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Development and application of molecular-based diagnosis for '*Candidatus* Liberibacter asiaticus', the causal pathogen of citrus huanglongbing

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Conventional PCR and two real-time PCR (RTi-PCR) methods were developed and compared using the primer pairs CQULA03F/CQULA03R and CQULA04F/CQULA04R, and TaqMan probe CQULAP1 designed from a species-specific sequence of the rplJ/rplL ribosomal protein gene, for diagnosis of citrus huanglongbing (HLB) disease in southern China. The specificity and sensitivity of the three protocols for detecting '*Candidatus* Liberibacter asiaticus' in total DNA extracts of midribs collected from infected citrus leaves with symptoms in Guangxi municipality, Jiangxi Province and Zhejiang Province, were tested. Sensitivities using extracted total DNA (measured as copy number, CN per μL of recombinant plasmid solution) were 439·0 ($1{\cdot}30 \times 10^5$ CN μL^{-1}), 4·39 ($1{\cdot}30 \times 10^3$ CN μL^{-1}) and 0·44 fg μL^{-1} ($1{\cdot}30 \times 10^2$ CN μL^{-1}) for conventional PCR, TaqMan and SYBR Green I (SGI) RTi-PCR, respectively. SGI RTi-PCR was the most sensitive, but its specificity needed to be confirmed by running a melt-curve assay. The TaqMan RTi-PCR assay was rapid and had the greatest specificity. Concerning the correlation of PCR detection results with the various HLB symptoms, uneven mottling of leaves had the highest positive rate (96·50%), indicating that leaf mottling was the most reliable symptom for field surveys. Dynamic analysis results from the TaqMan assays showed that the titre (CN) g^{-1} citrus tissue of '*Ca.* L. asiaticus' was highest between October and December (threshold cycle (C_t) average = 29·3, CN = $3{\cdot}35 \times 10^7$) and lowest between March and May (C_t average = 32·0, CN = $5{\cdot}10 \times 10^6$) in 2004 and 2005. The optimized molecular-based assays should prove useful for presymptom diagnosis of HLB disease, monitoring and identification of '*Ca.* L. asiaticus', and field epidemic regulation.

Keywords: '*Candidatus* Liberibacter asiaticus', *Citrus*, citrus greening, end-point PCR, TaqMan real-time PCR, α-Proteobacteria

Introduction

Huanglongbing (HLB, ex-greening), one of the most devastating citrus diseases, was first reported in southern China (Lin, 1956) and can be transmitted by grafting infected scions. The causal agent was identified as a phloem-restricted bacterium belonging to a new genus in the alpha-subdivision of the α-Proteobacteria (Jagoueix *et al.*, 1994). The '*Candidatus*' generic name 'Liberobacter' was defined by Murray & Stackebrandt (1995), but since 2000 the nomenclature of the species has been revised from 'Liberobacter' to 'Liberibacter' following the rules of the International Code of Nomenclature of Bacteria (Garnier *et al.*, 2000). Three species have been proposed: '*Ca.* L. asiaticus' in Asia; '*Ca.* Liberibacter africanus' in Africa (Da Graca, 1991; Planet *et al.*, 1995); and '*Ca.* L. americanus' in Brazil (Teixeira *et al.*, 2005b). However, the organism has not been cultured, nor have Koch's postulates been satisfied. HLB is transmitted by grafting, or by the psyllid vectors *Diaphorina citri* in Asia (Zhao, 1981) and *Trioza erytreae* in Africa (Da Graca, 1991). HLB was not formally reported in the USA until September 2005 (Knighten *et al.*, 2005). Although the inability of the psyllid vectors to survive in temperatures <5°C may play a key role in limiting the spread of HLB to areas with cold winter climates, such as Chongqing, China and central California, USA, the northern limit at which psyllid vectors overwinter has gradually moved from the southern to the northern citrus plantation areas in southern China during the past 30 years. Field diagnosis of HLB is difficult because symptoms are often confused with those of disorders such as Zn^{2+} deficiency, and because the organism has not been cultured. The disease can be diagnosed using electron microscopy, but such assays are time-consuming and require expensive equipment and appropriate sample preparation. Large-scale production of pathogen antibody

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and reliable serological assays have not been developed because of difficulties in raising specific antibodies to an uncultivated organism, and the variation of antigen traits of the target pathogen. Several pairs of conventional PCR primers have been developed, based on sequences from a cloned gene cluster of ribosomal protein and 16S rDNA (Jagoueix *et al*., 1996; Hocquellet *et al*., 1999; Teixeira *et al*., 2005a), but the resulting amplicons are quite long and do not result in stable, sensitive PCR reactions. Also, a RFLP analysis of the resulting amplicons must be performed to differentiate Liberibacter spp.

The aim of this study was to develop reproducible conventional PCR with several sets of primers and two quantitative real-time (RTi)-PCR methods for specific detection and monitoring of '*Ca.* L. asiaticus'. The following steps are described: (i) development of a DNA marker specific to '*Ca.* L. asiaticus' from ribosomal protein genes and 16S rDNA followed by its transformation into a plasmid pMD18-T; (ii) design and validation of a specific primer set and TaqMan probe for RTi-PCR monitoring; (iii) comparison of end-point PCR, SYBR Green I (SGI) and TaqMan RTi-PCR for detection and identification of '*Ca.* L. asiaticus' of HLB; and (iv) application of optimized methods to monitor the population titre of pathogenic bacteria in sieve tubes of citrus and provide guidance for field surveys and integrated pest management of HLB disease.

Materials and methods

Citrus sample collection and DNA extraction

Citrus leaves with typical yellow mottling symptoms of HLB and other suspected HLB samples were collected from groves in Guangxi Municipality, Fujian, Sichuan, Jiangxi and Zhejiang provinces, in China. DNA was extracted from midribs of leaves by a rapid soaking-filtration method (Wang & Gast, 2000), in which leaf midribs were ripped off and chopped, then 200-mg leaf midribs were soaked in TES buffer 30 min at 65°C. Samples were centrifuged for 5 min at 10 000 ***g*** and the supernatant was transferred into a UNIQ-10 minicolumn (Sangon). After the minicolumn was rinsed with isopropanol twice and briefly spun, 50 μL ddH_2O was added into the minicolumn to elute total DNA. After a brief spin, the extract was stored at –20°C for PCR assay.

A recombinant plasmid pUCLA10 was included as a positive control. Negative controls for PCR assays consisted of (i) DNA template extracted from healthy citrus leaves; (ii) DNA of saprophytic bacteria isolated from citrus leaves; (iii) DNA of tested plant pathogenic bacteria; (iv) cDNA of *Citrus tristeza virus*; (v) ddH_2O (Millipore). Saprophytic bacteria isolated from citrus leaf surfaces were used directly for PCR amplification via the boiling method described previously (Wang *et al*., 1999).

PCR protocols for detection of Liberibacters

Apart from three sets of primers (OI1/OI2c, LAA2/LAJ5 and OL1/OL2, which had been published before: Bove *et al*., 1996; Jagoueix *et al*., 1996; Hocquellet *et al*., 1999), the other primer sets were designed using the software PRIMER PREMIER (Premier Bio-soft International) and PRIMER EXPRESS ver. 2 (ABI). All primers and the probe were synthesized and fluorescence-labelled by the Sangon Company, Shanghai. Sequences are shown in Table 1.

Conventional PCR

For the two published sets of primers (OI1/OI2c and LAA2/LAJ5), the GeneAmp PCR System 9700 (Perkin Elmer Cetus) was used for DNA amplification following Jagoueix *et al*. (1996) and Hocquellet *et al*. (1999), respectively.

For newly designed primer pairs, the iCycler PCR DNA System (Bio-Rad) was used for DNA amplification. The conventional PCR reaction was performed in a 25-μL reaction mixture containing 1 × PCR buffer, 0·25 μM of each primer, 0·3 mM dNTP, 2·0 mM $MgCl_2$, 1 U Taq polymerase and the appropriate amount of DNA template. The program consisted of 94°C for 2 min, 30 cycles of

Table 1 Tested HLB primers and probes used for detection of '*Candidatus* Liberibacter asiaticus'

Primer pairs/probe	Sequences of primer pairs and probe	Product (bp)	Source
OI1/OI2c	5′-GCGCGTATGCAATACGAGCGGCA-3′ 5′-GCCTCGCGACTTCGCAACCCAT-3′	1160	16S rDNA[a]
LAA2/LAJ5	5′-TATAAAGGTTGACCTTTCGAGTTT-3′ 5′-ACAAAAGCAGAAATAGCACGAACAA-3′	703	U09675[b]
CQULA01F/CQULA01R	5′-TGAATTCTTCGAGGTTGGTGAGC-3′ 5′-AGAATTCGACTTAATCCCCACCT-3′	535	This test[c]
CQULA03F/CQULA03R	5′-CAAGGAAAGAGCGTAGAA-3′ 5′-CCTCAAGATCGGGTAAAG-3′	382	This test[c]
CQULA04F/CQULA04R	5′-TGGAGGTGTAAAAGTTGCCAAA-3′ 5′-CCAACGAAAAGATCAGATATTCCTCTA-3′	87	This test[c]
CQULAP10	5′-ATCGTCTCGTCAAGATTGCTATCCGTGATACTAG-3′		This test[c]

[a]Designed from the 16S rDNA sequence of '*Ca.* Liberibacter asiaticus' (Jagoueix *et al*., 1996).

[b]From rplA/rplJ gene sequence both located on the In2·6 and AS1·7 inserts (Hocquellet *et al*., 1999).

[c]Designed in this test based on the cloning sequence of 382-bp specific fragment located in ribosomal protein L10 (rplJ) and L12 (rplL) of '*Ca.* Liberibacter asiaticus' GXLa10.

Table 2 Strains and DNA used to determine specificity of the RTi-PCR assay

Strain no.	Species	Source[a]	RTi-PCR result[b]
Xcc01	*Xanthomonas campestris* pv. *campestris*	1	N/A
Xoo10	*X. oryzae* pv. *oryzae*	1	N/A
Xooc02	*X. oryzae* pv. *oryzicola*	1	N/A
FJXac01	*X. smithii* subsp. *citri*	1	N/A
Cmm01	*Clavibacter michiganensis* sp. *michiganensis*	1	N/A
JM109	*Escherichia coli*	1	N/A
CVC01	*Xylella fastidiosa*[c]	1	N/A
FJXac02	*Xanthomonas smithii* subsp. *citri*	2	N/A
FJXac03	*X. smithii* subsp. *citri*	2	N/A
FJXac04	*X. smithii* subsp. *citri*	2	N/A
FJXac05	*X. smithii* subsp. *citri*	2	N/A
FJXS06	*X. smithii* subsp .*citri*	2	N/A
FJXS01	*Xanthomonas* sp.	2	N/A
FJXS02	*Xanthomonas* sp.	2	N/A
FJXS03	*Xanthomonas* sp.	2	N/A
FJXS04	*Xanthomonas* sp.	2	N/A
FJXS05	*Xanthomonas* sp.	2	N/A
FJXS06	*Xanthomonas* sp.	2	N/A
CTV02	*Citrus tristeza virus*[c]	2	N/A
CQNCK	*Citrus* sp.[c]	2	N/A
Pss20	*Pseudomonas syringae* pv. *syringae*	3	N/A
GXPCK	*Candidatus* Liberibacter asiaticus	2	Ct 27·5

[a]1, Tested isolates or DNA from N. W. Schaad, USDA-ARS, Foreign Disease–Weed Science Research Unit, Fort Detrick, MD, USA; 2, isolated at the Genetic Engineering Center, Chongqing University; 3, from G. Ma, Southwest Agricultural University, Chongquing.
[b]N/A, Ct value not available in RTi-PCR assay.
[c]Pathogen or plant tissue DNA used.

92°C for 20 s, 56°C for 30 s and 72°C for 45 s. The specificity of PCR was tested using the plant pathogens and nonpathogens listed in Table 2.

Real-time PCR

For RTi-PCR, the FAM and TAMRA fluorescence-based TaqMan and SGI systems were used. For TaqMan, the primer pairs (CQULA04R/CQULA04F) and probe (CQULAP10) were designed from the target sequence of ribosomal protein genes of '*Ca.* L. asiaticus'. The probe was labelled with 6-FAM as a reporter fluorescent dye at the 5′ end and with TAMRA at the 3′ end as a quencher dye. The TaqMan PCR reaction was performed in a 25-μL reaction mixture containing 1 × PCR buffer (Bio-Rad Master Mix), 0·8 μM of each primer, 0·2 μM TaqMan probe and the appropriate amount of DNA template. The iCycler IQ Multiplex Real Time PCR DNA System (Bio-Rad) was used with the following program for DNA amplification: 95°C for 1 min, 45 cycles each of 95°C for 15 s, 59°C for 15 s and 72°C for 45 s. During the extension step (72°C for 45 s) of each cycle the fluorescent signal was generated from the excited reporter dye-labelling probe.

For SYBR Green, the PCR reaction was performed in a 25-μL reaction mixture containing 1 × PCR buffer (SYBR Green Master Mix; Bio-Rad); 0·8 μM of each primer (CQULA04R/CQULA04F) and the appropriate amount of DNA template. The iCycler IQ Multiplex Real Time PCR DNA System was used with the following programme: 95°C for 1 min, 45 cycles each of 95°C for 15 s, 59°C for 15 s and 72°C for 45 s. During the extension step (72°C for 45 s) of each cycle the instrument collected fluorescent signal generated from SGI fluorescent dye nonspecifically bound to any dsDNA. To analyse the specificity of the PCR amplification, a melt-curve programme was subsequently conducted as follows: 95°C for 1 min, 55°C for 1 min, then the temperature was increased by 0·5°C every 10 s from 55 to 95°C. The melt curve was plotted according to the manufacturer's instructions (Bio-Rad).

Construction of recombinant plasmid and standard curve

The target fragment (382 bp) of '*Ca.* L. asiaticus' amplified by primer set CQULAF3/CQULAR3 was electrophoresed on 2% agarose gel with GoldView (SBS Genetech). The expected target band was cut out *in situ* and purified with DNA recover kit (Promega) according to the manufacturer's instructions. The DNA was eluted in 20 μL Millipore pure water and 8 μL were used for ligation with the pMD18-T vector. Recombinant plasmid solution (2 μL) was used to transform competent *Escherichia coli* string JM109 cells (Takara). The recombinant plasmid, designated pUCLA10, was extracted with a UNIQ-10 mini-column following the instructions for the plasmid DNA extraction kit (Sangon), and was sequenced and aligned using BLASTN. The original plasmid standard solution was quantified with a spectrophotometer (Beckman), then diluted in 10-fold serial dilutions, from the original solution of target DNA to 10^{-10}, the dilutions being used in RTi-PCR to generate a standard curve. With the

standard-curve method, the iCycler iQ could calculate the titre of pathogen in tested field samples automatically. The unit of detection limitation was fg μL^{-1}, which was converted into copy number (CN; not bacteria number) per g citrus tissue. The following formula was used to calculate CN:

$$CN = (M \times N)/(L \times D)$$

where M = minimum concentration of nucleic acid detected (g mL^{-1}); N = Avogadro's number ($6{\cdot}022 \times 10^{23}$ molecules $mole^{-1}$); L = length of nucleic acid in kbp (total length of plasmid + insert of LaGX10 fragment); and D = conversion factor from 1 kb nucleic acid to Daltons (dsDNA = $6{\cdot}6 \times 10^5$ g $mole^{-1}$ kb^{-1}). For example, for the standard plasmid samples the concentration of the original plasmid solution (relative dilution rate 1×10^0) was $4{\cdot}39 \times 10^{-4}$ g mL^{-1}, which was equal to $1{\cdot}30 \times 10^{11}$ CN μL^{-1}.

Practical monitoring of HLB pathogen in citrus plantations

Conventional and RTi-PCR methods were applied to determine the population titre of '*Ca.* L. asiaticus' in infected Ponkan mandarin and Shatian pumello trees during different growth seasons. Samples were collected monthly from newly matured leaves in the east, south, west and north from July 2004 to May 2005 in Zheguhu citrus orchard, Liuzhou, Guangxi Municipality. The total DNA extracted from leaf midribs by the chopping and soaking–filtration method was identified using conventional PCR. The DNA samples tested were then stored at –20°C and reanalysed with TaqMan RTi-PCR to investigate changes in the titre of '*Ca.* L. asiaticus' in the phloem of the host throughout the year.

Results

Detection with conventional PCR

With the newly designed primer sets for '*Ca.* L. asiaticus', the target 382-bp band was amplified from the DNA extracts of infected plants (Fig. 1a, lane 10). In contrast, DNA samples extracted from the several saprophytic bacteria on citrus leaves (Fig. 1a, lanes 3–8); the cDNA obtained from reverse transcription of *Citrus tristeza virus* ssRNA (lane 2); DNA from *E. coli* string JM109 (lane 9); and DNA extracted from healthy citrus leaves (lane 1) were all negative and showed no target bands.

With regard to detection sensitivity (Fig. 1b), the standard samples containing 439·0, 43·9, 4·39 or 0·439 pg μL^{-1} all produced the specific target band (lanes 1–4, respectively), while the following three standard samples, with 4·39, 0·44 and 0·04 fg μL^{-1} (Fig. 1b, lanes 6–8) were negative and the fifth dilution sample, containing 43·9 fg μL^{-1} DNA, resulted in only a very weak band (lane 5). The no-template negative control (lane 9) and filtrated Millipore water (lane 10) showed no amplified DNA bands.

To compare different sets of primers, conventional PCR based on OI1/OI2c (1160 bp) and LAA2/LAJ5 (703 bp) primers were used to detect '*Ca.* L. asiaticus' of HLB in samples from the Beihai citrus plantation. The primer pairs tested were able to amplify expected fragments of ribosomal protein and the β-operon gene of liberibacters from citrus leaf midrib samples (Fig. 2). Amplification was not observed when negative control DNA extracted from healthy leaves of Valencia sweet orange grown in the greenhouse was used in conventional PCR. The target DNA band was always obtained with purified DNA from leaves with mottling, which was set as the positive control for PCR, but the rate of detection in citrus leaf samples without symptoms was relatively low (3·28%).

Practical detection of HLB liberibacter with conventional PCR

HLB routine field surveys were performed based on symptoms such as mottling, yellowing and small leaves each week during April–May 2000 (first round) and October–November 2000 (second round) in Beihai citrus plantation, Guangxi Municipality. Leaf samples with or without symptoms of HLB were collected and, after washing with tap water, leaf midribs were removed

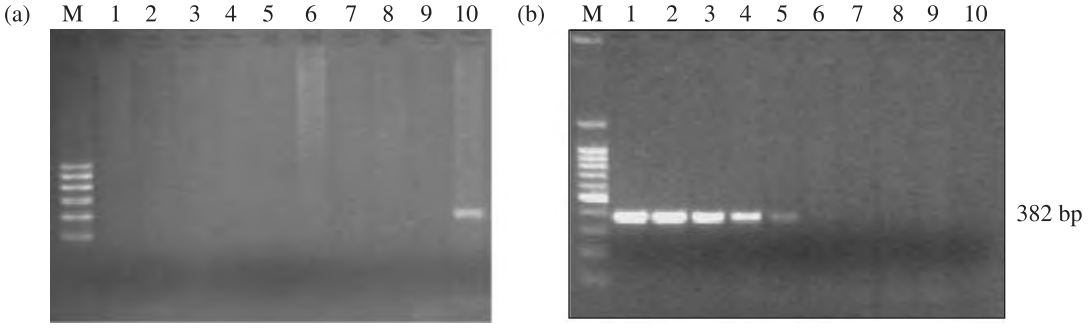

Figure 1 (a) Specificity of conventional PCR assay for detection of citrus huanglongbing agent, '*Candidatus* Liberibacter asiaticus'. M, DNA ladder (300–800 bp); lane 1, DNA extracted from healthy citrus leaves; lane 2, cDNA reverse transcripts from *Citrus tristeza virus* ssRNA; lanes 3–8, DNA extracted from saprophytic bacteria on citrus leaves; lane 9, DNA extracted from untransformed *Escherichia coli*; lane 10, DNA extracted from midrib of HLB-infected leaf sample. (b) Sensitivity of conventional PCR assay. M, DNA ladder; lanes 1–8, amounts of plasmid DNA of target fragment, 439 pg, 43·9 pg, 4·39 pg, 0·44 pg, 43·90 fg, 4·39 fg, 0·44 fg, 0·04 fg μL^{-1}; lane 9, tested sample without template as negative control; lane 10, ddH_2O.

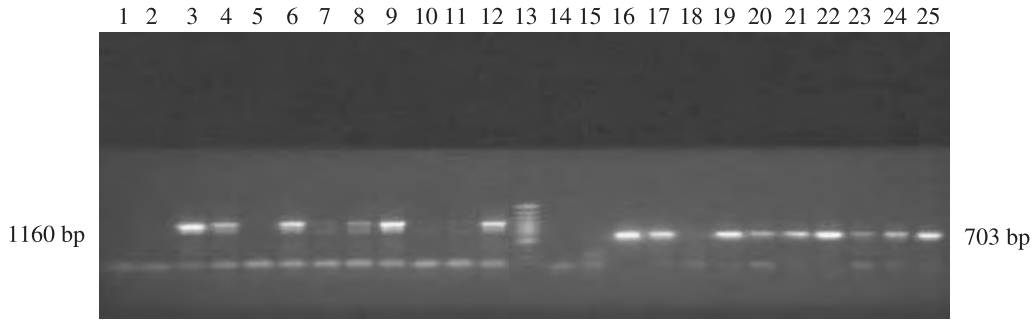

Figure 2 Results of analysing citrus leaf samples by PCR for the presence of '*Candidatus* Liberibacter asiaticus' based on primer pair OI1/OI2c (lanes 1–12, 1160 bp) and LAA2/LAJ5 (lanes 14–25, 703 bp). Lanes 1 and 14, filtered sterilized distilled water; lanes 2 and 15, negative control; lanes 3 and 16, positive control; sample with: lanes 4 and 17, both mottling and small, zinc-deficient leaves; lanes 5 and 18, yellow-vein symptom; lanes 6 and 19, leaf mottling; lanes 7 and 20, light mottling and yellowing of veins; lanes 8 and 21, light mottling and yellowing of veins; lanes 9 and 22, mottling; lanes 10 and 23, light yellowing of veins; lanes 11 and 24, yellow veins and slight mottling of leaves; lanes 12 and 25, typical mottling symptoms; lane 13, 100-bp DNA ladder.

Table 3 Comparing routine survey and conventional PCR detection for citrus HLB disease

	Routine survey[a]	PCR detection[b]	Agreement (%)
April–May 2000			
No. positive/no. total	106/217	109/217	
Positive result (%)	48·39	50·23	96·34
No. negative/no. total	111/217	108/217	
Negative result (%)	51·61	49·77	96·43
October–November 2000			
No. positive/no. total	88/182	98/182	
Positive result (%)	48·35	53·85	89·79
No. negative/no. total	94/182	84/182	
Negative result (%)	51·65	46·15	89·35

[a]Routine survey conducted using typical leaf mottling and yellowing or HLB-like symptoms of citrus trees.
[b]PCR amplification performed based on LAA2/LAJ5 set primers for the same batch samples as the routine survey, conducted in a reaction volume of 25 μl per tube, 703-bp target DNA using agarose gel electrophoresis is positive result; *vice versa*, negative result.

and 200 mg leaf midribs chopped into fine mince using single-face disposable blade. To extract DNA of the HLB pathogen, a slightly modified cetyltrimethylammonium bromide (CTAB) method was employed (Wang *et al*., 1999). The survey results were verified by conventional PCR using LAA2/LAJ5 primers. A total of 443 leaf samples of citrus with varied symptoms were analysed in two disease-occurrence peaks: the target band of DNA was amplified among 113 mottled leaf samples (96·50%). Otherwise, the positive percentage of disease detection for samples with Zn^{2+} deficiency, yellowing, small leaves and no symptoms was relatively low (data not shown). The percentage agreement between routine survey results and positive PCR detection reached up to 96·34 and 89·79% during the two rounds of field surveys (Table 3). The DNA band generated had a well defined size and was visualized via agarose gel electrophoresis. Positive results were obtained with various sets of primer pairs; however, a smaller target fragment (382 bp) was more reproducible and sensitive than larger fragments (data not shown).

Detection with RTi-PCR

TaqMan

A specific fluorescent signal for the HLB pathogen was obtained reproducibly using primer pair CQUCLA04F/CQUCLA04R and the TaqMan probe together with an annealing temperature of 59°C and an annealing time of 15 s. Some other annealing temperatures (50·0, 50·8, 52·0, 53·7, 56·2, 58·1 and 60·0°C) and times (10 and 20 s) were tested with the same primer pair, but none led to a better result (not shown). Regarding the specificity of the TaqMan RTi-PCR assay, only the DNA template from recombinant plasmid pUCLA10 was positive (Fig. 3), with a low threshold cycle (C_t) value (19·0), an amplifying curve plot, and a unique band obtained. In contrast, all other isolates of plant pathogenic bacteria or citrus saprophytes listed in Table 2 did not yield signals, with no C_t values or amplifying curves. This result confirmed the specificity of the primers and probe for the HLB pathogen.

The standard curve was calculated on the serial dilution of total DNA [from 0·439 ng μL^{-1} ($1{\cdot}30 \times 10^8$ CN μL^{-1}) to

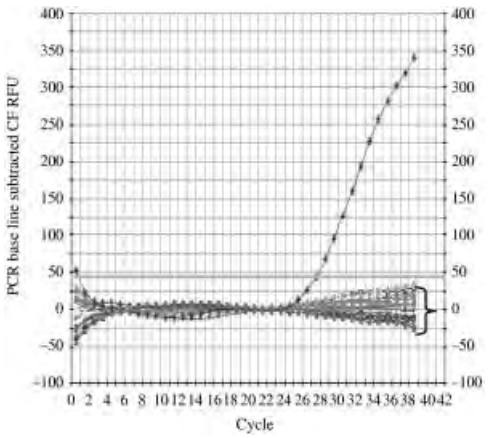

Figure 3 Specificity of the TaqMan real-time PCR assay for '*Candidatus* Liberibacter asiaticus'. Target pathogen positive control shows a remarkable increase in fluorescent signal, while the others, including 23 bacteria and plant pathogens tested (Table 2) and a negative control, show no increase.

0·04 fg μL^{-1} ($1{\cdot}30 \times 10^{1}$ CN μL^{-1})]. A linear relationship was observed between C_t values and the log concentrations of recombinant plasmid DNA (not shown). The results for TaqMan detection sensitivity (Fig. 4a) showed that the samples containing DNA template from $1{\cdot}30 \times 10^{8}$ to $1{\cdot}30 \times 10^{3}$ CN μL^{-1} had C_t values ranging from 17·0 to 38·5, all below the acceptable upper threshold limit C_t of 40, which was established by repeated experiments with various samples of target pathogen. However, the copy numbers of target DNA template $<1{\cdot}30 \times 10^{2}$ CN μL^{-1} or dilution in 1×10^{-9} gave negative reactions, (non-C_t values obtained after 40 cycles), and no amplification curve of fluorescent signal. The detection sensitivity of TaqMan PCR for HLB pathogens was therefore 4·39 fg μL^{-1}, equal to $1{\cdot}30 \times 10^{3}$ CN μL^{-1}.

In RTi-PCR (Fig. 4a), six samples with DNA concentrations of 10^{-3} to 10^{-8} exceeded the threshold, whereas when the same products were analysed by gel electrophoresis, only the first four dilution samples could be amplified and observed in agarose gel; the other four samples and the negative control gave only the weak bands of primer. This showed that the TaqMan RTi-PCR assay was about 100-fold more sensitive than conventional PCR (Fig. 4b).

SYBR Green

The detection limit of SGI-PCR was 0·44 fg μL^{-1} (plasmid dilution 1×10^{-9}) and was the most sensitive of all protocols tested. Although some of the other 23 plant pathogenic and saprophytic bacteria in SYBR Green PCR showed pseudopositive reactions, with increasing curves and across the baseline after 35 cycles (data not shown), the melt curve-verifying test indicated that negative sample and negative control have a lower melting temperature (Fig. 5).

Practical application of conventional PCR and TaqMan RTi-PCR

From July 2004 to May 2005 in Zheguhu citrus orchard, Liuzhou, Guangxi Province, samples were tested by conventional PCR then retested by TaqMan RTi-PCR (Table 4). The percentage of positive detection with conventional PCR clearly varied with the different seasons of citrus growth: the highest percentage of positive detection was obtained in October (100%) and the lowest in April (37·5%). There were slight differences in detection rate between Ponkan mandarin (86·4%) and Shatian pumello (79·6%) trees year-round from July 2004 to May 2005. The population titre of the '*Ca.* L. asiaticus' pathogen showed distinct dynamic changes during citrus-growing periods. The average pathogen copy numbers calculated

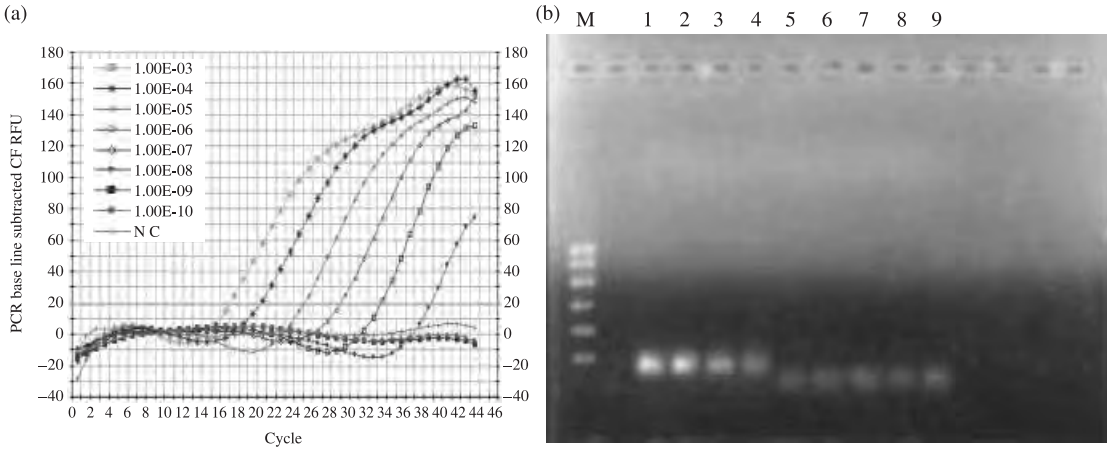

Figure 4 (a) Detection sensitivity of TaqMan RTi-PCR for '*Candidatus* Liberibacter asiaticus' using 10-fold serial dilution of recombinant plasmid DNA. (b) Products of TaqMan RTi-PCR analysed by agarose gel electrophoresis. M, DNA marker (100–600 bp); lanes 1–4, 10-fold serial dilution of recombined plasmid of '*Ca.* L. asiaticus' from 10^{-3} to 10^{-6} to ($1{\cdot}30 \times 10^{5}$ CN μL^{-1}) produce a band (87 bp); lanes 5–8, dilution from 10^{-7} to 10^{-10} ($1{\cdot}30 \times 10^{1}$ CN μL^{-1}); lane 9, negative control.

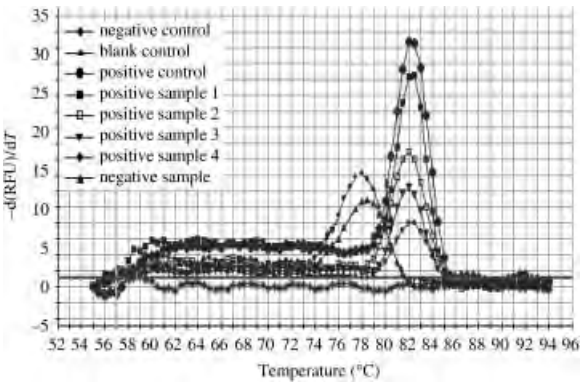

Figure 5 Melting curve of SYBR Green I assay for '*Candidatus* Liberibacter asiaticus'. The group peaking at 82°C shows the specific amplicons produced; the group peaking at 78°C included negative sample and negative control; straight line below baseline, blank control.

from the sample C_t value obtained by TaqMan RTi-PCR revealed that the population of '*Ca.* L. asiaticus' pathogen in sieve tubes of host phloem tissues had a peak titre between October and December (C_t values 28·0–30·2) and was lowest between March and May (C_t values 32·5–34·8), coinciding with the incidence of HLB disease in the orchard over a year. The maximum and minimum amounts of pathogen detected during the year with TaqMan RTi-PCR showed >100-fold difference in CN (Table 4).

Discussion

Detection results showed that conventional PCR was inferior to the RTi-PCR assays. Conventional PCR would be expected to lead to some degree of false-negative results, especially when sample preparation is inappropriate. In addition, conventional PCR requires time-consuming Southern blotting or alignment of target sequence to confirm the similarity or identity of the amplified product. Sensitivities using extracted prokaryotic purified DNA were 439·0 ($1{\cdot}30 \times 10^5$), 4·39 ($1{\cdot}30 \times 10^3$) and 0·44 fg μL^{-1} ($1{\cdot}30 \times 10^2$ CN μL^{-1}) for conventional PCR, TaqMan PCR and SGI RTi-PCR, respectively. The most sensitive assay was the SGI RTi-PCR protocol. However, the SGI dye-based PCR assay may react with many other nontarget bacteria, and requires a DNA melt-curve to confirm the test and its accuracy, especially in the case of a high C_t value. The TaqMan PCR approach has several advantages over conventional PCR and SGI RTi-PCR, including specificity, robustness and speed (Schaad & Frederick, 2002). The TaqMan assay is somewhat less sensitive than the SGI RTi-PCR, but has superior specificity and is considerably faster. Compared with molecular beacons and Scorpion probes, the TaqMan probe provides a simple, straightforward design (Ginzinger, 2002). This newly described TaqMan assay should provide an accurate, reliable method for the early diagnosis of HLB in suspicious cases, and could be used to distinguish '*Ca.* L. asiaticus' from '*Ca.* L. africanus' and '*Ca.* L. americanus' (N. W. Schaad, USDA-ARS, Foreign Disease–Weed Science Research Unit, Fort Detrick, MD, USA, personal communication).

Many of the currently used sample-preparation methods currently used are technically challenging and time-consuming. Rapid and robust preparation methods are needed before PCR can be used for routine analysis to facilitate detection of targets in varied biological samples (Templeton & Claas, 2003). The application of

Table 4 Year-round detection of HLB pathogen by PCR

	Conventional PCR/Ct values of real-time PCR[b]									CN/GCT of RTi-PCR[c]	
Date	Po1	Po2	Po3	Po4	Pu1	Pu2	Pu3	Pu4	Positive C-PCR[d]	Po	Pu
07/04	+/29·2[c]	+/31·7	+/30·7	+/29·0	+/30·9	+/29·9	+/31·0	–/31·9	87·5	186	108
08/04	+/32·2	+/31·7	+/31·7	+/32·0	–/29·9	+/30·9	+/29·0	+/30·9	87·5	55	179
09/04	+/34·0	+/32·7	+/30·2	+/30·9	+/29·4	+/29·9	–/30·1	+/30·9	87·5	55	192
10/04	+/28·8	+/28·1	+/27·4	+/27·5	+/28·7	+/28·6	+/27·8	+/27·4	100·0	828	758
11/04	+/30·0	+/30·6	+/29·4	+/29·7	+/29·8	+/29·2	+/30·1	+/28·3	100·0	206	330
12/04	+/31·4	+/30·2	+/29·6	+/29·8	+/29·9	–/31·0	+/29·0	+/32·1	87·5	179	146
01/05	+/32·2	+/31·6	+/29·9	+/30·5	+/29·8	+/30·9	+/30·5	–/32·6	87·5	118	103
02/05	+/31·6	+/32·2	+/31·6	–/30·7	+/29·3	+/30·6	+/30·4	+/32·0	87·5	73	135
03/05	+/31·6	+/32·5	–/33·9	+/32·2	+/32·5	+/32·2	–/33·0	+/33·0	75·0	36	32
04/05	–/35·0	–/34·3	+/35·6	–/34·4	+/35·0	–/33·9	–/35·0	+/34·9	37·5	7	8
05/05	+/31·9	–/31·5	–/31·8	+/32·2	+/32·8	+/31·9	–/32·2	+/33·1	62·5	55	36

[a]Tested citrus samples were collected from infected Ponkan mandarin 'Po' and Shatian pumello 'Pu' trees at Zheguhu Citrus Orchard, Liuzhou, Guangxi municipality, each with 40 pieces of newly mature leaf from the surrounding canopy, without typical symptoms, from Liuzhou, Guangxi Province.

[b]Detected rate percentage, average of tested samples each month.

[c]CN/GCT, copy numbers g^{-1} citrus tissue with HLB pathogen samples, calculated following the formula given in the text.

[d]+, Sample with target band of HLB pathogens; –, tested samples without target band of pathogens. Ct values were detected by the iCycler IQ Multiplex Real-time PCR DNA System (Bio-Rad) with TaqMan RTi-PCR assay with specific primers and probe.

conventional and RTi-PCR is, to some extent, restricted by the presence of PCR inhibitors such as proteins, IgG, EPS, phenol, Zn^{2+}, Ca^{2+}, EDTA and heparin (Wilson, 1997). Citrus plant pigments, phenolic materials or bactericidal copper ions can often lead to false negatives. To remove PCR inhibitors efficiently the use of Bio-PCR, with or without agar absorption, is an alternative (Wang *et al*., 1999; Schaad *et al*., 2002; Fatmi *et al*., 2005), but is not suitable for the uncultured liberibacter of citrus HLB, for which the modified soaking and filtration method with a minicolumn (Wang & Gast, 2000) worked well in the present experiments.

Among the 113 citrus leaf samples with typical mottling symptoms in 2000 [from pumello (*Citrus maxina*) and sweet orange (*Citrus sinensis*) varieties Hamlin, Valencia and Pineapple], 96·5% yielded the target band of DNA and positive detection. These results indicate that diagnosis and identification of HLB based on leaf mottling is reliable and should be recommended as the main diagnostic characteristic of HLB in eradication campaigns. In contrast, only 43 samples out of the 330 tested with other varied symptoms – such as leaf or vine yellowing (28/205, 13·66%), zinc deficiency (11/48, 22·90%), small leaves (2/16, 12·50%) and symptomless leaves (2/61, 3·28%) – gave only 13·0% positive detection by conventional PCR. For the same batch of symptomless samples, the detection rate increased to 13·11% using the TaqMan quantitative approach. The low detection rate with conventional PCR demonstrates the need for more sensitive methods such as quantitative PCR.

Dynamic monitoring of the HLB pathogen in the host plant showed distinct changes in titre of the pathogen in different seasons. Results of the TaqMan assays revealed that the titre of the '*Ca.* L. asiaticus' pathogen in sieve tubes of phloem tissues peaked between October and December, and was lowest between March and May. As the HLB pathogen is presently uncultured, these monitoring results provide basic information about the HLB population in different growing seasons, useful for appropriate integrated control planning. The trend obtained from the present work basically agrees with the infection rate of HLB disease in the Beihai citrus plantation, and probably reflects changes in sap, and the population and feeding activities of psyllid vectors, as well as environmental factors such as temperature. The titre in the phloem tissues of citrus apparently varied with growing season and the reproductive location of pathogens. According to previous dissections of citrus tissue with the chemical staining method, there are more liberibacter pathogens in sieve-tube cells of citrus leaf midribs than in lamina tissue (Da Graca, 1991).

Evaluating the population of HLB pathogen with PCR techniques not only shows their dynamic variation with different seasons, but also provides suggestions for integrated field disease management. The most important aspect is to offer researchers an efficient technique to confirm the identity of the HLB pathogen, with a view to its potential cultivation *in vitro* on semiselective media for liberibacters.

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References

Bove JM, Chau NM, Trung HM, Bourdeaut J, Garnier M, 1996. Huanglongbing (greening) in Vietnam: detection of *Liberobacter asiaticum* by DNA-hybridization with probe In2·6 and PCR-amplification of 16S ribosomal DNA. In: Da Graca J, Moreno P, Yokomi RK, eds. *Proceedings of the 13th Conference of the International Organization of Citrus Virologists*. Riverside, CA, USA: University of California, 258–66.

Da Graca JV, 1991. Citrus greening disease. *Annual Review of Phytopathology* **29**, 109–36.

Fatmi M, Damsteeg VD, Schaad NW, 2005. A combined agar-absorption and BIO-PCR assay for rapid sensitive detection of *Xylella fastidiosa* in grape and citrus. *Plant Pathology* **54**, 1–7.

Garnier M, Eveillard SJ, Cronje PR, Le Roux HF, Bove JM, 2000. Genomic characterization of a liberibacter present in an ornamental rutaceous tree, *Calodendrum capense*, in the Western Cape province of South Africa. Proposal of '*Candidatus* Liberibacter africanus subsp. capensis'. *International Journal of Systematic and Evolutionary Microbiology* **50**, 2119–25.

Ginzinger DG, 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Experimental Hematology* **30**, 503–12.

Hocquellet A, Toorawa P, Bove JM, Garnier M, 1999. Detection and identification of the two *Candidatus* Liberobacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the β-operon. *Molecular and Cellular Probes* **13**, 373–9.

Jagoueix S, Bove JM, Garnier M, 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the Proteobacteria. *International Journal of Systematic Bacteriology* **44**, 379–86.

Jagoueix S, Bove JM, Garnier M, 1996. PCR detection of the two '*Candidatus*' Liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes* **10**, 43–50.

Knighten C, Redding J, Feiber D, Compton E, 2005. *US Department of Agriculture and Florida Department of Agriculture confirm detection of citrus greening*. Press release. http://www.doacs.state.fl.us/press/2005/09022005_2.html

Lin KX, 1956. Study on citrus yellow shooting (huanglongbing) disease. *Acta Phytopathologia Sinica* **2**, 1–4.

Murray RG, Stackebrandt E, 1995. Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described prokaryotes. *International Journal of Systematic Bacteriology* **45**, 186–7.

Planet P, Jagouxie S, Bove JM, Garnier M, 1995. Detection and characterization of the African citrus greening liberobacter by amplification, cloning and sequencing of the rplKAJL-ropBC operon. *Current Microbiology* **30**, 137–41.

Schaad NW, Frederick RD, 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology* **24**, 250–8.

Teixeira DA, Eveillard S, Martins EC, Jesus WC, Jr, Yamamoto PT, Lopes SA, Bassanezi RB, Ayres AJ, Saillard C, Bove JM, 2005a. Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the '*Candidatus*' Liberibacter species associated with the disease. *Molecular and Cellular Probes* **19**, 173–9.

Teixeira DA, Saillard C, Eveillard S, Danet JL, da Costa PI, Ayres AJ, Bove J, 2005b. '*Candidatus* Liberibacter americanus' associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1857–62.

Templeton KE, Claas EC, 2003. Comparison of four real-time PCR detection systems: Bio-Rad I-Cycler, ABI7700, Roche LightCycler, and the Cepheid Smartcycler. In: Dieffenbach CW, Dveksler GS, eds. *PCR Primer: A Laboratory Manual.* Plainview, NY, USA: Cold Spring Harbor Laboratory Press, 187–97.

Wang ZK, Gast TC, 2000. Rapid detection of *Liberibacter asiaticus* in citrus by PCR amplification of 16s rDNA and ribosomal protein. In: *Proceedings of the First Asian Conference on Plant Pathology, Beijing, China, 25–28 August.* Beijing, China: China Agricultural Scientech Press, 180.

Wang ZK, Comstock JC, Hatziloukas E, Schaad NW, 1999. Comparison of PCR, BIO-PCR, DIA, ELISA and isolation on semi-selective medium for detection of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane. *Plant Pathology* **48**, 245–52.

Wilson IG, 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63**, 3741–51.

Zhao XY, 1981. Citrus yellow shoot disease (huanglongbing). A review. *Proceedings of the International Society of Citriculture* **1**, 466–9.