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Cloning and Sequencing of *Candidatus liberibacter asiaticus* Isolated from Citrus Trees in Malaysia

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ABSTRACT

C*andidatus liberibacter asiaticus*, a causal agent of huanglongbing (HLB) disease of citrus in Malaysia, was detected in infected leaf samples collected from citrus growing areas in Selangor and Terangganu. The HLB bacterium was cloned and sequenced. The nucleotide sequences were 1324 and 1337 bp for GFB (greening fastidious bacterium) -T and-S isolate, respectively including the upstream and downstream nucleotides. It was confirmed that both sequences were full of 16S rDNA gene of *C. liberibacter asiaticus*. The sequences of 16S rDNA gene of Malaysian isolates are identical to each other, and had close relationship with isolates or strains from China. However, it showed slight difference from those African and American species.

Key Words: Citrus greening disease; Huanglongbing disease; Greening fastidious bacterium

INTRODUCTION

Huanglongbing (HLB), which is occurring widely in citrus growing areas in Africa and Asian Tropics (Garnier & Bove, 1993) is one of the most destructive diseases of citrus in Malaysia and it known as 'Penyakit Greening Limau'. According to Lim *et al.* (1990), citrus decline in Malaysia was caused by various factors such as *Phytophthora* sp., citrus tristeza virus (CTV) and micronutrient deficiency. HLB disease was generally at that time overlooked although the vector has already been recorded in the seventies (Lim *et al.*, 1990). Even large areas were destroyed six to seven years after cultivation, but the major cause was put on CTV severe strain. HLB disease was confirmed to occur in Malaysia in 1989, when typical pleomorphic HLB organism were observed in the sieve tubes of diseased fruits and leaves and significant recovery of disease plants were recovered after treated with antibiotics (Lim *et al*., 1990).

The causal agent of HLB disease was identified as a phloem-restricted bacterium belonging to a new genus in the alpha-subdivision of the α-Proteobacteria (Jagoueix *et al*., 1994). The genus *Candidatus* specific epithet '*liberobacter*' was given by Murray and Schleifer (1994) to the bacterium, but later, the specific epithet was renamed as '*liberibacter*' (Garnier *et al*., 2000). To date three species name have been identified namely *C. liberibacter asiaticus* in Asia, *C. liberibacter africanus* in Africa (Da Graca, 1991) and *C. liberibacter americanus* in Brazil (Teixeira *et al*., 2005).

Field diagnosis of HLB disease is difficult because of the symptoms are often confused with those of disorders and micronutrient deficiency such as Zn^{2+}. Therefore, it is essential that a reliable assay procedure be available to distinguish between nutritional or stress related symptoms and HLB disease. Detection of the HLB bacterium was based on biological indexing (grafting & vector), transmission electron microscope (TEM) mainly. However, TEM methods are less practical, because ultra-thin sectioning is tedious and required expensive equipment. Transmission tests are of limited value due to the latency and the long incubation period in insect and plant (Nakashima *et al.*, 1996). Development of conventional polymerase chain reaction (PCR) test has great advantages of understanding the bacterium at genetic level. The comparison of 16S ribosomal DNA is a useful tool for deducing phylogenetic and evolutionary relationship among bacteria and other prokaryotes (Weisburg *et al*., 1991). Previous study on 16S rDNA gene towards Poona strain, India and Nelspruit strain, Africa showed high homology up to 97.7%. Study conducted by Subandiyah *et al*. (2000) also showed high homology between Asian isolates (Thai-99.4 to 100%, Nepalese- 100% & Indian-98.8%) and less compared to African strain (97.5%).

In the present study, we attempt to detect several Malaysian isolates collected from two major citrus growing areas in Peninsular Malaysia. This study also determined the sequences of amplified 16S rDNA fragment of GFB-S and GFB-T isolates to define the phylogenetic position of Malaysian isolates.

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MATERIALS AND METHODS

Inoculums source. Two isolates of *Candidatus liberibacter asiaticus* used in this study were collected from two different locations i.e., Terengganu (Eastern part of Peninsular Malaysia) and Selangor (Western part of Peninsular Malaysia) (Fig. 1). These two isolates were isolated from infected *Citrus reticulata* (honey mandarin) trees that showed classical symptoms of huanglongbing disease such as leaves mottling and yellowing, green veins of leaf, twigs dieback and leaves defoliation. These were referred to as GFB-T isolate and GFB-S isolate for Terengganu and Selangor origin, respectively.

Polymerase chain reaction. The presence of GFB isolates was detected by PCR test. Nucleic acid was extracted from symptomatic leaves collected from the field sites by using the method of Hung *et al*. (1999). Citrus leaf midribs from each plant were chopped into small pieces and left to air dried for 2 days. Approximately 250 mg of the midrib per sample was powdered in liquid nitrogen, and then suspended in 1.5 mL of DNA extraction buffer [100 mM Tris-HCL (pH 8.0), 250 mM NaCl, 10% N-Lauroylsarcosine] and transferred to a 1.5 Eppendorf tubes. After 1 h incubation at 55°C, the sample was centrifuged at 4000 g (± 6000 rpm) for 5 min. The supernatant (±800 µL) was collected and 100 µL 5 M NaCl and 100 µL 10% CTAB (hexadecyl-trimethyl-ammonium-bromide) in 0.7 M NaCl was added. The mixture was incubated at 65°C for 10 min, followed by one cycle of chloroform: isoamyl alcohol (24:1) extraction. The aqueous supernatant was then re-extracted by an additional cycle of phenol:chloroform: isoamyl alcohol (25:24:1). The nucleic acids were precipitated by mixing 600 µL of the supernatant and 360 µL isopropanol, followed by centrifugation at 12000 g (±15000 rpm) for 10 min. The pellet was washed with 70% ethanol, dried and resuspended in 50 µL Tris-EDTA (TE) buffer. The concentration of the nucleic acid was estimated by using spectrophotometer. About 200 ng of the purified DNA was used for the PCR test. Specific primer pairs for the amplification of segment of the 16S rDNA of GFB isolates were as the following, forward primer OI1: 5'-GCG CGT ATG CAA TAC GAG CGG CA-3' and reverse primer of OI2c: 5'-GCC TCG CGA CTT CGC AAC CCA T-3' (Jagoueix *et al*., 1994). PCR was performed using 25 µL of reaction mixture containing 20 mm Tris-HCl (pH 8.0), 50 mm KCl, 4 mm $MgCl_2$, 0.2 mm each dATP, dTTP, dCTP and dGTP, 25 um Forward primer, 25 um Reverse primer, 0.75 units of Taq DNA polymerase and 200 ng of nucleic acid preparation as a template. The thermal cycle conditions were: one cycle at 95°C for 2 min. followed by 35 cycles at 95°C for 40 sec, 60°C for 1 min and 72°C 1 min then followed by a 72°C extension for 10 min. The PCR products were identified by gel electrophoresis using 1.2% agarose (Boehringer Mannheim, Mannheim, Germany) in TBE buffer: 40 mm Tris-acetate (pH 8.0), 1 mm EDTA. The 1000 bp DNA Ladder set (Promega, Madison, WI, USA) was included as size markers. The electrophoresis was run for 30 to 40 min using a high voltage (100 V). After electrophoresis, the gel was stained with ethidium bromide (0.5 ug mL^{-1}) and photographed under the UV illuminator.

Purification of amplified DNA products. PCR products (15 µL) were electrophoresed on 1.2% agarose gel. The bands at the correct size were excised from the gel and subsequently purified using DNA extraction kit (Fermentas). The DNA was eluted with three volumes of binding solution (6 M sodium iodide) to one volume of gel inside a microfuge tube. The solution was then incubated 5 min. at 55°C to dissolve agarose. After that silica powder was added (10 µL) and mixed by vortexing every 2 min. to keep silica powder in suspension and spun (13000 rpm) the DNA complex for 5 sec. to form pellet and remove supernatant. Ice cold wash buffer (500 µL) was added, mixed and spun (13000 rpm) for 5 sec. and supernatant was discarded off. The procedure was repeated 3 times. After the supernatant from the last wash has been removed, the tube was spun again (13000 rpm) for 30 sec. and the remaining liquid was removed from pellet. The pellet was air-dry for 15 min. The pellet was dissolved in 15 µL of sterile distilled water (dH_2O) and incubated at 52ºC only for 5 min, spun at 13000 rpm for 1 min. The supernatant was removed to a new tube and used for cloning.

Cloning of PCR product and DNA sequencing. Purified DNA was cloned and transformed into *Escherichia coli* using TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. White colonies were picked and transferred into Luria-Bertani (LB) medium containing 100 µg/mL ampicillin and incubated overnight at 37°C. The plasmid DNA was purified using the QIAprep Spin Miniprep Kit protocol (QIAGEN). The purified plasmids were used for sequencing using automated DNA sequencer. Results of DNA sequences were analyzed. DNA sequences were aligned using Bio-Edit softwere Version 7 (www.mbio.ncsu.edu/bioEdit/bioEdit). The nucleotides sequences of two Malaysian isolates were compared to other accessions of *C. liberibacter asiaticus* and *C. liberibacter africanus* available in the NCBI databases using Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using the CLUSTAL W software. Phylogenetic studies were done with Ali-Bee program provided by the Gene-bee Software (http://www.genebee.msu). The tree was constructed using the following 16S rDNA sequences: *C. liberibacter asiaticus* isolate Chongqing-ZG 16S ribosomal RNA gene, (ACCESSION no. DQ432004), *C. liberibacter asiaticus* isolate Fujian-NHE 16S ribosomal RNA gene, (ACCESSION no. DQ431998), *C. liberibacter asiaticus* isolate Kumquat 1 16S ribosomal RNA gene, (ACCESSION no. DQ302750), *C. liberibacter americanus* 16S ribosomal RNA gene, (ACCESSION no.AY742824), *C. liberibacter africanus* ssp. *capensis* 16S ribosomal RNA

gene, (ACCESSION no. AF137368), *C. liberibacter africanus* 16S ribosomal RNA gene, (ACCESSION no. L22533).

RESULTS

Detection of *Candidatus liberibacter asiaticus*–Malaysian isolates. Polymerase chain reaction amplification with primer pairs OI1 and OI2 c was performed on sample extracts obtained from field infected *C. reticulata* trees at different localities i.e., Terengganu and Selangor. Fig. 2 showed the amplified DNA after electrophoresis on 1.2% agarose gel. The 16S rDNA gene fragments of about 1160 bp were amplified from infected trees and no amplification was observed when water was used (S1 & S13). Twenty leaf samples from each location showing the symptoms of HLB disease such as leaf mottling and green vein and twigs dieback were tested. Results showed that both locations showed high percentage of disease incidence of 70% and 65% for Terengganu and Selangor, respectively (Table I).

Sequence of 16S rDNA gene of Malaysian isolates. Three clones from each of the two Malaysian isolates namely Terengganu (GFB-T) and Selangor (GFB-S) isolate were amplified using the OI1/OI2 c primers pair together with template DNA obtained from diseased trees were sequence using automated sequencer in both orientation (M 13 forward & M 13 reverse primer pairs). Sequencing of the PCR products and further alignment and edited using Bio-Edit program resulted sequences of about 1324 bp and 1337 bp for GFB-T and GFB-S isolate, respectively including the upstream and downstream nucleotides (Fig. 3). Further confirmation of sequences using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/blast.cgi) suggested and confirmed that both sequences isolates are the full sequence of 16S rDNA gene of *Candidatus* Liberibacter asiaticus. These 16S rDNA gene sequences were deposited in the Genbank database with the following accession numbers; EU371106 (GFB-T) and EU371107 (GFB-S).

The 16S rDNA sequences of the two Malaysian isolates i.e., GFB-T and GFB-S showed percentage nucleotide similarity of 96% (Table II). In this study, about 98% nucleotide similarity was observed between GFB-T isolate with 16S rDNA gene of *C. liberibacter asiaticus*-Chongqing and *C. liberibacter asiaticus*-Fujian strain. Interestingly, less percentage nucleotide similarity was observed on African species (*C. liberibacter africanus*) and African-subspecies (*C. liberibacter africanus* ssp. *capensis*) were about 85% and 93%, respectively. Malaysian isolates were also showed low similarity with American species (*C. liberibacter americanus*), 82%. Based on the evolutionary distance value, the 16S rDNA gene sequences of the two Malaysian isolates were 3.24. The GFB-T isolate give low distance value with other Asian strains (1.48 to 1.66) except for Kamquat strain (4.94). This isolate was also gave high distance value with African and American species of 7.70 and11.09, respectively. Similar trend was observed on GFB-S isolate (Table II). These results suggested that sequences of 16S rDNA gene of Malaysian isolates were identical to each other and has close relationship with those isolates or strains from China. The Malaysian isolates showed distantly relationship to those species from Africa and America. A phylogenetic tree constructed using distance matrix method displaying three way relationships as shown in Fig. 4. Both Malaysian isolates are cluster together and showed close relationship with Asian strains.

Fig. 1. Map of Peninsular Malaysia showing the location of sampling areas i.e. Selangor and Terengganu conducted in this study

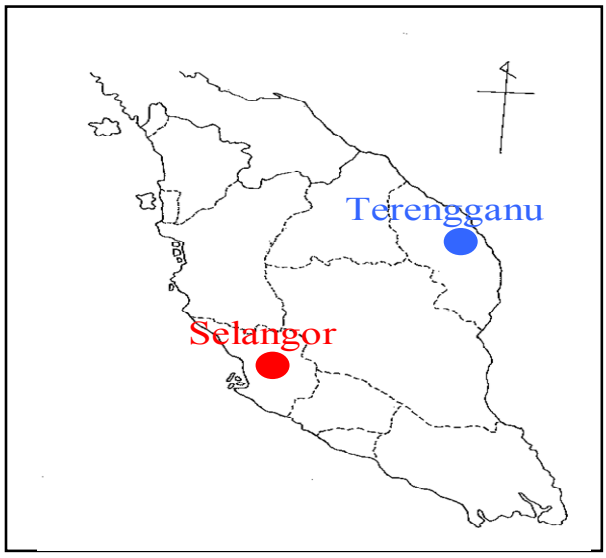
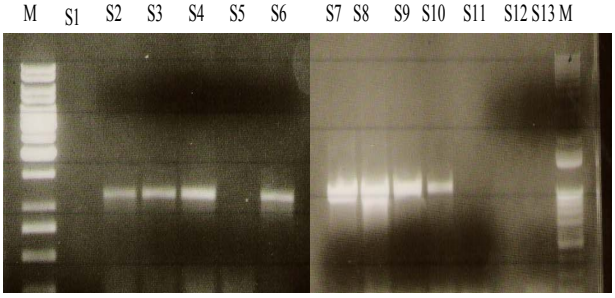


Fig. 2. 16S rDNA fragments with molecular weight of 1160 bp were successfully amplified from citrus leaf samples collected from Terengganu and Selangor

M= Marker;
S1, S13 = water;
S2, S3, S4, S5 andS6 = Terengganu;
S7, S8, S9, S10, S11 and S12 = Selangor.



DISCUSSION

In Malaysia, conventional PCR test is routinely used for detection of HLB disease. Unfortunately, detection is not always reliable. Sometimes infected citrus trees with classic HLB symptoms such as leaf mottling, green vein and twigs dieback tested are negative with PCR as explained by

Fig. 3. Nucleotide sequence of 16S rDNA gene of Malaysian isolates (GFB-T and GFB-S) with comparison to the other six species/strains of *Candidatus liberibacter* spp. available in Gene-Bank accessions

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Aficanus      GCGGCAGGCCTAACACATGCAAGTCGAGC--GCGTATT-TTA-TACGAGC
Americanus    GCGGCAGGCTTAACACATGCAAGTCGAGC--GAGTACG-CAAGTACTAGC
As. Kumquat   ------------ACACATGCAAGTCGAGC--GCGTATG-CGAATACGAGC
Af.capensis   --------------------AATTCGATT--GCGCGTATCGAATACGACG
As.chongqing  ----------------------------GC--GCGTATG--CAATACGAGC
As. fujian    ----------------------------GC--GCGTATG--CAATACGAGC
GFB-T         GTGTGATGGATATC-TGCAGAATTCGCCCTTGCGCGTATGCAATACGAGC
GFB-S         GTGTGATGGATATC-TGCAGAATTCGCCCTTGCGCGTATGCAATACGAGC
                                                * * * *** * *** *

Aficanus      GGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAAC
Americanus    GGCAGACGGGTGAGTAACGCGTGGGGATCTACCTTTTTCTACGGGATAAC
As. Kumquat   GGCAGACGGGTGAGTAACGCGTAGGAATCCACCTTTTTCTACGGGATAAC
Af.capensis   GGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAAC
As.chongqing  GGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAAC
As. fujian    GGCACACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTTCGGGATAAC
GFB-T         GGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAAC
GFB-S         GGCAGACGGGTGAGTAACGCGTAGGAATCTACCTTTTTCTACGGGATAAC
              **** ***************** ** *** ********** *********

Aficanus      GCATGGAAACGTGTGCTAATACCGTATACGCCCTATTGGGGGAAAGATTT
Americanus    GCACGGAAACGTGTGCTAATACCGTATACGCCTT-TAGAGGGAAAGATTT
As. Kumquat   GCATGGAAACGTGTGCTAATACCGTATACGCCCTATTGGGGGAAAGATTT
Af.capensis   GCNTGGAAACGTGTGCTAATACCGTATACGCCCTATT--GGGAAAGATTT
As.chongqing  GCATGGAAACGTGTGCTAATACCGTATACGCCCTATTGGGGGAAAGATTT
As. fujian    GCATGGAAACGTGTGCTAATACCGTATACGCCCTATTGGGGGAAAGATTT
GFB-T         GCATGGAAACGTGTGCTAATACCGTATACGCCCTATTGGGGGAAAGATTT
GFB-S         GCATGGAAACGTGTGCTAATACCGTATACGCCCTATTGGGGGAAAGATTT
              ** ****************************  * *   ***********

Aficanus      TATTGGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTAGGGTAAAG
Americanus    TATTGGAGAAAGATGAACCCGCGTTAGATTAGCTAGTTGGTGGGGTAAAG
As. Kumquat   TATTGGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTAGGGTAAGA
Af.capensis   TATTGGAGAGAGATGAGCCTGCTGTGGATTAGCTAGTTGGTAGGGTAAAG
As.chongqing  TATTGGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTAGGGTAAGA
As. fujian    TATTGGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTAGGGTAAGA
GFB-T         TATTGGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTAGGGTAAGA
GFB-S         TATTGGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTAGGGTAAGA
              ********* ****** ** **  * *************** ******

Aficanus      GCCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
Americanus    GCCTACCAAGGCGATGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
As. Kumquat   GCCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
Af.capensis   GCCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
As.chongqing  GCCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
As. fujian    GCCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
GFB-T         ACCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
GFB-S         GCCTACCAAGGCTACGATCTATAGCTGGTCTGAGAGGACGATCAGCCACA
               *********** * ************************************

Aficanus      CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
Americanus    CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
As. Kumquat   CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
Af.capensis   CTGGGACTGAGACACG--CCAGACTC-TACGGA--GCAGCAGTGGGGAAT
As.chongqing  CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
As. fujian    CTGGGACTGAGACACGGCCCAGCCTCCTACGGGAGGCAGCAGTGGGGAAT
GFB-T         CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
GFB-S         CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
              ***************  **** *** *****   ***************

Aficanus      ATTGGACAATGGGGGCAACCCGAATCCAGCCATGCCGCGTGAGTGAAGAA
Americanus    ATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
As. Kumquat   ATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
Af.capensis   ATTGGACAATGGGGGCAACC-TGATCCAGCCATGCCGCGTGAGTGAAGAA
As.chongqing  ATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
As. fujian    ATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
GFB-T         ATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
GFB-S         ATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
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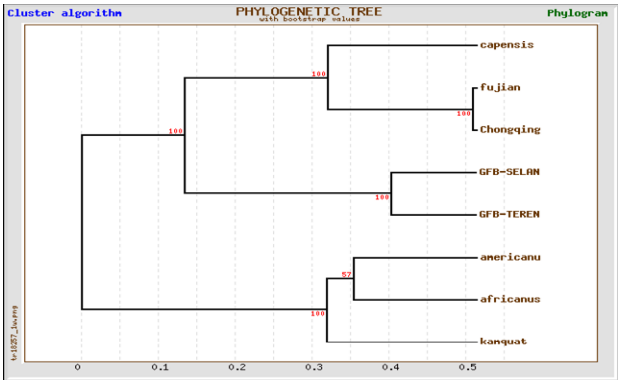
Toorawa (1998) and Hoy *et al.* (2001). According to Wang *et al.* (2006) conventional PCR was inferior to the real time-PCR; the former is expected to lead to some degree of false negative results, especially when sample preparation is inappropriate. According to Hoy *et al.* (2001), false negative could have been due to the fact that the 16S rDNA primers are relatively short (20 mers) and thus potentially less sensitive than long primers (30 mers). Similar experience was also faced by Colletta-Filho *et al.* (2005), when they managed to detect only 15 out of 53 samples, even though the trees displayed classic HLB symptoms. In order to further confirm the presence of HLB disease in Malaysia,

Fig. 4. Phylogenetic trees based on 16S rDNA gene sequences, showing the positions of all the Malaysian isolates and representatives of *Candidatu liberibacter* spp. and strains available in the Genbank

Capensis= *C. liberibacter africanus* subsp. capensis
Fujian= *C. liberibacter asiticus*-Fujian
Chongqing= *C. liberibacter asiaticus*-Chongqing
GFB-Selan= GFB-S
GFB-Teren= GFB-T
Americanu= *C. liberibacter americanus*
Africanus= *C. liberibacter africanus*
Kamquat= *C. liberibacter asiaticus*-Kamquat



the amplified 16S rDNA gene that previously collected from two localities i.e., Selangor and Terengganu were sequenced and compared to reported 16S rDNA gene in the Genbank database. This data gave strong evidence that the symptomatic citrus leaf samples collected from the surveyed areas were infected with *Candidatus liberibacter asiaticus* and not due to micronutrient deficiencies or disorder. Huanglongbing bacterium has historically been a difficult organism to study because of the lack of a culture system as explained by Weisburg *et al.* (1991) for *Anaplasma marginale* (alpha-subdivision of purple bacteria). Amplification of 16S rDNA gene is one way to characterize the bacterium and obviously the sequence of the gene provides basic information useful for designing probes or PCR primers specific for the detection of this disease. Many studies reported that the sequence of the 16S rDNA genes are quite conserved among bacteria but present sufficient variation to design primers for specific diagnostic for species or strains (Jagoueix *et al.*, 1996; Colletta-Filho *et al.*, 2005). Genetic variation among the Asian isolates were observed for the different strains and they also can be distinguished into several groups such as from the Pacific area (Japan, Philippines, Indonesia, Thailand & Nepal) and from India and China (Subandiyah *et al.*, 2000; Colletta-Filho *et al.*, 2005). Based on this data, it is suggested that the Malaysian isolates could probably belong to the group of the Pacific area.

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Table I. Detection of *Candidatus liberibacter asiaticus* by PCR test from two locations in Peninsular Malaysia

Location	Types of HLB symptom	Number of sample	% of disease incidence
Terengganu	Leaf mottling and green vein, Twigs dieback	14/20	70
Selangor	Leaf mottling and green vein, Twigs dieback	13/20	65

Table II. Percentage of nucleotides similarity and evolutionary distance of Malaysian isolates compared with six *Candidatus liberibacter* spp. and strains available in NCBI database

	GFB-T	GFB-S	*C. liberibacter africanus*	*L.a. Chongqing*	*L.a. Kamquat*	*L.a. Fujian*	*C. liberibacter americanus*	*C. liberibacter aficanus ssp. capencis*
GFB-T	-	96%	85%	98%	87%	98%	82%	93%
GFB-S	3.24	-	85%	97%	87%	97%	82%	92%
C. liberibacter africanus	7.70	9.04	-	97%	97%	96%	93%	94%
L.a.Chongqing	1.48	2.66	2.38	-	99%	99%	94%	94%
L.a.Kamquat	4.94	6.87	2.29	0.43	-	99%	94%	94%
L.a.Fujian	1.66	2.84	2.55	0.52	0.60	-	94%	94%
C. liberibacter americanus	11.09	12.88	4.38	4.22	3.98	4.51	-	90%
C. liberibacter aficanus- ssp. capencis	5.26	6.06	4.39	2.79	4.89	3.08	7.28	-

Number below the diagonal indicate evolutionary distance

REFERENCES

Colletta-Filho, H.D., M.A. Takita, M.L.P.N. Targon and M.A. Machado, 2005. Analysis of 16S rDNA sequences from citrus huanglongbing bacteria reveal a different Ca. Liberibacter strain associated with citrus disease in Sao Paulo. *Plant Dis*., 89: 848–52

Da Graca, J.V., 1991. Citrus greening disease. *Ann. Rev. Phytopathol*., 29: 109–36

Garnier, M. and J.M. Bove, 1993. Citrus greening disease and the greening bacterium. *In*: Moreno, P., J.V. Da Graca and L.W. Timmer (eds.), *Proceedings 12th Conference Internatinal Organization Citrus Virologists (IOCV)*, pp: 212–9. University of California, Riverside

Garnier, M., S.J. Eveillard, P.R. Cronje, H.F. Le Roux and J.M. Bove, 2000. Genomic characterization of a liberibactert present in an ornamental rutaceous tree, *Calodendrum capense,* in the Western Cape province of South Africa. Proposal of *Candidatus* Liberibacter africanus subsp. Capensis. *Int. J. Syst. Evol. Microbiol*., 50: 2119–25

Hoy, M.A., A. Jeyaprakash and R. Nguyen, 2001. Long PCR is a sensitive method for detecting *Liberobacter asiticum* in parasitoids undergoing risk assessment in quarantine. *Biol. Contr.*, 22: 278–87

Hung, T.H., M.L. Wu and H.J. Su, 1999. Development of rapid method for the diagnosis of citrus greening disease using polymerase chain reaction. *J. Phytopathol*., 147: 599–604

Jagoueix, S., J.M. Bove and M. Garnier, 1996. PCR detection of the two *Candidatus* Liberobacter species associated with greening disease of citrus. *Mol. Cell. Probe,* 10: 43–50

Jagoueix, S., J.M. Bove and M. Garnier, 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha-subdivision of proteobacteria. *Int. J. Syst. Bacterial*., 44: 379–6

Lim, W.H., O.M. Shamsudin and W.W. Ko, 1990. Citrus greening disease in Malaysia: Status report. *In*: *Proceeding of the 4th International Asia Pacific Conference on Citrus Rehabilitation*. Chiang Mai, Thailand, 4-10 February

Murray, R.G.E. and K.H. Schleifer, 1994. Taxonomic notes: A proposal for recording the properties of putative taxa of prokaryotes. *Int. J. Syst. Bacteriol*., 44: 174–6

Nakashima, K., M. Prommintra, Y. Ohtsu, T. Kano, J. Imada and M. Koizumi, 1996. Detection of 16S rDNA of Thai isolates of bacterium like organisms associated with greening disease of citrus. *JIRCAS J*., 3: 1–8

Subandiyah, S., T. Iwanami, S. Tsuyumu and H. Ieki, 2000. Comparison of 16S rDNA and 16S/23S Intergenic Region sequences among citrus greening organisms in Asia. *Plant Dis*., 84: 15–8

Teixeira, D.A., C. Saillard, S. Eveillard, J.L. Danet, P.I. Da Costa, A.J. Ayres and J.M. Bove, 2005. Candidatus Liberibacter americanus associated with citrus huanglongbing (greening disease) in Sao Paulo State, Brazil. *Int. J. Syst. Evol. Microbiol*., 55: 1857–62

Toorawa, P., 1998. La Maladie du hunglongbing (greening) des agrumes a l'lle Maurice. Detection de 'Candidatus Liberobecter asiaticum' et 'Candidatus Liberobacter africanum'dans les agrumes et les insects vecteurs. *Ph.D. Thesis*, p: 186. Universite Victor Segalen, Bordeaux 2, Bordeaux, France

Wang, Z., Y. Yin, H. Hu, Q. Yuan, G. Peng and Y. Xia, 2006. Development and application of molecular based diagnosis for candidates Liberibecter asiaticus, the causal pathogen of citrus huanglongbing. *Plant Pathol*., 55: 630–8

Weisburg, W.G., S.M. Susan, D.A. Pelletier and D.J. Lane, 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*., 173: 697–703

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