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A new diagnostic system for ultra-sensitive and specific detection and quantification of *Candidatus* Liberibacter asiaticus, the bacterium associated with citrus Huanglongbing

Hong Lin [a,*], Chuanwu Chen [b], Harshavardhan Doddapaneni [c], Yongping Duan [d], Edwin L. Civerolo [a], Xianjin Bai [e], Xiaolong Zhao [b]

[a] *USDA ARS PWA, Parlier, CA, USA*
[b] *Guangxi Citrus Research Institute, Guangxi, China*
[c] *Roy J. Carver Center for Genomics, Department of Biology, University of Iowa, Iowa City, USA*
[d] *USDA-ARS, Fort Pierce, FL, USA*
[e] *Guangxi Academy of Agricultural Sciences, Guangxi, China*

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ABSTRACT

An ultra-sensitive and quantitative diagnostic system by combining nested PCR and TaqMan® PCR in a single tube was developed for detection of “*Candidatus* Liberibacter asiaticus”. The procedure involves two PCR steps using the species-specific outer and inner primer pairs. Different annealing temperatures allow both the first and the second rounds of PCR to be performed sequentially in the same closed tube. The first PCR with outer primers was performed at a higher annealing temperature and with limited amount of primers to prevent interference with the inner primers during the second round of PCR. The specificity of the dual primer TaqMan® is high because the fluorescent signal can only be generated from the TaqMan® probes that are homologous to the product amplified by the outer and inner primers. This new detection system can reliably detect as few as single copies of target DNA. The sensitivity of the dual primer system is comparable to the conventional two-tube nested PCR, but it eliminates the potential risk of cross contamination commonly associated with conventional nested PCR. This one-tube dual primer TaqMan® PCR method is gel-free with reduced handling time and is cost effective. At the same time, this system provides significantly increased sensitivity, improved reliability and high through-put capability suitable for routine, large scale diagnoses of clinical plant tissue and insect samples. The technique described here is generic and can be applied to the detection of other plant pathogenic bacteria.

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1. Introduction

Citrus Huanglongbing (HLB, also known as greening disease) is one of the most devastating diseases that threaten citrus production worldwide (Bové, 2006; Halbert and Manjunath, 2004). Three “*Candidatus* Liberibacter” species, “*Candidatus* Liberibacter asiaticus” (Las), “*Ca.* L. africanus” (Laf) and “*Ca.* L. americanus” (Lam), are associated with various forms of HLB disease (Bové, 2006; Bové et al., 1974; Gottwald et al., 2007). The Gram-negative bacterium is phloem-limited and belongs to the α subdivision of the *Proteobacteria*. These HLB-associated prokaryotes can be transmitted by dodder as well as by budding or grafting with propagative material from HLB-affected plants. Under natural conditions, the HLB-associated Liberibacter species are transmitted by the psyllid vectors, *Diaphorina citri* (Kuwayama) in the Asia and America (Halbert and Manjunath, 2004), and *Trioza erytreae* (Del Guercio) (Homoptera: Psyllidae) in Africa (Bové, 2006; Catling, 1969; McClean, 1974; McClean and Oberholzer, 1965). Among the three HLB-associated Liberibacter species, Las is the most widespread and is responsible for increasing economic losses worldwide in the recent years. The disease has been known in Asian countries since the 1870s (Beattie et al., 2008; Bové, 2006; Lin, 1956) but has been emerging as a serious economic threat to citrus production in the western hemisphere in recent years (Bové, 2006; Gottwald et al., 2007). HLB, associated with Las, was recently reported in São Paulo State (Brazil) (Coletta-Filho et al., 2004; Teixeira et al., 2005), Florida (USA) (Halbert, 2005), Cuba in 2006 (Martínez et al., 2008) and subsequently in the Dominican Republic in 2008 (Matos et al., 2009). More recently, the disease was also confirmed to be present in the states of Yucatán, Nayarit and Jalisco, Mexico in 2009 (NAPPO, 2009).

Studies of HLB have been impeded because Koch's postulates *sensu stricto* have not been conclusively fulfilled (Sechler et al., 2009). Early disease detection relies primarily on scouting for disease symptoms in the field, such as yellow shoots, blotchy mottle leaf and lopsided fruit with green color remaining at the stylar end and aborted seeds (Bové, 2006; Gottwald et al., 2007). However, HLB diagnosis based on disease

* Corresponding author. Crop Diseases, Pests and Genetics Research Unit, San Joaquin Valley Agricultural Science Center, USDA-ARS, 9611 S. Riverbend Ave, Parlier, CA, 93648, USA. Tel.: +1 559 596 2933; fax: +1 559 596 2921.
E-mail address: hong.lin@ars.usda.gov (H. Lin).

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symptoms can be difficult because they may be complicated by other biotic and/or abiotic plant health related problems (Bové, 2006). While diagnostic methods, such as microscopy, serology and DNA–DNA hybridization, have been used for detection purposes (Varma et al., 1993; Villechanoux et al., 1990), these methods are either time consuming or lack appropriate sensitivity. PCR-based molecular diagnostic methods, which are rapid, sensitive, specific and reliable, have been widely used for clinical diagnosis of HLB-associated Liberibacters (Hocquellet et al., 1999; Hung et al., 1999; Kunimasa et al., 2006; Li et al., 2006; Okuda et al., 2008; Teixeira et al., 2008; Villechanoux et al., 1993, 1992). These methods have also been used for Liberibacter detection in psyllid vectors (Manjunath et al., 2008). However, application of these methods for HLB detection, particularly for early detection, is inconsistent due to low bacterial titers in citrus and uneven distribution of Las in symptomatic and non-symptomatic field-grown trees (Li et al., 2006; Manjunath et al., 2008). The sensitivity of detection of HLB-associated Liberibacters, therefore, has been improved by adapting a nested PCR procedure (Deng et al., 2007; Ding et al., 2004; Li and Ke, 2002). Introduction of a second pair of primers, resulting in amplification of the previously amplified material, could lead to a significant increase in sensitivity of detection. Nested PCR has been used to improve the sensitivity of HLB-associated Liberibacter detection in clinically asymptomatic trees, especially in asymptomatic nursery plants, citrus relatives with low HLB-associated Liberibacter titer and for possible seed transmission (Benyon et al., 2008; Ding et al., 2004). However, the procedure is time consuming, as it involves two sequential rounds of PCR. The operational cost of nested PCR is nearly twice that of standard PCR. More importantly, the introduction of first round PCR amplification products to another tube for the second round of PCR amplification could potentially lead to significant percentage of false positives due to cross-contamination rendering this approach too risky for practical applications (Llop et al., 2000).

To maintain high sensitivity while overcoming these drawbacks, we designed and developed an ultra-sensitive "single closed tube dual primer" (STDP) TaqMan® PCR system. In contrast to conventional two-tube nested PCR, this detection method is based on two sequential PCR amplifications in a single closed tube, thereby eliminating the potential risk of cross-contaminations commonly associated with conventional two-tube nested PCR. The new system is a TaqMan®-based quantitative PCR which is gel-free, and easier to perform than two-tube nested PCR. The only extra cost is for the outer primers compared to standard TaqMan® PCR. The assay system provides ultra-sensitivity and reliability for HLB-associated Liberibacter detection. The system is useful for year-around disease or HLB-associated Liberibacter surveys and epidemiological studies. The assay system is also suitable for a large scale sample screening in HLB-affected areas, as well as in areas where HLB is a potential threat, but has not yet been found.

2. Materials and methods

2.1. Sample sources and DNA preparation

Leaf samples from 250 citrus trees with variable blotchy mottle symptom severity were collected from 11 citrus orchards and from 20 1–2 year symptomless nursery trees in Nanning City, China. In addition, leaf samples were also collected from 20 *Murraya paniculata* (orange jasmine) and 20 *Clausena lansium* (Chinese wampee) in Guilin City, China, respectively. Leaves were washed under running tap water and blotted dry with paper towels. The midribs were then excised from the leaf blade. Total genomic DNA was extracted from 4–5 midribs per sample. Samples were ground in liquid nitrogen and DNA was extracted using the CTAB method as previously described (Lin et al., 2008). Precipitated DNA was dissolved in 100 µl of TE buffer. The quality of DNA samples was checked by electrophoresis in 1.2% agarose gels. DNA concentrations were determined spectrophotometrically and adjusted to 50 ng/µl. Due to the select agent status of the "*Candidatus* Liberibacter" species at that time, HLB-infected DNA samples from São Paulo, Brazil, India, Florida and China were extracted in their respective origins and sent to California as microbially-sterile, non-infectious DNA samples. HLB-associated Liberibacter-free DNA samples used as negative controls were prepared from citrus leaves collected in an orchard at the USDA-Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center in Parlier, CA.

2.2. Primer and probe designs

"*Ca*. Liberibacter asiaticus" sequences representing a single copy in the Las genome were selected (Duan et al., 2009) for designing TaqMan® primers and probe for STDP TaqMan® PCR. This region has 8371 bp, of which 3701 bp were previously reported in a region containing the genes, "outer membrane protein and UDP-3-O-3-hydroxymyristoyl glucosamine N-acyltransferase" (accession # AY642159) (Bastianel et al., 2005) and 4150 bp were obtained using the genomic walking method (accession # EU523377 and EF164804) containing the genes, "elongation factor Ts", "uridylate kinase", "ribosome recycling factor", and "undecaprenyl diphosphate synthase" (Lin et al., 2008; Doddapaneni et al., 2008). The optimum TaqMan® probe and inner primers were designed using Beacon Design Software v7.0 (Premier Biosoft International, CA, USA) with the following criteria: GC% ≥ 40–50, Tm = 55 °C ± 2, primer length = 18–22 bp with amplicon size ranging from 120 to 200 bp. The melting temperature (Tm) for the TaqMan® probe was set 10 °C higher than the Tm for inner primer. To ensure amplification efficiency, the primers and probes were designed in a region where no secondary structures have been observed. Among the designed primers and probes, only those having the least possibility of forming a hairpin, self/cross dimer structures were selected for further validation. For designing the outer primers, the same criteria were applied, except that a higher Tm of 65 °C (10 °C higher than inner primer Tm) and longer amplicon size (i.e., 300–500 bp) flanking upstream and downstream of forward and reverse inner primers were selected. A computational algorithm was then performed to conduct pair-wise comparisons of all primer/primer and primer/probe and to select the best primers/probe set combination that had the least stability of forming self/cross dimers between inner and outer primers and between primer and probe ($\Delta G \geq -2$ kcal/mol). These sequence analyses resulted in identification of three sets of inner/outer primers and TaqMan® probes. The fluorescent reporter dye, 6-carboxyl-fluorescein (FAM) was labeled at the 5′ end of the TaqMan® probe. A non-fluorescent quencher, minor groove binder (MGB) was labeled on the 3′ end of probe. The probe was synthesized by Applied Biosystems Inc. (ABI, Foster City, CA).

2.3. Validation of primer and probe specificity

To ensure that the designed primers and probes were unique to "*Ca*. Liberibacter asiaticus", an *in silico* search was performed against available microbial sequences in GenBank. The specificities of the inner and outer primers were also PCR-evaluated with DNA prepared from a number of citrus-related pathogens, including *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker; *Xylella fastidiosa* strain 9a5c, the causal agent of citrus variegated chlorosis; and *Spiroplasma citri*, the causal agent of citrus stubborn disease. Further, Laf, Lam which are other citrus HLB-associated Liberibacter species, and "*Ca*. L. solanaceraum" that is associated with solanaceous plant diseases, were also included for specificity evaluation.

2.4. Optimizations of PCR conditions

For an efficacious, reliable single closed tube dual primer PCR, the annealing temperatures for inner and outer primers need to be optimized. The first PCR round should be performed at a higher

annealing temperature at which only the outer primers work. To prevent the outer primers from interfering with the inner primers when the annealing temperature is lower during the second PCR round, the amount of outer primers should be adjusted so that they are nearly depleted at the end of the first round of PCR. We therefore optimized primer annealing temperatures and inner and outer primer concentrations as described below.

2.4.1. Optimization of the primer annealing temperatures

To determine optimal annealing temperatures for the inner and outer primers, PCR experiments were conducted using an ABI Veriti thermal-cycler with a temperature gradient heating block (ABI, Foster City, CA). For the inner primers, annealing temperatures were set from 55 to 65 °C with 2 °C increments per well. For the outer primers, however, the annealing temperatures were set from 65 to 75 °C with 2 °C increments per well. PCR experiments for annealing temperature optimization were carried out in 20 µl of PCR mixture containing 2 µl of HLB-infected DNA, 1 × PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM each of dNTP, 5 pmol of inner or outer primer pairs, and 0.2 U of AmpliTaq Gold® DNA Polymerase (ABI, Foster City, CA). The PCR amplification conditions were 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 55–65 °C (for inner primer) or 65–75 °C (for outer primers) and 72 °C for 45 s. HLB-free citrus DNA was included as a negative control. PCR products were separated by electrophoresis in 1.2% of agarose gels and assessed after staining with ethidium bromide.

2.4.2. Optimization of the primer concentration

To determine the optimal concentration of inner and outer primer pairs, forward and reverse outer primers at the concentrations of 0.05, 0.1, 0.5 and 1.0 pmol were combined with either 5 or 10 or 20 pmol of the inner forward and reverse primers in 20 µl PCR reaction mixture mentioned above. PCR was carried out at 95 °C for 10 min followed by two concatenate rounds of PCR. The first round was performed for 20 cycles at 95 °C for 30 s, 67 °C for 45 s and 72 °C for 45 s. The second round of PCR was run for 35 cycles at 95 °C for 30 s, 57 °C for 45 s and 72 °C for 45 s. The optimal annealing temperatures were determined to be 67 °C for outer primers and 57 °C for inner primers based on the optimization annealing temperature experiments described above. The products were visualized in 1.2% agarose gels after staining with ethidium bromide.

2.4.3. Comparison of sensitivity of conventional two-tube nested PCR and one-tube closed nested PCR

We evaluated the sensitivity of the newly designed primers using conventional PCR. The experiments were conducted to compare two-tube nested PCR and single closed tube nested PCR. For this, serially-diluted plasmid DNA containing target sequences in a range of 10^6, 10^5, 10^4, 10^3, 10^2, 50 and 5 copies were used as template DNA. We used the outer primer pair PCR as reference. 20 µl standard PCR conditions were set for single primer pair PCR (outer primers) and for two-tube nested PCR (inner and outer primers). For single closed tube nested PCR, 0.5 pmol outer and 10 pmol inner primers were mixed in 20 µl reaction mixture in the same tube and run in concatenate PCR with annealing temperature of 67 °C for 20 cycles in the first round of PCR and 57 °C annealing temperature for 35 cycles in the second round of PCR. PCR products were separated by electrophoresis in 1.2% of agarose gels and visualized after ethidium bromide staining.

2.5. Single tube dual primer TaqMan® PCR

To quantitatively evaluate the sensitivity of STDP for TaqMan® PCR, an annealing temperature of 67 °C was used for the outer primers during the first round of PCR and 57 °C for the inner primers during the second round of PCR. The outer primer pair (0.5 pmol) was combined with the inner primer pair (at 5, 10 or 20 pmol) and 5 or 10 pmol of TaqMan® probe. The PCR mixture (20 µl) was prepared using TaqMan® master mixture (ABI, Foster City, CA) and amplification was carried out using the following parameters: 50 °C for 2 min and 95 °C for 10 min and followed by 20 cycles at 95 °C for 30 s, 67 °C for 45 s and 72 °C for 45 s for the first round of PCR and 35 cycles at 95 °C for 30 s, 57 °C for 45 s and 72 °C for 45 s for the second round of PCR. The fluorescence signal was recorded at the end of each step of 57 °C during the second round of PCR.

2.6. Amplicon cloning and molecular standard curve

A 470 bp DNA fragment amplified with the outer primers using conventional PCR was purified and ligated with the pGEM®-T Easy vector (3015 bp) (Promega, CA). The ligated vector was transformed into JM109 competent cells following the manufacturer's protocol (Promega, CA). Plasmid DNAs were purified from transformed cells using QiAprep Miniprep kit (Qiagen). The cloned plasmid DNA contains a total of 3485 base pairs, equivalent to a molecular weight of 2,117,339 Da of double strand DNA. The purified plasmid DNA was quantified using PicoGreen method. According to Avogadro's number, "6.022×10^{23} molecules/mol", each µg of cloned plasmid DNA is therefore mathematically equivalent to 2.84×10^{11} target DNA copies. To prepare a standard curve for absolute target DNA copy estimation, the cloned plasmid DNA with 1.0×10^7 copies/µl was sequentially diluted at 10-fold intervals to 1.0×10^1, 5 and 1 copies/µl. Due to the stochastic behavior when molecules are extremely diluted, there were three replications for each dilution. The same series of dilutions was also made by mixing cloned plasmid DNA with healthy citrus genomic DNA (50 ng/µl) to mimic naturally infected HLB samples. Both molecular standards were used to generate standard curves using STDP TaqMan® PCR by a Bio-Rad IQ5 Real-time PCR cycler.

3. Results

3.1. Primers and probe selected for dual primer TaqMan® detection system

Each of three primer/probe sets, designed from sequence analyses, was evaluated by conventional PCR. All three sets of primers produced clean target products without visible primer dimers. However, primers designed with the locus of the "elongation factor Ts" gene region gave the strongest band compared to the other two sets of primers under the same conditions. Therefore, this primer/probe set was selected for this study (Table 1). The forward and reverse inner primers were "Las-I-F" (5′-CGATTGGTGTTCTTGTAGCG-3′) and "Las-I-R" (5′-AACAATAGAAGGATCAAGCATCT-3′), respectively. The

Table 1
Sequences of inner primers (Las-I-F and Las-I-R), outer primers (Las-O-F and Las-O-R) and TaqMan® probe (Las-P) designed within the '*Candidatus* Liberibacter asiaticus' "elongation factor Ts" sequence locus.

Name	Length (bp)	Tm (°C)	Amplicon (bp)
Las-O-F/Las-O-R	28/33	65	470
Las-I-F/Las-I-R	20/23	55	170
Las-P	28	65	

CGGTGAATGTATTAAGCTGAGGCGTTCCGCTCTTCTGTGTGTTTCGGAAGGGGTCATCTCTTCGTATCTT
Las-O-F →
ATGCTTCTCCTTCTGAGGGTTTAGGAT***CGATTGGTGTTCTTGTAGCG***TTGCAGTCTTCTGCGGAAGATAAG
Las-I-F →
GAATTGCTTTCTGCGATTGGAGAGAAGATTGCAGTGCA***TGTAATGCTGGCTTCTCCTTCGGTGATT***TCTGT
← Las-P
GC***AGATGCTTGATCCTTCTATTGTT***GCTAATAAACGTGCCCATTACATGACAGAAGCACTTGATTCTGGGA
← Las-I-R
AATCAGGTAATATTGTTGAAAAGATCGTAAATGGAAAGATGCAAAGCTTTTGTAAGGAATGTGTTCTTTTG
CATCAGGGGTTTGTTGTTGATCCTTCCAAAACTGTATCAGATTTTTTGAAAGAATCTGAAAAATCAATCGG
TGCTTCTATT***GAAGTTGTTGGTGTATCTCATTTTGTTGTGGGTA***
← Las-O-R

forward and reverse outer primers were "Las-O-F" (5′-CGGTGAATG-TATTAAGCTGAGGCGTTCC-3′) and "Las-O-R" (5′-TACCCACAA-CAAAATGAGATACACCAACAACTTC-3′). The TaqMan® probe designed in this locus is "Las-P" (5′-AATCACCGAAGGAGAAGCCAG-CATTACA-3′). The amplification products are 470 bp for the outer primers and 140 bp for the inner primers.

3.2. Optimal annealing temperatures and primer concentration determination

To achieve dual primer PCR in a single tube PCR, both primer annealing temperature and concentration were optimized. The outer primers were designed to have a higher annealing temperature than that of the inner primers. This precluded interference by the inner primers during the first round of PCR. The inner primers work at temperatures ranging from 55 to 63 °C (Fig. 1). No product was observed at annealing temperatures above 63 °C. Strong, single bands were observed at the annealing temperatures between 55 and 57 °C (Fig. 1). Therefore, 57 °C was considered to be the optimal annealing temperature for the inner primers. The outer primers were designed with an annealing temperature 10 °C higher than that of the inner primers. The annealing temperatures tested for the outer primers ranged from 65 to 75 °C. No amplification product was observed at annealing temperature higher than 69 °C. An annealing temperature of 67 °C resulted in the strongest band (Fig. 1). Therefore, 67 °C was considered to be the optimal annealing temperature for the outer primers (Fig. 1). To minimize the competition of the outer primers with the inner primers during the nested amplification process, the amount outer primer concentrations were limited so that most of the outer primers were incorporated into PCR products at the end of the first round PCR and no extra outer primers were carried over into the second PCR round. With the outer primer concentrations of 0.05, 0.1, 0.5 and 1.0 pmol, after 20 cycles of amplification, no visible outer primer products were observed at 0.05, 0.1, and 0.5 pmol regardless of the inner primer concentration used (data not shown). Thus, 0.5 pmol appeared to be the highest concentration that could be used for the outer primers, and therefore, was set as the optimal concentration for the outer primer pairs. To further evaluate the sensitivity and efficiency of amplification for the STDP detection system, the experiments were carried out using conventional gel-based PCR and quantitative real-time PCR.

3.3. Conventional two-tube and single closed tube nested PCR comparison

The comparative sensitivity of detection by the conventional two-tube nested PCR and STDP PCR methods was evaluated using the selected outer and inner primer pairs. The lowest detectable level for a single primer pair PCR (outer primers only) was 10^6–10^3 copies of target DNA (Fig. 2). However, both two-tube nested PCR and STDP PCR detected as few as 5 copies demonstrating that the STDP PCR described in this study has comparable sensitivity of detection as conventional two-tube nested PCR (Fig. 2).

3.4. Sensitivity and amplification efficiency of STDP TaqMan®

Plasmid DNA cloned with target DNA amplicon fragments was sequentially diluted 10-fold in water or in HLB-free citrus genomic DNA, representing 10^7 to 1 copies of target DNA. This set of target DNA copies served as a molecular standard to evaluate the amplification efficiency and the sensitivity of detection. Increase in the probe concentration or *Taq* polymerase did not alter Ct values, indicating both were not limiting factors under the test conditions. However, in comparative tests with inner primer concentrations at 5, 10 or 20 pmol, the STDP TaqMan® PCR with 20 pmol inner primers resulted in the lowest Ct values. With 20 pmol inner primer, STDP TaqMan® detected 10^7 copies of target DNA with a Ct value at 5, 10^3 copies with Ct value at 18.5 and a single copy with Ct value at 27.4 (Fig. 3). With the same target DNA copy numbers, approximately 2–4 more Ct values were observed when 10 and 5 pmol inner primers were used. Thus, the increase in inner primer pair concentration appeared to be necessary for increased detection sensitivity. When assays were compared between the molecular standards diluted with citrus genomic DNA and the molecular standards diluted in water, only a slightly higher Ct value (~0.3–0.6) was observed with the molecular standards diluted in citrus genomic DNA, indicating that extracted genomic DNA did not have significant inhibitory effect on detection. A standard curve generated under the test conditions gave a linear regression $R = 0.98$ (Fig. 4). This indicated that under the concatenate dual primer PCR process, the quantitative relationship between log target DNA copy numbers and PCR cycles is retained. The standard curve in this study had an average slope value of −3.33. The amplification efficiency (AE) of STDP TaqMan® PCR was therefore estimated to be about 0.99 based on the equation $AE = [10^{-1/slope} - 1]$.

3.5. Specificity

The *in silico* BLAST searches against all available microbial sequence databases in NCBI did not identify any homologous sequences suggesting that the sequence locus selected for primers and probe designed are specific to Las. The specificity was further confirmed by PCR experiments using either inner or outer primer pairs against several citrus pathogens including *Xanthomonas citri*

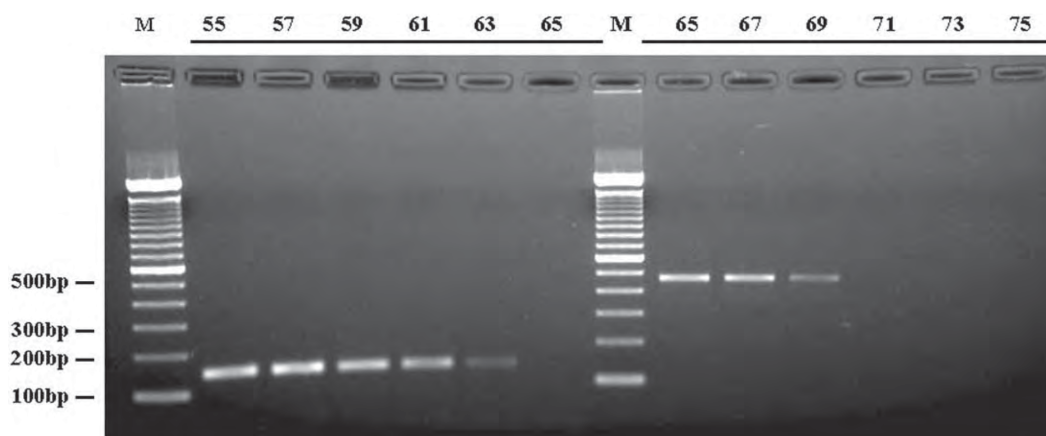

Fig. 1. Optimization of inner primer (left) with 140 bp PCR product at annealing temperatures from 55 °C to 65 °C and outer primer (right) with 470 bp PCR product at annealing temperatures from 65 °C to 75 °C. M is 100 bp molecular markers.

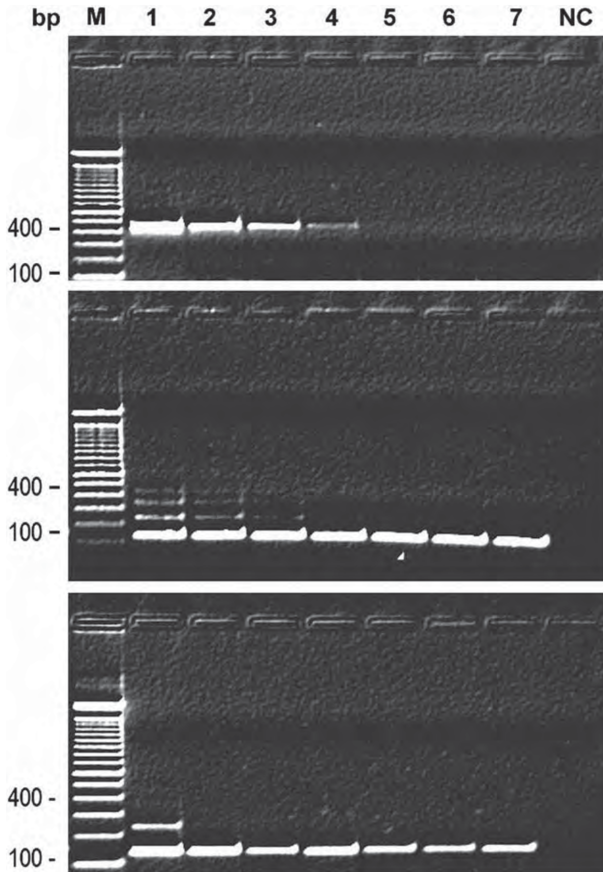

Fig. 2. Conventional single primer pair with outer primer PCR (top), two-tube nested PCR (middle) and one-tube dual primer PCR (bottom). Lanes 1–7 represent 10^6, 10^5, 10^4, 10^3, 10^2, 50 and 5 copies of target DNA. NC is negative control.

subsp. *citri*; *Xylella fastidiosa* strain 9a5c, *Spiroplasma citri*, Laf, Lam and "*Ca.* L. solanaceraum". As expected, all assays yielded negative results. To confirm if the sequence locus selected for this detection is conserved and shared among "*Ca* Liberibacter asiaticus" isolates, DNA from 34 Las sources from Brazil, 30 from India, 3 from Taiwan, 1 from Thailand and 1 from Japan, in addition to the samples from Guangxi, China and Florida, U.S. were tested using the USDA APHIS-approved diagnostic HLBasrp primers (Li et al., 2006) and STDP primers in this study. All sources that were tested positive with HLBasrp primers were also positive with the STDP primers (date not shown) indicating that the sequence locus selected for this STDP diagnosis system is species-specific and conserved among Las sources from Asia, as well as from North and South America.

3.6. Diagnostic evaluations of field samples

To evaluate the suitability of a new assay system for routine assay, two experiments were conducted to compare this dual primer pair system with the standard single primer pair system. In the first experiment, 169 suspect HLB field samples collected from 14 counties in Florida, U.S. and 216 samples collected from four cities in Guangxi province, China were assayed. Both single primer pair TaqMan® PCR (with the inner primers only described in this study and HLBasrp TaqMan®) resulted in 24% and 34% positives among the Florida and Guangxi samples, respectively (Fig. 5). When STDP TaqMan® PCR was compared with single primer pair TaqMan® methods, 36% and 47%, of the samples, respectively, were positive. About ~12–13% more positives were obtained using the STDP TaqMan® PCR than when the single primer pair TaqMan® PCR method was used (Fig. 5). To confirm the results, those identified as positive by STDP TaqMan® PCR only were reconfirmed by sequencing PCR amplicons. Ten PCR products representing relatively bright bands and 10 representing faint bands were excised from 1.2% of gel and cloned into pGEM vector. Re-sequencing of these amplicons confirmed that they matched the target sequences 100%.

In the second experiment, asymptomatic citrus nursery samples (sweet orange and pummelo), as well as ornamental citrus relatives, *Murraya paniculata* (orange jasmine) and *Clausena lansium* (Chinese wampee) were assayed for Las HLB. Using STDP TaqMan® PCR, 7 of 9 (77.7%) sweet orange samples and 9 of 9 (100%) pummelo samples were positive while both single primer pair real-time PCRs detected 4 of 9 (44.4%) sweet orange and 5–6 of 9 (55.5%–66.6%) pummelo positive (Table 2). With STDP TaqMan® PCR, 8 of 15 (53%) Chinese wampee and 8 of 12 (67%) orange jasmine samples were identified as positive. In contrast, only 13.3% and 8.3% of the Chinese wampee and orange jasmine samples, respectively, were positive when both single primer pair real-time PCRs were used (Table 2). Among these 45 tested samples, the results of two-tube conventional nested PCR

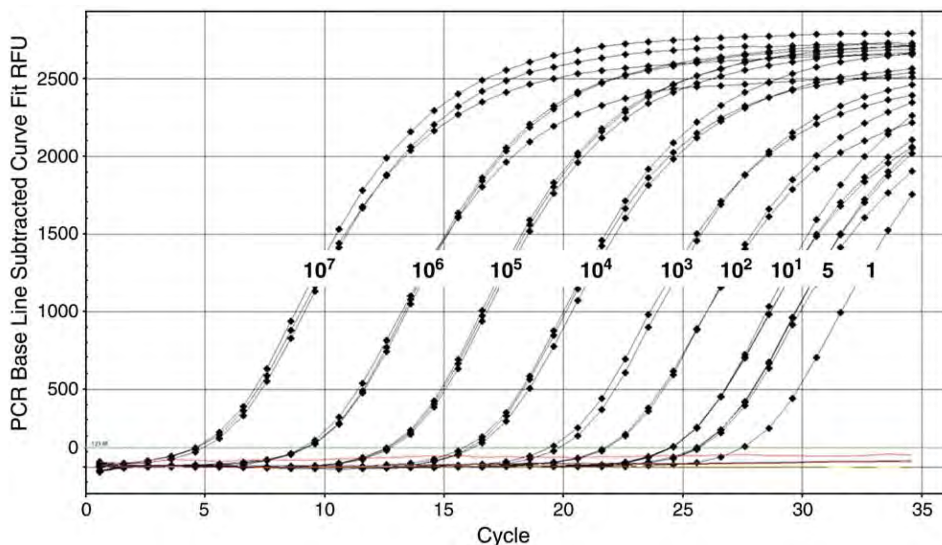

Fig. 3. STDP TaqMan® PCR with 0.5 pmol outer primers, 20 pmol inner primers and 5 pmol TaqMan probe in 25 µl of reaction. The amplification plot represents a dilution series of molecular standards ranging from 10^7 to 10^0 copies.

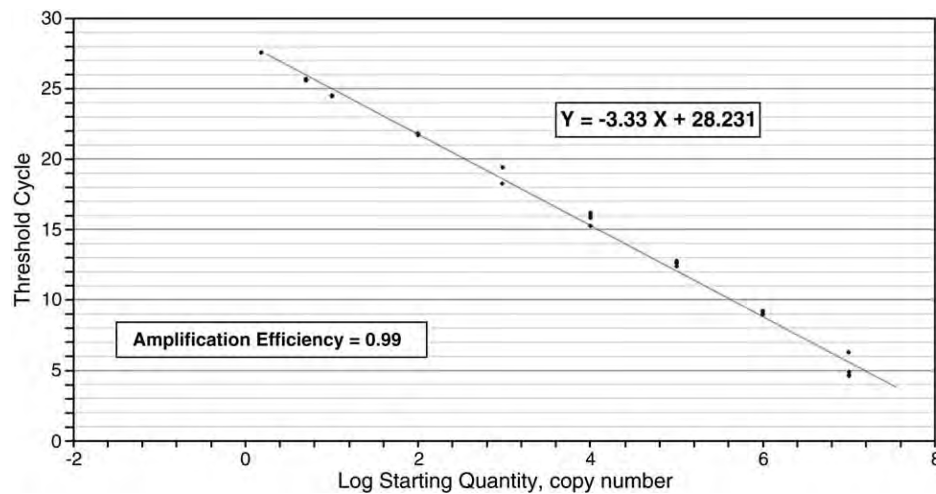

Fig. 4. A molecular standard curve of STDP TaqMan® PCR using the target DNAs ranging from 10^7 to 10^0 copies with 0.99 amplification efficiency and 0.98 correlation coefficient.

were generally comparable with the results obtained by the STDP TaqMan® PCR except for 5 samples. Of these 5 samples, two (GL-06-09-2 and GL-06-32) were only identified as positive using the two-tube nested PCR while three samples, (GL-06-20-3, GL-06-30 and GL-06-31) were only identified as positive by STDP TaqMan® PCR (Table 2). Again, sequencing of the positive amplicons detected by STDP TaqMan® PCR confirmed that they were true positives.

4. Discussion

Sensitive and accurate detection is essential for efficient management and regulatory responses to prevent the introduction into, and spread of HLB-associated "*Candidatus* Liberibacter" species in, unaffected areas. Since HLB-associated asiaticus may present in low titer and unevenly distributed in citrus, and because the disease has a prolonged latent period before symptoms appear, current diagnostic methods cannot reliably detect early infections. This is particularly critical in areas where psyllids have been introduced but HLB has not yet been reported. Recent emerging HLB diseases in Brazil and Florida are good examples where the confirmation of HLB apparently occurred late, probably a few years after the establishment of the pathogen (Coletta-Filho et al., 2004; Halbert, 2005). Clearly, the causal agent(s) of disease became widespread prior to detection, highlighting the lack of adequate monitoring tools. Consequently, by the time HLB was confirmed the critical time for early disease detection and eradication of infected plants had passed (Gottwald et al., 2006).

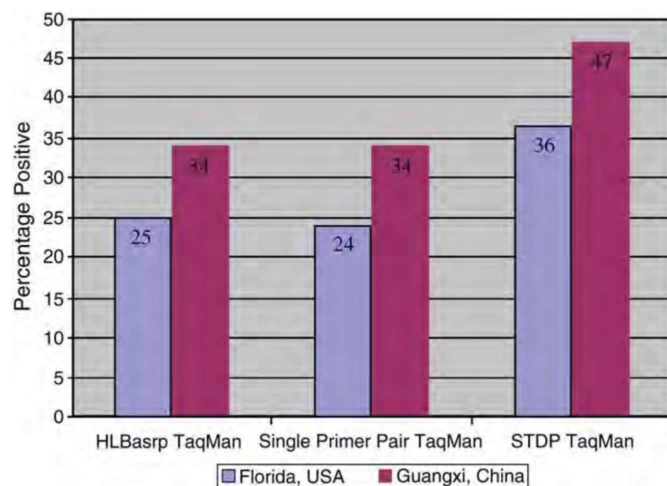

Fig. 5. Comparative detection of HLB-associated "*Candidatus* Liberibacter asiaticus" with HLBasrp TaqMan® (Li et al., 2006), Single Pair TaqMan® (inner primer pairs in this study) and STDP TaqMan®. Both HLBasrp TaqMan and Single Pair TaqMan identified 25% positive samples from Florida and 34% samples from Guangxi, while STDP TaqMan identified 36% positive samples from Florida and 47% positive samples from Guangxi, respectively.

Various molecular diagnostic methods have been developed since "*Ca*. Liberibacter" species were identified to be associated with HLB. While these methods generally work well with symptomatic samples, it has often been observed that the methods are not reliable for consistently detecting "*Ca*. Liberibacter"-infected, but asymptomatic, field or nursery trees or in psyllids (Li et al., 2006; Manjunath et al., 2008; Teixeira et al., 2008). Therefore, most currently available HLB tests can be used only as confirmatory tests with limited early detection value. Improved methods with enhanced reliability and sensitivity for HLB-associated Liberibacters in plant and insect samples are needed (Manjunath et al., 2008).

In the case of extremely low Las titer, nested PCR was essential for detection (Benyon et al., 2008; Deng et al., 2007). This concatenate PCR has a higher sensitivity than that of the real-time PCR (Anderson et al., 2003). However, this procedure requires that the tubes containing high concentrations of the first amplification product be opened and transferred to a separate tube for the second amplification. This procedure is time consuming and nearly doubles the operation cost. More importantly, the enhanced sensitivity of nested PCR increases the risk of potential false positives due to possible cross-contamination, rendering this method less practical unless extreme care is taken. These limitations above were overcome by developing an innovative system based on differential Tm between the first and the second round PCR primers. Llop et al (2000) designed a single closed-tube nested PCR detecting system which was able to detect 20% more positive plants naturally infected with *Erwinia amylovora* than conventional PCR and found that the sensitivity of the detection system was comparable to the conventional two-tube nested PCR.

To improve the current detection limit of the HLB-associated "*Ca*. Liberibacter" asiaticus, we developed a novel ultra-sensitive dual primer TaqMan® PCR for molecular diagnosis of HLB. This new detection method is conceptually analogous to conventional nested PCR, but incorporates quantitation and automation features of real-time PCR. Detection and identification of the target DNA are performed by monitoring the fluorescence signal emitted as the result of the disassociation of fluorescent molecules from TaqMan® probes. Therefore, post-PCR analysis steps, such as electrophoresis, are avoided. As a result, this detection system is highly sensitive and is adaptable for large scale high through-put sample screening.

Both well-designed primers/probe and optimization of the PCR conditions are critical in order to achieve ultra-sensitivity and

Table 2
Comparisons of single inner primer pair TaqMan (SPP TaqMan), single closed-tube dual primer TaqMan (STDP TaqMan), HLBasr/HLBp TaqMan (Li et al., 2006) and two-tube conventional nested PCR. Samples identified as positive are shown in shaded areas. + = positive, ND = none detected.

Sources	Host	Tissue	Ct value			Two-tube nested PCR
			HLBasr/HLBp TaqMan	Single primer pair TaqMan	STDP TaqMan	
Nan-38-1	Sweet orange	Midrib	27.74	29.77	19.68	+
Nan-38-2	Sweet orange	Midrib	ND	ND	ND	ND
Nan-38-3	Sweet orange	Midrib	ND	ND	27.83	+
Nan-38-25	Sweet orange	Midrib	ND	ND	ND	ND
Nan-40-3	Sweet orange	Midrib	ND	ND	26.55	+
Nan-40-4	Sweet orange	Midrib	ND	ND	26.73	+
Nan-40-12	Sweet orange	Midrib	29.07	30.25	23.31	+
Nan-40-13	Sweet orange	Midrib	30.48	31.8	24.82	+
Nan-42-4	Sweet orange	Midrib	31.28	32.63	25.75	+
Nan-42-5	Pummelo	Midrib	30.65	32.2	25.6	+
Nan-42-10	Pummelo	Midrib	31.94	33.48	25.81	+
Nan-42-14	Pummelo	Midrib	30.18	31.53	24.65	+
Nan-42-15	Pummelo	Midrib	ND	ND	27.66	+
Nan-44-6	Pummelo	Midrib	28.48	29.67	23.54	+
Nan-44-8	Pummelo	Midrib	32.93	ND	25.78	+
Nan-44-14	Pummelo	Midrib	ND	ND	27.12	+
Nan-44-15	Pummelo	Midrib	ND	ND	26.96	+
Nan-44-16	Pummelo	Midrib	33.57	ND	25.54	+
GL-06-09-1	Chinese wampee	Midrib	ND	ND	26.45	+
GL-06-09-2	Chinese wampee	Green stem	ND	ND	ND	+
GL-06-09-3	Chinese wampee	Root	ND	ND	ND	ND
GL-06-12-1	Chinese wampee	Midrib	ND	ND	ND	ND
GL-06-12-2	Chinese wampee	Green stem	29.12	29.71	19.82	+
GL-06-12-3	Chinese wampee	Root	ND	ND	ND	ND
GL-06-14-1	Chinese wampee	Midrib	ND	ND	ND	ND
GL-06-14-2	Chinese wampee	Green stem	ND	ND	27.72	+
GL-06-14-3	Chinese wampee	Root	ND	ND	ND	ND
GL-06-18-1	Chinese wampee	Midrib	ND	ND	28.22	+
GL-06-18-2	Chinese wampee	Green stem	ND	ND	28.68	+
GL-06-18-3	Chinese wampee	Root	ND	ND	ND	ND
GL-06-20-1	Chinese wampee	Midrib	30.51	31.12	23.79	+
GL-06-20-2	Chinese wampee	Green stem	ND	ND	27.92	+
GL-06-20-3	Chinese wampee	Root	ND	ND	28.39	ND
GL-08-24	Orange jasmine	Midrib	ND	ND	28.67	+
GL-06-25	Orange jasmine	Midrib	ND	ND	ND	ND
GL-06-26	Orange jasmine	Midrib	ND	ND	27.08	+
GL-06-27	Orange jasmine	Midrib	ND	ND	28.04	+
GL-06-28	Orange jasmine	Midrib	33.01	33.89	25.99	+
GL-06-29	Orange jasmine	Midrib	ND	ND	ND	ND
GL-06-30	Orange jasmine	Midrib	ND	ND	28.66	ND
GL-06-31	Orange jasmine	Midrib	ND	ND	28.42	ND
GL-06-32	Orange jasmine	Midrib	ND	ND	ND	+
GL-06-33	Orange jasmine	Midrib	ND	ND	ND	ND
GL-06-34	Orange jasmine	Midrib	ND	ND	28.86	+
GL-06-35	Orange jasmine	Midrib	ND	ND	28.12	+

diagnostic reliability of STDP real-time PCR. Due to the complexity of the multiple primers and probe involved, the interference between primer–primer and primer–probe should be minimized. We verified that the STDP real-time PCR system described in this study achieved 0.99 amplification efficiency (Fig. 4).

PCR optimization is also critical to ensure the highest sensitivity. This includes not only optimal annealing temperatures for inner and outer primers, but also coordinately optimizing reaction agents including appropriate ratios of amounts of inner and outer primer and probe (Forsman et al., 2003). Our results indicate that 0.5 pmol outer primers with 20 cycles for the first round amplification followed by 20 pmol inner primers with 35 cycles of subsequent nested PCR are optimal conditions for the most sensitive detection. Optimal amounts of outer primers cannot be determined exclusively as copies of target bacterial DNA vary from sample to sample. Most clinical samples from HLB-affected trees have bacteria concentrations ranging from 10^4–10^7 per gram midrib tissue in symptomatic samples to 10^1–10^4 in asymptomatic samples (Li et al., 2006; Teixeira et al., 2008). The amount of outer primers (0.5 pmol) was, therefore, experimentally determined for a wide range of target DNA copies (10 to 10^7) and reconfirmed with naturally-affected HLB samples. With this amount of outer primers, 20 cycles of the first round PCR will incorporate most of outer primers into amplified products that subsequently served as templates for second round of PCR. In addition, 20 pmol of inner primers appeared to be necessary to achieve the highest sensitivity. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production in each PCR cycle. As amplified target DNAs generated from the first round PCR increase, the higher the concentration of inner primers are provided for the nested PCR cycle, the earlier the accumulative fluorescent signal reaches to the threshold, and therefore, the earlier the Ct values are recorded. 20 pmol of inner primer in this case confirmed to be necessary to achieve the highest sensitivity.

Reliable detection is also dependent on the specificity of the detection method. Conserved regions, such as 16S rRNA, have been used for designing PCR-based Liberibacter detection protocols. While 16S rRNA contains important genetic signatures of evolutionary histories and relationships among bacterial species, molecular diagnostic

assays based on this region may have limit specificity due to conserved sequence (Ali et al., 2006; Farrell 1999; Rolain et al., 2007). To ensure reliable detection, other species-specific sequencing loci should be used to avoid ambiguous PCR results (Tjhie et al., 2001). In this study, a specific-gene region, the "elongation factor Ts" (EF-Ts) was selected for primer/probe designs. The EF-Ts gene is conserved among phylogenetic related bacteria in general; however, the sequencing region that was selected for primers and probe design is unique in Liberibacter asiaticus. The specificity of this STDP TaqMan® detection system is high because the target DNA amplicon is amplified sequentially by species-specific dual primers and the fluorescent signal is detected only from the probe which specifically hybridizes with the target DNA amplicon. The STDP TaqMan® PCR assay has a broad detection range from 1 to 10^7 copies of target DNA with equal sensitivity and efficiency. The relationships between the threshold cycles and the log of target DNA copies are also linear with the average a correlation coefficient of 0.98. The ultra-sensitivity, high specificity and reliability of this detection system provide an improved tool for HLB diagnosis and research. This ultra-sensitive assay is a valuable tool for early detection of Las prior to development of HLB symptoms. The STDP TaqMan® PCR identified ~12% more HLB-positive field-collected citrus samples from Florida and China than single primer pair real-time PCR (Fig. 5). We further validated STDP TaqMan® PCR with other real-time PCRs for detecting Las in the ornamental rutaceaous relatives, *M. paniculata* and *C. lansium*, that have been shown to be potential inoculum sources of the HLB causal agents (Ding et al., 2005; Li and Ke, 2002). Both of these species tend to have lower titers of Las and could only be reliably detected by nested PCR (Deng et al., 2007; Ding et al., 2005). Using these samples, we confirmed that the sensitivities of the STDP TaqMan® PCR and two-tube nested PCR were comparable except for five samples, of which two were identified as positive by two-tube nested PCR and three were identified as positive by STDP TaqMan® PCR. The discrepancy between the two methods was likely due to pipetting errors as these five samples had extremely low titers (Ct value > 28) while all samples with Ct value 27 or less were consistent in both tests. Therefore, use of this novel technique overcomes the limitations of two-tube nested PCR and provides an improved method for HLB diagnosis. To our knowledge, this is the first development of such a methodology for detection of a phytopathogen, and can be adapted for other pathogenic disease diagnosis.

This new diagnostic system can be used for high through-put assay of symptomatic or asymptomatic clinical samples and monitor vectors for the HLB-associated Liberibacter species. Accordingly, this pathogen diagnostic system can enhance the effectiveness of quarantine surveys that are critical for effective HLB management, especially for areas in which the disease has not yet been reported. This method could also be used all year round to obtain information about the population dynamics of HLB-associated Liberibacter species and the epidemiology of HLB disease under various environmental conditions.

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