ×*g* for 1 min was conducted to pull off residual ethanol prior to DNA elution with 35 μL of AE buffer (10 mM Tris, 0.5 mM EDTA, pH 9.0). Eluted DNA was quantified spectrophotometrically using a NanoDrop 2000 (ThermoScientific, Wilmington, DE). To reduce risk of cross-contamination, all surfaces were treated with DNase/RNase solution (Eliminase, 04-355-32) and disposable sterile, DNase–RNase-free microcentrifuge tubes and filter tips were used for this and all subsequently described procedures.

2.3. *Candidatus Liberibacter asiaticus 16S rDNA sequence and primers*

GenBank entry DQ778016.1 was used to design primers for the *16S* rDNA plasmid for the qPCR standard curve (CLas-16S-Temp-F1 and CLAS-16s-Temp-R1, Table 1; Fig. A2) and the external set of primers for nested-qPCR (CLas-16S-Ex-F1 and CLas-16S-Ex-R1, Table 1; Fig. A2). Upon inspection of this GenBank entry and subsequent BLASTn searches, it was noted that the forward qPCR primer designed by Li et al. (2006) contained several mismatches in the forward primer at the 3′ end due to a gap in the original sequence (GenBank: L22532.1). This mismatch was verified upon sequencing of the plasmids for standard curves (see Section 2.5) and was corrected from 5′ TCGAGCGCGTATGCAATACG 3′ to 5′ TCGAGCGCGTATGCGAATAC 3′ (Fig. A2). All primers were designed using Primer3 *v.* 0.4.0 (Untergrasser et al., 2012).

2.4. *D. citri Wingless sequence and primers*

To obtain a full-length *Wingless* (*Wg*) gene sequence for use as a template to generate a plasmid for the qPCR standard curve, the partial sequence for the *Wingless* gene (*Wg*) from *D. citri* (GenBank: AF231365.1) was retrieved from NCBI and used as a BLASTn query against a downloaded and formatted database of the draft *D. citri* genome (*v.* 1.0; February, 2013) using default parameters. The top hit was found in scaffold 5281.1 and shared 98% identity with the query sequence, with mismatches being ambiguous nucleotides in the query. The scaffold which contained the top hit was retrieved from the database and the *Wg* coding sequence (CDS) was determined using a BLASTx search in the NCBI nr database. Forward and reverse primers (Wng-Temp-F1 and Wng-Temp-R1, Table 1, Fig. A3) were designed using Primer3 (Untergrasser et al., 2012) to generate a *Wg* plasmid for the qPCR standard curve as described in Section 2.5.

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[1] CLas: *Candidatus* Liberibacter asiaticus.

[2] HLB: Huanglongbing.

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Table 1
Sequences and details of primers and probes used in this study.

Primer/probe	Sequence (5′–3′)	Target gene	Amplicon size (bp)	Purpose
CLas-16S-Temp-F1	TGACGTTGGAAGATGTTTGTAGC	CLas 16S rRNA	1402	Standard curve template
CLas-16S-Temp-R1	GCCCGAGAACGTATTCACC	CLas 16S rRNA	1402	Standard curve template
CLas-16S-Ex-F1	TGACGTTGGAAGATGTTTGTAGC	CLas 16S rRNA	263	External nested set
CLas-16S-Ex-R1	ACGCAGGCTCATCTCTCTCC	CLas 16S rRNA	263	External nested set
HLBas_c[a,b]	TCGAGCGCGTATGCGAATAC	CLas 16S rRNA	79	qPCR
HLBr[a]	GCGTTATCCCGTAGAAAAAGGTAG	CLas 16S rRNA	79	qPCR
HLBp[a]	AGACGGGTGAGTAACGCG	CLas 16S rRNA	79	qPCR
Wg-Temp-F1	TTTACTGCCATGATTGTGAACC	Wingless	638	Standard curve template
Wg-Temp-R1	GGATCAATAGGCACAGTTCTGG	Wingless	638	Standard curve template
WgF[c]	GCTCTCAAAGATCGGTTTGACGG	Wingless	69	qPCR
WgR[c]	GCTGCCACGAACGTTACCTTC	Wingless	69	qPCR
WgP[c]	TTACTGACCATCACTCTGGACGC	Wingless	69	qPCR

[a] Li et al. (2006).
[b] The HLB primers designed by Li et al. (2006) were based on GenBank: L22532.1, which contains ambiguous and miscalled nucleotides. As a result, the original forward primer contains gap against its cognate target. HLBas_c primer reflects the corrected sequence. The "_c" is to denote this correction and distinguish it from the original forward primer, HLBas.
[c] Pelz-Stelinski et al. (2010).

2.5. Molecular cloning and sequencing of the CLas 16S rDNA and Wg plasmids

To generate plasmids for qPCR standard curves, genomic DNA was isolated from five adult *D. citri* individuals as described above and used as templates in PCR using TaKaRa *Taq* recombinant Polymerase (Clontech, R001A; Mountain View, CA). Primer, dNTP and $MgCl_2$ concentrations were 0.5 μM, 200 μM and 1.5 mM, respectively. For the CLas *16S* plasmid, the following PCR scheme was used: two cycles of 94 °C for 90 s, 63 °C for 45 s, and 72 °C for 90 s followed by 34 cycles of 94 °C for 30 s, 63 °C for 45 s, and 72 °C for 90 s, with a final extension time of 72 °C for 5 min. For *Wg*, the same scheme was used except for the annealing temperature and extension time, which were 60 °C and 40 s, respectively. PCR reactions were run through a 1.0% agarose gel and all reactions resulted in a single amplicon of the correct size (Table 1). Amplicons were gel purified using Promega's Wizard® SV Gel and PCR Clean-Up System (A9281; Madison, WI) and were ligated into the cloning vector pGEM-T Easy (A1360). JM109 *Escherichia* cells were transformed with ligation reactions and plated using standard microbial protocols. Two bacterial colonies for each of *16S* and *Wg* cloning reactions were selected for overnight growth in 3 mL LB/Amp and plasmids were isolated using Promega's Wizard Plus Miniprep Kit (A7510). The plasmids were submitted to the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR; Gainesville, FL) for Sanger sequencing from both directions using SP6 and T7 universal primers. Sequences were aligned using CLUSTALX 2.1 (Larkin, et al., 2007) and inspected for mismatches, which were verified by inspecting respective chromatograms. The two *16S* sequences from the plasmids were 99% identical to one another and to the template GenBank entry DQ778016.1 (Table A1, Fig. A2). Sequences from both *Wg* plasmids were identical to another and to scaffold 5281.1, with a representative sequence submitted to GenBank under accession number KF876339 (Fig. A3). All inserts were oriented in the pGEM-T Easy vector such that the *Pst*I restriction enzyme site used to linearize the plasmids was located at the 3′ end.

2.6. Quantitative PCR

Quantitative PCR was conducted with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) based on a multiplex qPCR assay described previously with modifications (Pelz-Stelinski et al., 2010; USDA, 2012). Briefly, the *Wingless* gene from *D. citri* was used as an endogenous control for psyllid DNA and *16S* rDNA was used for the detection of *Candidatus* Liberibacter asiaticus. The qPCR reaction volume was 20 μL containing 0.1 μM of each primer and probe (Table 1) in 1× *Taq*Man Universal PCR Master Mix containing ROX™ as the passive internal reference dye (Applied Biosystems, 4304437). The probe for *Wg* was labeled with 4,5-dichloro-dimethoxy-fluorescein (JOE™) and *16S* with 6-carboxyfluorescein (FAM™). Standards and samples were added to individual wells of the qPCR plate in a 2 μL volume and 18 μL of the master mix was added over the sample to wash the entire mixture to the bottom of the well. Other parameters, such as amount of template, are indicated in relevant sections below. All qPCR reactions were performed in triplicate except in the APHIS presence/absence screening assays to identify negative and positive samples, which were performed in duplicate. All plates included a non-template control in triplicate with 2 μL Qiagen Elution Buffer (EB, 10 mM Tris, pH 8.5). Plates were prepared in a sterile, biological PCR laminar flow cabinet. The qPCR scheme for all qPCR reactions was 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, with fluorescence data collected at the last step. Sample amplification plots were inspected using the Applied Biosystems 7500 software (*v.* 2.0.1) and raw data were exported as a tab-delimited file and analyzed in Microsoft Excel (2013).

2.7. Validation of external primers for nested-qPCR and verification of the specificity of the nested-qPCR 16S amplicon

Samples used to test the external set of primers for the nested-qPCR assay were selected from psyllid DNA isolates collected in September 2013. These samples were isolated from a CLas-positive colony to estimate the rate of infection for routine monthly screening, and had been categorized as positive ($C_q < 32$), negative ($C_q > 37$ or undetected), or inconclusive ($32 \leq C_q \geq 37$) based on APHIS guidelines (USDA, 2012) with a presence/absence qPCR assay (see Results section). Eleven samples were selected for initial testing: four positive, two inconclusive, and five negative. Out of the negative samples, one had a C_q value too high to be considered positive (39.04) while the other four had no amplification signal (undetected, UD). Using 1 μL of each sample, pre-amplification was conducted with conventional PCR using the primers (CLas-16S-Ex-F1 and CLas-16S-Ex-R1, Table 1) with the following PCR routine: two cycles of 94 °C for 90 s, 62 °C for 45 s, and 72 °C for 20 s followed by 34 cycles of 94 °C for 30 s, 62 °C for 45 s, and 72 °C for 20 s, with a final extension time of 72 °C for 3 min. Non-template control (NTC) contained all components of the reaction with 1 μL of EB used in place of DNA template. As a negative control for non-specific amplification, genomic DNA isolated from the sharpshooter, *Cuerna costalis*, was used as a template. Following PCR, samples were diluted 1:5, including the NTC from conventional PCR, and 2 μL of each dilution was used as a template in nested-qPCR with the thermocycling routine as described above. Following nested-qPCR, amplicons from positive reactions were purified using a Qiagen MinElute Reaction Cleanup Kit (Qiagen, 28204) and TA-cloned into pGEM T-Easy as described in section 2.5.

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Plasmids were submitted for Sanger sequencing at the University of Florida ICBR Genomics Core (Gainesville, Florida) using the T7 universal primer. Sequences were aligned and evaluated with CLUSTALX 2.1 (Larkin, et al., 2007).

2.8. Optimization of nested-qPCR

Because the 1:5 dilution resulted in very low C_q values, the optimal dilution factor was determined by diluting conventional PCR reactions from two samples; one positive and one negative from the qPCR presence/absence assay, in a ten-fold serial dilution from 10^{-1} to 10^{-8} and used as templates in nested-qPCR. A positive sample was run to estimate the lower C_q boundary of the range in each dilution series that might be observed. It was determined that a dilution of 10^{-2} to 10^{-3} produced average C_q values of 4.1 and 6.7, and 13.9 and 17.3, for positive and negative samples, respectively, from the initial qPCR screening assay (Fig. A4). Although the dilution of 10^{-2} still resulted in low C_q values for the positive sample, the 10^{-2} dilution produced C_q values high enough to allow the instrument to accurately set baseline fluorescence, and thus was adopted in the subsequent assay for determining an estimate for false-negative rates.

2.9. Estimates of the false-negative error rate in the 16S presence/absence qPCR assay

DNA isolated from psyllids for routine monthly screening for infection rate of a *C*Las-positive colony was used to determine the rate of false-negatives in the presence/absence qPCR assay as compared to the nested-qPCR assay. DNA samples from five months were analyzed, September through November, 2013; and January through February, 2014. Twenty psyllids were screened for each month except for November, for which 18 psyllids were screened. An average of 65 ng was used in a presence/absence qPCR assay to determine initial estimates for *C*Las infection. One microliter of from each negative sample was used in conventional PCR as described above. Reactions were diluted 1:1000, including the NTC from the conventional PCR, and 2 μL of each dilution was used as template in nested-qPCR.

2.10. Performance evaluation of the Wg and 16S qPCR assays

To investigate the efficiency and sensitivity of the *Wg* and *C*Las *16S* *Taq*Man qPCR assays, calibration curves were generated from serial dilutions of linearized *Wg* and *16S* plasmids. One microgram of each plasmid was digested with *Pst*I restriction endonuclease (Promega, R6111) in a 20 μL reaction volume and subsequently purified using a MinElute Reaction Cleanup Kit (Qiagen, 28204). Samples were eluted in 10 μL EB. Quantification of purified, linearized plasmids was conducted spectrophotometrically with a NanoDrop 2000. Two microliters of each purified plasmid was run through a 1.0% agarose gel to confirm complete digestion and the presence of only one *Pst*I restriction endonuclease recognition site within each plasmid. Seven, ten-fold serial dilutions were made with each plasmid ranging from 5 pg/μL to 5 ag/μL. The amplification efficiency of *Wg* and *16S* was also determined for genomic DNA isolated from individual psyllids using four, ten-fold serial dilutions ranging in concentration of 20 ng/μL to 20 pg/μL. To reduce potential effects on efficiency due to a buffer concentration gradient across dilutions, EB was used make serial dilutions. Five independent calibration curves were generated for each plasmid, and 15 *Wg* and *16S* sample curves were generated from DNA isolated from independent psyllids previously been shown to be positive for CLas. Averaged raw C_q data were plotted against log input DNA (log pg or log ng) to determine slope of each regression line. Quantitative PCR efficiencies were calculated using the basic equation: $\text{efficiency} = -1 + 10^{(-1/\text{slope})}$. Molecular weights of plasmids were calculated using Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/dna_mw.html). An analysis of deviance was conducted to determine statistical differences between plasmid and sample calibration curves using GenStat, 15th Edition, Vsn International.

2.11. Secondary structure prediction of Wg and 16S qPCR amplicons

Predictions of secondary structures of *Wg* and *16S* amplicons were determined using the DINAMelt Web Server (http://mfold.rna.albany.edu/?q=DINAMelt; Markham and Zuker, 2005) using the following parameters: Molecule type, DNA; temperature, 60 °C; $[Na^+]$, 50 mM; and $[Mg^{2+}]$, 1.5 and 4.0 mM. The concentration of magnesium is proprietary in the *Taq*Man™ Universal Master Mix but is reported as being between 1.5 and 4.0 mM, thus secondary structure was determined at these two concentrations.

3. Results

3.1. Validation of external primers for nested-qPCR and verification of the specificity of the nested-qPCR 16S amplicon

DNA samples from 20 psyllids collected in September 2013 from a *C*Las positive colony were evaluated for *C*Las in a presence/absence qPCR assay. Results were categorized as positive, negative or inconclusive according to APHIS guidelines (USDA, 2012) as described above (Fig. 1 and Table A2). The C_q values obtained for the endogenous control, *Wg*, for all 20 samples ranged between 22.8 and 24.3 with an average of 23.5 (SD 0.4). No amplification signals were detected for *Wg* or *16S* in the non-template control (NTC). Out of 20 samples, 11 were positive for *16S*, with C_q values ranging from 18.3 to 26.9, and four samples were classified as inconclusive (C_q 32.1, 34.3, 35.5 and 36.2). Five samples were negative, four that were undetected and one with a C_q value too high to be considered inconclusive (39.0). Based on the presence/absence qPCR assay, the breakdown of infection was: 55% positive, 20% inconclusive, and 25% negative (Fig. 1). From this screening, eleven

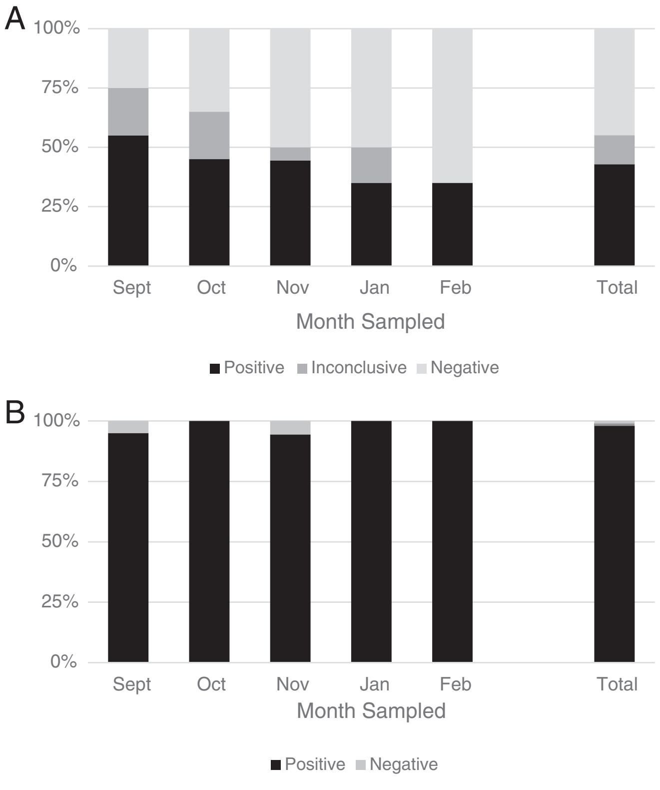

Fig. 1. *C*Las infection rate of psyllids surveyed by month as determined by A. positive/negative qPCR. Positive = $C_q \leq 32$; inconclusive $32 < C_q < 37$; negative $C_q > 37$; and B. nested-qPCR. *n* = 20 except for November, which *n* = 18. Total: summarized total for all five months.

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samples were selected for pre-amplification in conventional PCR and subsequent nested-qPCR to validate the nested primers and test the assay: five negative samples; two inconclusive samples (C_q 34.3 and 35.5); and four positive samples (C_q 18.3, 19.5, 25.7, 25.8). Ten samples tested positive with positive C_q values ranging from 2.3 to 10.2. One of the negative samples, Sept igc 1, resulted in a nested-qPCR C_q value of 38.7, which raises questions about appropriate cutoffs for C_q values in this assay that remain to be decided, but for the purposes of this study, this sample was considered to be negative (Table A2). Importantly, no signal was detected in the conventional PCR or nested-qPCR NTC, supporting the notion that contamination did not play a role in observed results. No signal was observed using DNA isolated from the sharpshooter, *C. costalis*, as a template. The sequences from all eleven amplicons were 100% identical to one another and to the expected sequence for *16S* as represented by GenBank entry DQ778016.1 (Fig. 2). The remaining samples that were initially categorized as inconclusive were tested in a nested-qPCR assay and found to be positive, for a final infection rate of 95% (Fig. 1).

3.2. Estimates of the false-negative error rate reported by the presence/absence qPCR assay

Comparison of the two methods, the presence/absence qPCR and nested-qPCR, was conducted over the course of five months starting with the data collected in September 2013. *Wg* was detected in all 98 samples with an average C_q of 24.9 (SD 1.0; Table A2). Infection rates of 55, 45, 44, 35 and 35% were determined by the presence/absence qPCR assay for the months of September, October and November 2013, and January and February 2014, respectively (Fig. 1A). Collectively, 42 samples out of 98 were positive by APHIS guidelines, with a final infection rate of 42.9%. Out of the remaining 56 samples, 8 were inconclusive and 44 were negative (Fig. 1A, Table A1). In comparison, the infection rate as determined by nested-qPCR was 95% for September, 94.4% for November, and 100% for October, January and February. The final infection rate for all psyllids sampled across the five months using nested-qPCR was 97.9% (Fig. 1B, Table A2), resulting in an overall false-negative rate of 54.9%.

An unexpected observation was made in analyzing psyllid samples collected over the course of several months, and that is that the rate of infection that was detected by the presence/absence qPCR assay dropped gradually as the months progressed into the winter (Fig. 1A), presumably as an indicator of dropping *C*Las titers.

Whether this observation is biologically relevant or is an artifact of rearing insects in an artificial environment is unknown, but is consistent with the finding that *C*Las titer within the insect host is affected by temperature (Kingdom Gibbard et al., unpublished results).

3.3. Evaluation of qPCR assays: dynamic range, sensitivity and efficiency

The dynamic range, sensitivity and efficiency of the *Wg* and *16S* qPCR assays were evaluated using calibration curves generated with ten-fold serial dilutions of linearized plasmid templates. These curves were then compared to the *Wg* and *16S* calibration curves generated with 10-fold serial dilutions of genomic DNA isolated from individual psyllids harboring *C*Las. Five independent trials were conducted for plasmid DNA and 15 for psyllid DNA, the results of which are summarized in Fig. 3 and Tables A3 and A4. The dynamic range of the *Wg* plasmid qPCR assay spanned seven orders of magnitude, with an average R^2 value of 0.993 (SD 0.002), with the reproducibility between trials relatively high (Fig. 3A). The estimated minimum sensitivity of the assay was 2.7 starting molecules of template (Table A3). The range of efficiency for the *Wg* linearized plasmid across the five trials was 97.4–103%, with an average of 99.2% (SD 2.3%). The *16S* plasmid assay was less robust than that for *Wg* (Fig. 3B). The average R^2 value of calibration curves across the five trials was 0.983 (SD 0.019), with two trials falling below the desired R^2 cutoff of 0.99. The assay spanned seven orders of magnitude in two trials (sensitivity: 2.21 molecules; data not shown) and six in the remaining three (sensitivity: 22.1 molecules) (Table A3). Efficiencies ranged from 93.4 to 103.2% with an average amplification efficiency of 98.9% (SD 3.6%). While the overall performance of the *16S* assay was well within the guidelines for qPCR, it is not as sensitive as *Wg* (Fig. 3A vs B; Table A3). Maximal sensitivity is particularly important given that the *C*Las DNA template will be in low abundance relative to that of the host DNA. Low abundant templates are particularly sensitive to stochastic error that is inherent in every qPCR assay, and run the risk of being underestimated or undetected, even in well-performing assays. Any factor that reduces qPCR efficiency or sensitivity will exacerbate this phenomenon. In many cases, plasmid calibration curves perform better than those made from samples, with two main causes being non-specific binding of primers to non-target DNA, and inhibitory contaminants that are co-isolated during DNA purification. Therefore, it was not unexpected that the parameters of the calibration curves made using psyllid DNA were not as robust as the *Wg* and *16S* those made from plasmid DNA (Fig. 3C and D vs. Fig. 3A and B; Tables A3 and A4). The variability of the slopes, which correspond directly to qPCR efficiency (see formula in Materials and methods section), of the sample calibration curves were significantly greater than that of the plasmids (*Wg*: $F_{1,20}$ 72.30, $p = 0.001$; *16S*: $F_{1,20}$ 20.50, $p = 0.006$). The *Wg* qPCR efficiencies for psyllid DNA samples ranged from 73.4 to 101.2% with an average efficiency of 91.1% (SD 7.8%) and nine out of the 15 standard curves had efficiencies that fell within the accepted range of 90–110% (Fig. 4). The average R^2 value for all curves was 0.996 (SD 0.004), with all but two making the 0.99 cutoff (Table A4). The corresponding *16S* assays performed less optimally. Efficiencies for nine out of the 15 calibration curves for *16S* from the same psyllid samples fell outside of the accepted range (Fig. 4, Table A4), four of which had nonsensical efficiencies of 150% and above (Table A4). The corresponding *Wg* standard curves from the same psyllid samples for these four assays were as expected and within acceptable parameters for qPCR assays (Figs. 3 and 4, Table A4), with low standard deviations between technical replicates (≤0.5; data not shown), showing that these results were not due to error in serial dilution. This, coupled with normal reactions for *16S* positive controls in the assay that rule out errors made with master mixes or degradation of primers and/or probes, suggests that stochastic error or other unknown factor, to which the *16S* assay is particularly sensitive, contributed to these aberrant results, rather than technical error (Fig. 3D; Table A4). The remaining six *16S* efficiencies ranged from 76.0 to 100.25% with an average of

```
Nested-1       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-2       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-3       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-4       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-7       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-8       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-9       TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-10      TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-12      TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-17      TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
Nested-18      TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
CP001677.5     TCGAGCGCGTATGCGAATACGAGCGGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAACGC
```

Fig. 2. *Candidatus* Liberibacter asiaticus *16S* sequence alignment of nested-qPCR products with reference *16S* rDNA sequence CP001677.5.

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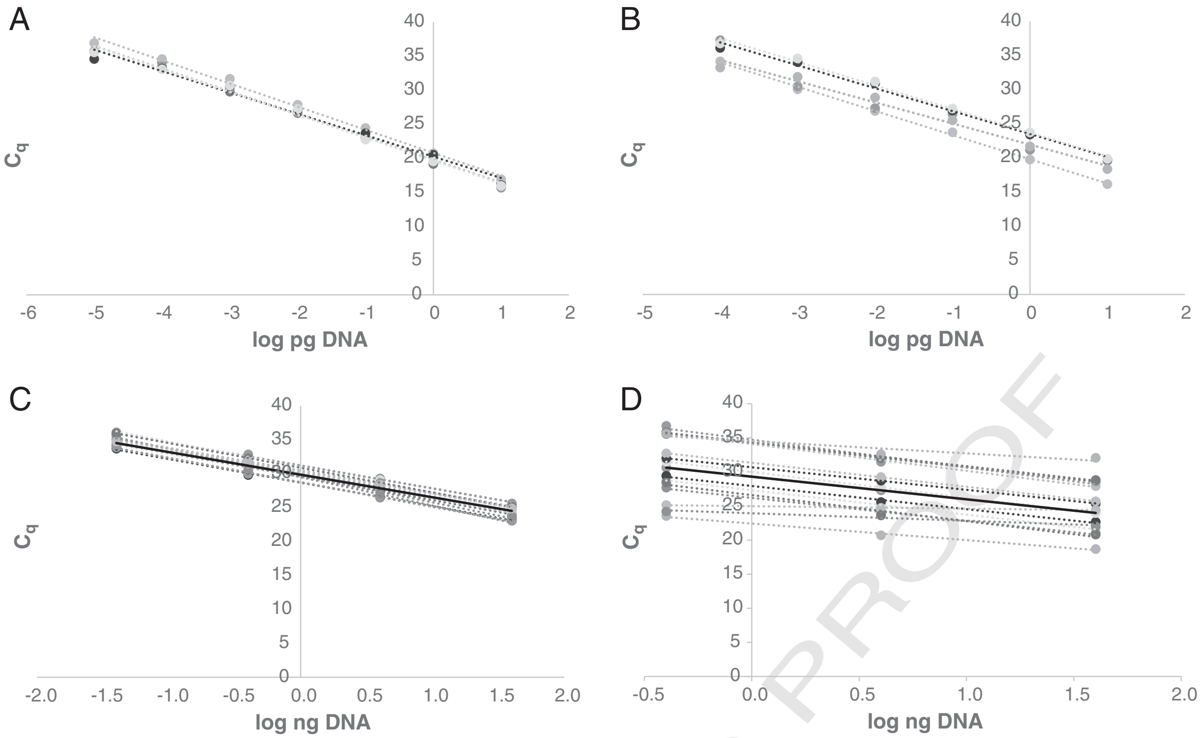

Fig. 3. *Wingless* and *16S* 10-fold serial dilution standard curves. Standard curves of five independent *Wg* (A) and *16S* (B) plasmid preparations. *Wg* (C) and *16S* (D) standard curves from 15 psyllid samples. *Wg* and *16S* were determined from the same psyllid sample for each of the 15 curves. Heavy regression lines in C and D represent optimal amplification with a slope of −3.321 and the average intercept for the data set. Note that variability of intercepts for 16S plots is expected due to the fact that samples have varying titers of *C*Las. Q2

95.9% (SD 4.2%). It is interesting to note that in all but two of the *16S* assays, it would require greater than 50 cycles of qPCR to detect 22 molecules of template, the highest reliable dilution determined in the corresponding *16S* assay using plasmid (Table A3). All but four regression lines met the required cutoff of 0.99 with an overall average R^2 of 0.997 (SD 0.004; Table A4). As would be expected for samples with varying titers of *C*Las, the intercept of the calibration curves varies widely as this parameter is directly affected by starting copy number of template. The most striking result is that in some cases, the efficiencies of *16S* vs *Wg* from the same psyllid sample differed significantly, suggesting that the *16S* assay may be more sensitive than the *Wg* counterpart to inherent differences between DNA isolations. As stated above, contamination co-isolated with the DNA such as salt, buffer or carbohydrates, or other contaminant can negatively impact qPCR efficiency. However, no correlation was found between the measured A_{260}/A_{230} ratio and efficiency for either gene target (data not shown). Interestingly, in some cases, the standard deviations between technical replicates were well below the 0.3 cutoff for one target, while exceeding the cutoff for the other target from within the same sample, again suggesting that stochastic error, rather than pipetting or other technical error, contributed to variation across replicates (data not shown).

3.4. Secondary structure predictions of qPCR Wg and 16S amplicons

To determine if secondary structure could potentially contribute to greater variability in efficiencies of the *Wg* and *16S* qPCR assays, structures and their thermodynamic parameters were predicted using the DINAMelt Webserver (Markham and Zuker, 2005). The thermodynamic parameters for *Wg* were: $\Delta G = 0.1$ kcal/mol, $\Delta H = -18.9$ kcal/mol, $\Delta S = -56.9$ e.u., $T_m = 59.1$ °C; and $\Delta G = -0.1$ kcal/mol, $\Delta H =$

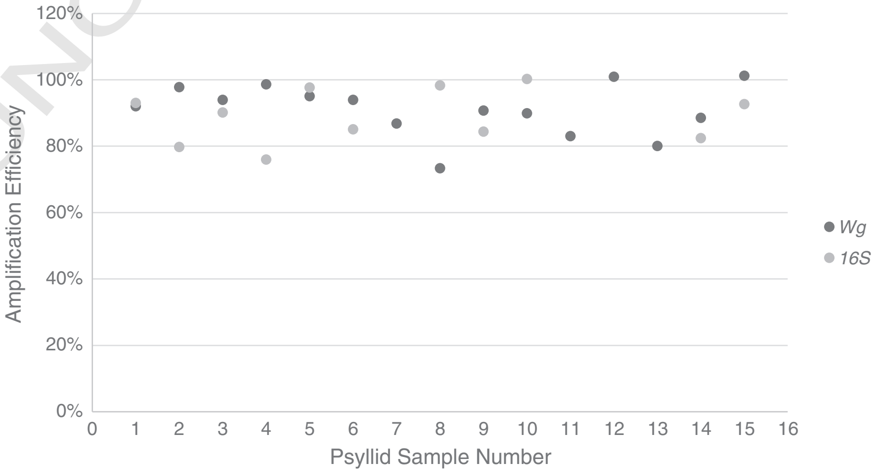

Fig. 4. Percent efficiency for *Wg* and *16S* qPCR assays from 15 independent psyllid samples. *16S* efficiencies for samples 7 and 11–13 were greater than 150%, and are not plotted.

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-18.9 kcal/mol, $\Delta S = -56.5$ e.u., $T_m = 61.5$ °C for 1.5 and 4.0 mM Mg^{2+}, respectively (where ΔG = Gibb's Energy, ΔH = enthalpy, ΔS = entropy). The predicted secondary structures at both Mg^{2+} concentrations are the same for *Wg* and *16S*, and are shown in Fig. 5. The predicted secondary structure for *Wg* had one small loop at the 3′ end of the amplicon, which partially blocks the binding site for the reverse primer (center, gray shading, Fig. 5A). However, the free energy associated with this structure would likely be overcome at the temperatures used in the qPCR assay. In contrast, the thermodynamic parameters for *16S* suggested greater stability of secondary structure, with predictions of: $\Delta G = -1.8$ kcal/mol, $\Delta H = -94.4$ kcal/mol, $\Delta S = -278.0$ e.u., $T_m = 66.5$ °C; and $\Delta G = -2.4$ kcal/mol, $\Delta H = -94.4$ kcal/mol, $\Delta S = -276.1$, e.u. $T_m = 68.8$ °C for 1.5 and 4.0 mM Mg^{2+}, respectively. The secondary structure for *16S* contained two loops at the 5′ end of the amplicon; one blocking the binding site for the forward primer and the second blocking the binding site for the probe (gray shading, Fig. 5B). These results suggest that secondary structure could have a negative impact on *16S* qPCR amplification efficiency.

4. Discussion

Here we describe a nested-qPCR assay that can be used in conjunction with the *C*Las *16S* presence/absence qPCR assay to reduce the number of false-negatives obtained when using the presence/absence assay alone. It is simple, inexpensive, and can be used in series with existing assays without disrupting workflow. The decision to employ the companion assay will depend on the question being addressed, and whether or not false-negatives will affect the interpretation of the data collected to address that question. In many cases, a few false-negatives will not change the outcome and the extra step to investigate them would be wasted effort. Examples include the detection of one positive sample within a batch that calls for the destruction of the entire batch, or making the determination if any insects within a colony test positive for *C*Las. However, there are cases where false-negatives could have serious consequences. For example, the use of an assay with high rates of false-negatives is inappropriate for addressing complex biological, molecular and biochemical questions such as: the localization of *C*Las within plant and insect tissues, the elucidation of transmission rates given certain conditions, or the determination of what titer levels constitute an infection. False-negatives are also problematic in the evaluation of new methods to control or combat the disease, such as treatments to clear the insect or plant of *C*Las, or the identification of resistant cultivars. False negatives are of the most serious consequence in instances when the tolerance of a disease-causing agent is zero. A relevant example of this is the screening of budwood to be certified as free of viruses and other graft-transmissible diseases. No method of detection can say with absolute certainty that all negatives are genuinely negative, but for the purposes described above, the method of detection must be optimized to provide the greatest sensitivity possible, i.e., optimized to provide the lowest rate in false-negative reporting achievable. As seen in this study, the rates of false-negative reporting can be significantly high in the presence/absence qPCR assay — up to more than half of the samples tested. We propose that two characteristics inherent in the *16S* target contribute to problem of underestimation made by this assay: low abundance and sensitivity to suboptimal conditions. Both *Wg* and *16S* showed greater variability in performance using DNA isolated from psyllids over that observed for plasmid DNA, in some cases performing below acceptable parameters. For *Wg* that is not as problematic because this gene target is in abundance. However, *16S* can be in low abundance, especially in samples where bacterial titer is low. It is well demonstrated that the stochastic error inherent in any qPCR assay, even one that has been developed to perform optimally, has a greater effect on the amplification of low-abundant targets (<1000 copies) than those in greater copy numbers, and that the lower the starting copy number, the less likely the abundance will be reflected in the final analysis of the qPCR (Bustin and Nolan, 2004; Karrer et al., 1995). The implication of this is that for some low-abundance templates, the accumulation of sufficient product for signal detection above the fluorescent background noise might not occur, and template within samples goes undetected. Any factor that negatively impacts efficiency will magnify this limitation of qPCR. Several observations made in this study suggest that the efficiency of the *16S* assay is variably affected by some component present DNA samples isolated from host tissue. First, the *Wg* and *16S* qPCR assays perform well under the conditions of pure template in highly purified conditions. This demonstrates that overall, the qPCR assays are optimal for the basic parameters of the reaction, such as magnesium, primer and probe concentrations. However, it was noted that the *16S* assay is on average an order of magnitude less sensitive than the *Wg* counterpart. In using calibration curves with psyllid DNA, differences in performance between the *Wg* and *16S* start to diverge further. Most of the *Wg* assays performed within acceptable efficiency range for qPCR assays, the majority of the *16S* assays did not, with 60% falling outside the acceptable range of 90–110%. In four cases, calculated efficiencies for *16S* were nonsensical values of greater than 150%, while the corresponding *Wg* assays were normal; suggesting something within the isolated DNA sample is affecting *16S* more profoundly than *Wg*. In one case, C_q values for *16S* were about 24.5 for all four, ten-fold serial dilutions of the psyllid sample. This was not an error in dilution or pipetting because the serial dilution for the *Wg* counterpart from the same psyllid sample was normal with a calculated efficiency of 100.9%. In most cases, there was a lack of correlation in the efficiencies for most of psyllid samples between *Wg* and *16S*. In summary, *16S* qPCR assay is not as sensitive or consistent in its performance as compared to the *Wg* counterpart, and can demonstrate aberrant behavior using psyllid DNA as template. Our current hypothesis is that contaminants

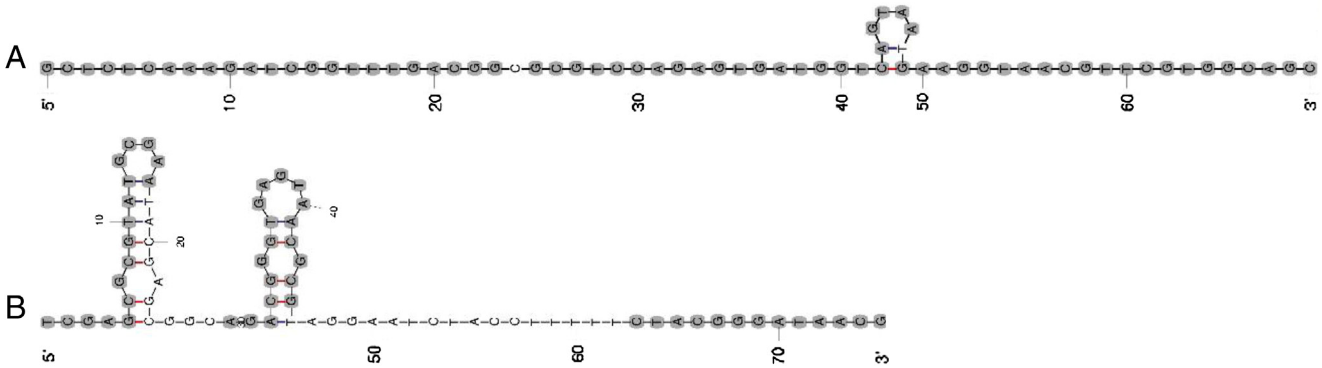

Fig. 5. Predicted secondary structures of A. *Wg* and B. *16S* amplicons at 60 °C. Predicted structures were the same for both 1.5 and 4.0 mM Mg2+. Gray shading are primer (5′ and 3′ ends) and probe binding (center) sites.

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co-isolated with sample DNA are contributing to these results, which is consistent with the observations in other systems (e.g., Hargreaves et al., 2013). In addition, the two relatively stable secondary structures that are thermodynamically favorable at the thermocycling temperatures used in the *16S* qPCR assay may contribute to less-than-optimal amplification as these stem/loop structures may block the primer binding site of the forward primer. Secondary structures formed in an amplicon are known to cause reduction of PCR efficiency (McDowell et al., 1998). While the utility of *16S* in PCR-based methods for the discovery, identification and detection of pathogens are undeniable (for a historical overview, see Sontakke et al., 2009), the propensity to form secondary structure, on which its biological function depends, needs to be considered when designing primers. It is also possible that the introduction of host DNA provides template for non-specific binding of the *C*Las primers, thus forming non-specific products, resulting in decreased efficiency of the qPCR reaction. Most likely, the observations made in this study are a result of a complex combination of underlying factors, and it is not surprising that other gene targets for *C*Las detection are now being used (Ananthakrishnan et al., 2013). However, we chose to develop a nested-qPCR assay to be compatible with previous studies conducted by our group that utilized the *16S* assay, and for researchers who are not able to shift to different gene targets for various reasons. While other methodologies with even greater sensitivity than qPCR are available and are being used for screening for disease-causing agents, such as Droplet Digital™ PCR (ddPCR™, Bio-Rad; Carlsbad, CA; White et al., 2012), they are not currently mainstream. Therefore, building on commonly available instrumentation and methodology is a necessity for many laboratories. Currently, we are using nested-qPCR to begin addressing the potential of cryptic and/or latent infection of the psyllid with *C*Las, and to further understand the transmission dynamics that facilitate the spread of HLB in citrus. In addition, we have determined that this method is not only applicable to studies in the psyllid, but in plant tissue as well. We have used this method to screen plant tissue, and found it to be of high utility in detecting early *C*Las infection (data to be published in the future). In addition, we are evaluating methods to enrich for bacterial DNA isolation from host tissue, both of psyllid and host origin, in order to increase the ratio of *C*Las to host DNA to reduce stochastic effects of low-abundance templates in qPCR assays.

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5. Uncited references

Koressaar and Remm, 2007
Thompson et al., 1997

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2014.04.007.

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