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Improved real-time PCR detection of '*Candidatus* Liberibacter asiaticus' from citrus and psyllid hosts by targeting the intragenic tandem-repeats of its prophage genes

J. Kent Morgan [a], Lijuan Zhou [b], Wenbin Li [c], Robert G. Shatters [a], Manjunath Keremane [d], Yong-Ping Duan [a,*]

[a] USDA-ARS, U.S. Horticultural Research Laboratory, 2001 South Rock Road, Fort Pierce, FL 34945, USA
[b] University of Florida, IFAS-IRREC, Fort Pierce, FL 34945, USA
[c] USDA-APHIS-PPQ-CPHST, Beltsville, MD 20705, USA
[d] USDA-ARS, National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA 92507, USA

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ABSTRACT

'*Candidatus* Liberibacter asiaticus' (*C*Las) is a Gram-negative α-proteobacterium, and the prominent species of Liberibacter associated with a devastating worldwide citrus disease known as huanglongbing (HLB). This fastidious bacterium resides in phloem sieve cells of host plants and is vectored by the Asian citrus psyllid (*Diaphorina citri*). Due to its uneven distribution *in planta* and highly variable bacterial titers, detection of HLB bacteria can be challenging. Here we demonstrated a new utility of nearly identical tandem-repeats of two *C*Las prophage genes for real-time PCR by SYBR Green 1 (LJ900fr) and TaqMan® (LJ900fpr). When compared with conventional 16S rDNA-based real-time PCR, targeting the repeat sequence reduced the relative detectable threshold by approximately 9 and 3 real-time PCR cycles for LJ900fr and LJ900fpr, respectively. Additionally, both LJ900 methods detected *C*Las from otherwise non-detectable samples by other methods. *C*Las was also detected from globally derived samples including psyllids, various citrus varieties, periwinkle, dodder, and orange jasmine, suggesting the new detection method can be applicable worldwide. Additionally, we demonstrated the presence of the hyv_I/hyv_{II} repeat sequence within the '*Ca.* Liberibacter americanus' strain. The method thereby provides sensitive HLB detection with broad application for scientific, regulatory, and citrus grower communities.
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1. Introduction

Citrus huanglongbing (HLB), also known as citrus greening, is a destructive disease that was first noted in the early 20th century in China [1]. This disease has spread throughout the global citrus producing regions and has recently been found in North America with first detection in Florida in 2005 [2]. Three fastidious α-Proteobacteria species of '*Candidatus* Liberibacter,' namely '*Ca.* Liberibacter asiaticus' (*C*Las) (the only species currently found in the U.S.), '*Ca.* Liberibacter americanus' (*C*Lam) and '*Ca.* Liberibacter africanus' (*C*Laf) [3,4] are associated with HLB. These bacteria have been shown to reside within sieve tube cells (sieve elements) of infected plants [5] and to be vectored by psyllids, *Diaphorina citri* [6] and *Trioza erytreae* [3,7,8].

Although HLB presents systemically, low titer and uneven distribution of these bacteria within infected plants [5,9,10] can make reliable detection difficult. Many methods have been developed including biological indexing using graft and dodder transmission [4], light or electron microscopy [3], loop-mediated isothermal amplification [11], polymerase chain reaction (PCR) [12–14], and real-time PCR [9,10,15–18] to detect '*Ca.* Liberibacter' bacteria.

Currently, real-time PCR has become the preferred detection method of Liberibacter species [9,10,15–18]. Relative to conventional PCR, real-time PCR offers both sensitive and rapid detection of these bacteria. Real-time PCR is reported to increase the sensitivity for Liberibacter detection by 10 times relative to nested PCR [9], and 100 to 1000 times relative to conventional PCR [9,18] for these bacteria. These real-time PCR methods target genes with low copy number: three copy 16S rDNA [17], single copy β-operon [9], or single copy elongation factor Ts (EF-Ts) [19]. The reported real-time PCR low threshold limits are approximately ten gene copies

* Corresponding author. Tel.: +1 772 462 5840; fax: +1 772 462 5962.
E-mail address: yongping.duan@ars.usda.gov (Y.-P. Duan).

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for 16S rDNA and β-operon methods [9,15], and single gene copy for the elongation factor Ts (single closed tube with dual sets of primers) [19]. However, since '*Ca*. Liberibacter' bacteria can exist at extreme low titer in their host plant and insects, and because samples contain host DNA that can interfere with reaction efficiency, the targeted DNA may often be present at sub-amplification levels by current PCR detection methods [20].

The recent sequencing of the *C*Las genome using a metagenomics approach [21], has revealed two unique hypothetical genes located within a prophage region of the genome that are designated as hyv_{I} (YP_003084345) and hyv_{II} (HQ263703) [22]. These genes contain multiple nearly identical tandem-repeat sequences of 132 base pairs (bp) for each full-length repeat [22]. The 100 bp core sequence of each repeat provides ideal targets for development of a real-time PCR method, as hyv_{I}/hyv_{II} may contain up to a combined fifteen nearly identical repeats (Fig. 1). In this study, we used these intragenic repeats of the prophage sequence to develop and validate new real-time PCR methods with improved assay sensitivity for detection of *C*Las in both host plants and vector insects.

2. Materials and methods

2.1. hyv_{I} and hyv_{II} gene identification and PCR primer and probe design

The hyv_{I} and hyv_{II} genes were identified by analyzing the PCR amplicons from psyllid 62 (the genomic DNA source used to produce the *C*Las genomic sequence that were generated during the gap closing process of the *C*Las genome sequence study [21]), and were verified using our BAC clones of the *C*Las genome.

Primers LJ900f (forward), LJ900r (reverse) and LJ900p (TaqMan® probe) (Table 1), were designed based on the internal 100 bp core sequence of the 132 bp full repeat shared by the hyv_{I}/hyv_{II} genes (Fig. 1) using OLIGO 7 Primer Analysis Software version 7.23 (Molecular Biology Insights, Cascade, CO). The probe was labeled 5′ with 6-carboxylfluorescein (6-FAM™) reporter and 3′ with Iowa Black FQ quencher (Table 1). Integrated DNA Technologies (IDT, Coralville, IA) synthesized all primers and probes (Table 1) used in this study. A series of *in silico* evaluations of primers LJ900f and LJ900r and probe LJ900p were carried out, using the NCBI BLAST megablast algorithm parameters for highly similar sequence alignment against the nucleotide (nr/nt) database having either the *C*Las genome included or excluded in separate searches respectively for each. The specificity of these primers and probe was also evaluated by real-time PCR assays against a variety of DNA extracts from plant pathogens, *Xanthomonas citri* subsp. *citri*, *Xanthomonas axonopodis* pv. *citrumelo*, *Ralstonia solanacearum*, *Escherichia coli* DH5α, soil bacteria from the USHRL Picos farm and the USHRL facility and from numerous plant species.

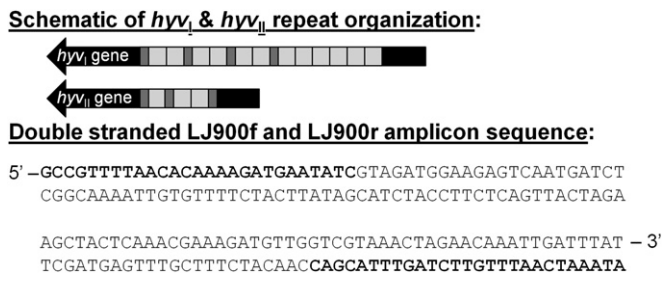

Fig. 1. Top is a representative (not to scale) hyv_{I} and hyv_{vII} gene repeat sequence schematic with light and dark gray boxes representing full and partial *repeat* sequences, respectively. Arrow direction indicates gene orientation [22]. Bottom, indicates the 100 bp double stranded amplicon sequence of the LJ900f and LJ900r primers as bolded sequences respectively.

2.2. Real-time PCR

The optimized concentrations of the primer set LJ900fr (with SYBR Green 1) and LJ900fpr (with TaqMan®) were 600 and 900 nanomolar (nM) of LJ900f and LJ900r respectively, with an addition of 500 nM of LJ900p to the LJ900fpr. The optimized annealing temperature was 62 °C for both LJ900fr and LJ900fpr methods. Unless otherwise indicated, 2 μL of each DNA preparation (described below) was used per 15 μL reaction. The SYBR Green 1 real-time PCR was run at 95 °C for 3 min, followed by 40 cycles at 95 °C for 3 s and 62 °C for 30 s, with fluorescence signal capture at the end of each 62 °C step, followed by a default melt (disassociation) stage. TaqMan® real-time PCR was carried out at 95 °C for 30 s, followed by 40 cycles at 95 °C for 3 s and 62 °C for 30 s, with fluorescence signal capture at the end of each 62 °C step. All reactions were run on the Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems (ABI), Foster City, CA). Cycle threshold (Ct) values were analyzed using ABI 7500 Software version 2.0.1 with a manually set threshold at 0.1 and automated baseline settings. The PCR amplification efficiency was evaluated by standard curves established on serial dilutions of the pLJ153.1 plasmid DNA (10^6 copies to single copy, Supplementary Fig. 1A) in triplicate using water or background plant DNA at 50 ng/15 μL reaction (Supplementary Fig. 1C and D), respectively.

2.3. Real-time PCR

All real-time PCR reactions were performed in MicroAmp® Fast Optical 96 well reaction plates (ABI) with MicroAmp Optical Adhesive Film (ABI) plate coverings using the same 7500 Fast real-time PCR system (ABI) at 15 μL total reaction volume. For TaqMan® real-time PCR reactions with the LJ900fpr method and the 16S rDNA standard for *C*Las detection HLBaspr, HLBampr (*C*Lam), HLBafpr (*C*Laf), or COXfpr (plant cytochrome oxidase) methods [17] the ABI TaqMan® Fast Universal PCR Master Mix (2x) No AmpErase® UNG (Applied Biosystems Inc., Foster City, CA) was used. The EF-Ts gene targeting Single Tube Dual Primer (STDP) detection strategy was done using ABI TaqMan Universal PCR Master Mix [19]. The SYBR Green 1 (SG1) real-time PCR reactions with LJ900fr, β-operon *rpl*JAm (*C*Lam) and *rpl*LAs (*C*Las) [9], or HLBasr/SG1 detection strategies employed PerfeCTa™ SYBR® Green FastMix™ 2 × master mix (Quanta Biosciences, Inc., Gaithersburg, MD) and for comparison Promega GoTaq® real-time PCR Master Mix (Promega) was used.

2.4. Samples and DNA extraction

Total DNA was extracted from the leaf midribs of citrus samples collected from the U.S. Horticulture Research Laboratory (USHRL) Picos Farm or USHRL maintained greenhouses in Fort Pierce, FL (Table 2, Table 3 and Table 4), and from samples representing different counties in Florida (Table 5) and *Murraya paniculata* plants (Table 6) in accordance with standard DNeasy® Plant Mini Kit isolation protocols (Qiagen Inc., Valencia, CA). Briefly, midrib tissues were finely chopped and 0.2 g was placed into a sterilized 2 mL screw cap tube containing two 4 mm Silicone-Carbide Sharp Particles and four 2.3 mm Chrome-Steel Beads (BioSpec Products, Inc, Bartlesville, OK). To this 800 μL of DNeasy® AP1 lysis buffer was added, and the tissues were homogenized by a FastPrep-24 System (MP Biomedicals, Solon, OH) for two successive rounds at 6.5 M/S for 45 s. Subsequent steps followed pursuant with the DNeasy® Plant Mini Kit protocol (Qiagen) from the 'RNase A' step.

Table 1
PCR and real-time PCR primers.

Name	Sequence (5′ → 3′)	Purpose	Source
LJ900f_f	GCCGTTTTAACACAAAAGATGAATATC	LJ900fr repeat sequence real-time PCR	*This study*
LJ900r_r	ATAAATCAATTTGTTCTAGTTTACGAC		
LJ900p_p	[a]ACATCTTTCGTTTGAGTAGCTAGATCATTGA[b]	LJ900fpr TaqMan® real-time PCR probe	*This study*
Las-O-f_f	CGGTGAATGTATTAAGCTGAGGCGTTCC	STDP (nested) real-time PCR	H. Lin *et al* [19]
Las-O-r_r	ACCCACAACAAAATGAGATACACCAACAACTTC		
Las-I-f_f	CGATTGGTGTTCTTGTAGCG		
Las-I-r_r	AACAATAGAAGGATCAAGCATCT		
Las-p_p	[a]AATCACCGAAGGAGAAGCCAGCATTACA[b]		
HLBas$_f$	TCGAGCGCGTATGCAATACG	HLBaspr real-time PCR	W. Li *et al* [17]
HLBr$_r$	GCGTTATCCCGTAGAAAAAGGTAG		
HLBp$_p$	[a]AGACGGGTGAGTAACGCG[b]		
HLBaf$_f$	CGAGCGCGTATTTTATACGAGCG	HLBafpr real-time PCR[e]	W. Li *et al* [17]
HLBam$_f$	GAGCGAGTACGCAAGTACTAG	HLBampr real-time PCR[e]	W. Li *et al* [17]
COXf$_f$	GTATGCCACGTCGCATTCCAGA	COXfpr (multiplex) real-time PCR	W. Li *et al* [17]
COXr$_r$	GAATGCCCTTAGCAGTTTTGGC		
COXp$_p$	[c]ATCCAGATGCTTACGCTGG[d]		
EUB338$_f$	ACTCCTACGGGAGGCAGCAG	Universal Eubacterial 16S real-time PCR	D. Lane [32]
EUB518$_r$	ATTACCGCGGCTGCTGG		G. Muyzer *et al* [33]
ALF685$_f$	TCTACGRATTTCACCYCTAC	Universal α-proteobacteria real-time PCR[f]	D. Lane [32]
f-*rpl*JAm$_f$	GGACAAGGGGATATTGGATAATGATG	'*Ca.* Liberibacter americanus' β-operon real-time PCR	D. Teixeira *et al* [9]
r-*rpl*JAm$_r$	ATTAAGAGTTCTAAGCAACCTGACAG		
f-*rpl*LAs$_f$	CGCCCGTTTCCGTTGT	SYBR® Green real-time PCR of '*Ca.* Liberibacter asiaticus' β-operon	D. Teixeira *et al* [9]
r-*rpl*LAs$_r$	AGCCTCTTTAAGCCCTAAATCAG		

f = Forward, r = Reverse, p = TaqMan® Probe.
[a] 6-FAM™.
[b] Iowa Black FQ.
[c] TET.
[d] BHQ-2.
[e] Use with common probe (HLBp) and reverse primer (HLBr).
[f] ALF685$_f$ is used in combination with EUB518$_r$ universal 16S reverse primer.

Table 2
Real-time PCR data comparison of hyv_I/hyv_{II} detection by LJ900fr, LJ900fpr or 16S by HLBaspr.

Citrus host	Sample		Mean Ct value by method (± St. dev. mean ct)			ΔCt		
	#	Name	LJ900fr	LJ900fpr	HLBaspr	LJ900fr−HLBaspr	LJ900fpr− HLBaspr	LJ900fpr −LJ900fr
Blood orange	1	R2T6	18.70 (± 0.16)	26.30 (± 1.81)	29.89 (± 0.15)	−11.19	−3.59	−7.60
Trifoliate	2	09−002	27.26 (± 0.29)	32.76 (± 4.71)	ND	N/A	N/A	−5.50
Sour orange	3	R7T6	19.31 (± 0.17)	26.29 (± 0.18)	30.26 (± 0.13)	−10.95	−3.97	−6.98
Sweet orange	4	R3T7-G	19.93 (± 0.07)	26.86 (± 0.45)	29.47 (± 0.06)	−9.54	−2.61	−6.93
Sweet orange	5	R3T7-Y	15.01 (± 0.31)	20.93 (± 1.01)	22.90 (± 0.02)	−7.89	−1.97	−5.92
Dancy tangerine	6	R10T6 (N)	11.20 (± 1.02)	16.92 (± 0.17)	22.61 (± 0.06)	−11.41	−5.69	−5.72
Lemon	7	R11T11	12.76 (± 0.21)	17.93 (± 0.81)	23.16 (± 0.14)	−10.40	−5.23	−5.17
Orangequat	8	R12T9	12.70 (± 0.07)	18.50 (± 1.31)	21.85 (± 0.10)	−9.15	−3.35	−5.80
Pomelo	9	R8T1-GY	19.44 (± 0.20)	25.26 (± 3.06)	29.58 (± 0.06)	−10.14	−4.32	−5.82
Pomelo	10	R8T1-M	14.52 (± 0.16)	20.04 (± 1.52)	24.80 (± 0.12)	−10.28	−4.76	−5.52
Pomelo	11	R8T1-Y	26.16 (± 0.26)	32.45 (± 0.30)	36.47 (± 0.55)	−10.31	−4.02	−6.29
Pomelo	12	R8T4-Y	12.90 (± 0.35)	18.87 (± 1.29)	21.89 (± 0.07)	−8.99	−3.02	−5.97
Pomelo	13	R8T4-M	18.16 (± 0.19)	25.83 (± 1.67)	27.80 (± 0.10)	−9.64	−1.97	−7.67
Pomelo	14	R8T1-11	27.43 (± 0.24)	35.50[a]	37.99 (± 0.82)	−10.56	−2.49	−8.07
Pomelo	15	R8T1-14	30.48 (± 1.67)	35.56 (± 0.41)	ND	N/A	N/A	−5.08
Pomelo	16	R8T1-15	28.78 (± 0.33)	34.08 (± 0.71)	ND	N/A	N/A	−5.30
Pomelo	17	R8T1-31	26.82 (± 0.21)	34.80 (± 1.38)	ND	N/A	N/A	−7.98
Pomelo	18	R8T1-72	26.60 (± 0.29)	33.04 (± 0.71)	ND	N/A	N/A	−6.44
Pomelo	19	R8T1-129	27.39 (± 0.76)	34.27 (± 0.93)	ND	N/A	N/A	−6.88
Pomelo	20	R8T1-130	24.89 (± 0.11)	30.20 (± 0.30)	ND	N/A	N/A	−5.31
Melogold hybrid	21	R8T3-M	15.84 (± 0.87)	21.03 (± 0.70)	24.93 (± 0.17)	−9.09	−3.90	−5.19
Melogold hybrid	22	R8T3-Y	15.03 (± 0.43)	18.99 (± 0.20)	23.15 (± 0.14)	−8.12	−4.16	−3.96
Melogold hybrid	23	R8T3-4	27.58 (± 0.12)	35.07 (± 0.92)	ND	N/A	N/A	−7.49
Melogold hybrid	24	R8T3-12	28.05 (± 0.31)	34.81[a]	ND	N/A	N/A	−6.76
Melogold hybrid	25	R8T3-13	26.58 (± 0.24)	33.47 (± 0.31)	ND	N/A	N/A	−6.89
Melogold hybrid	26	R8T3-101	13.66 (± 0.19)	18.85 (± 0.74)	24.25 (± 0.08)	−10.59	−5.40	−5.19
Melogold hybrid	27	R8T3-111	18.03 (± 0.43)	25.08[a]	27.77 (± 0.23)	−9.74	−2.69	−7.05
Melogold hybrid	28	R8T3-NT	26.32 (± 0.28)	33.47 (± 0.23)	ND	N/A	N/A	−7.15
[b] CA Rep. Citrus	29	#67	ND	ND	ND	N/A	N/A	N/A
Mean ΔCt→						−9.88 (± 1.00)	−3.71 (± 1.16)	−6.27 (± 1.04)

N/A = Not applicable.
ND = No amplification detected.
[a] Insufficient DNA precluding technical replicates, no reportable St. dev.
[b] CA Rep. Citrus (#67) = California Citrus Repository sample #67 is representative of >68 California Citrus repository samples tested, each being negative by these methods.

Table 3
Real-time PCR comparison of '*Ca.* Liberibacter asiaticus' dilution sample detection by LJ900fr, LJ900fpr, and HLBaspr methods.

Sample 'VPCQ' dilutions	Mean Ct value by method (± St. dev. mean Ct)		
	LJ900fr Ct	LJ900fpr Ct	HLBaspr Ct
10^{-1}	15.90 (± 0.05)	21.65 (± 0.38)	25.64 (± 0.06)
10^{-2}	19.30 (± 0.09)	24.93 (± 0.07)	28.88 (± 0.08)
10^{-3}	22.94 (± 0.06)	29.11 (± 0.02)	32.29 (± 0.05)
10^{-4}	26.00 (± 0.03)	32.67 (± 0.21)	34.98 (± 0.08)
10^{-5}	28.78 (± 0.10)	35.72 (± 0.29)	ND
10^{-6}	32.70 (± 0.23)	ND	ND
10^{-7}	+/−	ND	ND
10^{-8}	ND	ND	ND

ND = No amplification detected.
+/− = greater than 50% amplification detected within replicates but less than 100% positive.

Citrus DNA samples from the USDA National Clonal Germplasm Repository for Citrus and Dates in Riverside, California, citrus DNA were isolated using either Plant DNeasy or MagAttract® 96 DNA Plant mini-prep systems pursuant with manufacturer's directions (Qiagen). Global citrus DNA samples (Table 6) were received directly as total DNA extracts by collaborators using a CTAB method [23].

Total DNA of the Asian citrus psyllids, *D citri* (Table 4 and Table 6) was extracted with phenol/chloroform as described by Hung et al., 2004 [24]. DNA from bacterial strains: *X. citri* subsp. *citri* (Citrus Canker agent), *X. axonopodis* pv. *citrumelo* (agent of citrus bacterial spot), *R. solanacearum* (multi-host bacterial plant pathogen), and *E. coli* DH5α was isolated using the Promega Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI) in accordance with manufacturers' protocols. Total soil DNA was extracted from 1 g of soil in 9 mL of 1 × PBS buffer that was vigorously vortexed for 1 min. From this, a 1 mL aliquot was transferred to a sterile 1.5 mL micro-centrifuge tube and centrifuged at low speed (1000 × *g*) for 10 min to pellet soil debris. Supernatant was transferred to a new sterile 1.5 mL micro-centrifuge tube and centrifuged at high speed (20,000 × *g*) for 10 min to pellet bacteria. Total DNA was extracted from the bacterial pellet using the Promega Wizard® Genomic DNA Purification Kit (Promega) in accordance with manufacturer's protocols. All DNA extracts were stored at −20 °C until use.

Table 4
Representative results from a larger ongoing study detecting the '*hyvI*/*hyvII*' repeat by LJ900fr from citrus seedlings or seedling fed psyllids.

Host	Sample		LJ900fr	
	#	Name	Ct value	Tm °C
Pomelo	1	Pomelo G7	23.88	74.86
Pomelo	2	Pomelo H3	25.31	74.33
Pomelo	3	Pomelo H5	34.38	73.97
Pomelo	4	Pomelo E5	34.39	75.40
Pomelo	5	Pomelo F6	ND	63.43[a]
Trifoliate	6	TF-#33	30.41	74.65
Trifoliate	7	TF-#31	31.22	74.65
Trifoliate	8	TF-#25	35.52	75.00
Trifoliate	9	TF-#32	36.48	75.35
Trifoliate	10	TF-#37	ND	63.41[a]
Grapefruit	11	I-GF-#13	27.96	74.16
Grapefruit	12	I-GF-$H_2$0 07.20.09 #3	28.94	74.33
Grapefruit	13	I-GF-#8	36.46	75.04
Grapefruit	14	I-GF-#28	ND	63.41[a]
Sweet orange	15	I-SO-Anti 01.21.10 #17	29.47	74.51
Sweet orange	16	1-SO-H_2O 01.21.10 #14	30.39	74.15
Sweet orange	17	I-SO-H_2O 07.10.09 #10	30.60	75.02
Sweet orange	18	I-SO-Anti 07.14.09 #10	31.27	74.11
Sweet orange	19	I-SO-H_2O 07.10.09 #8	31.40	75.21
Sweet orange	20	I-SO-Anti 07.14.09 #3	35.54	75.21
Sweet orange	21	I-SO-Anti 07.14.09 #16	36.80	75.21
Sweet orange	22	I-SO-Anti 01.21.10 #14	ND	63.61[a]
Seedling fed psyllid (*D. citri*)	23	#22 14-6-11-5	28.29	74.80
Seedling fed psyllid (*D. citri*)	24	#23 14-6-11-5	30.86	74.99
Seedling fed psyllid (*D. citri*)	25	#34 14-6-11-5	31.82	75.35
Seedling fed psyllid (*D. citri*)	26	#21 14-6-11-5	34.17	75.44
Seedling fed psyllid (*D. citri*)	27	#31 14-6-11-5	34.62	75.35
Seedling fed psyllid (*D. citri*)	28	#14 14-6-11-5	34.84	74.80
Seedling fed psyllid (*D. citri*)	29	#3 14-6-11-5	35.35	74.62
Seedling fed psyllid (*D. citri*)	30	#4 14-6-11-5	ND	63.46[a]

ND = No amplification detected.
[a] Indicates a null melt value returned by the ABI 7500 Fast System for non-amplicon reactions.

Table 5
Comparison of multiplex TaqMan real-time PCR-based on hyv_I/hyv_{II} repeat and 16S rDNA genes of '*Ca.* Liberibacter asiaticus'.

[a]Florida citrus sample	TaqMan qPCR Ct		16S rDNA TaqMan qPCR Ct	
	LJ900fpr	[b]COXfpr	HLBaspr	[b]COXfpr
1	20.71	18.12	24.93	18.46
2	19.67	17.50	23.69	17.77
3	18.72	17.77	22.15	17.59
4	19.99	16.81	22.32	17.28
5	20.26	20.18	22.16	20.30
6	17.86	17.93	22.18	18.16
7	23.44	19.51	25.83	20.07
8	39.05	17.59	34.89	17.70
9	20.43	18.26	23.50	18.36
10	22.40	18.72	25.19	18.62
11	36.50	18.92	37.12	18.74
12	38.71	17.72	34.03	17.42
13	37.59	18.20	0.00	19.30
14	23.36	18.46	27.07	18.57
15	37.28	19.53	39.54	19.17
Mean Ct	*25.59*	*18.35*	*27.47*	*18.50*

[a] DNA extracts are from foliar midrib of HLB-symptomatic sweet orange trees from field in 15 counties of Florida.
[b] The TaqMan primer/probe set COXfpr was based on plant cytochrome oxidase (COX).

2.5. Target detection with elevated background DNA's

To test the effect of increasing background DNA on target template PCR amplification, host DNA was increased to 100 and 200 ng/15 μL reaction using *C*Las negative citrus DNA that was added to serial dilutions of pLJ153.1 (plasmid containing a single copy of the hyv_I/hyv_{II} repeat) from 10^6 to 1 copy at each background DNA level. Additionally, to increase the background DNA to 500 and 1000 ng/15 μL, reactions were tested using salmon sperm DNA (Invitrogen) instead of citrus DNA.

2.6. Statistical analysis

A single factor ANOVA at 95% ($P = 0.05$) confidence interval with MS Excel 2007 (Microsoft, Redmond, WA) was used to determine statistical significance of observed differences in measured amplification parameters where $P < 0.05$ was considered statistically significant.

3. Results

3.1. LJ900 primer specificity analyses

To validate the different LJ900 primer specificities, we undertook a series of *in silico* and real-time PCR analyses against related and random sequences and/or samples to determine the

Table 6
LJ900fr real-time PCR hyv_I/hyv_{II} repeat detection within citrus, psyllid, and Murraya hosts from global origins.

Host	Origin	Sample		Mean Ct value (±St. dev. mean Ct)
		#	Name	LJ900fr Ct
Psyllid (*D. citri*)	Brazil	1	[a]Psy-Br12	17.41
Psyllid (*D. citri*)	Brazil	2	[a]Psy-Br17	19.13
Psyllid (*D. citri*)	Brazil	3	[a]Brazil-Amer.11	27.82
Citrus	Brazil	4	Brazil 'AM'	23.33 (± 0.15)
Tangerine	Fujian, China	5	C18-CH	17.14 (± 0.17)
Tangerine	Fujian, China	6	C2-CH	22.09 (± 2.01)
Kumquat	Fujian, China	7	C3-CH	23.71 (± 0.09)
Citrus	Fujian, China	8	Cha12	21.37 (± 2.86)
Psyllid (*D. citri*)	Fujian, China	9	[a]Ch.Psy1-1	28.28
Psyllid (*D. citri*)	Fujian, China	10	Ch.Psy1-10	18.69 (± 1.14)
Psyllid (*D. citri*)	Fujian, China	11	[a]Ch.Psy1-2	22.31
Psyllid (*D. citri*)	Philippines	12	F3957.1	18.84 (± 0.02)
Psyllid (*D. citri*)	Philippines	13	F3957.18	11.91 (± 7.83)
Psyllid (*D. citri*)	Philippines	14	F3957.2	14.80 (± 0.74)
Psyllid (*D. citri*)	Philippines	15	F3957.21	19.82 (± 0.27)
Psyllid (*D. citri*)	Philippines	16	F3957.4	10.58 (± 0.97)
Citrus	India	17	[a]#25	20.91
Citrus	India	18	#17	30.74 (± 0.12)
Citrus	India	19	#18	28.86 (± 0.50)
Psyllid (*D. citri*)	India	20	01.01.10 #1	18.82 (± 0.16)
Psyllid (*D. citri*)	India	21	01.01.10 #2	19.53 (± 0.38)
Tangerine	Thailand	22	08.14.09.2	11.41 (± 5.52)
Psyllid (*D. citri*)	Thailand	23	[a]Thai Psy.2	25.49
Psyllid (*D. citri*)	Thailand	24	[a]Thai Psy.4	24.64
Psyllid (*D. citri*)	Thailand	25	[a]Thai Psy.26	21.50
Psyllid (*D. citri*)	Thailand	26	[a]Thai Psy.28	25.54
Psyllid (*D. citri*)	Thailand	27	[a]Thai Psy.32	24.69
Psyllid (*D. citri*)	Thailand	28	[a]Thai Psy.38	24.86
Psyllid (*D. citri*)	Thailand	29	[a]Thai Psy.39	24.95
Psyllid (*D. citri*)	Thailand	30	[a]Thai Psy.41	25.15
Murraya (*M. paniculata*)	Florida, USA	31	M3	33.12 (± 0.55)
Murraya (*M. paniculata*)	Florida, USA	32	M14	33.61 (± 1.76)
Murraya (*M. paniculata*)	Florida, USA	33	M16	33.06 (± 0.33)
Murraya (*M. paniculata*)	Florida, USA	34	M62	32.55 (± 1.90)

[a] Insufficient DNA quantities precluding technical replicates, therefore no St. Dev. is reported.

uniqueness and fidelity of LJ900 primers to the repeat target. *In silico* analysis of the LJ900 primers demonstrated strict alignment of the hyv_I and hyv_{II} repeat sequence to *C*Las when analyzed against the NCBI nr/nt database. Specific sequence alignments of the hyv_I and hyv_{II} genes were made against the related '*Ca.* Liberibacter solanacearum' bacterium. From this, only an alignment to a 130 bp segment of the hyv_I gene was indicated. However, the region of alignment was 5′ of the multiple tandem-repeat section of the hyv_I gene and no observation of hyv_I and hyv_{II} gene repeat regions exist in '*Ca.* Liberibacter solanacearum'.

Specificity testing in real-time PCR reactions using the LJ900 primers and probe was evaluated against DNA extracts from plant pathogens, *X. citri* subsp. *citri*, *X. axonopodis* pv. *citrumelo*, *R. solanacearum*, and *E. coli* DH5α, as well as total DNA extracted soils from the USHRL Picos Farm and USHRL grounds. No amplicon was produced in any of these samples (data not shown). We have also identified a set of negative control HLB negative USHRL greenhouse maintained citrus plants and Asian citrus psyllid colonies that always test negative for the presence of *C*Las using the LJ900 hyv_I/hyv_{II} primers. Moreover, in excess of 50 individual LJ900 primer amplicon products (from multiple global sources and hosts varieties) having *C*Las were cloned and sequenced. Each product had the same sequence indicating primer fidelity to the hyv_I/hyv_{II} 100 bp target (data not shown). In addition to these, there was no LJ900-targeted amplification in greater than one-hundred putative HLB negative DNA citrus 'repository' samples of multiple citrus varieties received as isolated DNA's from the USDA National Clonal Germplasm Repository for Citrus and Dates (as example see Table 2, sample 29). To verify amplifiable DNA within these 'repository' samples, real-time PCR was also conducted using universal 16S rDNA primers for eubacteria and α-proteobacteria populations, respectively. Universal 16S rDNA Ct values were in the low to mid teens with low thirty Ct values for the α-proteobacteria primers (data not shown), indicating viable DNA was present within these samples for testing. These data indicate target specificity for hyv_I/hyv_{II} for LJ900 methods.

To investigate the presence of the hyv_I/hyv_{II} repeat sequence in other HLB-associated species of '*Ca.* Liberibacter' (americanus and africanus), DNA samples from Brazil (americanus species) and South Africa (africanus species) were analyzed using LJ900fr in comparison with standard '*Ca.* Liberibacter americanus or africanus' real-time PCR protocols: HLBampr [17] and *rpl*JAm [9] for *C*Lam, and HLBafpr [17] for *C*Laf. In addition to LJ900fr analysis, the use of alternate *C*Las detection methods HLBaspr [17], STDP [19], and *rpl*LAs [9] were performed to aid in differentiating mixed Liberibacter populations. As shown in Table 6, Brazilian samples tested positive for hyv_I/hyv_{II} by LJ900fr. For these samples, Psy-Br12, Psy-Br17, Brazil-Amer.11, Ct values of 32.98, 21.74, 21.70 and 37.51, 25.35, 26.34 by *rpl*JAm and HLBampr were obtained respectively; however, no detectable Ct values were observed by *C*Las methods *rpl*LAs, STDP, and HLBaspr (data not shown). These data show that samples Psy-Br12, Psy-Br17, and Brazil-Amer.11 contained exclusive *C*Lam populations and that the presence of the hyv_I/hyv_{II} repeat was indicated.

The investigation of 'hyv_I/hyv_{II}-like' repeat sequence within '*C*Laf was limited to a single African sample – 'Laf 2'. The presence of *C*Laf within the 'Laf 2' sample was confirmed by HLBafpr with a Ct value of 23.77 (st. dev. ± 0.13); however, LJ900fr testing failed to produce amplification for the hyv_I/hyv_{II} sequence. These indicate that the hyv_I/hyv_{II} repeat region appears to be lacking from the *C*Laf genome.

3.2. LJ900 PCR efficiency

Primer efficiency was determined by ten-fold serial dilutions from 10^6 to 1 copy per 15 μL reaction of pLJ153.1 in water (Supplementary Fig. 1A) and subsequently with the addition of 50 ng per reaction of total *C*Las negative citrus DNA included. Without addition of citrus DNA, the LJ900 primers had an efficiency of 100.91% (Slope = −3.300, $R^2 = 0.999$) (Supplementary Fig. 1C) and with citrus background DNA present of 100.59% (Slope = −3.308, $R^2 = 0.999$) (Supplementary Fig. 1D).

As *C*Las resides within its citrus host or psyllid vector, total DNA extracts from these sources contain a mixed population with host DNA being the most abundant. It was previously demonstrated for detection of *C*Las, that the addition of non-target background DNA at 50 ng/μL in serial dilutions of target DNA effected real-time PCR detection thresholds at low target levels [15]. As host to target ratios for *C*Las samples may exceed these tested levels, we extended these experiments using elevated background DNA and LJ900fr detection using the plasmid pLJ153.1 as the template at a concentration of from 10^6 to single copy quantities.

At single copy levels we observed no significant difference in Ct at 0 and 50 ng/15 μL reactions for LJ900fr detection, while an increase of one and two Ct's was observed with background DNA levels of 100 and 200 ng/15 μL, respectively. To evaluate the influence of even greater background DNA levels relative to single copy pLJ153.1 for LJ900 detection salmon sperm DNA was used. Repeating experiments using salmon sperm DNA at 0, 50, 100, and 200 ng/15 μL reaction, the results indicated equivalent effects as *C*Las negative citrus DNA values. Salmon sperm DNA levels were then subsequently increased to 500 and 1000 ng/15 μL for testing 10^6 to single copy pLJ153.1 dilutions at these elevated background DNA levels by LJ900fr. LJ900fr detected the repeat target within all replicates from 10^6 to 10 copies for all background levels tested (0, 50, 100, 200, 500, and 1000 ng/15 μL reaction). At 500 ng total DNA/15 μL reaction (500 ng/15 μL:1 copy, salmon sperm DNA:pLJ153.1) 100% of replicates amplified the repeat target at ∼36 Ct's, whereas, for the 1000 ng/15 μL reactions the limit of detection was exceeded for single copy detection by LJ900fr (data not shown) in a standard 40 cycle reaction.

3.3. Detection by SYBR Green 1 method LJ900fr

Despite the established use of SG1 in real-time PCR [25,26], concerns arise with respect to potential signal interference of SG1 bound to non-targeted dsDNA species as SG1 binds all dsDNA. For this reason, SG1 allows a subsequent melt analysis to validate amplicon fidelity. Supplementary Fig. 1B demonstrates associated curve profiles of multiple sample well analyses for LJ900fr using PerfeCTa SYBR Green FastMix. Under these conditions the LJ900fr mean melt peak occurred at ∼75 °C for viable amplification, while non-amplification wells returned essentially a flat derivative reporter (-rn) value (Supplementary Fig. 1B Neg. ctrl.) with a null melt peak reading of ∼63 °C (Table 4, samples 5, 10, 14, 22, and 30) on the ABI 7500 Fast real-time PCR system. The melt curve remained consistent with little to no variance regardless of variable background DNA levels when amplification was detected by these methods as described.

3.4. Comparison of 'Ca. Liberibacter asiaticus' detection methods

Comparisons between LJ900fr (SYBR Green I), LJ900fpr (TaqMan®), and HLBaspr (standard 16S rDNA TaqMan® based *C*Las detection) protocols with a standardized (equal samples and volume normalized quantities tested) sample set (Table 2) were performed on samples from trees expressing typical HLB symptoms (Table 2, samples 1–28). *C*Las was detected by all three methods in 18 of 29 samples (Table 2). Significant differences ($P < 0.05$) were shown using single factor ANOVA Ct values between LJ900fr and LJ900fpr ($P = 3.9 \times 10^{-4}$) and LJ900fr relative to HLBaspr with ($P = 7.7 \times 10^{-7}$) and between LJ900fpr and HLBaspr methods ($P = 4.1 \times 10^{-2}$). In Table 2, the average Ct difference between LJ900fpr and LJ900fr relative to HLBaspr was approximately −3.71 (St. dev. ± 1.16) and −9.81 (St. dev. ± 1.02) Ct's, respectively.

The STDP method showed no significant difference ($P > 0.05$, $P = 0.993$) with LJ900fr for *C*Las detection with samples: 1, 3–13, 21–22, and 26–27; however, STDP did not amplify a product (data not shown), for samples 2, 14–20, 23–25, and 28 all of which were amplified with both LJ900fr and LJ900fpr with average Ct's of 27.46 and 33.92, respectively.

Ten-fold serial dilution of a *C*Las positive citrus sample 'VPCQ' were comparatively tested by LJ900fr, LJ900fpr, and HLBaspr methods (Table 3). From 10^{-1} to 10^{-4} dilutions, each method detected the presence of *C*Las. At 10^{-5}, only LJ900fr and LJ900fpr were capable of detecting *C*Las. At 10^{-6} and beyond, only LJ900fr was able to detect *C*Las. For 10^{-8}, none of these methods was capable of detecting *C*Las, indicating the greater sensitivity/reliability in low level detection by LJ900 methods compared to HLBaspr.

3.5. Multiplex TaqMan PCR with LJ900fpr

COXfpr, a primer/probe set targeting the plant cytochrome oxidase, provides a reliable positive internal control targeting host plant DNA when used in multiplex real-time PCR for *C*Las detection [17]. As shown in Fig. 2 lower gel, when LJ900fpr (TaqMan®) was used in multiplex real-time PCR in combination with the COXfpr primer/probe set (Table 1), the COXfpr produced a band of 68 bp while LJ900fpr produced a single repeat band of 100 bp. Neither PCR amplification efficiency, nor specificity relative to standard singleplex real-time PCR with LJ900fpr was affected by the multiplex reactions (data not shown).

A comparison of multiplex reactions using the internal plant control COXfpr with LJ900fpr or HLBaspr in multiplex reactions indicated no significant differences of COXfpr Ct values (Table 5). Consistent with the singleplex results of Table 2, LJ900fpr (in multiplex) yielded significantly different Ct values for *C*Las compared with HLBaspr for 15 DNA extracts from suspected HLB field-grown sweet orange trees from 15 counties in Florida

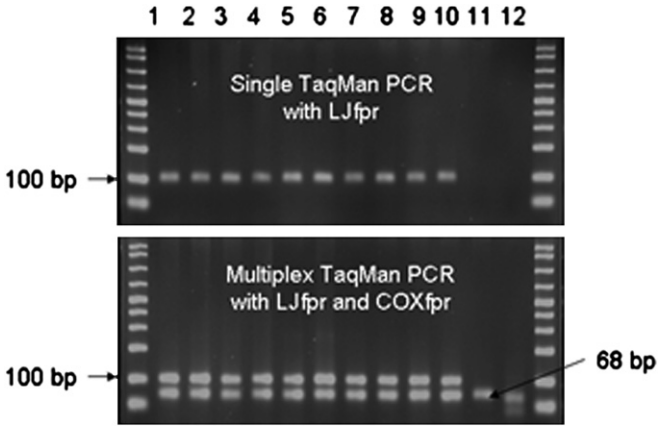

Fig. 2. 2.5% agarose gel image of singleplex LJ900fpr (upper gel) and multiplex LJ900fpr with COXfpr (lower gel) indicating amplicon products of single 100 bp (LJ900fpr) or 68 bp (COXfpr) bands. Lanes 11 and 12 (upper and lower gels) are *C*Las negative citrus controls.

(Table 5). Thirteen DNA extracts yielded lower Ct values for LJ900fpr than for HLBaspr, while two DNA extracts (#8 and #12) produced higher Ct values for LJ900fpr than for HLBaspr.Sample #13 tested negative for *C*Las using HLBaspr but yielded a Ct of 37.59 by LJ900fpr.

3.6. hyv_I/hyv_{II} detection of *Ca. Liberibacter asiaticus* from global origins and alternate hosts

To evaluate the potential of LJ900 primers in worldwide *C*Las detection, globally derived DNA samples, including psyllids and citrus varieties from HLB infected regions of Brazil, China, the Philippines, India, and Thailand were tested. Table 6 indicates detection of *C*Las within these samples by LJ900fr. Additionally, tests from alternate *C*Las-infected hosts including periwinkle, dodder, and orange jasmine (*M. paniculata*) were performed. Positive detection of *C*Las in these samples indicated the presence of the hyv_I/hyv_{II} repeats in all DNA isolates of various origins and hosts evaluated (Table 6, or data not shown).

3.7. LJ900fpr probe based and HLBasr/SG1 analyses

The addition of an internal probe to forward and reverse primer pairs for real-time PCR are generally accepted as providing superior specificity as opposed to non-probe based methods. To determine the relative detection of the LJ900fpr, a comparative analysis of selected samples (Table 2) with LJ900fpr verses LJ900fr and HLBaspr methods was performed. LJ900fpr amplified with an average Ct value 3.71 (St. dev. ± 1.04) lower than HLBaspr but an average 6.27 (St. dev. ± 1.04) Ct's higher than LJ900fr (Table 2). For general comparison of SG1 verses TaqMan® probe, a comparison of the HLBaspr primers with either SYBR Green 1 dye or using the TaMan probe was done. The SYBR Green 1 reactions produced a product with an average Ct 4.29 (St. dev. ± 0.13) lower than that observed for the TaqMan® probe. Further testing of HLBasr/SG1 with for previously undetectable Table 2 samples 17, 18, and 20 (by HLBaspr), resulted in a mean cycle threshold detection of 35.51 (St. dev. ± 0.39) for sample 17; while samples 18 and 20 remained non-detectable even by HLBasr/SG1.

3.8. Comparative dsDNA binding master mix chemistries using LJ900fr

As the proprietary contents of commercial master mixes vary in formulation from one company to another, a comparison was made with respect to LJ900fr using two alternate mixes. Testing of pLJ153.1 as the template at dilutions of 10^5 to 10^3 with the BRYT Green® based Promega GoTaq® real-time PCR master mix verses PerfeCTa SYBR Green FastMix from Quanta Biosciences under the same conditions (on the same plate) indicated a statistically significant difference in detection Ct. The GoTaq® at 10^5, 10^4, and 10^3 dilutions returned Ct's of: 16.83 (St. dev. ± 0.09), 20.32 (St. dev. ± 0.07), and 24.28 (St. dev. ± 0.05), respectively. Quanta FastMix returned Ct's of: 16.53 (St. dev. ± 0.13), 19.85 (St. dev. ± 0.10), and 23.32 (St. dev. ± 0.30) for these same samples. Single factor ANOVA at 95% confidence interval showed a statistically significant difference between these mixes ($P < 0.05$) at each dilution (10^5 at $P = 8.0 \times 10^{-3}$, 10^4 at $P = 6.6 \times 10^{-6}$, and 10^3 at $P = 7.4 \times 10^{-5}$, respectively) with the Quanta returning the lowest thresholds under these conditions. However, the Promega GoTaq melt curve analyses indicated a greater than 2 × derivative reporter (-rn) value relative to the same melt analyses of the Quanta FastMix comparative samples (Supplementary Fig. 2), a potentially useful attribute for resolving melt analyses.

4. Discussion

Accurate detection of citrus HLB-associated bacteria can be challenging because of their low titers and uneven distribution within the infected plants [5,9,10]. Current real-time PCR methods target low copy genes, which have detection limits of one to ten gene copies [9,15,17,19]. However, due to the low ratio of *C*Las DNA relative to background DNA within samples, detecting these low titer levels can be unreliable, partly due to issues associated with sampling when the target is very dilute (inability to perform reproducible sampling from a dilute solution). Therefore, we tested the PCR amplification of a newly discovered highly repetitive region of the *C*Las genome as a target for PCR-based *C*Las detection methods. For purposes of this discussion we are using Ct value as a measure of PCR assay sensitivity. We define an assay as providing a lower Ct for a given sample as more sensitive since it will allow more reproducible detection at low template genome copy number. Our results show that by using the higher copy number genetic marker (hyv_I/hyv_{II}) we were able to improve the sensitivity of detection of *C*Las as compared to current standard real-time PCR detection methods.

In this study, targeting of the *C*Las genes hyv_I and hyv_{II} multiple tandem-repeats using LJ900 primer real-time PCR demonstrates a significantly earlier threshold detection value for *C*Las bacteria relative to alternate real-time PCR methods. Each LJ900 method, SYBR Green 1 (LJ900fr) and TaqMan® (LJ900fpr), resulted in lower mean Ct values with 9.88 (SG1) and 3.71 (TaqMan®) less than the 16S rDNA HLBaspr method for *C*Las real-time PCR detection (Table 2). These indicate an increased likelihood for detection by ~10 (LJ900fpr) to >100 (LJ900fr) fold (Tables 2 and 3). Additionally, LJ900 primers detect single copy template (Supplementary Fig. 1A), although other real-time PCR methods [9,19] also indicate this limit of detection for *C*Las targets. However, the reduction in cycle detection threshold by LJ900 may reduce the potential for PCR artifact accumulation that may result from extended real-time PCR reactions that may be required by alternate methods for *C*Las detection.

We systematically evaluated the effect of increasing non-target background DNA levels up to a maximum of 1000 ng/reaction against a serial dilution of pLJ153.1 plasmid (the single copy repeat containing plasmid). These data show an increasingly delayed detection threshold of greater than five Ct values up to the 1000 ng/non-target background reaction level relative to control samples. This indicates that the ratio of target to host (non-target) DNA can have a dramatic effect on low level detection. As *C*Las exists at variable titers within different locations of its host(s) [5,9,10] and enrichment for *C*Las DNA is not presently viable (due to a lack of culturing capability for *C*Las), the level of non-target background DNA can be problematic. In incipient infections, where the ratio of non-target background DNA is high relative to *C*Las target, the probability of positive target detection within a typical real-time cycle window of 40 cycles can be low, in these instances extended cycling (>40 cycles) may be required (validated by appropriate controls [27]). However, the use of these LJ900 primers should reduce the need for extended cycling and improve the detection of early and/or low level infections within a standard 40 cycle run.

Both SYBR Green 1 (LJ900fr) and TaqMan® (LJ900fpr) chemistries using LJ900 primers demonstrated enhanced detection from known HLB-affected samples that tested negative by HLBaspr (Tables 2 and 3). In Table 3, dilutions of a known *C*Las positive citrus sample, showed that HLBaspr reached a limit of detection at serial dilution decade 10^{-4}. LJ900fpr and LJ900fr each detected an additional dilution decade beyond the HLBaspr limit of detection. While LJ900fr further obtained an additional decade dilution beyond

LJ900fpr before reaching a limit of detection. These indicate approximately 10 (LJ900fpr) to greater than 100 (LJ900fr) fold increase in detection sensitivity compared to HLBaspr.

Our primers, which target the multiple tandem-repeats of the *C*Las hyv_{I} and hyv_{II} prophage genes, provide reliable and versatile detection of *C*Las. In Table 2, HLB-symptomatic Pomelo (R8T1) and Melogold hybrid (R8T3) trees, comparative tests from multiple leaf isolates from these tree canopies indicated 100% positive identification for *C*Las by LJ900 real-time PCR (using both SG1 and TaqMan® methods). However, when these same samples were tested using alternate 16S rDNA (HLBaspr) and EF-Ts (STDP) real-time PCR target methods, each returned the identical 50% positive results for the same tested samples (Table 2 and data not shown). As bacterial levels can be variable within a sample source, false negative results might occur from an otherwise HLB positive tree, as is indicated here by 16S rDNA and EF-Ts targets underrepresenting *C*Las prevalence by half of the actual levels. These data serve to underscore the reliability of the LJ900 primers for detecting *C*Las in extreme low titer samples that may otherwise go undetected.

By comparing Ct values of the same samples run using the two LJ900 methods, the SYBR Green 1 based LJ900fr assay provided the greatest reduction in cycle threshold detection for *C*Las over the LJ900fpr TaqMan® protocol (Table 2). Interestingly, a reduction in the cycle threshold detection was shown when the internal TaqMan® probe from HLBaspr method was replaced with SYBR Green 1. This reduction may be attributed to the binding of multiple SG1 molecules to single amplicon, as opposed to the single TaqMan® reporter fluorescence signal per amplicon. In certain instances, the substitution of SG1 for the TaqMan® probe of HLBaspr returned a detectable signal for *C*Las detection in samples where previously with TaqMan® it had been undetectable.

As SG1 is non-discriminatory for DNA species, appropriate controls must be included (positive, negative, and no template controls) combined with DNA melt analysis to verify primer/amplicon specificity for each real-time PCR run. As each unique amplicon returns a signature melt profile [28], this attribute (melt) may be used to discriminate between amplification species within a real-time PCR run. Typical melt profiles for LJ900fr, under the conditions tested here, are indicated in Supplementary Fig. 1B and Table 4 with typical positive amplification peak melt temperatures at ~74–75 °C while by comparison, non-amplification sample wells return a null Tm value of ~63 °C on the ABI 7500 Fast real-time PCR system. Melt profiles from the multiple samples types tested in this study (data not shown) indicated target specific amplification for the hyv_{I}/hyv_{II} repeat by LJ900fr.

The newly developed TaqMan hyv_{I} and hyv_{II} based method could be well multiplexed with the established internal (plant) control 'COXfpr' (Fig. 2, Table 5). Table 5 indicates twelve out of 15 DNA samples from suspected HLB infected field citrus plants from around Florida that produced Ct values of one to five cycles earlier relative to a similar multiplex reaction using HLBaspr. In addition, the multiplexed LJ900fpr produced a high Ct value for DNA sample #13 that tested negative for the bacterium in the HLBaspr multiplex reaction. This gap between relative target detection was attributable to a higher frequency of the tandem-repeats per bacterial genome and the potential for elevated copies of these targeted genes should the prophage become lytic [29].

However, multiplex LJ900fpr samples #8 and #12 (Table 5) produced higher Ct values than multiplex HLBaspr. One possibility for the increased Ct values of these two samples may be non-perfect priming or probing resulting from SNP variation on the binding sites of the primer(s) or probe. Additionally, based on differences in Ct values between LJ900fpr and HLBaspr assays (Tables 2 and 4), the number of the tandem-repeats within the hyv_{I} and hyv_{II} genes may be variable among these bacterial isolates (although there is sequence conservation among the individual repeats). Different repeat numbers were found not only in samples of distinct geographical origins but also in samples from a single origin and even from a single *C*Las-infected sample [22]; therefore, a diagnostic multiplex reaction using LJ900fpr and HLBaspr could be developed to not only provide more reliable detection, but may also indicate genetic diversity within *C*Las.

Samples from HLB infected regions of Brazil, China, the Philippines, India, and Thailand were tested using the LJ900 primers (Table 6). Ct values from the samples indicate the global presence of the hyv_{I}/hyv_{II} repeats, suggesting the worldwide application of these primers for bacterial detection. However, as previously discussed, the number of the tandem-repeats might differ among samples (Table 5, samples 8 and 12) precludes using either LJ900fr or LJ900fpr for absolute real-time PCR quantification.

The hyv_{I}/hyv_{II} repeats also appear present within *C*Lam (Table 6). Samples confirmed to contain the americanus strain while lacking the asiaticus strain tested positive with LJ900 primers (Table 6 samples 1–3). However, sample 4 indicated a mixed americanus/asiaticus population illustrating that the LJ900 primers do not distinguish between these bacterial strains. For *C*Laf, the hyv_{I}/hyv_{II} repeats appear to be lacking in the 'Laf 2' sample, as real-time PCR using the LJ900 primers did not result in an amplicon. This may be due to specificity issues or that the repeat containing genes are not found in *C*Laf.

These data serve to underscore the reliability of these LJ900 methods for detecting *C*Las in extreme low titer samples and have been used in extensive testing to reliably detect *C*Las within thousands of samples (data not shown) from various citrus varieties and psyllids from both Florida and global sources. The addition of these LJ900 methods to current HLB detection protocols, along with the apparent presence of these targets within *C*Lam, warrants further study into the significance of these prophage genes and will enhance research aiding in elucidation of the HLB complex. The use of multiple tandem-repeat sequences exemplified here should serve as an example for alternate method development to exploit the enhanced detection potential for other organisms containing similar multiple and nearly identical tandem-repeat sequences, such as the *avrBS3*/*pth*A gene family of *Xanthomonas* plant pathogens [30,31].

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture and by the Specialty Crop Block Grant, FDACS contract #015579.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.mcp.2011.12.001.

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