

Comparison of Two Huanglongbing Detection Methods in Samples with Different Symptom Severity

Perbandingan Dua Metode Deteksi Huanglongbing pada Sampel dengan Tingkat Keparahan Gejala yang Berbeda

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ABSTRACT

Huanglongbing or citrus greening in Asia caused by the pathogen *Candidatus Liberibacter asiaticus* (CLAs) is one of the most devastating citrus diseases worldwide. This disease is one of the causes of decreased citrus production in Indonesia. Symptoms of huanglongbing in citrus plants in the field and greenhouses have different levels of severity. This study was conducted to detect CLAs in several types of samples based on leaf symptoms using conventional and real-time PCR (qPCR). Three pairs of primers were used in this study, a pair of Las606/LSS for conventional PCR and two pairs for qPCR, namely Las931/LSS and Lj900F/Lj900R. The results showed that blotchy mottle is the most easily detected symptom of huanglongbing and found in fields and greenhouses. The Lj900F/Lj900R primer pair is more suitable for detecting CLAs pathogens using qPCR than Las606/LSS based on the melting curve and Ct value that appear. qPCR detection is more accurate and sensitive even with lower DNA concentrations. The lower limit of Ct value of healthy leaf samples is 34.08. Citrus leaves are considered positive if the Ct value is less than 34.08. Ct value based on severity or scoring between HLB symptomatic leaves from the field and greenhouse showed a significant difference, i.e. the Ct value of symptomatic samples from the field was lower than that of greenhouse samples.

Keywords: citrus, CLAs, Ct value, qPCR, scoring

ABSTRAK

Huanglongbing atau *citrus greening* di Asia disebabkan oleh patogen *Candidatus Liberibacter asiaticus* (CLAs) merupakan salah satu penyakit jeruk yang paling menghancurkan di seluruh dunia. Penyakit ini menjadi salah satu penyebab penurunan produksi jeruk di Indonesia. Gejala huanglongbing pada tanaman jeruk di lapangan dan rumah kaca ternyata memiliki tingkat keparahan yang berbeda.

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Penelitian ini dilakukan untuk mendeteksi CLas pada beberapa jenis jeruk berdasarkan gejala pada daun menggunakan PCR konvensional dan *real-time* (qPCR). Tiga pasang primer digunakan dalam penelitian ini, sepasang Las606/LSS untuk PCR konvensional dan dua pasang untuk qPCR, yaitu Las931/LSS dan Lj900F/Lj900R. Hasil penelitian menunjukkan bahwa *blotchy mottle* adalah gejala huanglongbing yang paling mudah dideteksi dan ditemukan di kebun dan rumah kaca. Pasangan primer Lj900F/Lj900R lebih cocok untuk mendeteksi patogen CLas menggunakan qPCR daripada Las606/LSS berdasarkan kurva leleh dan Ct value yang muncul. Deteksi qPCR lebih akurat dan sensitif bahkan dengan konsentrasi DNA yang lebih sedikit. Batas bawah Ct value sampel daun sehat ialah 34.08. Daun jeruk dinyatakan positif jika Ct value kurang dari 34.08. Ct value berdasarkan tingkat keparahan atau dengan skoring antara HLB daun simptomatik dari lapangan dan rumah kaca menunjukkan perbedaan yang cukup besar, yaitu Ct value sampel simptomatik dari lapangan lebih rendah daripada sampel rumah kaca.

Kata kunci: CLas, Ct value, jeruk, qPCR, skoring

INTRODUCTION

Citrus is among the most popular fruits globally, cultivated in 11.42 million ha with a fruit production of 179 million tons (Dala-Paula *et al.* 2019; Deng *et al.* 2019; Bassanezi *et al.* 2020). China is the number one citrus-producing country globally with 35 601 million tons, followed by Brazil with 18.08 million tons. European countries are third-world citrus-producing countries with 11 497 million tons (Anonym 2021). In Indonesia, the total area of citrus plantations is more than 57 000 ha, producing 2.5 million tons (Anonym 2022). However, Indonesia exported citrus with an insignificant amount in 2019, i.e. 1752 tons. On the other hand, the value of citrus imports to Indonesia in the same year was 100 thousand tons or 4% of national production (Anonym 2022).

In its effort to increase national citrus production, the government has encountered several obstacles, including the low productivity and quality of citrus fruits. These challenges stem from several factors, among others are lack of awareness among farmers about the use of certified citrus seeds and insufficient skills in citrus cultivation among farmers. Following the recommended technology, some problems that farmers face are beyond their control, such as managing various pests and diseases such as Huanglongbing or *Citrus Vein Phloem Degeneration* (CVPD) and fruit fly pests.

Huanglongbing (HLB) or CVPD known also as citrus greening is one of the most

devastating diseases to the citrus industry in various parts of the world. *Candidatus Liberibacter asiaticus* (CLas) is known as the causal agent of HLB in Asia (Bove 2006; Wang *et al.* 2017; Yaqub *et al.* 2017; Ha *et al.* 2019). HLB spreads in nature through insect vectors, i.e. the Asian citrus psyllid (ACP), *Diaphorina citri* (Bove 2006; Lin *et al.* 2017; Pagliaccia *et al.* 2017; Ramsey *et al.* 2017; Li *et al.* 2019; Abreu *et al.* 2020).

Infection by HLB will drastically shorten production life and reduce yields of citrus trees (Pagliaccia *et al.* 2017; Li *et al.* 2019; Koh *et al.* 2020). Thus, HLB and the presence of ACP threaten citrus production and harm citrus farmers in various parts of the world (Paudyal 2016; Blaustein *et al.* 2018; Tirado-Corbalá *et al.* 2018), including in Indonesia. According to the Data and Information Center of the Ministry of Agriculture in 2014, there was a decrease in the citrus plantation area by approximately 7000 ha over five years (2008–2013), from 60 190 ha in 2009 to 53 517 ha in 2013 (Supriyanto *et al.* 2010; Dwiastuti *et al.* 2019).

One of the citrus producing areas affected by HLB in the past was Purworejo, Central Java. Bayan village in Purworejo was known as the center of citrus production as well as the largest producer of citrus seedlings in Indonesia. These seedlings are unlabeled and have spread to at least seven provinces in Indonesia (Supriyanto *et al.* 2010). The distribution of unlabeled seeds has led to a decline in the national citrus industry. Therefore, control techniques are needed to prevent the more widespread of HLB.

HLB symptoms were characterized by yellowing veins, blotchy mottle, vein corking, mottling, greening, and zinc/iron deficiency-like symptoms (Gopal *et al.* 2010; Valdés *et al.* 2016; Yaqub *et al.* 2017; Dala-Paula *et al.* 2019; Ajene *et al.* 2020). Symptoms of HLB are similar to symptoms of other diseases, such as those caused by viruses or micronutrient deficiencies. Therefore, confirming that the symptomatic plant was the HLB in the field was difficult.

The most effective way to determine whether a plant is HLB positive is through molecular detection techniques using polymerase chain reaction (PCR). PCR is commonly used as a detection method for plant disease. Conventional PCR detects the DNA of any pathogens in the samples, while quantitative PCR (qPCR) is often used to measure the number of copies of a specific DNA sequence of the pathogen, corresponding to its titer (Zhang *et al.* 2010; Gardner *et al.* 2016). Based on qPCR estimates of gene copy number, the number of cells of CLAs per g of plant tissues can be predicted as described in the work of Zhang *et al.* (2010). Based on the cycle threshold (Ct) values obtained from qPCR, the relative change in the pathogen population in response to treatment can be quantified (Paglialiccia *et al.* 2017).

This research aimed to detect CLAs from citrus leaves showing HLB symptoms of varying severity based on disease score. The samples were examined using both conventional PCR and quantitative PCR (qPCR). Evaluation of differences in Ct value of positive and negative HLB samples is discussed in this paper.

MATERIALS AND METHODS

Samples Collection

Observation of HLB diseases in the field was conducted in three citrus orchards owned by farmers. The orchards are located in one of the citrus-producing areas in Bayan Village, Purworejo, Central Java which has been known affected by HLB in the past. Symptomatic leaves were separated into five groups based on the severity and then assigned to disease scores ranging from zero to four, as illustrated in Table 1. Symptoms corresponding to each score are shown in Figure 1.

Leaf samples from the greenhouse were obtained from the Plant Protection Research Station, Faculty of Agriculture, Gadjah Mada University (UGM). The symptomatic leaves come from plants that have been inoculated with CLAs pathogens by grafting using HLB-positive bark (after five months of inoculation). Leaves were also collected from healthy plants grown from seeds which will be used as negative controls.

The leaf samples were stored in plastic bags and kept at -20 °C until they were used for DNA extraction. DNA extraction and molecular detection were conducted at the Plant Clinic Laboratory, Department of Plant Protection, Faculty of Agriculture, UGM.

DNA extraction

The DNA was extracted from the midribs and petioles using Genomic DNA Kit (Plant) following the manufacturer's instructions (Geneaid Biotech Ltd., Taiwan). Before the extraction process, the leaves were washed

Table 1 Huanglongbing severity rating scale (score) based on leaf symptom

Severity score	Description
0	No symptoms
1	< 25% yellowing veins and blotchy mottle
2	25%–50% yellowing veins and blotchy mottle
3	> 50%–75% yellowing veins, blotchy mottle and vein corking symptoms
4	> 75% yellowing veins, blotchy mottle, and vein corking symptoms



Figure 1 Citrus leaves showing Huanglongbing symptoms in different rating scale. a, Natural infected leaves from the field and b, Artificial inoculated leaves from the greenhouse.

with 70% ethanol and distilled water. Leaf samples were ripped off and cut by sterilized scissors. Approximately 0.1 g of leaf midribs were ground in the mortar by adding liquid nitrogen. After extraction, the DNA was stored at $-20\text{ }^{\circ}\text{C}$ to be used as the template for PCR amplification. DNA concentration was measured using nanodrop (Biodrop, $\mu\text{LITE}+$) and all samples were equalized to $10\text{ ng }\mu\text{L}^{-1}$ for amplification by qPCR.

Conventional PCR Amplification

Amplification of DNA target was carried out using a conventional PCR method (Biorad T100 Thermal Cycler, US). Each amplification reaction consisted of $5\text{ }\mu\text{L}$ PCR mix (MyTaq HS Red, Bioline Meridian Bioscience, Memphis, US), $1\text{ }\mu\text{L}$ Las606 (5'-GGA GAG GTG AGT GGA ATT CCG A-3') as forward primer, $1\text{ }\mu\text{L}$ LSS (5'-ACC CAA CAT CTA GGT AAA AAC C-3') as reverse primer (Fujikawa and Iwanami 2012), $1\text{ }\mu\text{L}$ DNA template, and $2\text{ }\mu\text{L}$ ddH₂O up to $10\text{ }\mu\text{L}$ of final volume. Amplification was performed including one cycle of initial denaturation at $94\text{ }^{\circ}\text{C}$ for three minutes followed by 40 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for one minute, annealing at $55\text{ }^{\circ}\text{C}$ for one minute, extension at $72\text{ }^{\circ}\text{C}$ for two minutes, and final extension at $72\text{ }^{\circ}\text{C}$ for 10 minutes. Amplicons were visualized with UV transilluminator after electrophoretic migration on 1.5% agarose gels.

Quantitative PCR (qPCR) Amplification

Amplification was carried out in quantitative real-time PCR (AriaMX, Agilent, US) using SYBR Green (Life Technologies, Carlsbad, US). Each amplification reaction was performed in $10\text{ }\mu\text{L}$, consisted of $5\text{ }\mu\text{L}$ qPCR mix, $1\text{ }\mu\text{L}$ forward primer, $1\text{ }\mu\text{L}$ reverse primer, $2\text{ }\mu\text{L}$ sample DNA, and $1\text{ }\mu\text{L}$ nuclease free water (NFW). Two pairs of specific primers, Las931 (5'-CAG CCC TTG ACA TGT ATA GGA CG-3')/LSS (5'- ACC CAA CAT CTA GGT AAA AAC C-3') and Lj900F (5'-GCC GTT TTA ACA CAA AAG ATG AAT ATC-3')/Lj900R (5'-ATA AAT CAA TTT GTT CTA GTT TAC GAC-3') were used in qPCR amplification. The amplification was run at $95\text{ }^{\circ}\text{C}$ for 3 minutes, followed by 40 cycles at $95\text{ }^{\circ}\text{C}$ for 5 second, annealing at $55\text{ }^{\circ}\text{C}$ for 30 second for Las931/LSS primers and at $62\text{ }^{\circ}\text{C}$ for 30 second for Lj900/Lj900 primers, with fluorescence signal capture at the end of each $62\text{ }^{\circ}\text{C}$ step, followed by a default melt (disassociation) stage at $95\text{ }^{\circ}\text{C}$ for 30 second, $65\text{ }^{\circ}\text{C}$ for 30 second, and final extension at $95\text{ }^{\circ}\text{C}$ for 30 second.

Nucleotide sequencing and phylogenetic tree analysis

DNA fragments successfully amplified in conventional PCR were subjected for sequencing at 1st BASE (Malaysia). The bioinformatics analysis of nucleotide sequences

involved blasting consensus of the sequences in NCBI, and alignment using Bioedit software. The eleven highest percent identity species in the NCBI GenBank Blast was selected to construct phylogenetic trees using MEGAX software with statistical method maximum likelihood. Bootstrap phylogeny test and construction was executed using the Kimura-2 model with gamma distributed.

RESULTS

Symptoms Variations of Huanglongbing in the Farmer's Orchards

In Purworejo, numerous leaves displaying symptoms of HLB have been discovered. This region has indeed suffered severe damage due to HLB, prompting many “*Siam Purworejo*” farmers to abandon citrus plants in favor of other crops. Symptoms of HLB on trees and fruits include a declining citrus tree with a thinning canopy and branch dieback (Figure 2). Generally, affected trees display yellow shoots with upright and blotchy-mottle symptoms. Citrus plants with symptoms of HLB will appear languishing and most of the leaves turn yellow or chlorosis. Other types of HLB symptoms were veins corking, symmetrical

leaves, yellowing, manganese, zinc deficiency-like, dark green, elongated, and constricted leaves (Figure 3).

Molecular Detection of HLB using Conventional PCR

DNA target was successfully amplified from HLB-infected leaves using a specific primer for CLas, Las606/LSS (Figure 4). The presence of HLB natural infection was revealed from three citrus orchards in Purworejo. In Orchard A, 83.88% of samples tested positive for HLB, while Orchard B had 100% positive samples, and Orchard C had 50% positive samples (Figure 4). The percentage of positive samples was highest in Orchard A, where HLB symptoms appeared most varied. On the other hand, only two out of eight samples from the greenhouse showed positive HLB (Figure 5).

Molecular Detection of HLB by qPCR

The results of qPCR using the primary pair Las931/LSS with an annealing temperature of 55 °C for 30 s did not yield the expected amplification results. Amplification plots and melt curves showed unsatisfactory outcomes, with melting temperatures varying between samples (Figure 6a), and the Ct value obtained



Figure 2 HLB symptoms on trees, fruits, and leaves in Purworejo, Central Java, Indonesia.

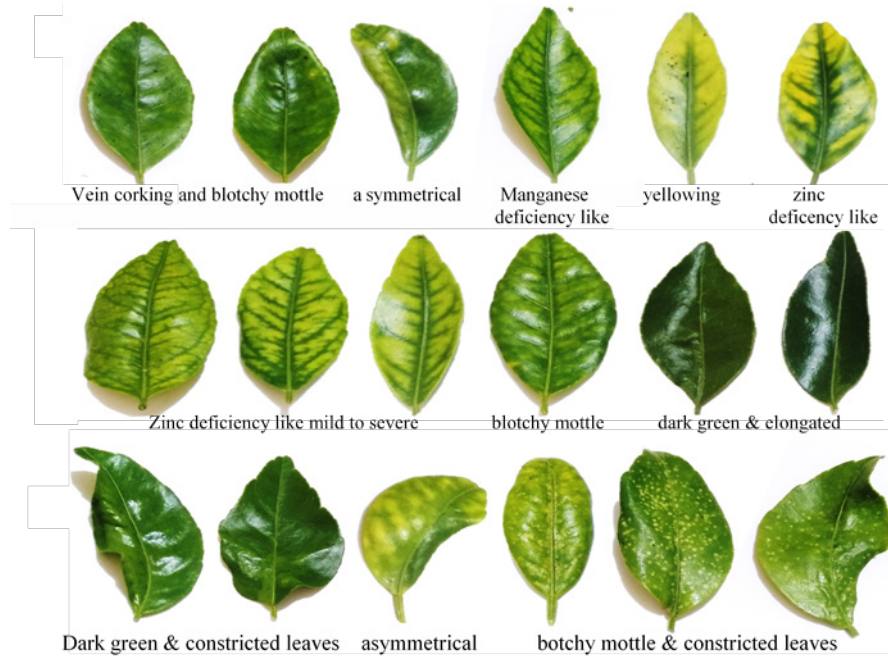


Figure 3 Leaves symptoms confirmed positive HLB.

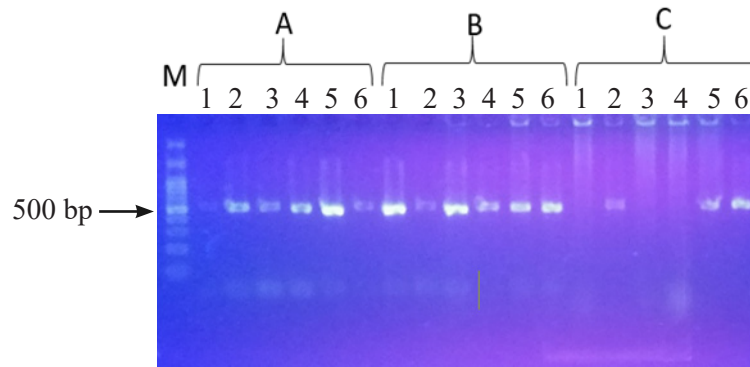


Figure 4 Visualization of UV transilluminator of amplified product of HLB natural infection from the field. M, 100 bp DNA ladder (Geneaid, US). A–C, samples from each orchards A, B, and C.

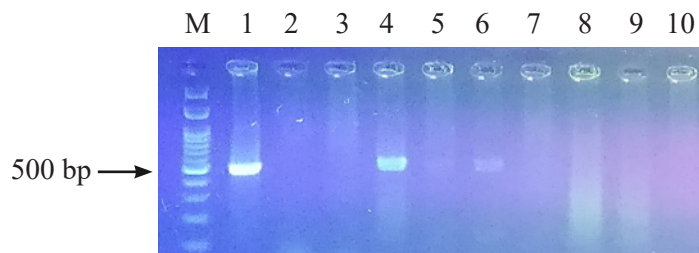


Figure 5 Visualization of UV transilluminator of amplified product of HLB artificial infection from greenhouse. M, 100 bp DNA ladder (Geneaid, US). Lane 1, positive control from orchard; lane 2 to 10, greenhouse samples.

accounted for only 20% of all samples. Subsequently, we attempted the same samples using another pair of primers, Lj900F/Lj900R with an annealing temperature of 55 °C for 30 s. Based on the amplification plot and melting

temperature results, the anticipated outcomes were achieved, with all melting temperatures converging at one point and forming a consistent curve for all samples (Figure 6b). Following these qPCR results, the next step

involves using Lj900F/Lj900R primers to determine the Ct value for all samples from the field and the greenhouse. The Ct values of healthy and HLB-positive plants were compared to observe the differences (Figure 7). The minimum Ct value for healthy plants establishes the threshold for determining positive or negative HLB samples, with a Ct value of 34.08 (Figure 7).

The results of amplification by PCR conventional and qPCR are shown in Table 2. Based on the Ct value obtained in the amplification with qPCR, more accurate data was obtained than the results of conventional PCR. The percentage of positive HLB with

qPCR from orchards A and B has reached 100% and in orchard C reached 66.67%. The lowest Ct value is in orchard B, with the lowest Ct value of 21.00 and the highest is 28.68. The lowest and highest Ct value of orchard A is 23.30 and 31.57, respectively; while the lowest and highest Ct value of orchard C is 26.41 and 36.42, respectively (Table 2).

Ct Value Based on Disease Severity Rating Scale

The severity of HLB symptoms differed significantly between field samples and greenhouse samples. Symptomatic leaves from the field displayed more pronounced symptoms,

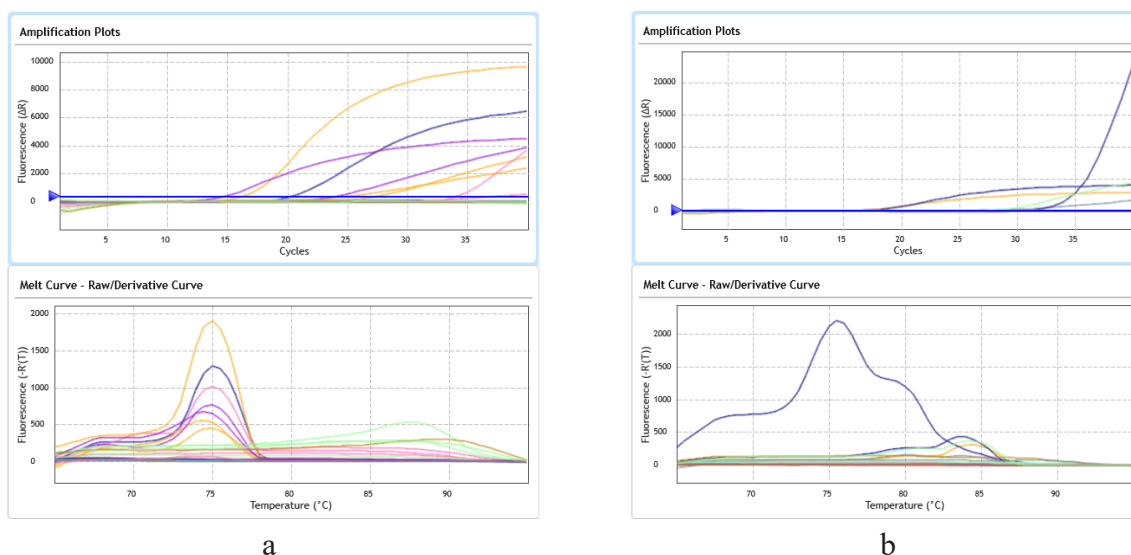


Figure 6 Amplification plots and melt raw derivative curve of qPCR-DNA binding dye including standard melt using a, Las931/LSS primers with an annealing temperature at 55 °C and b, Lj900F/Lj900R primers with an annealing temperature at 62 °C.

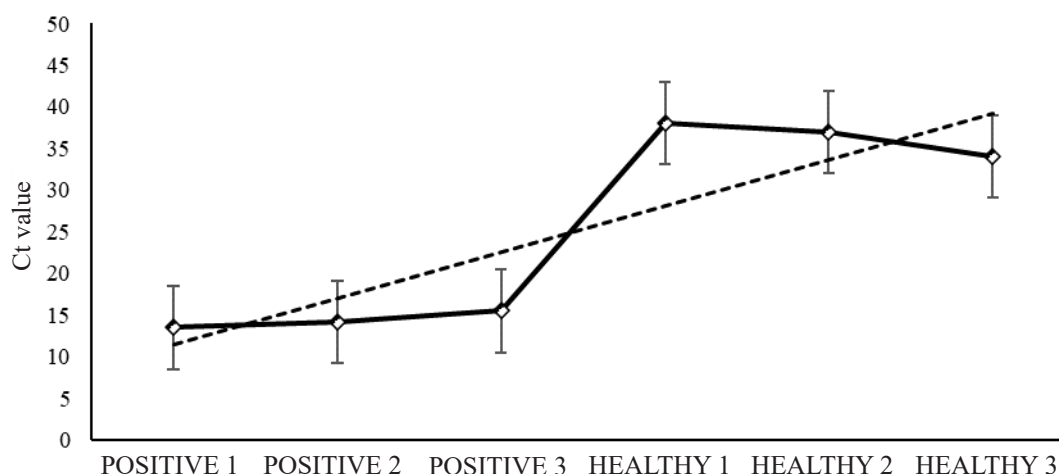


Figure 7 The Ct value from HLB positive and healthy leaf samples.

Table 2 Results of HLB detection using conventional PCR and qPCR from symptomatic leaves collected from the field and the greenhouse

Sampel code	Conventional PCR	Real-time (qPCR)	Ct Value of qPCR
Orchard A			
1A	+	+	23.94
2A	+	+	31.57
3A	+	+	26.82
4A	+	+	26.41
5A	+	+	28.43
6A	+	+	23.20
Orchard B			
1B	+	+	28.68
2B	+	+	22.35
3B	+	+	24.20
4B	+	+	28.44
5B	-	+	21.00
6B	+	+	24.96
Orchard C			
1C	-	-	35.66
2C	-	+	26.41
3C	+	+	26.88
4C	+	+	32.82
5C	-	+	32.98
6C	-	-	36.42
Greenhouses			
1 Positive control	+	+	31.51
2	-	+	31.20
3	-	-	35.62
4	+	+	32.45
5	-	+	33.29
6	+	+	33.48
7	-	+	31.05
8	-	+	31.39
9	-	-	36.32
10	-	-	35.94

with chlorosis being more severe than greenhouse leaves. The results of the calculated Ct value confirmed the visual symptoms (Figure 8). The Ct value of symptomatic leaf samples of natural infection from the field was lower than the Ct value of HLB symptomatic leaf samples of artificial inoculation from the greenhouse. This suggests that the titer of CLAs pathogens on leaves from the field was higher than on leaves from green the greenhouse. It

was also known that leaves with a score of one in the greenhouse did not show positive results because the Ct value was still above 34.08.

Sequences and phylogenetic tree of CLAs from Purworejo

The analysis of nucleotide sequences from the Purworejo samples demonstrated a high percentage identity with CLAs species from various countries. According to the

phylogenetic tree (Figure 9), the Purworejo sample is closely related to the *CLas* haplotype H24Y, with an accession number JQ867421.1, which was registered in GenBank in 2012. The *CLas* species with sequence number JQ867421.1 originated from Mexico, with the host *Citrus latifolia*.

DISCUSSION

Citrus greening is one of the oldest diseases in citrus. In Chaozhou, farmers call this disease “*huang long bing*”, *huang* means yellow, *long* means shoot, and *bing* means disease, yellow shoot disease. In Indonesia, HLB is a major

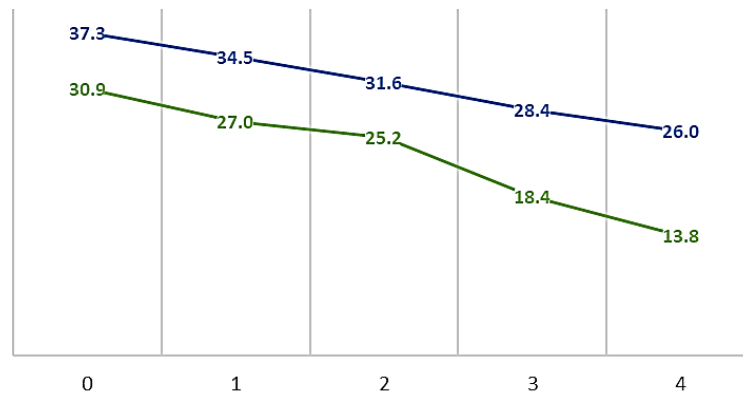


Figure 8 CT-Value based on HLB severity rating scale (score). —, Field sample and —, greenhouse sample.

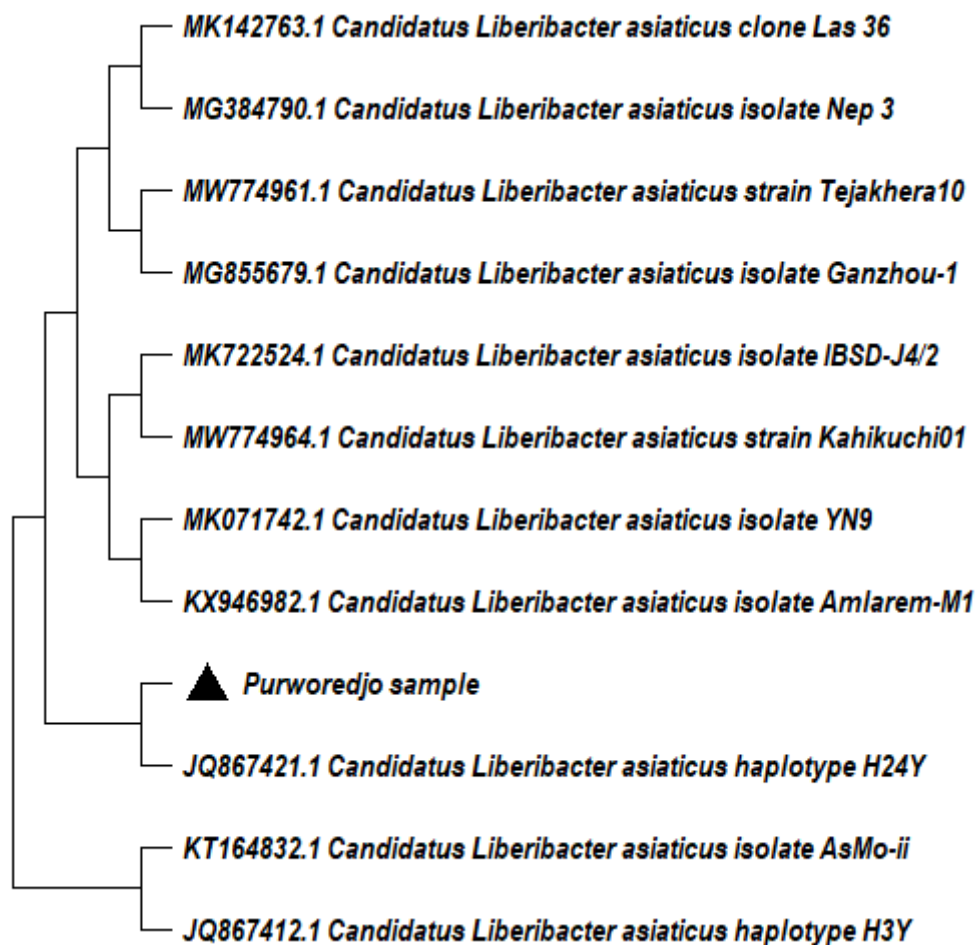


Figure 9 Phylogenetic tree of HLB sample from Purworejo and CLAs from NCBI.

problem in citrus production and was given the name of “*Citrus Vein Phloem Degeneration*” from the name CVPD by Tirtawidjaja in his research report in 1965 (Nurhadi 2015). At the study site, symptoms of HLB were found with the main symptoms consisting of yellow shoots, leaves with small yellow spots, and small and asymmetrical fruits. In addition, citrus branches infected with CLas will usually die (Lee *et al.* 2015).

Our observation also confirmed that HLB affects the fruit quality or morphology. Fruits produced from HLB-infected trees are small, asymmetric, and have a bitter taste (McClelland and Schwarz 1970) due to increased concentrations of limonin and nomilin, which negatively impact sensory attributes of juice (Bove 2006; Dala Paula *et al.* 2018; 2019). In addition, such fruits lopsided with inverted color and aborted seeds are common symptoms of HLB (Dala-Paula *et al.* 2019; Barus *et al.* 2021). Although the detrimental flavour attributes of symptomatic fruits would be largely diluted in commercial juice blends from fruits of several varieties, locations, and seasons, those symptomatic fruits are likely rejected by the industry since weight and juice content are reduced (Dala Paula *et al.* 2018; 2019). Furthermore, a positive relationship between the number of fruits and yield per tree has suggested that yield reductions are primarily caused by the lack of fruit set or the early fruit drop on affected branches (Bassanezi *et al.* 2020). Thus, depending on the disease severity, a 30%–100% yield reduction can be observed in HLB-affected plants (Bove 2006; Dala-Paula *et al.* 2019; Bassanezi *et al.* 2020).

Despite the symptoms of HLB being well described, it was difficult to visually screen for HLB-symptomatic trees in the fields because the symptoms looked like nutrient deficiency such as manganese and zinc. Therefore, the PCR method was used for amplifying the 16S rDNA fragment of the HLB pathogen by using CLas specific primers (Fujikawa and Iwanami 2012). The organism can be detected in samples from diseased trees but not from healthy-looking trees. The present study also

showed that PCR-based diagnosis using primers specific to rDNA and the 16S-23S spacer region worked well. Due to its specificity the primers were used regularly in surveys and screening of citrus crops for HLB (Gopal *et al.* 2007). Conventional PCR using primer Las606/LSS and qPCR using primer Lj900F/Lj900R are a highly sensitive and robust method for the detection of CLAs. These results confirm the phloem was inhabiting the bacterium pathogen's nature (CLas).

Candidatus Liberibacter asiaticus (CLas) (Jagoueix *et al.* 1994) is a species that has taxonomy ID 34021 in NCBI. *Candidatus* is a provisional name for well-characterized but as-yet uncultured organisms which are equivalent to *Liberibacter asiaticus*. NCBI BLAST name for this organism is a-proteobacteria with details classification: Bacteria; Proteobacteria; Alphaproteobacteria; Hyphomicrobiales; Rhizobiaceae; *Liberibacter*. DNA sample from Purworejo, Central Java, Indonesia, was proved to be one of the CLas, which has similarities with species of CLas haplotype H24Y from Mexico. This research shows that this class of pathogens spreads widely throughout the world across different continents and becomes very dangerous for the citrus industry if not managed and controlled properly.

The diagnosis of HLB by PCR was found to be excellent and essential for screening disease-free plants and establishing disease-free citrus nurseries. In addition, the outbreak of HLB has significantly impacted the citrus industry worldwide, including Indonesia. Based on visual observations and surveys, uncertified seedlings are used in many citrus orchards in Indonesia (Barus *et al.* 2021). An effective agricultural policy needs to be enacted and put into practice. The government should, by any means, improve the farmer's awareness of the importance of good quality seedlings, endorse proper agricultural practices and improve breeder facilities as those are the initial capitals for preventing HLB disease.

This study concludes that blotchy mottle is the best diagnostic and earliest leaf symptom. The Lj900F/Lj900R primers were more suitable for detecting CLas pathogens using

qPCR than Las606/LSS, as indicated by the melt curve and the emerging Ct value. qPCR detection demonstrated higher accuracy and sensitivity even with a lower DNA concentration. The lower limit of the Ct value for healthy leaf samples was 34.08; therefore, citrus leaves were considered positive if the Ct value was less than 34.08. A significant difference was observed when considering the Ct value based on severity or scoring between HLB symptomatic leaves of natural infection from the field and artificial inoculation from the greenhouse. The Ct value of symptomatic samples from the field was lower than that of the greenhouse samples. The lower the Ct value, the higher the titer of the pathogen present in the sample.

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