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Detection of 16S rDNA of *Candidatus* Liberibacter asiaticus in Orange Juice by Quantitative Real-time PCR

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Orange juice processed from Huanglongbing (HLB) affected fruit is often associated with bitter taste and/or off-flavor. HLB disease in Florida is associated with *Candidatus* Liberibacter asiaticus (*C*Las), a phloem limited bacterium. The current standard to confirm *C*Las for citrus trees is to take samples from midribs of leaves, which are rich in phloem tissues, and use quantitative real-time polymerase chain reaction (qPCR) test to detect 16S rDNA gene of *C*Las. It is extremely difficult to detect *C*Las in orange juice because of the low *C*Las population, high sugar and pectin concentration, low pH and possible existence of an inhibitor to DNA amplification. The objective of this research was to improve extraction of DNA from orange juice, and detection of *C*Las by qPCR.

HLB disease in Florida is widespread and associated with *Candidatus* Liberibacter asiaticus (*C*Las), a phloem limited bacterium. This disease can kill a tree in 5–10 years and orange juice processed from Huanglongbing (HLB) affected fruit is often associated with bitter taste and/or off-flavor (Baldwin et al., 2010; Plotto et al., 2010). *C*Las population has been shown to correlate with HLB symptoms in that leaves with serious symptoms had higher *C*Las population (Gottwald et al., 2012; Stover and McCollum, 2011; Trivedi et al., 2009). Among numbers of diagnostic methods to detect *C*Las, quantitative real-time polymerase chain reaction (qPCR) analysis targeting the *C*Las 16S rDNA gene using TaqMan protocol and specified primers and probes has been recommended by the US Department of Agriculture, Animal and Plant Health Inspection Service (USDA, APHIS) (Li et al., 2006). In comparison to midribs of leaves, which are rich in phloem vessels and used as a standard for HLB diagnostics, juice vesicles contain much lower *C*Las cells (Li et al., 2008; Liao and Burns, 2012), and this increases the difficulty to detect *C*Las in orange juice. Standard deviation of the cycle threshold (Ct) value in real-time PCR increases as target DNA decreases, indicating a higher risk of error at low target DNA concentrations (Liu et al., 2006). Preliminary experiments showed that, although midribs of leaves yield a large amount of DNA and the extracts are pure enough to detect16S rDNA by qPCR, even without elution column purification, attempts to isolate DNA from orange juice using Qiagen's DNeasy Plant mini kit, Food kit, QIAmp Blood kit, or Promega's Wizard® Genomic DNA purification kit were unsuccessful.

One of the difficulties for isolating DNA from plant material and pathogen cells is the presence of rigid polysaccharide cell wall and capsules, which physically inhibit DNA liberation (González-Mendoza et al., 2010; Noor Adila et al., 2007; Varma et al., 2007). *C*Las bacteria are localized to phloem sieve cells, which increase the difficulty of lysing the bacterial DNA (Bové, 2006). The most widely used method for tissue disruption for DNA extraction from plant tissue has been by grinding tissue with mortar and pestle under liquid nitrogen: the finer the grind, the greater the amount of DNA extracted (Rogstad et al., 2001). Special materials, such as microorganisms in plant tissues usually require additional lysis steps, such as mechanical disruption or sonication, enzymatic digestion or use of toxic chemicals (Al-Samarrai and Schmid, 2000; Alaey et al., 2005; González-Mendoza et al., 2010). The sonicator, or so called ultrasonic homogenizer, uses sonic waves to disrupt or deactivate a biological material, thus often can be used to disrupt cell membranes and release cellular contents (de Lipthay et al., 2004; Picard et al., 1992; van Burik et al., 1998).

Co-purification of pectin and DNA has long been recognized (Scott and Playford, 1996). Pectin becomes apparent when it co-precipitates with DNA following the addition of alcohol during the DNA extraction process. A number of DNA extraction methods have been developed to avoid the co-precipitation of pectin, such as use of high concentration NaCl (Varma et al., 2007), modified cetyl trimethyl ammonium bromide (CTAB) (Shepherd and McLay, 2011), phenol, ethylene glycol monoethyl ether and pectinase (Okada et al., 1997; Rether et al., 1993; Rogstad et al., 2001). Orange juice contains an extremely high content of pectin, measured as galacturonic acid (0.037–1.433 $mg \cdot g^{-1}$), depending on variety and harvest time (Baldwin et al., 2010), in comparison to 0.3–0.5 $\mu g \cdot g^{-1}$ of DNA, based on the following estimation: nucleic DNA 0.68–0.98 pg per citrus cell (Pessina

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et al., 2011; Seker et al., 2003); <600 juice vesicles per segment (Bain, 1958; Tisserat et al., 1990); 8 segments per orange fruit to produce 100 g of juice. To avoid hazardous organic solvents or large quantities of reagents, the pectinase method is efficient, environmentally friendly and economical.

Another common challenge for extracting quality DNA is that the extract is often contaminated with secondary metabolites and/or humic acid, which can inhibit PCR reaction (Deng and Cliver, 2000; Kim and Cho, 2010; Ogunjimi and Choudary, 1999; Tsai and Olson, 1992; Wilson, 1997). Orange juice is rich in secondary metabolites, including alkaloids, limonoids, and flavonoids (Baldwin et al., 2010; Justesen et al., 1998). Appropriate ion exchange columns or chelating agents can be used to remove contaminants (Saunders, 1999). Kim and Cho (2010) successfully removed PCR inhibitors from apple, grape and water melon juices using Chelex treatment and Sephadex column filtration. However, they did not succeed by this method for orange juice (Kim and Cho, 2010). Inhibitors from citrus plant tissues affect the results of conventional PCR assays (Hartung et al., 1993; Hocquellet et al., 1999; Jagoueix et al., 1996). However, Li et al. (2006) showed that TaqMan assays were not inhibited when the samples were extracted with the standard cetyltrimethyl ammonium bromide (CTAB) or the DNeasy plant kit (Qiagen), indicating that TaqMan assays with a small amplicon (about 70 bp) perhaps are less vulnerable to inhibitors of the amplification reaction in comparison with the conventional PCR assays with a large amplicon (about 1200 bp) (Mackay et al., 2002).

Low DNA content, including 16S rDNA, increases the difficulty to extract sufficient amount of target DNA to detect *C*Las in orange juice in comparison with midribs of orange leaves. Liao and Burns (2012) reported that expression of 16S rRNA in juice vesicles, which make up the major part of orange juice, is only 0.25% that of leaf midribs.

A number of protocols have been established for extraction of DNA from orange juice without the need for enrichment or isolation of microbial targets (Kim and Cho, 2010; Ros-Chumillas et al., 2007). However, the procedures included precipitation of bacteria or spores prior to extracting DNA (Kim and Cho, 2010; Ros-Chumillas et al., 2007). Because the low ratio of live cells (17% to 31% in citrus samples) (Kim and Wang, 2009; Trivedi et al., 2009), and because dead *C*Las cells had already caused metabolic changes in plants and fruit (Fan et al., 2010; Liao and Burns, 2012), a new method to extract *C*Las DNA from whole juice, including DNA in dead and broken cells, is considered necessary. The primary objective of this study was to develop a streamlined protocol to efficiently extract genomic DNA from processed orange juice from fruit harvested from HLB diseased trees and detect *C*Las population in the juice.

Multiplex-qPCR is a variant of qPCR that enables amplification of many targets in one reaction by using more than one pair of primers/probes. However, the technique is subject to certain difficulties related in principle to the availability of primers (Martin et al., 2000; Schaad and Fredric, 2002) and the formation of primer dimmers (Jannine et al., 1997). Li et al. (2006) successfully developed a multiplex system to detect *C*Las and the citrus reference, cytochrome oxidase (COX). However, our preliminary experiment showed that 16S rDNA amplification was remarkably inhibited by the multiplex method. This study will discuss the importance of using a relative Ct value by comparing the reference Ct with the target Ct. However, a separate simple qPCR was used to avoid the interference between 16S rDNA and COX DNA.

Materials and Methods

Plant materials and juice processing. HLB symptomatic (HLBs), asymptomatic (HLBa) and healthy 'Valencia' and 'Hamlin' oranges [*Citrus sinensis* (L.) Osbeck] were harvested at commercial maturity from a commercial grove located in south Florida from 3 Feb. 2010 to 21 Apr. 2011. Both HLBs and HLBa fruit were harvested from HLB infected trees which were visually symptomatic for the disease (Bové, 2006) and real-time PCR was used to detect *C*Las in leaf midribs (Li et al., 2006). HLBs fruit were misshapen, small size and green in skin color. HLBa fruit were similar to healthy fruit in size, color and shape, and usually were located on the asymptomatic sectors of HLB infected trees.

Fruit were processed into juice directly after harvest by standard industry procedure. Briefly, fruit were fed into an industrial cup extractor (JBT 391, JBT Food Tech, Lakeland, FL), pulp was reduced using a pressure filtration finisher with screen size of 0.51 mm (JBT), and then thermo-pasteurized using a pilot pasteurizer (UHT/HTST Lab 25EHV Hybrid, Microthermics, Inc., Raleigh, NC) at 90 °C for 10 s with a flow rate of 1.2 $L \cdot min^{-1}$. Each treatment contained at least 100 kg of fruit, and four replicate juice samples were taken upon exiting the pasteurizor at regular intervals. Juice samples were stored at –20 °C until used for DNA extraction.

HLBs and healthy juice samples processed from 'Valencia' oranges harvested on 10 May 2010 were used for the development of *C*Las DNA detection methodology. All other juices were only used to confirm the new protocol.

Genomic DNA extraction from juice. Figure 1 shows the newly developed standard extraction protocol for DNA extraction from orange juice. Components of Wizard® Genomic DNA purification kit (Promega, Madison, WI) and QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA) were used in multiple steps of the protocol. Briefly, juice samples were mixed with lysis Buffer AL (Qiagen) and disrupted using an ultrasonic homogenizer (Omni Sonic Ruptor 250, Omni International, Kennesaw, GA) for 10 min. Because the optimum pH for pectinase is 5, the mixture was adjusted to pH 5.0 by adding about 25 µL of 10 M NaOH solution prior to adding pectinase (from *Aspergillus niger,* Sigma-Aldrich, St. Louis, MO) to the mixture, and incubating at 37 °C to hydrolyze pectin molecules. Following pectinase treatment, more NaOH solution was added to neutralize the mixture to pH 7.0, and DNA-free RNase was added prior to incubation at 56 °C to hydrolyze RNA and improve DNA extraction. Then a protein precipitate solution (Promega) was added to the mixture, protein precipitate was separated by centrifugation and pellets were discarded. The supernatant containing DNA was mixed with isopropanol very gently to precipitate DNA. After centrifugation, the DNA mixture in the pellets was dissolved in water, and then isopropanol was added prior to loading the mixture onto a QIAmp Mini Spin column (Qiagen). AW 1 and AW 2 buffers (both from Qiagen) were used to wash the column. Finally DNA was eluted in 50 µL of Buffer AE (Qiagen).

Quantitative PCR (qPCR). The primer/probe sequences for *C*Las 16S rDNA and citrus reference, cytochrome oxidase (COX) gene were designed according to Li et al. (2006) and manufactured by Applied Biosystems (Foster City, CA). The 16S rDNA forward primer used was 5´-TCGAGCGCGTATGCAATACG-3´, and the reverse primer used was 5´-GCGTTATCCCGTAGAAAAAGG-TAG-3´. The probe used was 5´-6 FAM/AGACGGGTGAGTA-ACGCG/3´TAMRA. For COX, the forward and reverse primers

(In 50 mL centrifuge tube) Lysis and washing	7 g orange juice + 7 mL lysis buffer [a] ↓ Disruption to improve DNA extraction [b] Adjust pH to 5.0 using NaOH + Pectinase [c] ↓ Incubation for pectin degradation [d] Adjust pH to 7.0 by adding NaOH (10 M) + RNase 50 µL ↓ Incubation for nuclei lysis [e] 5 mL of protein precipitation solution [f] ↓ Centrifuge [g] Collect the supernatant containing DNA + 17 mL of cool isopropanol (for about 17.6 mL of samples) ↓ Mix very gently by inversion then centrifuge Keep the pellets + 0.8 mL water (resuspend) + 0.8 mL isopropanol
(In 2 mL centrifuge tube) Column purification	↓ Gently mix then load on to column [h] ↓ Centrifuge [i] 0.5 mL wash buffer [j] ↓ Centrifuge 15 s at 10,000 g (discard fluid)
(In 1.5 mL centrifuge tube) Elution	(after getting rid of ethanol, apply column to a clean 1.5 mL tube) 50 µl of DNA rehydration solution [k] (Buffer AE, QIAamp) ↓ Incubate in room temperature for 5 min ↓ Centrifuge 2 min at 10,000 x g DNA extraction (store at -20 ° C)

Fig. 1. A standard DNA extraction protocol from orange juice for preparation of quantitative real-time PCR samples: a) buffer AL from QIAamp DNA Blood Mini kit (Qiagen); b) sonic ruptor for 10 min (pulse 70, power 6.5) in ice bath; c) pectinase 380 units; d) 37 °C for 30 min; e) 56 °C for 10 min; f) protein precipitation solution from Wizard Genomic DNA Purification kit (Promaga); g) condition for this and next centrifuges: 5 min at 16,000 × g; h) QIAmp Mini Spin Column from QIAamp DNA Blood Mini kit (Qiagen); i) load the suspension to column → centrifuge for 15 s at 10,000 × g → discard the fluid → add the suspension to column and run the cycle again, until finishing all the suspension; j) AW1 once and AW2 twice – both AW1 and AW2 are from QIAamp DNA Blood Mini kit (Qiagen); and k) Buffer AE from QIAamp DNA Blood Mini kit (Qiagen).

are 5´-GTATGCCACGTCGCATTCCAGA-3´, and 5´-GCCAAAACTGCTAAGGGCATTC-3´, respectively. The probe was 5'-VIC/ATCCAGATGCTTACGCTGGA/3' TAMRA. Assays of qPCR were performed using ABI PRISM 7500 Sequence Detection Fast System (Applied Biosystems). Reactions of qPCR were performed in a 20 µL reaction using 10 µL TaqMan Universal Master Mix II (Applied Biosystems), 0.25 µM each of 16S rDNA primer, 0.3 µM of each COX primer, 0.15 µM of each probe, and 2.5 µL of template DNA. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of each 95 °C for 9 s and 60 °C for 1 min. Each plate, regardless of sample number, contained at least two negative control wells, and one positive control 16S rDNA well, and each sample contained at least three replicates. Results were analyzed using ABI PRISM software. Raw data were analyzed using the default settings (threshold = 0.2) of the software. Real-time PCR for COX and 16S rDNA were run separately (simgleplex) and simultaneously (complex), and the results were compared.

To determine the amplification accuracy, eight qPCR products were randomly selected from simgleplex amplification samples, cloned into the pGEM®-T Easy Vector System (Promega), and sequenced at ICBR (University of Florida, FL). The sequences (75 bp) showed 100% identities with *C*Las 16S rRNA (FJ750458.1) using BLAST search tool (www.ncbi.nlm.nih.gov/blast).

Results and Discussion

Sonication improved isolation of DNA from the plant tissue and *C*Las matrix. In comparison with tissue homogenization using a mortar and pestle, disruption with the sonicator increased DNA extraction about two-fold, reduced Ct values for COX and 16S rDNA by 0.7, and 1.3, respectively, when pH value was neutralized during extraction of DNA. Without adjustment of pH, the effect of sonication on DNA extraction was not evident because most of the DNA strands were lost during column filtration later on in the extraction method, regardless of the method of homogenization. By using sonication, extraction efficiency was greater for 16S rDNA than for COX, possibly indicating that *C*Las DNA was bound more tightly to plant tissue, perhaps because the bacteria are phloem delimited (de Lipthay et al., 2004; Picard et al., 1992; van Burik et al., 1998).

Pectin gelation and application of pectinase. Both pectin and DNA are soluble in aqueous solutions and precipitate in alcohol (Scott and Playford, 1996). When adding isopropanol and/or ethanol to precipitate DNA strands, pectin also co-precipitates. Pectin content in orange juice ranges from 0.037–1.433 $mg \cdot g^{-1}$, depending on cultivar and harvest time (Baldwin et al., 2010). On the other hand, DNA content in orange juice is estimated about to be 0.3–0.5 $\mu g \cdot g^{-1}$, calculated based on Bain (1958), Pessina et

al. (2011), Seker et al. (2003), and Tisserat et al. (1990). Without removal of pectin, the DNA could not be extracted because, presumably most of DNA is trapped in the pectin slug-gel mixture. To remove pectin, pectinase was added into the lysis buffer-juice mixture prior to precipitation of DNA using isopropanol. Before adding pectinase, a NaOH solution (10 M) was added to the mixture to adjust the pH to 5.0, the optimum pH for pectinase activity. Phenol and ethylene glycol monoethyl ether (Okada et al., 1997; Rether et al., 1993), modified CTAB (Shepherd and McLay, 2011) and a high concentration of NaCl (Varma et al., 2007) have been used to extract DNA from plant tissue without co-precipitation of pectin. However, these methods either require use of hazardous organic solvents, or require large quantities of agents. Our preliminary experiment also showed that the non-enzymatic methods were not powerful enough to remove such a large amount of pectin. Pectinase, on the other hand, is a powerful tool that can be used to remove pectin with a small amount of enzyme (15 µL per milliliter of juice sample).

Removal of DNA amplification inhibitor using elution column. Without passing through the ion exchange column, DNA was mixed with orange juice components, some of them having a maximum absorbance at 230 nm and 280 nm. Serial dilution of DNA showed that increase in Ct value inversely correlated with the dilution rate when extracted DNA was passed through the column (Table 1). However, without passing through the column, the increase of Ct value was not proportional to the dilution rate. When 16S rDNA (the target DNA) concentration was low, we expected to see an increase in the Ct value, but instead the dilution also caused a decrease in the concentration of the amplification inhibitor, which, in turn, off-set the effect of DNA decrease. The inhibitors in plant materials are often humic acid and other secondary metabolites (Deng and Cliver, 2000; Kim and Cho, 2010; Ogunjimi and Choudary, 1999; Tsai and Olson, 1992; Wilson, 1997). Since humic acid is usually found in soil, plant roots and other soil contacting organs, but not in fruit, especially juice, secondary metabolites are likely to be the major contaminant inhibitors (Kim and Cho, 2010; Renard et al., 2008; Ros-Chumillas et al., 2007; Scott and Knight, 2009; Wan et al., 2006). Kim and Cho (2010) showed that the contaminants in orange juice are more difficult to remove by column filtration than juices derived from other fruits, such as apples, grapes and water melons. Nevertheless, we successfully removed the inhibitors as confirmed by the qPCR results obtained in the serial dilutions of DNA extraction.

Possibly, the sensitivity of qPCR was not only influenced by PCR inhibitors discussed previously, but also by non-target DNA (Klerks et al., 2004; Li et al., 2008; Rousselon et al., 2004). That happens especially when the detection is SYBR-Green, an asymmetrical cyanine based dye, because all amplified double-stranded DNA, including non-specific reaction products have a response to the dye (Rousselon et al., 2004). However, TaqMan-based qPCR detects exclusively specific amplification products and had little interference (Rousselon et al., 2004). This study showed that the non-target DNA did not inhibit amplification of either 16S rDNA or COX when other inhibitors were not present (Table 1, "with elution column"). However, the amplification of 16S rDNA was remarkably influenced by inhibitors, whereas COX, with 212.4 – 212.7 times higher DNA template concentration than 16S rDNA, was not influenced by inhibitors, even when multiplex PCR was employed (Table 1, "non-column").

Within the same dilutions, the standard deviations among replicates (n = 3) also showed high variation when the DNA extraction did not pass through the elution column (Table 1), indicating the complicated interference in DNA amplification between concentrations of templates and inhibitors.

Effect of pH on DNA extraction. Orange juices are acidic with a pH range of 3.5–3.8 (Baldwin et al., 2010). After homogenizing with lysis buffer, the pH values were about 4.2–4.6. Without adjusting pH, the extraction after passing through the column showed absorbance spectrum peaks at 230 nm and 280 nm. However, there was no sound peak at 260 nm. Calculated DNA content, based on the reading of absorbance at 260 nm, was 10.7–12.3 ng·µL^{-1}, and both COX and 16S rDNA were not detected by qPCR. However, in the standard protocol adjusting pH to 7.0 and passing the extraction through the column, the extract showed a typical DNA absorbance spectrum with the absorption peak at 260 nm, A260 / A280 ratio of 1.94, and A260 / A230 ratio of 1.62. The effect of pH on DNA extraction can be caused by degradation of DNA or contaminants, or by loss of DNA due to the decreased binding force to the silicon column (Loftin et al., 2008; Wilfinger et al., 1997).

Effect of multiplex real-time PCR on COX and 16S rDNA

Table 1. Effect of elution column application and multiplex on PCR amplification of *C*Las 16S rDNA and plant COX[z]. Serial dilution with water was used.

Dilution	Ct		ΔCt	Ct		ΔCt
factor	16S rDNA	COX	($Ct_{16S\ rDNA} - Ct_{COX}$)	16S rDNA	COX	($Ct_{16S\ rDNA} - Ct_{COX}$)
	Non-column and simgleplex			With column and simgleplex		
× 2^0	35.2 ± 2.3	16.3 ± 0.2	18.9 ± 2.1	29.2 ± 0.1	16.7 ± 0.1	12.5 ± 0.2
× 2^2	31.1 ± 1.1	18.4 ± 0.1	12.7 ± 1.2	31.4 ± 0.2	18.7 ± 0.1	12.7 ± 0.1
× 2^4	32.9 ± 0.4	20.5 ± 0.1	12.4 ± 0.4	33.4 ± 0.1	20.8 ± 0.2	12.6 ± 0.2
× 2^6	35.0 ± 0.3	22.4 ± 0.1	12.6 ± 0.4	35.2 ± 0.3	22.8 ± 0.1	12.4 ± 0.3
	Non-column and multiplex			With column and multiplex		
× 2^0	nd[y]	16.5 ± 0.2	---	36.0 ± 2.1	16.8 ± 0.1	19.2 ± 2.0
× 2^2	39.3 ± 0.6[x]	18.6 ± 0.3	20.7	39.4 ± 0.5	19.0 ± 0.2	20.4 ± 0.4
× 2^4	nd	20.7 ± 0.1	---	nd	20.9 ± 0.1	---
× 2^6	nd	22.6 ± 0.2	---	nd	22.8 ± 0.2	---

[z]Juice samples were processed from HLB symptomatic 'Valencia' fruit harvested on 18 May 2010. DNA was extracted following the standard procedure showed in Fig. 1 except as described otherwise.

[y]nd: not detectable (Ct > 40).

[x]Average of three replicates. Ct value of the fourth replicate was not detectable.

amplification. When multiplex real-time PCR was applied for detection of COX and 16S rDNA, amplification of 16S rDNA was significantly influenced by COX as shown by an irregular amplification curve (not a regular steep curve), increased Ct value and a disproportional serial dilution vs. ΔCt ($Ct_{16S\ rDNA} - Ct_{COX}$) change (Table 1). On the other hand, amplification of COX was not influenced by 16S rDNA (Table 1). Results obtained from simgleplex real-time PCR for COX and 16S rDNA showed that copy number of COX is 2^{14} to $2^{24.6}$ times more abundant than that of 16S rDNA. It is likely because of this reason COX, which is more abundant due to greater starting quantity, causes the assay for that target to perform better than for 16S rDNA from the beginning, using up the deoxynucleoside triphosphates (dNTPs) in the reaction, and leaving little for the other assay (Henegariu et al., 1997). This problem should be overcome by limiting the amount of primer for the more abundantly expressed target (COX) (Henegariu et al., 1997).

Multiplex-PCR is a variant of PCR that enables amplification of multiple targets in one reaction by using more than one pair of primers. A multiplex assay can be tedious and time-consuming since it requires lengthy optimization procedures, although once optimized, it is a cost-saving technique used in many diagnostic laboratories (Elnifro et al., 2000). The technique is subject, however, to certain difficulties related in principle to the availability of primers for various plant pathogens (Martin et al., 2000; Schaad and Fredric, 2002) and the formation of primer dimers (Jannine et al., 1997). Thus, the optimization of reaction conditions should aim to minimize such non-specific reactions and avoid false results (De Lomas et al., 1992; Farag et al., 2010).

In conclusion, an effective DNA extraction method for qPCR detection of *C*Las in orange juice was developed. Juice samples were mixed with lysis buffer, homogenized using a sonicator, and then incubated with pectinase to hydrolyze pectin. The pH value was adjusted to neutral before proteins were denatured and precipitated by ammonium acetate. After removal of proteins, DNA was precipitated by isopropanol/ethanol, and further applied to an elution column-based purification. The role of sonication was to release *C*Las from plant cells and resulted in an increase of DNA yield by 86%. The role of pectinase was to eliminate pectin, which otherwise co-precipitates with DNA. Use of the elution column purification removed potential PCR enzyme inhibitors from the DNA extraction solution.

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