

## Occurrence and Spread of *Candidatus Liberibacter Asiaticus*, the Causal Agent of Huanglongbing Disease of Citrus in Malaysia

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**Abstract:** Huanglongbing (HLB) disease or citrus greening disease caused by nonculturable, phloem limited bacterium, *Candidatus Liberibacter asiaticus* is one of the most destructive disease in Asian countries. The occurrence and spread of the disease and their vector, *Diaphorina citri* in the major citrus orchards in Peninsular Malaysia has been evaluated and confirmed through polymerase chain reaction (PCR) and transmission electron microscope (TEM). Samples of DNA extracted from symptomatic leaves of putatively infected leaves collected from different range of citrus species were tested to PCR and TEM test. Infected samples produce amplicons with molecular weight of 1160 bp and electron micrographs of ultrathin section through a sieve tube of leaf midribs from infected leaves showing the elongated and spherical forms bodies. These results further confirming the presence of HLB disease in Malaysia.

**Key words:** *Candidatus Liberibacter asiaticus*, huanglongbing disease, citrus greening disease, Polymerase Chain Reaction, *Diaphorina citri*

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### INTRODUCTION

Citrus is believed to originate from the region within Northeast India, South China and Indonesia<sup>[18]</sup> and might be infected by several diseases, including the huanglongbing (HLB) disease. HLB disease, previously called citrus greening disease or locally known as “penyakit greening limau” is the most destructive disease to citriculture in Asia. This disease is caused by a phloem limited proteobacterium, *Candidatus Liberibacter asiaticus*<sup>[7]</sup>. It spreads through vegetative propagation and transmitted by an Asiatic citrus psyllid, *Diaphorina citri* as a vector<sup>[11,10,2]</sup>. No verified instance of seed transmission has been reported. Since there is no successful treatment either by using pesticides or antibiotics, then the removal and destruction of infected trees are the only practical method applied today to prevent disease spread in the orchards. *Candidatus Liberibacter* sp. is difficult to detect because it is present at a low concentration and is unevenly distributed in their host tissues<sup>[5,11]</sup>. It has a long incubation period and many infected plants were symptomless particularly those which were newly infected. The symptoms are almost the same as and can be confused with disorders and zinc or manganese deficiencies<sup>[8,17]</sup>. The disease kept on spreading all over the world. Amongst the newly listed areas included Brazil<sup>[16]</sup>, Timor-Leste and Papua New Guinea<sup>[17]</sup>.

In Malaysia, three important diseases of citrus are present namely citrus tristeza virus (CTV), citrus greening disease (CGD) and Phytophthora collar and root rot<sup>[13]</sup>. These diseases may be difficult to differentiate by ordinary growers because all diseases cause yellowing of leaves, mottling, stunting and dieback of twigs. Nutrient deficiencies also cause yellowing and this complicates in the diagnosis of these disease. Sometime more than one pathogen is present together in the same tree. In 1989 the occurrence of HLB disease in Malaysia was confirmed by field symptoms and transmission electron microscope (TEM)<sup>[14]</sup> but until now there is still little available information regarding this disease (local HLB disease) in Malaysia. Not much study has been done and previously only two agencies i.e. Universiti Putra Malaysia (UPM) and Malaysian Agricultural Research and Development Institute (MARDI) were involved in overcoming this disease in Malaysia. In early 2000 the Malaysian government gave more attention to cultivate fruit crop in order to reduce import bill for food. From that, an intensive survey for HLB disease was conducted by Department of Agriculture (DOA), Malaysia from 2001 to 2004. The results showed the presence of the disease all over the country included Sabah and Sarawak<sup>[4]</sup>. Studies by Azizah and Zazali<sup>[4]</sup> revealed that approximately 2,458 ha from the total of

3,526 ha of the citrus cultivation areas in Malaysia showed the presence of the HLB disease symptoms. In order to gain a complete picture regarding the status of HLB disease in Peninsular Malaysia, the present study is intended to further investigate the distribution of HLB disease in the major growing areas in Peninsular Malaysia, to assess the range of citrus species that were infected and finally to detect and confirm the presence of the causal agent of HLB disease.

## MATERIALS AND METHODS

**Survey Areas:** A series of survey for HLB disease were conducted from March to November, 2005 at four states in Peninsular Malaysia namely Selangor, Terengganu, Kelantan and Pahang (Figure 1 and Table 1). The survey areas were classified based on their altitudinal groups of either high (more than 700 m) or low altitudes (less than 700 m). Citrus growing areas in Terengganu and Selangor were located at low altitude while the citrus growing areas in Kelantan and Pahang were at high altitude. A total of nine locations were designated and at each location one to three sites were selected. The mean disease incidence for a location was evaluated based on average from the disease incidence of their three representative sites.

**Sampling and Data Collection:** The method of detection and delimiting survey for determining the presence of HLB disease on each site was carried out according to the method of Greesteranus and Stall<sup>[9]</sup> with minor modification. In order to have a better coverage and to minimize biasness in the estimation of actual disease incidence in the field, a systematic "X" sampling pattern was followed. Disease status was recorded from at least 50 plants per site. Disease incidence was assessed based on symptom appearance on the assessed plants after which further confirmation tests were carried out in the Microbiology Laboratory, Department of Plant Protection, Universiti Putra Malaysia. Samples were collected by using destructive sampling method where symptomatic leaves were cut and placed inside transparent plastic bags, labeled properly and kept in a cool box and brought to laboratory for disease assessment. Laboratory assessments were carried out by using polymerase chain reaction (PCR) and transmission electron microscope (TEM) tests.

**Field Disease Incidence Assessment:** In this study, field data such as types of HLB symptoms, number of healthy and symptomatic plants, types of cultivated citrus species and the presence of psyllid population

were recorded. Percentage of disease incidence was calculated based on the number of observed symptomatic plants over the total number of the assessed plants within study site as expressed by the formula below:

$$\% \text{ of disease incidence} = \frac{\text{Total infected citrus trees}}{\text{Total number of trees evaluated}} \times 100$$

Vector infestation was assessed through random sampling method, where by the numbers of adult psyllids on the young citrus shoots were counted from ten plants per site. The grading system was as followed:

- Highly infested = five and more vector per shoot
- Moderately infested = three to four vector per shoot
- Lowly infested = one to two vector per shoot
- Not infested = no vector found

## Detection and Identification of GFB (Greening fastidious bacterium):

### Polymerase Chain Reaction (PCR) test:

#### Nucleic Acid Extraction from Infected Citrus Leaf

**Midrib:** The extraction method used in this study was described by Hung *et al.*<sup>[11]</sup>. Infected citrus leaf midribs were chopped into small pieces and air dried for two days. Approximately 250 mg of midribs were powdered in liquid nitrogen and each sample was suspended in 1.5 ml of DNA extraction buffer [1M Tris-HCL (pH 8.0), 0.5M NaCl, 1% N-Lauroylsarcosine] and transferred to 1.5 Eppendorf tubes. After incubation at 55°C for 1 hour, the samples were centrifuged at 4000g (± 6000 rpm) for 5 min. The supernatant (±800 µl) was collected and 100 µl 5M NaCl and 100 µl 10% CTAB (hexadecyl-trimethyl-ammonium-bromide) in 0.7M NaCl were added and the mixture incubated at 65 °C for 10 min. The samples were subjected to one cycle of chloroform: isoamyl alcohol (24:1) extraction. The aqueous supernatant were then re-extracted by 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 pellets were washed with 70% ethanol, dried and resuspended in 100 µl TE buffer.

**Primer and PCR Conditions:** Specific primers pair, composed of the forward primer of 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') was used to amplify the 16S ribosomal DNA

**Table 1:** Locations covered during the HLB disease surveys in Peninsular Malaysia from March to November 2005

State	Location	Altitude
Selangor	Malaysian Agricultural Research and Development Institute (MARDI), Serdang	low
	Department of Agriculture (DOA), Serdang	low
	University Putra Malaysia (UPM), Serdang	low
Terengganu	Marang	low
	Dungun	low
Pahang	Cameron Highland	high
	MARDI, Cameron Highland	high
	DOA, Cameron Highland	high
Kelantan	Lojing Highland	high

Altitudes: Low < 700 meter < High



**Fig. 1:** Map of Peninsular Malaysia showing the location of sampling areas i.e. Selangor, Pahang, Terengganu and Kelantan was conducted in this study

(rDNA) fragment. PCR was performed using 25 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM each dATP, dTTP, dCTP and dGTP, 0.5 mM forward primer, 0.5 mM reverse primer, 0.75 units of Taq DNA polymerase and 200 ng template of nucleic acid preparation. The thermal cycle conditions were: one cycle at 95°C for 2 min. 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.

**Electrophoresis of PCR Product:** The PCR products were identified by gel electrophoresis using 1.4% agarose (Boehringer Mannheim, Mannheim, Germany)

in TBE buffer: 40mM Tris-acetate (pH 8.0), 1mM EDTA. After electrophoresis, the gel was stained with ethidium bromide (0.5 ug/ml) and photographed. 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 high voltage (100 V).

**Transmission Electron Microscope (TEM) test:** The TEM assay was carried out based on the method of Aubert *et al.*<sup>[3]</sup>. Fresh midribs samples sizes of 5 x 2 mm were fixed into 5% glutaraldehyde buffered with 0.1 M Phosphate buffer Saline (PBS) pH 7.4 and vacuumed in oven for two days. After that, the samples

were washed with 0.1 M Sodium Cacodylate (SC) buffer three times changes of 30 min respectively. Subsequently, the samples were postfixed with 1% Osmium Tetroxide for one day at 4° C. The samples were washed again for three times with SC buffer. After dehydration with a series of ethanol for one hour respectively, the samples were infiltrated and embedded in Epon 812. After polymerization, ultra thin sectioning were carried out by using diamond knife and ultra microtome. Golden sections were double stained with Uranyl acetate and Lead citrate for 15 and 30 min, respectively. The samples were then examined under transmission electron microscope for the GFB detection and identification.

## RESULTS AND DISCUSSION

**Field Symptoms of HLB Disease in Peninsular Malaysia:** Citrus plants showing symptoms of HLB disease were observed in all locations at both low and high altitudes areas. Citrus species encountered during the survey were pummelo (*C. grandis*), Mexican lime (*C. aurantifolia*), kaffier lime (*C. hystrix*), honey mandarin (*Citrus reticulata*), mandarin (*C. suhuiensis*), calamondin (*C. madurensis*), Cleopatra (*C. reticulata*), Troyer citrange (*Poncirus trifolia* x *C. sinensis*) and citrimelo (*P. trifolia* x *C. paradisi*).

HLB symptoms could be observed on the whole tree or more often as sectorial or confined to certain branches of the tree only. Symptoms on infected branches and leaves were leaf defoliation and varieties of mottling types. At an early stage, some leaves have a small size with up right orientation. This was followed intervienal chlorosis and green vein and resembles zinc deficiency pattern. Lastly, dieback of twigs occurs as an advance stage of the disease (Figure 2A. B. C. and D). Symptoms on fruit were lopsided shape, small fruit size and drop very easily. The infected seeds were brown in color and aborted.

### Detection and Identification of *Candidatus Liberibacter Asiaticus*:

**Polymerase Chain Reaction (PCR) test:** Data from PCR test showed that most of symptomatic citrus samples collected from Terengganu, Selangor, Pahang and Kelantan were showed positive reaction to HLB disease (Table 2). For instance, 71.4% of citrus samples collected from Pahang were positive with PCR tests and this was followed by Terengganu, Selangor and Kelantan. The amplified PCR product is about 1160 bp which is the targeted of 16S rDNA gene sequence region of the HLB pathogen amplified by the O11 and O12c primer sets (Figure 3 and 4). Based on these results, it is confirmed that the collected samples

that showed varied leaf symptoms such as mottling, green vein and dieback of the twigs on citrus plants in the surveyed areas were infected by *Candidatus Liberibacter asiaticus*, the causal agent of HLB disease in Peninsular Malaysia and not due to the micronutrient deficiencies or disorder.

### Transmission Electron Microscope (TEM) Test:

Electron micrographs of the serial ultrathin sections of the midribs of infected leaves with HLB symptoms further supported the above finding. Electron micrograph revealed that local GFB was pleomorphic and consisted of two types of bodies' i.e. the elongated and spherical forms and they are restricted in phloem tissues (Figure 5). However, no GFB bodies were observed in the phloem tissues of symptomless plants.

### Spread of HLB Disease in Peninsular Malaysia:

Kelantan recorded the highest mean percentage of disease incidence (80%) amongst the surveyed areas followed by Terengganu (63 %), Selangor (46.67 %) and Pahang (42%) (Table 3). Based on statistical analysis (LSD), significant differences were found between the surveyed areas such as Kelantan, Terengganu and Pahang. One of the reasons is the presence of HLB high vector population. Vector population plays vital role for disease spread in the orchards. Increase in vector population brought about an increase in disease incidence. This was proven by the finding of Yang *et al.*<sup>[19]</sup> showed that positive correlation observed between vector infestation and disease incidence in Guangxi, Guangdong, Fujian and Taiwan. The survey study showed that the level of vector infestation ranged from absent to moderate. For instance, moderate vector population was found at Kelantan and Terengganu areas as compared to high altitude areas, Cameron Highland wherein no vector was found (Figure 6 A and B). According to Aubert<sup>[2]</sup>, *D. citri* could not tolerate extremely low temperature and high humidity because these conditions would promote the growth of epizootic fungal, of which the nymphs are very susceptible. The vector was also not reported at the altitude of above 1300 m in various surveyed area in Asia<sup>[2]</sup>.

The incidence of HLB disease and level of vector infestation on different citrus species in Peninsular Malaysia were also studied (Table 4). Based on statistical analysis (LSD), there are significant differences of disease incidence between citrus species. The data showed that the highest disease incidence were recorded on pummelo and Troyer citrange (70%) followed by honey mandarin (68.33%), Cleopatra (50%), Mandarin (48.75%) and Citrimelo (30%). No disease incidence was recorded on kaffier lime (0%). The highest vector infestation was recorded on



**Table 2:** Detection of *Candidatus Liberibacter asiaticus* in infected citrus leaf from four localities in Peninsular Malaysia

Locations	HLB symptoms on leaf	Number of samples	% disease incidence
Terengganu	Mt, GV	14/20	70
Selangor	Mt, GV	13/20	65
Pahang	Mt, GV	5/7	71.4
Kelantan	Mt, GV	3/5	60

**Table 3:** Disease incidence and level of psyllid infestation at four different localities in Peninsular Malaysia from March to November 2005

Location	Mean* % disease incidence	Level of psyllid infestation
Terengganu	63b	Moderate
Selangor	46.67bc	Low
Pahang	42c	Not infested
Kelantan	80a	Moderate

\*Cultivar mean on a location

Means with in column followed by the same letters are not statically different at  $P \leq 0.05$



**Fig. 2:** Symptoms of HLB disease observed on honey mandarin collected from different locations in Peninsular Malaysia:

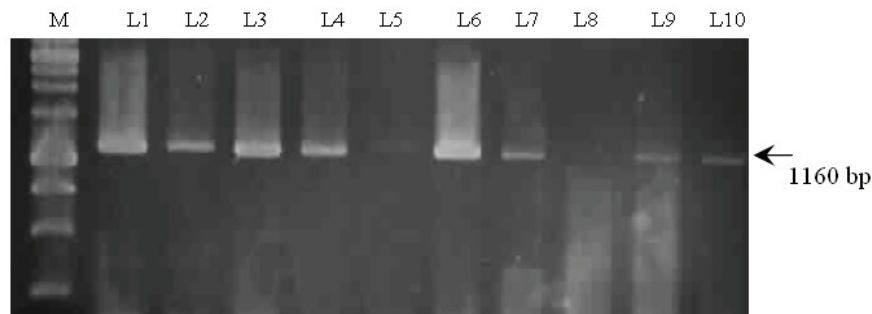
- (A) Sectorial leaf yellowing on infected citrus tree;
- (B) Infected tree showing defoliation, twigs dieback with small fruits size;
- (C) Green vein symptom of leaves;
- (D) Mottling symptom of leaves.

**Table 4:** Incidence of huanglongbing disease and level of vector infestation on different citrus species cultivated at surveyed areas in Peninsular Malaysia

Citrus Species	Mean* % Disease Incidence (Range)	Level of Vector Infestation
honey mandarin	68.33a (50-90)	Moderate
Mandarin	48.75b (10-65)	Moderate
Pummelo	70.0a (70)	Low
Kaffier lime	0 (0)d	Not infested
Cleopatra	50.0b (50)	Not infested
Citrimelo	30.0c (30)	Not infested
Troyer citrange	70.0a (70)	Not infested
Calamondin	n.d	Low
Mexican lime	n.d	Not infested

\* Location mean on a cultivar

Means with in column followed by the same letters are not statically different at  $P \leq 0.05$

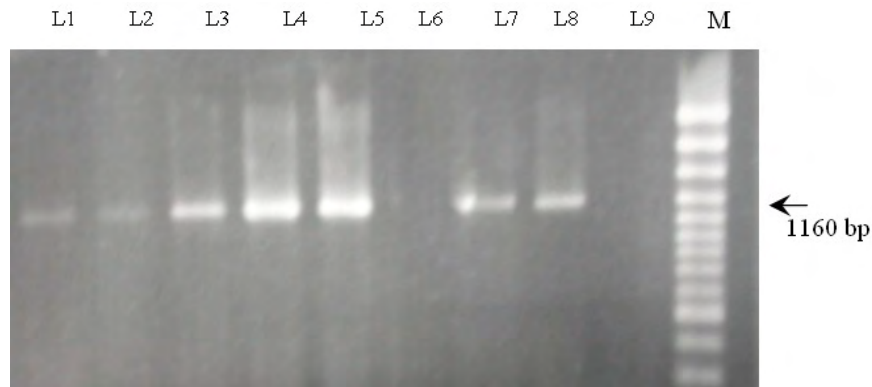


**Fig. 3:** 16S rDNA fragments with molecular weight of 1160 bp were successfully amplified from infected citrus leaves of sweet orange (LM) collected from Terengganu and pummelo and sweet orange from Selangor:

- M = Marker  
 Lane 1 = Honey mandarin/ Terengganu/ Mt\*  
 Lane 2 = honey mandarin/ Terengganu / Mt  
 Lane 3 = Honey mandarin/ Terengganu / Mt  
 Lane 4 = Honey mandarin/ Terengganu / Vy  
 Lane 5 = Pummelo/ Selangor/ Mt  
 Lane 6 = Pummelo/ Selangor/ Gv  
 Lane 7 = Honey mandarin/ Terengganu/ Vy  
 Lane 8 = Honey mandarin/ Terengganu/ NS  
 Lane 9 = Honey mandarin/ Selangor/ Vy  
 Lane 10 = Honey mandarin/ Selangor/ Gv  
 \*Vy = Vein yellowing, Mt= Mottling, Gv= Green vein; NS= no symptom

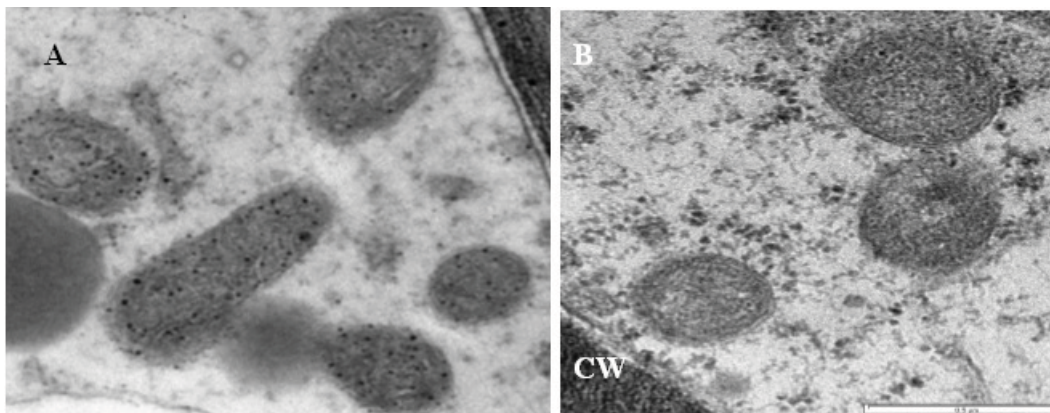
honey mandarin and mandarin (moderate level) followed by pummelo and calamondin (low level). No vector population was recorded on kaffier lime, Cleopatra, Citrimelo, Troyer citrange and Mexican lime (Table 4). Based on field observation, citrus trees can be infected at any stage from the nursery to the age of eight years old but the susceptibility differs depending on their age. In general, young trees especially at seedling stage are easily to be infected as observed in nursery at Lojing Highland, Kelantan. There are several factors contributed to disease spread and severity in the

orchards. The main factor is the lack of information of our farmers or field operators most of whom have no or little knowledge about the HLB disease. Their knowledge about disease spread and HLB vector were also deficient. As a consequence, they did not protect their seedlings and trees properly and also used infected seedlings as source of planting materials for new cultivation. Survey in Cameron Highland detected no vector but HLB incidence still occurred. Again, human factor contributed to increase disease spread, via introduction of infected seedlings which was brought in



**Fig. 4:** 16S rDNA fragments with molecular weight of 1160 bp were successfully amplified from infected citrus leaves of cleopatra (C), troyer citrange (T), citrimelo (CM) sweet orange (LM) collected from Pahang; Honey mandarin from Kelantan, Terengganu (Trg) and Selangor (Slr):

- M = Marker;  
 Lane 1 = Cleopatra/Pahang/Mt\*  
 Lane 2 = Troyer citrange/Pahang/Mt  
 Lane 3 = Sweet orange/Pahang/Mt  
 Lane 4 = Sweet orange/Kelantan/Mt  
 Lane 5 = Sweet orange/Kelantan/Mt  
 Lane 6 = Citrimelo/Pahang/Mt  
 Lane 7 = Citrimelo/Pahang/Mt  
 Lane 8 = Sweet orange/Terengganu/Mt  
 Lane 9 = Sweet orange/Selangor/Mt  
 \*Mt = Mottling



**Fig. 5:** Electron micrographs of ultrathin section through a sieve tube of leaf midribs from HLB infected citrus showing the elongated and spherical forms bodies (A). The bodies were bounded by a cell wall (CW) (B)

from infected areas such as Terengganu and Kelantan by local farmers and by using vegetative planting materials generated from infected mother plants.

Results of initial study were found significant differences of % disease incidence between locations surveyed and citrus species. This data suggested that the differences % disease incidence it could be due to different level of vector infestation and

different strains of GFB occurred at different localities in Peninsular Malaysia. Another reason, it could be due to possibility of local citrus species consisting of potential tolerant gene(s) against local GFB and their vector. Further study is required to determine occurrence of possible local GFB strains and tolerance level of local citrus species against HLB disease in Malaysia.



**Fig. 6:** (A) Survey of *D. citri* was conducted by the team in Citrus Valley, Dungun, Terengganu. (B) Close-up of adults *D. citri* on citrus shoots

**Conclusions:** Based on this study, it can be concluded that all the major citrus growing areas in Peninsular Malaysia such as Terengganu, Pahang, Selangor and Kelantan were infected with HLB disease confirming the report by Azizah and Zazali<sup>[4]</sup>. Each surveyed areas showed different percentage of disease incidence ranging from 42 to 80%. Varieties of foliar symptoms were observed and generally infected plants showed mottling symptom. In this study levels of psyllid infestation were also recorded. HLB vector are more abundant in lowland areas such as Selangor and Terengganu and it was not detected in highland areas such as Cameron Highland, Pahang. However, in Lojing Highland, Kelantan moderate level of vector infestation was observed. It could be due to the elevation of this area were below 1000m.

Among the nine citrus species that were assessed, the highest disease incidence was observed on pummelo and Troyer citrange. This was followed by honey mandarin, Cleopatra, mandarin and Citrimelo. No disease incidence was recorded on kaffier lime. The highest vector infestation was recorded on honey mandarin and mandarin followed by pummelo and calamondin while no vector population was recorded on kaffier lime, Cleopatra, Citrimelo, Troyer citrange and Mexican lime.

The presence of HLB pathogen, *Candidatus Liberibacter asiaticus* was confirmed by PCR and TEM tests. The 16S rDNA gene with molecular weight of 1160 bp was successfully amplified from infected citrus

leaves collected from all survey areas. Based on PCR test more than 60% of symptomatic leaf was positive. It was proved that the symptoms observed were not caused by nutrient deficiency. TEM study further confirmed the presence of HLB pathogen in infected citrus leaves. In this study local isolates were pleomorphic shape and consisted of two types of bodies i.e. the elongated and spherical forms. The characteristics of the bodies were similar to the description made by Agrios<sup>[1]</sup>.

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#### Abbreviations:

CGD = citrus greening disease  
CTAB = hexadecyl trimethyl ammonium bromide  
CTV = Citrus tristeza virus  
DNA = Deoxyribonucleic acid  
DOA = Department of Agriculture  
EDTA = Ethylenediaminetetraacetic acid  
GFP = Greening Fastidious Bactrium  
HLB = Huanglongbing  
LSD= Least Significant Difference test  
MARDI = Malaysian Agricultural Research and



Development Institute

PCR = Polymerase chain reaction

pH= Hydrogen ion concentration

rpm = Revolutions per minute

TBE = Tris-Boric-EDTA

TE= Tris-EDTA

TEM = Transmission electron microscope

UPM = Universiti Putra Malaysia

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