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Characterization of *Candidatus* Liberibacter asiaticus Isolated from *Citrus grandis* and *Citrus reticulata* Based on 16S rDNA and Outer Membrane Protein (OMP) Genes

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ABSTRACT

Molecular characterization of the local isolates of *Candidatus* Liberibacter asiaticus was carried out based on morphological properties, 16S rDNA and outer membrane protein (OMP) genes. A transmission electron microscopic (TEM) study revealed that both GFB-Pummelo and GFB-Mandarin (PK) were morphologically similar, suggesting that this method is not suitable to differentiate between them. A study on 16S rDNA genes of both isolates also showed no significant difference between them. Both isolates had very high nucleotide similarity (99%) and gave the lowest evolutionary value (0.30), suggesting that they are very close. However, significant difference was observed in their OMP gene sequences. Comparison of the nucleotide sequences of the OMP gene showed high nucleotide similarity (99%) but the evolutionary distance values was also high (0.68). Based on phylogenetic tree analysis, they were clustered in different groups, suggesting that some genetic variation occurred. Comparison of amino acids sequence also showed high amino acids similarity (98%) and high evolutionary distance value (3.41). They were clustered in different groups in the phylogenetic tree analysis. Further analysis of the amino acids sequence revealed 11 amino acids substitutions, which further proved that they belong to different strain of *Candidatus* Liberibacter asiaticus.

Key Words: Citrus greening disease; Huanglongbing (HLB); *Candidatus* liberibacter asiaticus; Molecular characterization; 16SrDNA gene; Outer membrane protein gene (OMP)

INTRODUCTION

Huanglongbing (HLB) disease previously called citrus greening disease or locally known as 'penyakit greening limau' is the most destructive disease to citriculture in Malaysia and other Asian countries. This disease is caused by phloem limited proteobacterium, *Candidatus* Liberibactor asiaticus (Garnier *et al.,* 1984) and spreads by vegetative propagation and Asiatic citrus psyllid, *Diaphorina citri* as a vector (Aubert, 1987; Gottwald *et al.,* 1989; Hung *et al.,* 1999). No confirmed instances of seed transmission have been reported. As there is no successful treatment available, removal and destruction of infected trees are the only effective methods of stopping the spread of the disease. However, most researchers reported that this pathogen is difficult to detect, because it occurs at low concentration and un-evenly distributed in it host tissues (Da Graca, 1991; Hung *et al.,* 1999). It is also difficult to control, because it spreads by vector and vegetative propagation. HLB disease has long incubation period and symptomless in certain host plants but infected citrus plants occur in fields. Another difficulties related to this disease is their symptoms. HLB symptoms are similar and can be confused with those of nutrient deficiencies or other disorders (Weiner *et al.,* 2004).

Information on the nucleotide sequence of the 16S ribosomal DNA gene is useful for deducing the phylogenetic and evolutionary relationship among bacteria and other prokaryotes (Weisburg *et al.*, 1991). Previous study on the nucleotide sequence of the 16S rDNA gene of *Candidatus* Liberibacter asiaticus of the Poona strain (India) and *Candidatus* Liberibacter africanus of the Nelspruit strain (South Africa) showed high similarity percentage (97.7%) (Subandiyah *et al.,* 2000). The percent similarity between the Okinawa isolate with those isolates from Thailand varied from 99.4 to 100%. The similarity toward Nepalese and Indian isolates was 100% and 98.8%, respectively while the similarity to the African strains was 97.5%.

The outer membrane protein (OMP) gene of the

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Candidatus Liberibacter asiaticus was first sequenced in 2005 (Bastianel *et al*., 2005). The OMP gene, which encodes for an outer membrane protein, was thought to be the most promising candidate gene for the study of inter-and intra-species variability. Results of their study revealed that the size of OMP genes of the African isolate (Nelspruit) and the Asian isolate (Poona) was 2,340 and 2,346 bp nucleotides, respectively. Their nucleotide sequences similarity was 72.2%, whereas their amino acid similarity was 86.5%. This finding opens a new sight to study variability of *Candidatus* Liberibacter sp at strain level.

Studies of HLB disease in Malaysia is at infant stage, only a few studies have been reported and most of them focus on epidemiological aspect. Most of the studies were carried out by researchers from MARDI and UPM in collaboration with UNDP-FAO. Limited studies are available on the characterization and strains discrimination of *Candidatus* Liberibacter asiaticus [known as greening fastidious bacterium (GFB) in Malaysia]. This study was carried out to characterize GFB-Pummelo and GFB-Mandarin (PK) isolates with the aim of identifying possible strain variation among the isolates collected from different host plants based on morphology, 16S rDNA and OMP genes.

MATERIALS AND METHODS

Source of inoculum. Infected *Citrus grandis* and *C. reticulata* trees with classical symptoms of HLB disease were previously isolated and maintained inside screen house at Universiti Putra Malaysia (UPM), Serdang, Selangor.

Transmission electron microscopy (TEM). The fresh midribs samples (5 x 2 mm) were fixed into 5% glutaraldehyde buffered with 0.1 M Phosphate buffer Saline (PBS), which had a pH of 7.4 and vacuumed in an oven for two days. Afterwards, the samples were washed three times with 0.1 M sodium cacodylate (SC) for 30 min. Subsequently, the samples were postfixed with 1% osmium tetroxide for one day at 4^0C. The samples were again washed three times with SC buffer. After dehydration with series of ethanol for one hour, the samples were infiltrated and embedded in Epon 812. After polymerization, ultra thin sectioning were carried out 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 detection and identification.

Polymerase chain reaction (PCR). The presence of greening fastidious bacterium (GFB) isolates was detected by PCR test. Nucleic acid was extracted from symptomatic leaves collected from field using the method of Hung *et al*. (1999). Citrus leaf midribs from each plant were cut into small pieces and left to air dry for two 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 [1 M Tris-HCL (pH 8.0), 0.5 M NaCl], 100 µL 10% N-Lauroylsarcosine and transferred to a 1.5 mL Eppendorf tubes. After one hour incubation at 55°C, the sample was centrifuged at 4000 *g* (±6000 rpm) for five minutes. 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 extraction of phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous supernatant was then re-extracted by an additional cycle of chloroform: isoamyl alcohol (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 80% ethanol, dried and resuspended in 50 µL Tris-EDTA (TE) buffer. The concentration of the nucleic acid was estimated using spectrophotometer. About 200 ng of the purified DNA was used for the PCR studies. 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 µM forward primer, 25 µM 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 two minutes followed by 35 cycles at 95°C for 40 seconds followed by 60°C for one minute and 72°C one minute 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 high voltage (100 V), after which the gel was stained with Ethidium bromide (0.5 µg/mL) and photographed under 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 the three volumes of binding solution (6 M sodium iodide) to one volume of gel inside a microfuge tube. The solution was then incubated for five minutes at 55°C to dissolve agarose. After which silica powder was added (10 µL) and mixed by vortexing every two minutes to keep the silica powder in suspension and spun (13000 rpm) the DNA complex for five seconds to form pellet and supernatant removed. Ice cold wash buffer (500 µL) was added, mixed and spun (13000 rpm) for five seconds and supernatant was discarded. The procedure was repeated three times. After the supernatant from the last wash had been removed, the

tube was spun again (13000 rpm) for 30 seconds and the remaining liquid was removed from the pellet. The pellet was air-dried for 15 min. The pellet was dissolved in 15 µL of sterile dH_2O and incubated at 52ºC for five minutes and spun at 13000 rpm for one minute. The supernatant was transferred to a new tube and used for cloning.

Cloning of PCR product and sequencing of 16S rDNA gene. Purified DNA was cloned and transformed into competent cell, *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 37ºC. The plasmid DNA was purified using the QIAprep Spin Miniprep Kit protocol (QIAGEN). The purified plasmids were used for sequencing using automated DNA sequencing. 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 the GFB-Pummelo and GFB-Mandarin (PK) isolates were compared with other accessions of *Candidatus* Liberibacter asiaticus and *Candidatus* 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 using Ali-Bee program provided by the Gene-bee Software (http://www.genebee.msu). The tree was constructed using the following 16S rDNA sequences: *Cand*. Liberibacter africanus (L22533), *Cand.* Liberibacter asiaticus-Kumquat (DQ302750), *Cand*. Liberibacter asiaticus-Fujian-NHE (DQ431998), *Cand*. Liberibacter asiaticus-Poona (L22532), *Cand*. Liberibacter asiaticus-Okinawa (AB008366), *Cand*. Liberibacter americanus (AY742824) and *Cand*. Liberibacter aficanus- subsp capensis (AF137368) as well as local Mandarin isolates such as, GFB-T (EU371106) and GFB-S (EU371107).

Cloning of PCR product and sequencing of outer membrane protein (OMP) gene. In this study, the outer membrane protein (OMP) gene of the GFB isolates was sequenced. The methodology for the total DNA extraction, PCR and purification of PCR product, ligation and transformation, mini-preparation of cloned plasmid and analysis of sequencing results were similar to those described above, except for the 16S rDNA gene, which was replaced by the OMP gene or otherwise described separately. However, for these studies the PCR conditions were as follows: one cycle at 92ºC, 40 cycles each consisting of 40 seconds at 92ºC, 40 seconds at 55ºC and three minutes at 72ºC, followed by extension at 72ºC for 10 min. Primer sets used in the amplification of the OMP gene fragments were listed in Table I. A phylogenetic tree was constructed using the following OMP sequences: GFB-Thailand (AY842432), GFB-Philippines (AY842431), GFB-China (AY842429).

RESULTS AND DISCUSSION

Molecular characterization of the local isolates of *Candidatus* Liberibacter asiaticus was carried out based on morphological properties, 16S rDNA and OMP genes. Two local isolates were successfully isolated from infected *Citrus grandis* and *C. reticulata.* These two local *Candidatus* Liberibacter asiaticus isolates were known as GFB-Pummelo and GFB-Mandarin (PK).

Transmission electron microscope. Electron micrographs of serial ultrathin sections revealed that both GFB-Pummelo and GFB-Mandarin (PK) were pleomorphic and consisted of two types of bodies' i.e., elongated and spherical forms (Fig. 1). The GFB-Pummelo bodies were restricted on phloem tissues. Based on electron micrographs, the GFB-Pummelo possessed two membranes namely inner membrane (IM) and outer membrane (OM). The bodies were bounded by a cell wall (CW) and the bodies had no flagella. The outer membrane of the bodies was undulating or rippled. These morphological properties of the GFB-Pummelo and GFB-Mandarin (PK) bodies clearly proved that they are very similar to each other.

Table I. Primer pairs and nucleotide sequences of OMP genes

Primer	Nucleotide Sequence	Fragment Size (bp)
OMP-F1 Forward	GATGATAGGTGCATAAAAGTACAGAAG	600
OMP-F1 Reverse	AATATTAAGCGTTGTCGGAG	
OMP-F2 Forward	ACACCATTCTATTTCTCCGA	600
OMP-F2 Reverse	TTAATTGGATCTCCCTCACTC	
OMP-F3 Forward	AAAGCGGATAGAAATTGAGG	600
OMP-F3 Reverse	GATATCAACATGCCTTTACGTG	
OMP-F4 Forward	ACTAGATAACCCAATTGTGCCA	600
OMP-F4 Reverse	CTACATGCGATTACCTATACGA	

Fig. 1. Electron micrographs of ultrathin section through sieve tube of leaf midribs from: HLB infected citrus showing elongated and spherical form (A). The bodies were bounded by a cell wall (CW) and the bodies have no flagella (B). GFB possess of two membranes namely inner membrane (IM) and outer membrane (OM) (B)

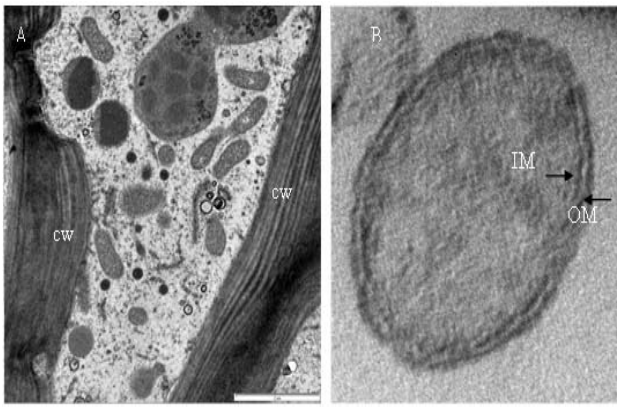


Table II. Comparison of nucleotide similarities (%) and evolutionary distance value between GFB-Pummelo and GFB-Mandarin (PK) isolates with other six *Candidatus* Liberibacter species accessions available in the Genbank database

Accessions	GFB-T	GFB-S	GFB-Pummelo	GFB-Mandarin (PK)	*La. poona*	*La. okinawa*	*L. africa*	*La. guangdong*	*La. kamquat*	*La. fujian*	*L. america*	L.aficanus-subsp capensis
GFB-T	-	96%	98%	98%	97%	98%	85%	98%	87%	98%	82%	93%
GFB-S	3.24	-	97%	98%	96%	96%	85%	97%	87%	97%	82%	92%
GFB-Pummelo	1.08	2.57	-	99%	99%	100%	84%	99%	86%	99%	81%	94%
GFB-Mandarin (PK)	1.15	2.72	0.30	-	98%	99%	85%	99%	87%	99%	82%	94%
La. Poona	1.49	2.68	0.35	0.34	-	99%	97%	98%	98%	98%	93%	93%
La. Okinawa	1.16	2.45	0.00	0.09	0.36	-	97%	99%	99%	99%	94%	94%
L. africa	7.70	9.04	6.74	7.07	7.87	1.98	-	97%	97%	96%	93%	94%
La.Guangdong	1.48	2.66	0.26	0.34	0.61	0.18	2.38	-	99%	99%	94%	94%
La.Kamquat	4.94	6.87	4.12	4.36	2.30	0.09	2.29	0.43	-	99%	94%	94%
La.Fujian	1.66	2.84	0.43	0.52	0.70	0.35	2.55	0.52	0.60	-	94%	94%
L. america	11.09	12.88	10.15	10.40	10.21	3.96	4.38	4.22	3.98	4.51	-	90%
L.aficanus-ssp. Capensis	5.26	6.06	3.96	4.05	4.91	2.78	4.39	2.79	4.89	3.08	7.28	-

Number below the diagonal indicate evolutionary distance

Table III. Percentage OMP gene similarities and evolutionary distance value of GFB-Pummelo and GFB-Mandarin (PK) isolates with the three *Candidatus* Liberibacter asiaticus isolates reported in the Genbank database

Isolates	GFB-Mandarin(PK)	GFB-Pummelo	GFB-Thailand	GFB-Philippines	GFB-China
GFB-Mandarin (PK)	-	99%	99%	99%	99%
GFB-Pummelo	0.68	-	99%	99%	99%
GFB-Thailand	0.30	0.56	-	99%	99%
GFB-Philippines	0.38	0.64	0.17	-	99%
GFB-China	0.34	0.60	0.13	0.21	-

Number below the diagonal indicate evolutionary distance value

Table IV. Percentage amino acid similarity and protein distance of GFB-Pummelo and GFB-Mandarin (PK) isolates with the three *Candidatus* Liberibacter asiaticus isolates available in the Genbank database

Isolates	GFB-Mandarin(PK)	GFB-Pummelo	GFB-Thailand	GFB-Philippines	GFB-China
GFB-Mandarin(PK)	-	98%	99%	99%	98%
GFB-Pummelo	3.41	-	98%	98%	98%
GFB-Thailand	1.70	2.86	-	99%	99%
GFB-Philippines	1.96	3.13	0.29	-	99%
GFB-China	2.29	3.41	0.57	0.85	-

Number below the diagonal indicate protein distance value

Table V. Amino acids substitution and their position of the GFB-Pummelo and GFB-Mandarin (PK) isolates with the three *Candidatus* Liberibacter asiaticus isolates available in the Genbank database as translated by OMP gene

Isolates	Amino acids position														
	53	66	73	76	108	113	115	178	196	288	333	475	556	560	600
GFB-China	I	S	G	I	I	R	I	S	I	S	R	R	R	L	S
GFB-Thailand	I	S	G	I	I	R	I	S	I	S	R	R	K	P	S
GFB-Philippines	I	S	G	I	I	R	I	S	I	S	R	C	K	P	S
GFB-Mandarin(PK)	I	S	G	I	L	K	N	S	K	S	G	R	K	P	S
GFB-Pummelo	T	F	R	F	I	R	I	F	I	F	G	R	K	P	T

I=Ile; T=Thr; S=Ser; F=Phe; G=Gly; L=Leu; K=Lys; N=Asn; R=Arg; C=Cys

Analysis of 16S rDNA gene sequence. Three clones of the local GFB isolates were sequenced in both orientations using M13 forward and M13 reverse primers. These sequences were further aligned and edited using Bio Edit program, which resulted in sequences of about 1167 bp of nucleotides. A search for sequence similarity and confirmation using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/blast.cgi) confirmed that these 1167 nucleotide sequences are full sequence of the 16S rDNA gene of the *Candidatus* Liberibacter asiaticus. These 16S rDNA gene sequences were deposited in the Genbank database with accession numbers of EU224393 and EU224394 for GFB-Pummelo and GFB-Mandarin (PK), respectively. Sequence similarity was compared between these isolates and with the other published isolates available in the Genbank database to determine position of the isolates.

Comparison of nucleotide sequences similarity among the Malaysian GFB isolates was between 96 to 99% (Table II). The highest nucleotide similarity (99%) was observed

between GFB-Pummelo and GFB-Mandarin (PK) and the lowest (96%) was between GFB-T and GFB-S. Similarity of the GFB-Pummelo to the Chinese isolates namely the Guangdong and Fujian were 97-99% but less than 87% similarity was observed when compared to the Kamquat isolate from Taiwan. Generally, Malaysian isolates had lower nucleotide similarity compared to the *Candidatus* Liberibacter africanus (84-85%) isolate or to the *Candidatus* Liberibacter americanus isolate (81-82%). On the other hand, the percent similarity was slightly higher with the *Candidatus* Liberibacter africanus subspecies capensis (92-94%).

The evolutionary distance values between the Malaysian isolates varied from 0.30 to 3.24 (Table II). The lowest (0.30) value was recorded between GFB-Pummelo and GFB-Mandarin (PK), which indicates that both were closely related. Malaysian GFB isolates also showed a slightly far distant relationship to the Asian strains except for Okinawa isolate. Their distance value to the Fujian and Guangdong isolates ranged from 0.26 to 2.84. However, distance values were higher with the Kumquat isolate (4.12 to 6.87) and also with isolates from Africa and America with distance values ranging from 6.74 to 9.04 and 10.15 to 12.88, respectively. These results suggested that sequences of the 16S rDNA gene of Malaysian isolates are almost identical to each other and are closely related with isolates and/or strains from China. This data also indicated that Malaysian GFB isolates were distantly related to the African and American GFB species.

Phylogenetic tree (Fig. 2) shows that the investigated isolates or strains were clustered into three main groups. The first group consisted of *Candidatus* Liberibacter africanus subsp. capensis and *Candidatus* Liberibacter asiaticus of the Fujian, Guangdong and Okinawa isolates. The second group consisted of *Candidatus* Liberibacter americanus, *Candidatus* Liberibacter africanus and *Candidatus* Liberibacter asiaticus of the Kamquat and Poona isolates. All the Malaysian GFB isolates were postulated together in the third group.

Candidatus Liberibacter species has been historically a difficult organism to study, because of the lack of a culture system as explained by Weisburg *et al.* (1991). Amplification of 16S rDNA gene is one way to characterize the bacterium and obviously the sequence of this gene provides basic genetic information and useful for designing probes or PCR primers specific for the detection of plants diseases. Many studies reported that the sequence of the 16S rDNA gene are quite conserved among bacteria but still may present a sufficient variation to be able to design primers for specific diagnostic for species or strains (Jagoueix *et al.*, 1996; Coletta-Filho *et al.*, 2005). Based on the present results, Malaysian GFB isolates originated from local areas, because citrus is a native plant in Malaysia and HLB disease has been present in Southeast Asian regions since 1970s (Schwarz *et al*., 1973). Based on the nucleotide sequences similarity (Table II) and cluster analysis (Fig. 2)

Fig. 2. Rooted phylogenetic trees based on 16S rDNA gene sequences, showing the positions of the GFB-Pummelo, GFB-Mandarin (PK) and representatives of related *Candidatus* Liberibacter sp. reported worldwide:

Subsp. Cap= *Cand*. Liberibacter aficanus- subsp capensis
Fujian= *Cand*. Liberibacter asiaticus-Fujian-NHE
Guangdong= *Cand*. Liberibacter asiaticus-Guangdong
Okinawa= *Cand*. Liberibacter asiaticus-Okinawa
Americanu= *Cand*. Liberibacter americanus
Kamquat= *Cand.* Liberibacter asiaticus- Kumquat
Africanus= *Cand*. Liberibacter africanus
Poona= *Cand*. Liberibacter asiaticus-Poona
GFB-Pumme=GFB-Pummelo
GFB-PK= GFB-Mandarin (PK)
GFB-S= GFB-S
GFB-T= GFB-T

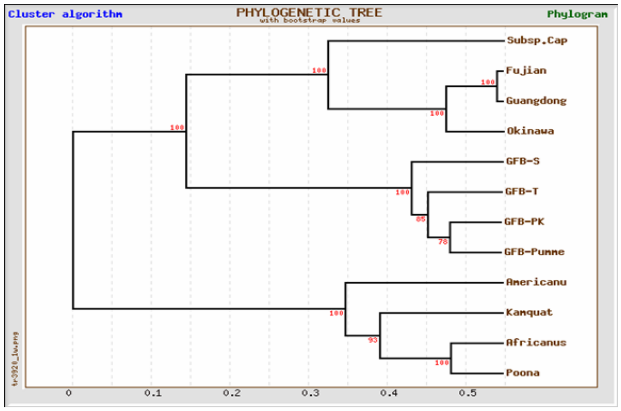
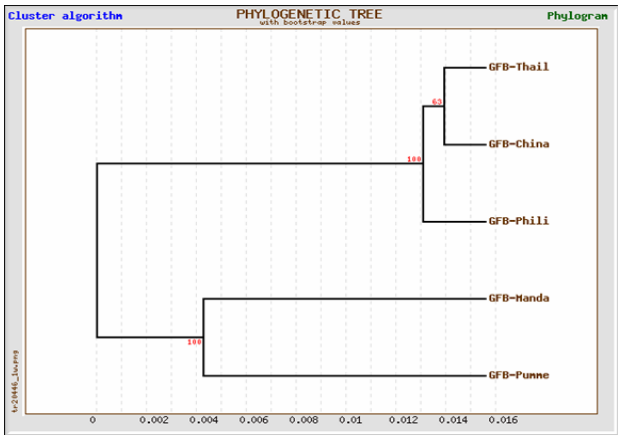


Fig. 3. Rooted phylogenetic trees based on nucleotide of the OMP gene, showing the positions of the GFB-Pummelo, GFB-Mandarin (PK) isolates and representatives *Cand.* Liberibacter asiaticus isolates

GFB-Manda= GFB-Mandarin (PK)
GFB-Pumme= GFB-Pummelo
GFB-Thail= GFB-Thailand
GFB-China=GFB-China
GFB-Phili=GFB-Philippines



it is believed that the Malaysian isolates were not introduced from foreign countries or regions. The present findings are further supported by the previous findings by Khairulmazmi *et al.* (2008). Malaysian isolates may be more similar or

closely related to isolates from Thailand and Indonesia as previously speculated by Subandiyah *et al.* (2000).

Analysis of OMP gene sequence. The outer membrane protein (OMP) genes of the Malaysian GFB isolates were amplified by PCR using four sets of primer pairs i.e., OMP-F1, OMP-F2, OMP-F3 and OMP-F4. Each primer pairs yielded PCR products of about 600 bp in size. Sequencing of all fragments and further alignment using Bio Edit software yielded the full sequence of OMP gene of about 2354-2356 bp in size. Sequences alignment and comparison using BLAST software confirmed that the generated nucleotide sequences were truly of the full sequence of the OMP gene of *Candidatus* Liberibacter asiaticus. These OMP gene sequences were deposited in the Genbank database with the following accession numbers, EU371108 and EU224395 for GFB-Pummelo and GFB-Mandarin (PK), respectively. The generated OMP gene sequences were also compared with the other OMP gene sequences that were available in the Genbank database.

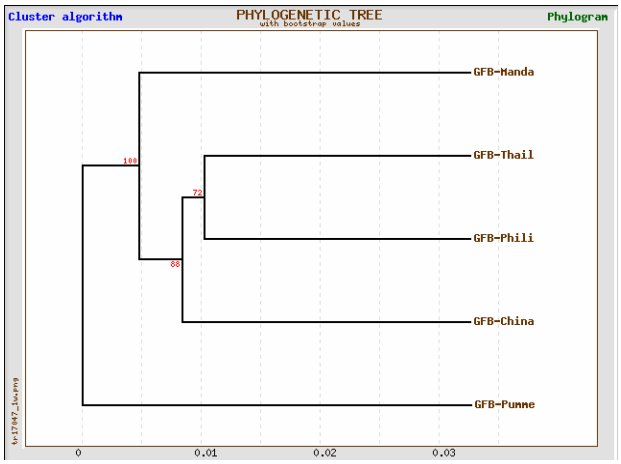
Comparison of the nucleotide sequences of the OMP gene showed that there was a high nucleotide similarity (99%) between the two Malaysian isolates (Table III). Comparison between their sequences with sequences of isolates from Thailand, Philippines and China also showed high nucleotide similarity (99%). Even though the percentage nucleotide similarities among them were high but the evolutionary distance values among them varied. The evolutionary distance value between GFB-Pummelo and GFB-Mandarin (PK) was 0.68. However, the values between GFB-Pummelo and the Thailand, Philippines and China isolates were slightly lower, ranging from 0.56 to 0.64. On the other hand, the evolutionary distances values between GFB-Mandarin (PK) and the three isolates were much lower than 0.38. These data suggest that GFB-Pummelo was distantly related to the GFB-Mandarin (PK), GFB-Thailand, GFB-Philippines and GFB-China. Phylogenetic trees based on the nucleotide sequences of the OMP gene (Fig. 3) showed that the isolates clustered into three main groups. The first group consisted of GFB-Pummelo alone. The second group was GFB-Mandarin (PK), while the third group comprises of GFB-China, GFB-Thailand and GFB-Philippines.

The OMP gene of the Malaysian GFB isolates translated a total of 781 amino acid sequences. This number was similar to those translated by GFB isolates from China, Thailand and Philippines. Interestingly, comparison of amino acid sequences between the OMP gene of the Malaysian isolates and others species reported from Asian countries showed differences between them.

Comparison of amino acids similarity between GFB-Mandarin (PK) and GFB-Pummelo showed high amino acid similarity (98%) and also high evolutionary distance value (3.41) as presented in Table IV. Even though similarity value was high, 11 amino acids substitutions were observed among them (Table V), which indicated possible genetic variation between them.

Fig. 4. Rooted phylogenetic trees based on amino acids sequences translated by OMP gene of respective GFB isolates

GFB-Manda= GFB-Mandarin (PK)
GFB-Pumme= GFB-Pummelo
GFB-Thail= GFB-Thailand
GFB-China=GFB-China
GFB-Phili=GFB-Philippines



High amino acid similarity (98-99%) was observed between the Malaysian isolates and isolates from China, Thailand and Philippines. However, variations in term of amino acids composition and evolutionary distance values were observed. About 15 amino acids substitutions were observed among them (Table V). Phylogenetic tree based on amino acids sequence showed three main clusters or groups. The first group consisted of GFB-Pummelo alone. The second group consisted of isolates from China, Thailand and Philippines and the third group consisted of GFB-Mandarin (PK) alone (Fig. 4). This phylogenetic tree indicated that the OMP gene of GFB-Pummelo is relatively far from the OMP gene of the other isolates tested in this study.

In bacteria, the OMP gene is encoded for the outer membrane protein (OMP). Very few studies had been done on the OMP gene of the *Candidatus* Liberibacter species. This gene was thought to be the most promising gene candidate for studying the inter-and intra-specific variability (Bastianel *et al*., 2005). However, many studies on the OMP gene have been done on other bacteria especially on the *Escherichia coli* (Nikaido, 2003; Guillier *et al.*, 2006). There are many OMP genes involved in the development of the outer membrane protein in *E. coli* such as the ompF, ompC, ompW and ompA. Interestingly, the ompA has been implicated in the pathogenicity of encapsulated *E. coli* strains (K1), which caused newborn meningitis (NBM) disease (Gophna *et al*., 2004). The ompA protein of the *E. coli* strain K1 and strain K12 differs only in three amino acids, whereas strains K12 ompA was found to possess a similar function in invasion of brain microvascular endothelial cells as strain K1 (Kim, 2001). From this information it is observed that a new strain of bacteria with different virulence level may have evolved as a consequence

of amino acids substitutions in their outer membrane protein.

In *Candidatus* Liberibacter spp., no study has been done on the role of OMP gene in the pathogenicity process. In the present study it was proved that the GFB-Mandarin (PK) and GFB-Pummelo belong to different strains based on amino acid sequence differences as translated by their OMP genes. This finding was in line with the pathogenicity reactions of certain GFB isolates in Taiwan as mentioned by Su (1998). In Taiwan, the GFB-Mandarin isolate induced severe HLB symptoms on mandarin and sweet oranges but produced only mild symptoms on pummelo. On the other hand, the GFB-Pummelo isolate was reported to induce severe HLB symptoms on pummelo and also on mandarin and sweet orange trees.

CONCLUSION

Characterization of the Malaysian *Candidatus* Liberibacter asiaticus isolates was carried out. This study was performed based on two methods namely morphological and molecular characterization. Characterization of their 16S rDNA gene sequences revealed that the Malaysian isolates have about 1167 bp. The nucleotide sequences of the 16S rDNA of Malaysian GFB isolates showed high similarity (96-99%). A similar trend was observed on their genetic distance, which indicated that their relationship was very close. Comparison with other reported GFB strains worldwide indicated that the Malaysian GFB isolates belong or closely related to the group of Asian species of Liberibacter asiaticus such as Fujian isolate (99%). However, lower percentage nucleotides similarity to the African and American species indicated that they may belong to separate groups. Even though the OMP gene of the GFB-Pummelo and GFB-Mandarin (PK) was high in their nucleotide similarity but some differences in the amino acids composition was observed. There are 11 amino acid substitutions between GFB-Pummelo and GFB-Mandarin (PK) isolates, which indicated that there is significant variation between these isolate. Since the OMP gene plays a vital role in the pathogenicity of *E. coli*, this may also true for *Candidatus* Liberibacter asiaticus. This may be the only explanation on the possible difference in the virulence level of the GFB-Pummelo and GFB-Mandarin strains in Taiwan or probably also in Malaysia.

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