Application of newly designed primer for Huanglongbing disease detection in citrus plants

Kristianto Nugroho[1, 3], *Agus* Purwito[21], *Dewi* Sukma[2], *Mia* Kosmiatin[3], *Tri Joko* Santoso[3], *Alina* Akhdiya[3], *Hadi Mohammad* Yusuf[3], *Redi* Aditama[4], and *Reflinur* Reflinur[5]

[1]Graduate Program of Plant Breeding and Biotechnology, Faculty of Agriculture, IPB University, Jalan Meranti, Dramaga Campus, Bogor 16680, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Jalan Meranti, Dramaga Campus, Bogor 16680, Indonesia
[3]Research Center for Horticulture, Research Organization for Agriculture and Food, National Research and Innovation Agency, Jalan Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, Indonesia
[4]PMB Lab, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Jalan Meranti, Dramaga Campus, Bogor 16680, Indonesia
[5]Research Center for Applied Botany, Research Organization for Life Sciences and Environment, National Research and Innovation Agency, Jalan Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, Indonesia

Abstract. The symptoms of Huanglongbing (HLB) disease are occasionally misunderstood with nutrient deficiency symptoms. The study aimed to develop HLB specific primer targeting the *CLIBASIA_RS01220* gene sequence for HLB detection in citrus plants collected around Cimanggu, Bogor. A total of 21 citrus leaves showing HLB symptoms were subjected to the bacterial DNA isolation and subsequently were amplified with the newly designed primer, namely YihY primers, and the reference A2/J5 primers. Of these, 15 out of 21 collected citrus leaves exhibited positive detection of HLB yielded a band size of 930 bp as targeted amplicon of the YihY primer. On the other side, the primer A2/J5 succesfully detected positive amplicon band with a size of 703 bp from 17 out of 21 citrus leaves. Based on the homology sequences analysis, all of the HLB isolate sequences both amplified by YihY and A2/J5 primers had similarity with *Candidatus* Liberibacter asiaticus. The phylogenetic analysis, both based on YihY and A2/J5 primers showed the mixed between bacterial isolates collected from Japansche citroen maintained in the Cikeumeuh greenhouse and lime trees in Cimanggu greenhouse, assuming the HLB isolate has been transferred between the two greenhouses by the ability of *Diaphorina citri* to fly in far distance.

[1] Corresponding author: apurwito@apps.ipb.ac.id

1 Introduction

Huanglongbing (HLB) also known as “citrus degreening” or “citrus vein phloem degeneration (CVPD)” is one of the most significant diseases in citrus plantations around the world. This disease caused by Gram-negative bacteria that unable to be cultured in artificial media, namely *Candidatus* Liberibacter, which infected citrus phloem tissue and causing the accumulation of callose that led to inhibition of the photosynthate transport [1-3]. The pathogen will affect the citrus production, by causing the shorter plant height, less fruit number with small fruit size, and the bitter fruit taste [4]. In Asia, the disease caused by a heat-tolerant species known as *Candidatus* Liberibacter asiaticus (*C*a L. asiaticus) that spread by Asian citrus psyllid (*Diaphorina citri*) as a vector [4]. This pathogen has been reported to cause several damage to citrus plantantion in Asia countries such as China, India, Japan, Iran, Nepal, Pakistan, Yemen, Vietnam, Thailand, Philippines, Taiwan, Malasia, and Indonesia [5].

The existence of HLB in Indonesia has been recorded since 1950 in Java Island [6]. From the last of 1980s to the early of 2000s, HLB has damaged several center citrus productions in Indonesia such as in Tulungagung-East Java, North Bali, Purworejo-Central Java, and Sambas-West Kalimantan, and caused enormous both yield and economic losses [7]. This condition occurred due to the inability of HLB detection from the visible symptoms at an early stage which essential for HLB disease prevention. HLB visual symptoms such as chlorosis, blotchy mottled leaf, and vein corking have been misunderstood with plant nutritional deficiencies of nitrogen (N), zinc (Zn), manganese (Mn), and iron (Fe) [8] or other diseases caused by virus [9] for a long time. The other phenotypic symptoms could be identified from fruit such as smaller size, the green color of stylar while peduncular end change color from green to yellow, and assymetrical locul [10]. However, citrus is a perennial plant that needs more than three years to produce fruits, so it will be challenging to detect the disease visually in juvenile phase. Therefore, a fast and accurate detection method is needed in order to prevent the damage of HLB disease as early as possible in citrus breeding program.

Several detection methods for HLB detection have been developed and previously reported in several studies, such as by using loop-mediated isothermal amplification (LAMP) [11, 12]; conventional Polymerase Chain Reaction (PCR) [13-15]; real-time PCR (RT-PCR) [16-19]; and Raman spectroscopy [20-22]. Conventional PCR using specific gene primer is currently a well known method for HLB detection. This method is more suitable for the small scale laboratories located in remote area, especially in developing countries because of its lower budget cost in comparison to RT-PCR method. Nevertheless, this method is less sensitive compared to RT-PCR, but produced more accurate results compared to LAMP method that occassionally more subjective in color changing determination.

Several primers for HLB detection have been reported previously with various types of gene target sequence [23]. The first primer was designed based on *16S rDNA* gene [24], followed by *β-operon* gene [25]. Subsequently, the primer based on *nusG–rplK* genes using long PCR [26] were developed. Several studies also reported the design of next specific primers based on *16S rDNA* gene [27-29], with more specificity and robustness. The successful of designing new primer based on hypervariable prophage gene (*hyvI* and *hyvII*) that plays crucial function in the bacterial pathogenicity evolution process also reported [30], especially for *Ca.* L. asiaticus diversity analysis. However, the development of new specific primers is still needed to complete the molecular marker database for better results in HLB detection. The focus of this study was to utlilized newly designed primer based on a gene sequence located in *CLIBASIA_RS01220* locus for HLB detection in citrus plants collected

around Cimanggu, Bogor. It is expected that the presence of newly developed primers conducted in present study could enrich the primer database and help the breeders for early detection of HLB in citrus plant, especially in small scale laboratories.

2 Materials and Methods

2.1 Genetic materials

A total of twenty one citrus leaves derived from plant trees that visually showed HLB symptoms, such as chlorosis, blotchy, and yellowing were used as genetic materials in this study (Figure 1). As many as thirteen leaves were collected from the Japansche citroen (*Citrus limonia* Osbeck) trees that grew around Cikeumeuh greenhouse (labeled as JC-1 to JC-13), while the rest were collected from the lime (*Citrus aurantifolia* Swingle) trees that grew around Cimanggu Greenhouse in Bogor (labeled as Lime-1 to Lime-8) (Figure 1)

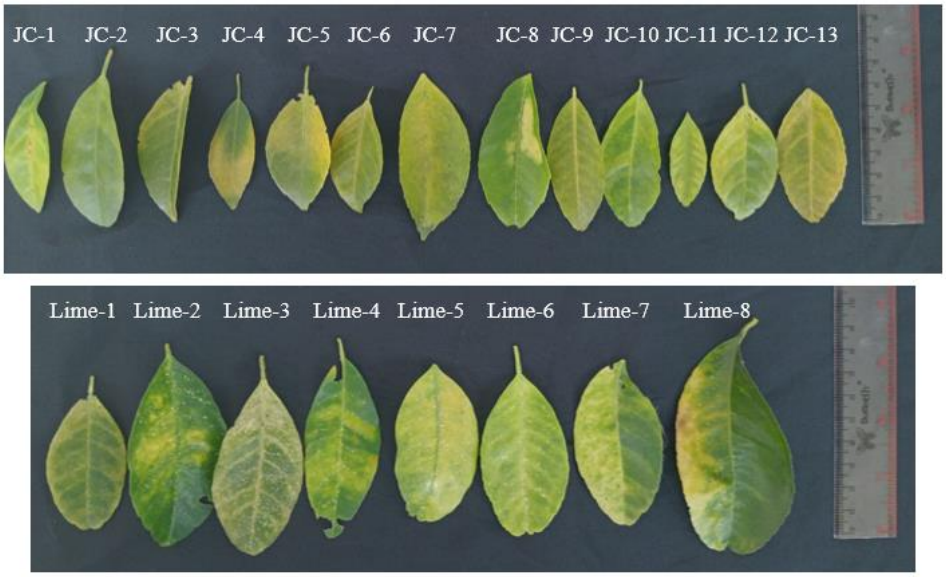

Fig. 1. Morphological visualization of symptomatic citrus leaf samples used in this study.

2.2 Primer design

The newly primer in this study was designed based on a gene that located in *CLIBASIA_RS01220* locus with the length of 846 bp (265,720 to 264,875) which encoded YihY/virulence factor BrkB family protein. The complete gene sequence was collected from *Candidatus* Liberibacter asiaticus psy62 genome from Citrus Genome Database (https://www.citrusgenomedb.org/). The primer was designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) [32] in the flanking area of the coding sequence and verified using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) [33] to assure that the primer only have one annealing site. The sequence of the primer was presented in Table 2. As the comparison, the A2/J5 primer that designed from partial sequence of *β-operon* (*rplKAJL-rpoBC* operon) genes and previously reported [25] was also used in this study (Table 1).

Table 1. List of specific gene primers for *C*Las detection used in this study.

Primer name	Sequence (5'-3')	Gene target	Product size	Reference
YihY-F	AAGCCTGAATTTATTTTGATCCCACA	*CLIBASIA_RS01220* locus which encoded YihY/virulence factor BrkB family protein	930 bp	This study
YihY-R	TTTGTCATCGCATGCCAATCC			
A2	TATAAAGGTTGACCTTTCGAGTTT	*β-operon* (*rpl*KAJL-*rpo*BC operon) gene which encode ribosomal protein	669 or 703 bp	[25]
J5	ACAAAAGCAGAAATAGCACGAACAA			

2.3 Genomic DNA extraction, DNA amplification, and Sanger sequencing

The genomic DNA of *C*Las was extracted using the modified method [31] from citrus leaf samples. A total of 0.5 gram blotchy or yellowing citrus leaf was ground using 500 µL of extraction buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% (w/v) polyvinylpyrrolidone (PVP), and 0.38% (w/v) sodium bisulfite] in sterile mortar. The grinding result was then put into a 2 mL microtube and the addition of extraction buffer was conducted until the volume reached 800 µL. Each samples were then incubated in 65 °C of waterbath for 15 min with homogenizing the samples thoroughly every 5 minutes. The samples were then extracted using 800 µL of chloroform: isoamyl alcohol solution (24:1 v/v) and centrifuged at 12,000 rpm at 20 °C for 10 minute. The supernatant was then transfered carefully into a new 1.5 mL microtube, followed by the addition of 3M sodium acetate (pH 5.2) at 1/10 of the supernatant volume and cold isopropanol in equal volume of the supernatant. The samples were then mix gently and incubated at −20 °C for 1 h and centrifugated at 12,000 rpm at 20 °C for 10 min. The supernatant was discarded and the DNA pellets were washed using 70% (v/v) cold ethanol and dried overnight in room temperature. Finally, the dried DNA pellets were dissolved in 100 µL of TE solution (10 mM Tris, pH 8.0, and 1 mM EDTA). The concentration and purity of each DNA stock solutions were measured using NanoDrop Spectrophotomer (ThermoScientific, USA) and followed by dilution to a concentration of 20 ng/µL for the amplification process.

DNA from each samples were then amplified in a total reaction volume of 40 µL containing 20 ng/µL DNA template to a volume of 2 µL, 2×MyTaqTM HS Red Mix (Bioline, UK) to a volume of 20 µL, 2 µL of forward and reverse primers each at a concentration of 10 µM, and sterile ddH_2O. The PCR reaction was conducted in a T100 Thermal Cycler (Bio-Rad, USA) with the following profile conditions such as: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 1 minutes, and extension at 72 °C for 1 minutes. The PCR reaction was completed with the final extension step at 60 °C for 15 min. The PCR products were then separated using 1% agarose gel in an electrophoresis tank containing 0.5× Tris Borate EDTA (TBE) buffer at 100 V for 25 minutes, stained using 10 mg/mL ethidium bromide, and visualized using UV Transilluminator. The samples with positive amplicon band were then sent to Genomic Laboratory of National Research and Innovation Agency (BRIN) in Cibinong Science Center for Sanger sequencing analysis.

2.4 Data analysis

The Sanger sequencing results were then analyzed using blastn (Basic Local Alignment Search Tool Nucleotide) menu on the NCBI (National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) utilizing highly similar sequences (megablast) program [34]. The aligned results which showed the homology of the sequences with the database collection, including informations such as species name, gene bank accession number, query cover, E-value, and percent identity, were collected. The phylogenetic tree was constructed using the Neighbor Joining method with Tamura-Nei model by 1000 times bootstrap on Mega X [35]. The sequence of *Candidatus* Liberibacter africanus isolate Ang37 (CP054879.1) was used as the outgroup for sequences amplified by YihY primer and the sequence of *Ca.* L. africanus subsp. Capensis strain Good Hope (JF419555.1) was used as the outgroup for sequences amplified by A2/J5 primer.

3 Results and discussions

3.1 HLB detection using conventional PCR method

The genomic DNA extraction results showed the DNA concentrations that ranging from 70.8 (JC-7) to 250.6 (Lime-8) ng/µL, with the average of 118.8 ng/µL (Table 2). The A_{260}/A_{280} ratio was ranging from 1.71 (Lime-3) to 2.04 (Lime-6), with the average of 1.89. There are several factors that affected the successful of DNA extraction method such as the genetic material conditions that were used and their handling process; the lysis of the tissue and elimination process of the contaminants such as polysacharides, phenolic compounds, protein, and RNA; also the composition of the solutions that were used in extraction process [36, 37]. However, *Ca.* L. asiaticus is a parasitic bacteria that distributed inside the citrus phloem tissue, so that the genomic DNA obtained in this study was a mix between the citrus DNA and the DNA of these bacteria. Therefore, the utilization of specific-gene primers for this bacteria as demonstrated in this study, is necessary to avoid the cross-amplification with the citrus DNA.

The PCR results using YihY primer showed that as many as 15 leaves from the total of 21 collected leaf samples showed positive amplicon band at a size of 930 bp as targeted. On the other side, the primer A2/J5 could detect 17 leaves with positive amplicon band from the total of 21 leaves with a size of 703 bp. Among 21 DNA samples, there were two samples namely JC-2 and Lime-2 that failed to be amplified by YihY primers. Nevertheless, both of these two samples were possessed good quality and quantity DNA, reflected by their DNA concentrations and purities as presented in Table 2. Additionally, both the newly designed primers and A2/J5 primers could not produced amplicon band from the DNA amplification of five samples namely JC-1, JC-4, JC-7, and JC-9. In this study, we assumed that the failed of DNA amplification process of those four samples was not affected by the DNA concentration or purity but caused by the non-existance of the bacteria in those leaf samples. The leaves symptoms that showed by those four samples might be similar and indistinguishable between nutrient deficiency and HLB disease symptoms. Nutrient deficiency symptoms in plants is usually misunderstood to HLB disease [8], therefore the detection using PCR method is necessary to ensure this.

Table 2. The measurement results of DNA concentration and purity using NanoDrop spectrophotometer.

No.	Samples Name	DNA concentrations (ng/µL)	A_{260}/A_{280}
1.	JC-1	114.4	1.95
2.	JC-2	117.3	1.96
3.	JC-3	168.3	2.02
4.	JC-4	105.8	1.94
5.	JC-5	65.3	1.97
6.	JC-6	180.8	1.92
7.	JC-7	70.8	1.84
8.	JC-8	115.9	2.03
9.	JC-9	156.1	2.02
10.	JC-10	145.2	1.87
11.	JC-11	91.8	1.76
12.	JC-12	112.3	1.91
13.	JC-13	80.4	1.77
14.	Lime-1	71.7	1.60
15.	Lime-2	141.4	1.90
16.	Lime-3	80.9	1.71
17.	Lime-4	91.2	1.92
18.	Lime-5	119.1	1.83
19.	Lime-6	165.5	2.04
20.	Lime-7	95.7	1.86
21.	Lime-8	205.6	1.84
Average		118.8	1.89

Previous study [25] reported that the amplicon band at a size of 703 bp indicated the HLB isolates are from the *Candidatus* Liberibacter asiaticus (*C*Las) species. On the contrary, when the amplicon band showed a size of 669 bp, the bacteria identified are from the *Candidatus* Liberibacter africanus (*C*Laf) species. *C*Laf caused HLB disease that attacked citrus plantation in the African region. On the opposite to *C*Las that was found in Asia region and heat tolerant, this bacteria had heat sensitive character and is spread by *Tryoza eritrea* as the vector [38]. Therefore, Sanger sequencing was necessary to assure the bacteria species that identified in this study.

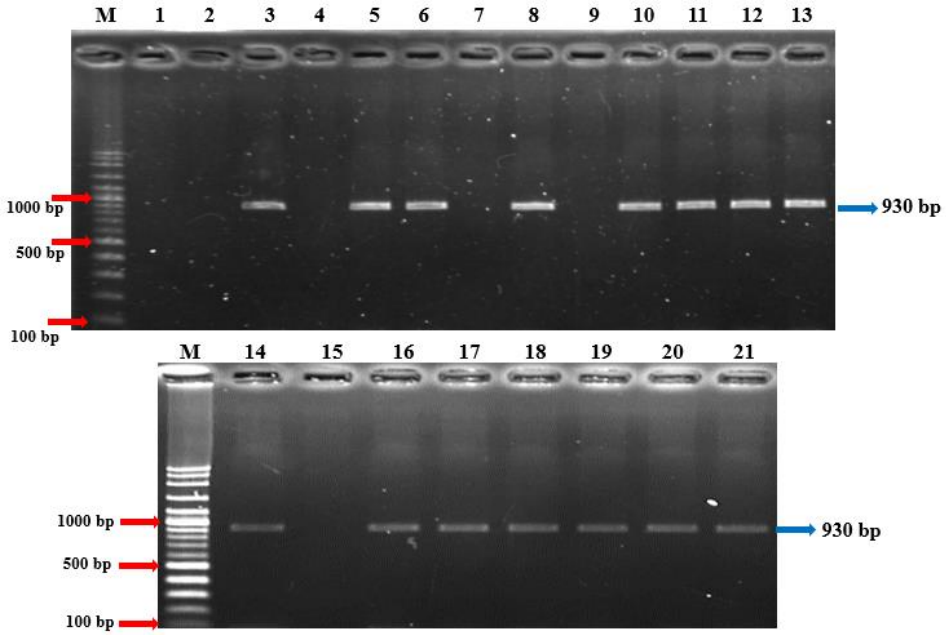

Fig. 2. Visualization of amplicon band from HLB bacteria DNA amplified using YihY primer. Information: M: 100 bp plus DNA ladder (Vivantis Technologies1-13: JC-1 to JC-13, 14-21: Lime-1 to Lime-8 as presented in Figure 1.

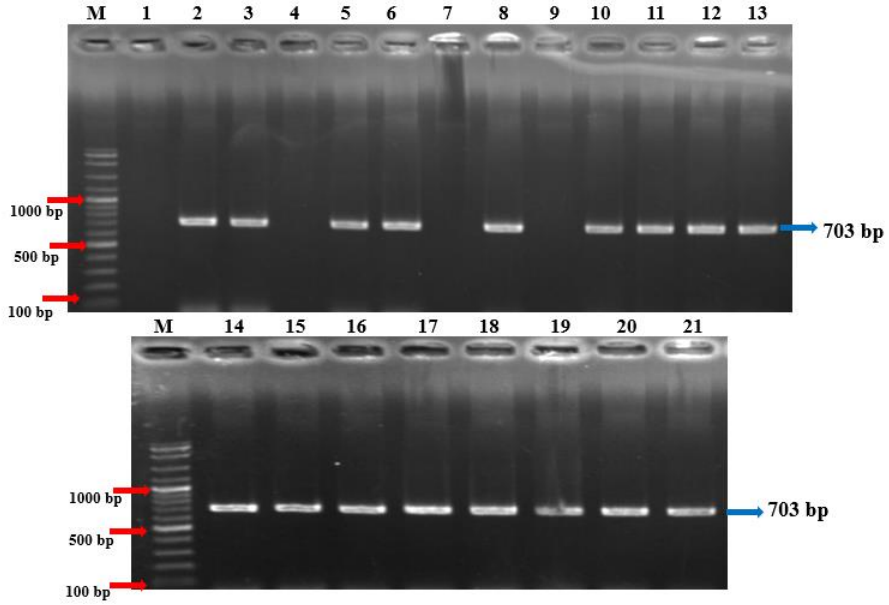

Fig. 3. Visualization of amplicon band from HLB bacteria DNA amplified using A2/J5 primer. Information: M: 100 bp plus DNA ladder (Vivantis Technologies), 1-13: JC-1 to JC-13, 14-21: Lime-1 to Lime-8 as presented in Figure 1.

The utilization of conventional PCR method as showed in this study still could be a potential tool to help the citrus farmers for identification of Huanglongbing disease in the future, because of its lower budget cost in comparison to RT-PCR and more accurate results compared to LAMP. The less sensitivity that possessed by conventional PCR compared to RT-PCR because of the different method in detection process. The conventional PCR is a gel-based detection under UV light using the ethidium bromide stained, while RT-PCR measurement is based on fluorescence in each single PCR plate well [39]. Previous study [9] demonstrated the accuracy and sensitivity of RT-PCR method in Huanglongbing detection using smaller amount of DNA. However, the conventional PCR method is suitable for the small scale laboratories located in remote area, especially in developing countries such Indonesia whereas the presence of RT-PCR in molecular laboratory are still limited.

3.2 Isolates identification using sequence homology to NCBI database

The homology analysis from HLB isolate sequence amplified by YihY primer showed that all of the sequences had similarity with *Ca* L. asiaticus isolate GDMMLM3 (Table 3). The query coverage obtained in this study ranged from 90 (Lime-4) to 98% (Lime-8) with all of the sequences showed E-value of 0.0. Menawhile, the percentage identity ranged from 95.98 (Lime-5) to 99.88% (Lime-6).

On the other side, the homology analysis from bacterial DNA sequence amplified by A2/J5 primer showed different results. A total of seven sequences (JC-3, JC-11, JC-12, JC-13, Lime-1, Lime-6, and Lime-8) showed homology to *Ca.* L. asiaticus strain MH 'B-L3P4-Mo', while five sequences (JC-5, JC-8, JC-10, Lime-3, and Lime-7) exhibited homology to *Ca.* L. asiaticus isolate GXHZ9, two sequences (JC-2 and Lime-2) showed homology to *Ca.* L. asiaticus isolate HANCM3b, one sequence (JC-6) showed homology to *Ca.* L. asiaticus strain KhM-ii, one sequence (Lime-4) showed homology to *Ca.* L. asiaticus clone L10, and one sequence (Lime-5) showed homology to *Ca*. L. asiaticus strain: 60 (Table 3). The query coverage ranged from 65 (Lime-4) to 99% (JC-3 and JC-10) with all of the sequences showed E-value of 0.0. The percentage identity ranged from 90.74 (Lime-4) to 100% (Lime-2 and Lime-8).

Both YihY and A2/J5 primers could similarly identify the HLB bacteria isolated from Cikeumeuh and Cimanggu greenhouse as *Ca.* L. asiaticus. Nevertheless, A2/J5 primers could detect higher variation among isolates used in this study. Taken together, it could be assumed that YihY primer amplified the more conserved gene target rather than A2/J5 primer, so its ability to detect variation among the HLB isolates was lower than A2/J5 primer. It is reported that, the markers that designed in relatively conserved gene is very useful for genetic variation studies between distantly related prokaryotes, but it less useful for distinguishing between closely related prokaryotes, as previously demonstrated by *16S rRNA* gene [40].

Table 3. Sequence homology among HLB isolates that amplified by YihY and A2J5 primers

No.	Samples Name	Accession Number	Species	Query coverage (%)	E-value	Identity (%)
Yihy Primer Amplification						
1.	JC-3	CP131347.1	*Ca.* L. asiaticus isolate GDMMLM3	95	0.0	99.42
2.	JC-5	CP131347.1	*Ca.* L. asiaticus isolate GDMMLM3	96	0.0	99.65
3.	JC-6	CP131347.1	*Ca.* L. asiaticus isolate GDMMLM3	94	0.0	99.30
4.	JC-8	CP131347.1	*Ca.* L. asiaticus isolate GDMMLM3	96	0.0	99.77

5.	JC-10	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	96	0.0	99.77
6.	JC-11	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	96	0.0	99.77
7.	JC-12	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	97	0.0	99.77
8.	JC-13	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	96	0.0	99.77
9.	Lime-1	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	96	0.0	99.54
10.	Lime-3	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	96	0.0	99.65
11.	Lime-4	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	90	0.0	98.41
12.	Lime-5	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	95	0.0	95.98
13.	Lime-6	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	96	0.0	99.88
14.	Lime-7	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	95	0.0	99.42
15.	Lime-8	CP131347.1	*Ca.*L. asiaticus isolate GDMMLM3	98	0.0	98.55
A2/J5 Primer Amplification						
1.	JC-2	CP131270.1	*Ca.*L. asiaticus isolate HANCM3b	97	0.0	99.54
2.	JC-3	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	99	0.0	99.55
3.	JC-5	CP131303.1	*Ca.*L. asiaticus isolate GXHZ9	97	0.0	99.69
4.	JC-6	KC477375.1	*Ca.*L. asiaticus strain KhM-ii	96	0.0	99.85
5.	JC-8	CP131303.1	*Ca.*L. asiaticus isolate GXHZ9	96	0.0	99.24
6.	JC-10	CP131303.1	*Ca.*L. asiaticus isolate GXHZ9	99	0.0	99.10
7.	JC-11	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	98	0.0	99.39
8.	JC-12	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	98	0.0	99.69
9.	JC-13	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	97	0.0	99.39
10.	Lime-1	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	97	0.0	99.10
11.	Lime-2	CP131270.1	*Ca.*L. asiaticus isolate HANCM3b	73	0.0	100
12.	Lime-3	CP131303.1	*Ca.*L. asiaticus isolate GXHZ9	96	0.0	99.39
13.	Lime-4	KR919750.1	*Ca.*L. asiaticus clone L10	65	0.0	90.74
14.	Lime-5	LC090236.1	*Ca.*L. asiaticus strain: 60	97	0.0	99.09
15.	Lime-6	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	98	0.0	99.40
16.	Lime-7	CP131303.1	*Ca.*L. asiaticus isolate GXHZ9	97	0.0	99.39

17.	Lime-8	JQ973890.1	*Ca.*L. asiaticus strain MH 'B-L3P4-Mo'	95	0.0	100

In this study, the YihY primer was designed based on a gene located in *CLIBASIA_RS01220* locus which encoded YihY/virulence factor BrkB family protein, that the function is still unclear in *Ca.* L. asiaticus. The selection of this locus is conducted due to its low similarity to *Citrus sinensis* genome sequence, in order to avoid the cross amplification to citrus DNA. Previous study showed that the YihY/virulence factor BrkB family protein in *Bordetella pertusis* is a new virulence factor that is functional in bacteria resistance [41]. This protein was also found in several bacteria such as *Streptomyces seoulensis* [42], *Klebsiella oxytoca* [43], *Mycobacterium tuberculosis* [44], *Mycoplasma hominis* [45], *Corynebacterium simulans* [46], *Orientia tsutsugamushi* [47], and *Turicibacter* sp. [48]. Meanwhile, A2/J5 primer was designed based on *β-operon* (*rplKAJL-rpoBC* operon) gene which encode ribosomal protein that played important role in protein synthesis [25]. This primer has been used in several studies [13, 14, 49-51] and proven its ability to detect HLB bacteria from citrus plants in several countries. The newly designed primer from this study still needs to be further validated using another HLB isolates derived from different regions and/or genetic background to evaluate its ability in following studies.

3.3 Phylogenetic analysis of HLB isolates identified in this study

The phylogenetic analysis, based on genotyping results using both YihY and A2/J5 primers showed the mixed between isolates collected from Japansche citroen (in Cikemeuh greenhouse) and lime trees (in Cimanggu greenhouse). In the phylogenetic tree which constructed based on YihY primer data, we found that a total of 15 isolates were existed in the similar cluster with *Ca*. L. asiaticus isolate GDMMLM3, separating from *Ca*. L. africanus as the outgroup (Figure 4). Similarly, that based on A2/J5 primer, a separation of the 17 isolates from *Ca*. L. Africanus (Figure 5) was also shown and this phenomenon confirming that all of the HLB isolates from this study were *Ca*. L. asiaticus species.

The mixing among isolates collected from Cikeumeuh and Cimanggu greenhouse in the similar cluster as demonstrated in this study was inseparable by the distance factor between those two greenhouses that relatively close in distance of approximately 200 m. Eventhough the greenhouses are separated by buildings and main street, but the Asian citrus psyllid as a vector had the ability to flight up to 2.4 km as previously reported [52]. This conditions could lead to the HLB isolate exchange among the greenhouses or fields that might be followed by cross infection. There are several efforts that could be performed to control HLB transmission such as geographical isolation and health certification programs for mother plant nurseries, geographical isolation for production nurseries, utilization of screenhouse to prevent the entry of insects, controlling insect vectors using insecticides or natural enemies, and removing citrus plants that have been infected with HLB from the screenhouse or field [53]. Besides, the molecular analysis using conventional PCR should be performed regularly in plantation as the supporting data to visual symptoms, to detect the HLB existance as early as possible.

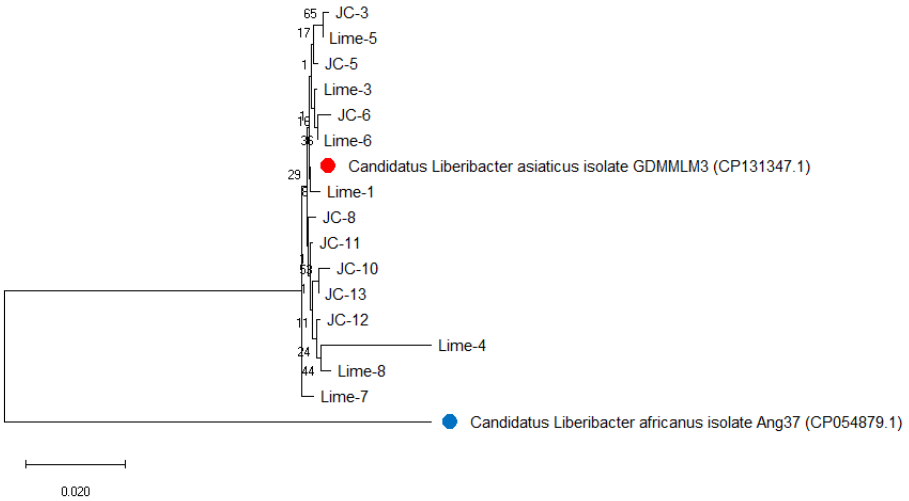

Fig. 4. Phylogenetic tree based on sequences amplified by YihY primer constructed using Neighbor Joining method with Tamura-Nei model. Information: red dot represented *Candidatus* Liberibacter asiaticus sequence collected from NCBI, while blue dot represented *Candidatus* Liberibacter africanus sequence collected from NCBI

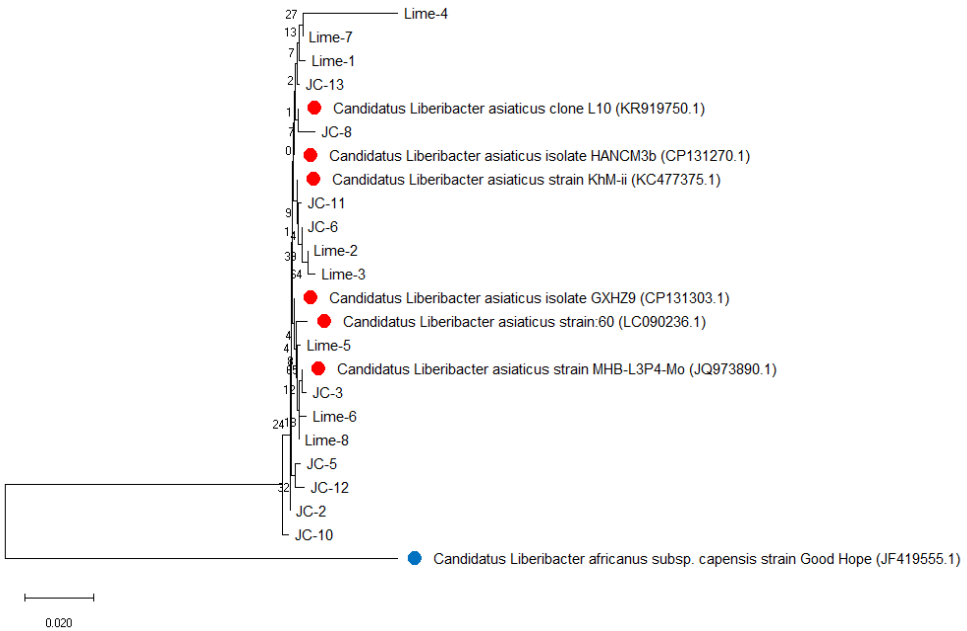

Fig. 5. Phylogenetic tree based on sequences amplified by A2/J5 primer constructed using Neighbor Joining method with Tamura-Nei model. Information: red dots represented *Candidatus* Liberibacter asiaticus sequence collected from NCBI, while blue dot represented *Candidatus* Liberibacter africanus sequence collected from NCBI

The presence of newly designed primers as demonstrated in this study could help to enrich the primer database for HLB detection and also help the breeders and farmers for early detection of HLB in citrus plantations, especially in remote area with small scale laboratories.

The early detection using the conventional PCR method is conducted to prevent the spread of HLB disease that usually occured due to misunderstood of the visual symptomps with nutrient deficiency. The preventive action is expected could help to reduce the economic and yield losses that caused by this disease, especially in developing contries.

4 Conclusions

Both the newly designed (YihY) and the reference (A2/J5) primer used in this study could identify the HLB isolates from Cikeumeuh and Cimanggu greenhouse as Candidatus Liberibacter asiaticus. The newly designed primer showed its ability to amplify the DNA and in the detection of variation among the isolates, eventhough with lower succesful percentage compared to the reference primer. The homology analysis from Huanglongbing isolate sequence amplified by newly designed primer also showed similarity with *Ca* L. asiaticus sequence in NCBI, as well as the reference primer. Overall, this primer could be a potential marker to be used as early detection to Huanglongbing symptoms especially in small scale laboratories.

This research was funded by In House (Rumah Program) research scheme in Research Organization for Agriculture and Food, National Research and Innovation Agency (BRIN). Authors gratefully acknowledged BBRMP Biogen for providing laboratory facilities. Authors also acknowledged the facilities, scientific, and technical support from Center Laboratory of Sequencing, National Research and Innovation Agency, through E- Layanan Sains (ELSA).

References

1. J.V. da Graça, G.W. Douhan, S.E. Halbert, M.L. Keremane, R.F. Lee, G. Vidalakis, H. Zhao, Huanglongbing: an overview of a complex pathosystem ravaging the world's citrus. J. Integr. Plant Biol. **58**, 373-387 (2016). https://doi.org/ 10.1111/jipb.12437
2. S. Munir, P. He, Y. Wu, P. He, S. Khan, M. Huang, W. Cui, P. He, Y. He Y, Huanglongbing control: perhaps the end of the beginning. Microb. Ecol. **76**, 192-204 (2018). https://doi.org/10.1007/s00248-017-1123-7
3. S. Welker, M. Pierre, J.P. Santiago, M. Dutt, C. Vincent, A. Levy, Phloem transport limitation in Huanglongbing-affected sweet orange is dependent on phloem-limited bacteria and callose. Tree Physiol. **42**, 379-390 (2022). https://doi.org/10.1093/treephys/tpab134
4. J.M. Bové, Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol. **88**, 7–37 (2006). https://doi.org/10.1371/journal.pone.0111032
5. B. Alquézar, L. Carmona, S. Bennici, M.P. Miranda, R.B. Bassanezi, L. Peña, Cultural management of Huanglongbing: current status and ongoing research. Phytopathology. **112**, 11-25 (2022). https://doi.org/10.1094/PHYTO-08-21-0358-IA
6. S. Tirtawidjaja, T. Hadiwidjaja, A.M. Lasheen, Citrus vein phloem degeneration virus, a possible cause of citrus chlorosis in Java. Proc. Amer. Soc. Hort. Sci. **86**, 235-243 (1965).
7. Nurhadi, Penyakit Huanglongbing tanaman jeruk (*Candidatus* Liberibacter asiaticus): ancaman dan strategi pengendalian. Pengemb. Inov. Pertan. **8**, 21–32 (2015).
8. M.M.H. Tipu, M.M. Masud, R. Jahan, A. Baroi, A.K.M.A. Hoque, Identification of citrus greening based on visual symptoms: a grower's diagnostic toolkit. Heliyon. **7**, e08387 (2021). https://doi.org/10.1016/j.heliyon.2021.e08387

9. Y. Sariasih, S. Subandiyah, S. Widyaningsih, T. Khurshid, J. Mo, N. Donovan, Comparison of two Huanglongbing detection methods in samples with different symptom severity. J. Fitopatol. Indones. **20**, 174–186 (2024). https://doi.org/10.14692/jfi.20.4.174–186
10. B.M. Dala-Paula, A. Plotto, J. Bai, J.A. Manthey, E.A. Baldwin, R.S. Ferrarezi, M.B.A. Gloria, Effect of Huanglongbing or greening disease on orange juice quality, a review. Front. Plant Sci. **9**, 1976 (2019). https://doi.org/10.3389/fpls.2018.01976
11. M.L. Keremane, C. Ramadugu, E. Rodriguez, R. Kubota, S. Shibata, D.G. Hall, M.L. Roose, D. Jenkins, R.F. Lee, A rapid field detection system for citrus Huanglongbing associated '*Candidatus* Liberibacter asiaticus' from the psyllid vector, *Diaphorina citri* Kuwayama and its implications in disease management. Crop Prot. **68**, 41-48 (2015). https://doi.org/10.1016/j.cropro.2014.10.026
12. C.W. Choi, J.W. Hyun, C.A. Powell, Loop-mediated isothermal amplification assay for detection of *Candidatus* Liberibacter asiaticus, a causal agent of citrus Huanglongbing. Plant Pathol. J. **34**, 499-505 (2018). https://doi.org/10.5423/PPJ.FT.10.2018.0212
13. M. Rizal, K.H. Mutaqin, G. Suastika, Deteksi dan evaluasi keragaman genetika *Candidatus* Liberibacter asiaticus sebagai penyebab penyakit Huanglongbing di Indonesia berdasarkan gen *β-operon*. J. Fitopatol. Indones. **12**, 168–177 (2016). https://doi.org/10.14692/jfi.12.5.168
14. E. Saberi, S.M. Alavi, N. Safaie, C. Moslemkhany, M. Azadvar, Bacterial pathogens associated with citrus Huanglongbing-like symptoms in Southern Iran. J. Crop Prot. **6**, 99-113 (2017).
15. M.S. Yaqub, I.A. Khan, M. Usman, I.A. Rana, Molecular detection of *Candidatus* Liberibacter asiaticus, the causal organism of Huanglongbing (citrus greening) in Faisalabad, Pakistan for Huanglongbing management. Pak. J. Agri. Sci. **54**, 21-26 (2017). https://doi.org/10.21162/PAKJAS/17.4455
16. I.G. Orce, L.N. Sendín, M.R. Marano, A.A. Vojnov, A.P. Castagnaro, M.P. Filippone, Novel set of real-time PCR primers for simultaneous detection of Liberibacter species associated with citrus Huanglongbing. Sci. Agric. **72**, 252-259 (2015). https://doi.org/10.1590/0103-9016-2013-0417
17. J.W. Park, E.S. Louzada, W.E. Braswell, P.A. Stansly, J.V. da Graça, G. McCollum, J.E. Rascoe, M. Kunta, A new diagnostic real-time PCR method for Huanglongbing detection in citrus root tissue. J. Gen. Plant Pathol. **84**, 359-367 (2018). https://doi.org/10.1007/s10327-018-0793-4
18. G. Cellier, C. Redondo, J. Cubero, M. Roselló, E. de Andrade, L. Cruz, E. Ince, H.N. Yildiz, P.G. Güler, A.M. D'Onghia, T. Yaseen, K. Djelouah, E. Metz-Verschure, F. Gaffuri, R.A. Gottsberger, B. Giovani B, Comparison of the performance of the main real-time and conventional PCR detection tests for '*Candidatus* Liberibacter'spp., plant pathogenic bacteria causing the Huanglongbing disease in *Citrus* spp. Eur. J. Plant Pathol. **157**, 919-941(2020). https://doi.org/10.1007/s10658-020-02052-3
19. M.L. Keremane, T.G. McCollum, M.L. Roose, R.F. Lee, C. Ramadugu, An improved reference gene for detection of "*Candidatus* Liberibacter asiaticus" associated with citrus Huanglongbing by qPCR and digital droplet PCR assays. Plants. **10**, 2111 (2021). https://doi.org/10.3390/plants10102111
20. M.R.V. Pérez, M.G.G. Mendoza, M.G.R. Elías, F.J. González, H.R.N. Contreras, C.C. Servín, Raman spectroscopy an option for the early detection of citrus Huanglongbing. Appl. Spectrosc. **70**, 829-839 (2016). https://doi.org/10.1177/0003702816638229

21. K. Wang, Y. Liao, Y. Meng, X. Jiao, W. Huang, T.C.Y. Liu, The early, rapid, and non-destructive detection of citrus Huanglongbing (HLB) based on microscopic confocal Raman. Food Anal. Methods. **12**, 2500-2508 (2019). https://doi.org/10.1007/s12161-019-01598-1
22. L. Sanchez, S. Pant, K. Mandadi, D. Kurouski, Raman spectroscopy vs quantitative polymerase chain reaction in early stage Huanglongbing diagnostics. Sci. Rep. **10**, 10101 (2020). https://doi.org/10.1038/s41598-020-67148-6
23. R.A. Valdés, J.C.D. Ortiz, M.B. Beache, J.A. Cabello, E.C. Chávez, Y.R. Pagaza, Y.M.O. Fuentes, A review of techniques for detecting Huanglongbing (greening) in citrus. Can. J. Microbiol. **62**, 803–811 (2016). https://dx.doi.org/10.1139/cjm-2016-0022
24. S. Jagoueix, J.M. Bové, M. Garnier, PCR detection of the two '*Candidatus*' Liberobacter species associated with greening disease of citrus. Mol. Cell. Probes. **10**, 43-50 (1996).
25. A. Hocquellet, P. Toorawa, J.M. Bové, M. Garnier. 1999. Detection and identification of the two *Candidatus* Liberobacter species associated with citrus Huanglongbing by PCR amplification of ribosomal protein genes of the beta operon. Mol. Cell. Probes. **13**, 373-379. https://doi.org/10.1006/mcpr.1999.0263
26. M.A. Hoy, A. Jeyaprakash, R. Nguyen, 2001 Long PCR is a sensitive method for detecting Liberobacter asiaticum in parasitoids undergoing risk assessment in quarantine. Biol. Control. **22**, 278-287 (2001). https://doi.org/10.1006/bcon.2001.0973
27. D.C. Teixeira, J.L. Danet, S. Eveillard, E.C. Martins, W.C. Jesus Jr., P.T. Yamamoto, S.A. Lopes, E.B. Bassanezi, A.J. Ayres, C. Saillard, J.M. Bové JM, Citrus Huanglongbing in São Paulo, Brazil: PCR detection of the '*Candidatus* Liberibacter' species associated with the disease. Mol. Cell. Probes. **19**, 173–179 (2005). https://doi.org/10.1016/j.mcp.2004.11.002
28. T. Fujikawa, T. Iwanami, Sensitive and robust detection of citrus greening (Huanglongbing) bacterium "*Candidatus* Liberibacter asiaticus" by DNA amplification with new 16s rDNA-specific primers. Mol. Cell. Probes. **26**, 194-197 (2012). http://dx.doi.org/10.1016/j.mcp.2012.06.001
29. M. Nageswara-Rao, M. Irey, S.M. Garnsey, S. Gowda, Candidate gene makers for *Candidatus* Liberibacter asiaticus for detecting citrus greening disease. J. Biosci. **38**, 229–237 (2013). https://doi.org/10.1007/s12038-013-9315-x
30. L. Zhou, C.A. Powell, M.T. Hoffman, W. Li, G. Fan, B. Liu, H. Lin, Y. Duan, Diversity and plasticity of the intracellular plant pathogen and insect symbiont "*Candidatus* Liberibacter asiaticus" as revealed by hypervariable prophage genes with intragenic tandem repeats. Appl. Environ. Microbiol. **77**, 6663–6673 (2011). https://doi.org/10.1128/AEM.05111-11
31. J.J. Doyle JJ, J.L. Doyle, Isolation of plant DNA from fresh tissue. Focus. **12**, 13-15 (1990).
32. A. Untergasser, H. Nijveen, X. Rao, T. Bisseling, R. Geurts, J.A.M. Leunissen, Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. **35**, 71–74 (2007). https://doi.org/10.1093/nar/gkm306
33. J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, T. Madden, Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. **13**, 134 (2012).
34. S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool. J. Mol. Biol. **215**, 403-410 (1990). https://doi.org/10.1016/S0022-2836(05)80360-2

35. S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. **35**, 1547–1549 (2018). https://doi.org/10.1093/molbev/msy096
36. A. Varma, H. Padh, N. Shrivastava, Plant genomic DNA isolation: an art or a science. Biotechnol. J. **2**, 386-392 (2007). https://doi.org/10.1002/biot.200600195
37. Syafaruddin, T.J. Santoso, Optimasi teknik isolasi dan purifikasi DNA yang efisien dan efektif pada kemiri sunan (*Reutalis trisperma* (Blanco) Airy Shaw). J. Littri. **17**, 11-17 (2011).
38. T.R. Gottwald, Current epidemiological understanding of citrus Huanglongbing. Annu. Rev. Phytopathol. **48**, 119–139 (2010). https://doi.org/10.1146/annurev-phyto-073009-114418
39. Z. Xia, M.L. Johansson, Y. Gao, L. Zhang, G.D. Haffner, H.J. MacIsaac, A. Zhan, Conventional versus real-time quantitative PCR for rare species detection. Ecol. Evol. **8**, 11799-11807 (2018). https://doi.org/10.1002/ece3.4636
40. Y. Lan, G. Rosen, R. Hershberg, Marker genes that are less conserved in their sequences are useful for predicting genome-wide similarity levels between closely related prokaryotic strains. Microbiome. **4**, 18 (2016). https://doi.org/10.1186/s40168-016-0162-5
41. M.P. Maela, M.H. Serepa-Dlamini, Genome sequence and characterisation of *Peribacillus* sp. strain AS_2, a bacterial endophyte isolated from *Alectra sessiliflora*. Microbiol. Res. **15**, 50-65 (2023). https://doi.org/10.3390/microbiolres15010004
42. J. Shin, S. Park, J.S. Lee, E.J. Lee, H.D. Youn, Complete genome sequence and comparative analysis of *Streptomyces seoulensis*, a pioneer strain of nickel superoxide dismutase. Genes Genom. **42**, 273-281 (2020). https://doi.org/10.1007/s13258-019-00878-8
43. L. Ni, Y. Xu, L. Chen L, First experimental evidence for the presence of potentially virulent *Klebsiella oxytoca* in 14 species of commonly consumed aquatic animals, and phenotyping, and genotyping of *K. oxytoca* isolates. Antibiotics. **10**, 1235 (2021). https://doi.org/10.3390/antibiotics10101235
44. T. Maeda, M. Kawada, N. Sakata, H. Kotani, C. Furusawa, Laboratory evolution of *Mycobacterium* on agar plates for analysis of resistance acquisition and drug sensitivity profiles. Sci. Rep. **11**, 15136 (2021). https://doi.org/10.1038/s41598-021-94645-z
45. D.V. Evsyutina, T.A. Semashko, M.A. Galyamina, S.I. Kovalchuk, R.H. Ziganshin, V.G. Ladygina, G.Y. Fisunov, O.V. Pobeguts, Molecular basis of the slow growth of *Mycoplasma hominis* on different energy sources. Front. Cell. Infect. Microbiol. **12**, 918557 (2022). https://doi.org/10.3389/fcimb.2022.918557
46. J.M. Schröder, Discovery of natural bispecific antibodies: is psoriasis induced by a toxigenic *Corynebacterium simulans* and maintained by CIDAMPs as autoantigens?. Exp. Dermatol. **33**, e15014 (2024). https://doi.org/10.1111/exd.15014
47. S. Giengkam, C. Kullapanich, J. Wongsantichon, H.E. Adcox, J.J. Gillespie, J. Salje. 2023. *Orientia tsutsugamushi*: comprehensive analysis of the mobilome of a highly fragmented and repetitive genome reveals the capacity for ongoing lateral gene transfer in an obligate intracellular bacterium. Msphere. **8**, e00268-23 (2023). https://doi.org/10.1128/msphere.00268-23
48. K.A. Klag, A.M. Weis, W.Z. Stephens, J.L. Round, Draft *Turicibacter* sp. genome isolated from a spore-forming community in mice. Microbiol. Resour. Announc. **13**, e00385-24 (2024). https://doi.org/10.1128/mra.00385-24

49. A.A. Arratia-Castro, M.E. Santos-Cervantes, E. Fernández-Herrera, J.A. Chávez-Medina, G.L. Flores-Zamora, E. Camacho-Beltrán, J. Méndez-Lozano, N.E. Leyva-López. 2014. Occurrence of '*Candidatus* Phytoplasma asteris' in citrus showing Huanglongbing symptoms in Mexico. Crop Pro.t **62**, 144e151 (2014). http://dx.doi.org/10.1016/j.cropro.2014.04.020
50. M.M.H. Tipu, M.M. Rahman, M.M. Islam, F.E. Elahi, R. Jahan, M.R. Islam, Citrus greening disease (HLB) on *Citrus reticulata* (Mandarin) caused by *Candidatus* Liberibacter asiaticus in Bangladesh. Physiol. Mol. Plant Pathol. **112**, 101558 (2020). https://doi.org/10.1016/j.pmpp.2020.101558
51. D.K. Ghosh, A.D. Kokane, S.B. Kokane, J. Tenzin, M.G. Gubyad, P. Wangdi, A.A. Murkute, A.K. Sharma, S. Gowda. 2021. Detection and molecular characterization of '*Candidatus* Liberibacter asiaticus' and Citrus Tristeza Virus associated with citrus decline in Bhutan. Phytopathology. **111**, 870-881 (2021). https://doi.org/10.1094/PHYTO-07-20-0266-R
52. X. Martini, A. Hoyte, L.L. Stelinski, Abdominal color of the Asian citrus psyllid (Hemiptera: Liviidae) is associated with flight capabilities. Ann. Entomol. Soc. Am. **107**, 842–847 (2014). https://doi.org/10.1603/AN14028
53. T.R. Gottwald, J.V. da Graça, R.B. Bassanezi, Citrus Huanglongbing: the pathogen and its impact. Plant Health Prog. (2007). https://doi.org/10.1094/PHP-2007-0906-01-RV