Diversity of '*Candidatus* Liberibacter asiaticus', the Causal Agent of Citrus Huanglongbing, in Psyllids (*Diaphorina citri*) Collected from *Murraya paniculata* and *Citrus spp*. in Thailand Revealed by Hypervariable Prophage Genes

A. Jantasorn[1], T. Puttamuk[1], Y. Duan[2], L. Zhou[3], S. Zhang[4] and N. Thaveechai[1,*]

[1]*Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand*
[2]*USDA-ARS-USHRL, Fort Pierce, FL, USA 34945*
[3]*University of Florida, IFAS-IRREC, Fort Pierce, FL, USA 34945*
[4]*University of Florida, IFAS-TREC, Homestead, FL, USA 33031-3314*

**Corresponding author. Email: agrnpt@ku.ac.th*

Abstract

'*Candidatus* Liberibacter asiaticus' (Las) is a phloem inhabiting bacterium that causes huanglongbing disease (HLB), also known as citrus greening associated with three species of α-Proteobacteria in the genus '*Candidatus* Liberibacter sp'. Prophage is an important genetic element of bacterial genomes and is involved in lateral gene transfer, pathogenicity, environmental adaptation, and interstrain genetic variability. Hypervariable gene *hyv*I was identified in the prophage region of the Las Psy 62 genome. In this study, the sequence of an *hyv*I gene from '*Candidatus* Liberibacter asiaticus' was selected as a molecular marker for the assessment of the genetic variation in two '*Ca.* Liberibacter asiaticus' populations infected by psyllid vector collected from *Citrus* sp. and *Murraya paniculata* in Thailand. The frequency of the *hyv*I gene was 81.25% (65/80) in HLB-infected psyllid fed on citrus and 90.91% (20/22) in HLB-infected psyllid fed on *M. paniculata*. Frequent deletions and insertions of these repeats did not disrupt the open reading frames. However, the hyv_I genes obtained from psyllids fed on Citrus and *M. paniculata* were identical or highly similar suggesting that, at least some bacterial strains in the two hosts, shared a common recent origin. This is the first report on diversity variation of '*Ca.* Liberibacter asiaticus' isolated from psyllids in Thailand. This understanding could be applied to studies on ecology and epidemiology of '*Ca.* Liberibacter asiaticus' for further effective disease management.

Keywords: *Candidatus* Liberibacter asiaticus, psyllid, *Diaphorina citri*, *Murraya paniculata*, prophage genes, diversity

Introduction

Huanglongbing (HLB) disease, previously known as citrus greening, is one of the most destructive diseases of citrus worldwide. HLB is associated with *Candidatus* Liberibacter, phloem limited, fastidious, gram negative bacteria belonging to the alpha subdivision of *Proteobacteria* (Damsteegt et al., 2010, Jagoueix et al, 1996), which has been known as uncultured bacterium until the first successful report on cultivation of HLB bacteria in 2009 by Sechler et al. (2009). The '*Candidatus* Liberibacter' genus contains three species of the expanding list, '*Candidatus* Li beribacter asiaticus'(Las), '*Candidatus* Liberibacter americanus' (Lam), and '*Candidatus* Liberibacter africanus' (Laf). The first two species are heat tolerant and transmitted by *Diaphorina citri,* while

the '*Candidatus* Liberibacter africanus' (Laf) is a heat sensitive species, which is transmitted by *Trioza erytreae*. These species of HLB exist in Asia and Florida, USA (Bruce et al., 2005), and in Brazil and South Africa (Halbert et al., 2004, Jagoueix et al., 1994, Li et al., 2006, Teixeira, 2005). The '*Candidatus* Liberibacter solanacearum', a newly recognized species isolated from tomato, is genetically related to the HLB bacteria. Although it is not naturally associated with HLB in citrus plants (Lin et al., 2009), this species is related to the emerging zebra chip disease of potato and tomatoes (Liefting et al., 2008). The *'Candidatus* Liberibacter asiaticus' has been shown to colonize plants, insect vectors, *Diaphorina citri*, periwinkles and dodders. Diagnostic tests using 16S rRNA and the *rpo*JL loci as target sequences for both classical polymerase chain reaction (PCR) and real-time PCR have consistently revealed a '*Candidatus* Liberibacter spp.' associated with the symptoms of HLB.

Annotation of *'Ca.* Liberibacter asia ticus' Psy62 genome did not reveal any known transposon or insertion sequence (IS) element (Duan et al., 2009). The potential contributions of prophage and phage-related sequences to the genetic diversity of *'Ca.* Liberibacter asiaticus' were then investigated. Prophages can constitute as much as 10 to 20% of a bacterial genome and are major contributors to genetic variability including virulence factors and pathogenicity islands between individuals within species (Casjens, 2003). Consequently, prophage DNA plays an important role in the evolution of bacterial pathogenicity (Boyd and Brussow, 2002). The *in silico* analyses revealed multiple regions of the Las Psy 62 genome containing prophage-related sequences, and two of them were identified as prophages in psyllids and as temperate phage in plants, which were similar to those described by Zhang et al., 2011. In these prophage regions, two candidate genes were identified and tentatively named as hyv_{I} and hyv_{II}. Investigation of genetic diversity provides understanding of the taxonomy, population structure, and dynamics of bacteria. The genetic diversity also provides the key for developing a sensitive, specific, and rapid method for pathogen detection, disease diagnosis, and disease risk management (Tomimura et al., 2009). Genetic variation of the pathogen is important for determining the source of the infection and epidemiology. Molecular techniques are the most accurate means of fingerprinting microorganisms on a species and subspecies level (Louws et al., 1999). Ribosomal DNA sequence profile is a powerful tool for deducing phylogenetic and evolutionary relationship among bacteria, archaebacteria, and eukaryotic organisms (Weisburg et al., 1991). Methods for identification of '*Ca.* Liberibacter asiaticus' isolates are fundamental for ecologic and epidemiologic studies. Knowledge about the genetic diversity of '*Ca.* Liberibacter asiaticus' from different geographical areas and citrus cultivars is useful for the assessment and management of disease risk. Different symptoms of HLB disease in different host species might be affected by genetic diversity. However, there have been only few reports related to the diversity of '*Ca.* Liberibacter asiaticus'.

The objective of this study is to assess characteristics and diversity of prophage gene (hyv_{I}) cloned from '*Ca.* Liberibacter asiaticus' isolates in psyllids (*Diaphorina citri*) collected from two host plants, citrus (*Citrus spp*.) and murraya (*Murraya paniculta*) from different geographic regions in Thailand. Hypervariations in these genes among '*Ca.* Liberibacter asiaticus' isolates imply a potentially important mechanism of adaptation available to '*Ca.* Liberibacter asiaticus'. The relationship between genetic diversity and the geographical regions of the strains and psyllids vector was investigated in order to elucidate population structure, ecology, and epidemiology of the pathogen for further effective disease management.

Materials and Methods

Collection of Samples and DNA Extraction

Adults of *D. citri* were collected from both visually healthy and symptomatic plants belonging to *Citrus* sp., *M. paniculata*, and related genera from diverse geographical regions in Thailand, such as commercial groves, retails, residential areas, and discount garden centers from different provinces of Thailand. Adult psyllids were collected using an aspirator. The insects were catalogued and stored in 2 mL of 75% ethanol for the analysis of Las and non-Las infections. The psyllids were assigned unique sample numbers and stored at -20°C until processed.

All DNA extractions were performed in a sterile laminar flow hood. Crude extracts of psyllid DNA were prepared using a modification of the method described in De Barro and Driver (1997). Psyllids were stored in 70% ethanol at 4°C from the time of collection until DNA extraction was performed. Individual psyllid was placed in a 2 mL screw-cap tube (USA Scientific, Ocala, FL) containing approximately twelve 1.6-1.8 mm zirconium silicate beads (Ceroglass, Columbia, TN) and 150 µL of lysis buffer (5% 1M KCl, 5% 1M Tris at pH 8.4, 0.45% Tween20, 0.45% NP40, 89.1% autoclaved deionized water). Tubes were placed in a FastPrep-24-system (MP Biomedicals, Solon, OH) and homogenized for 30 sec at 6 m s^{-1}. Following the homogenization, 100 µL of liquid was transferred to a clean 1.5 mL centrifuge tube (USA Scientific), and placed in a water bath at 65°C for 15 min. Samples were immediately placed on ice for at least 10 min, and then centrifuged at 14,000 rpm for 5 min. The supernatant was removed and stored at -20 °C until conventional and real time PCR were performed.

Real Time PCR Quantification of 'Candidatus *Liberibacter asiaticus'*

Real time PCR was performed using primers as listed in Table 1. Real time PCR amplifications were performed in Mastercycler Realplex Real time PCR system (Eppendorf inc., USA) using TaqMan Universal PCR Mastermix (Applied Biosystems) in a 15 µL reaction. The standard amplification protocol was 95°C for 15 minutes followed by 40 cycles at 94°C for 15 seconds and 58°C for 60 sec. All reactions were performed in triplicate including positive, negative, and water control. The mean value of the threshold cycle (Ct) was presented with standard deviation. For direct quantification of total DNA, each sample was measured using a Nano-Drop spectrophotometer (Thermo Scientific Inc., USA).

Conventional PCR Amplification

All primers used for conventional PCR in this study are listed in Table 1. DNA amplification was performed in a final volume of 20 µL containing 10 µL of 2X buffer D (Epicentre Biotechnologies, Madison, WI, USA), 250 nmol of each forward/reverse primer, 1.25 units of *Taq* DNA polymerase (New England BioLabs Inc., Ipswich, MA, USA), and 1 to 2 µL of genomic DNA template from '*Candidatus* Liberibacter asiaticus', which was extracted from midribs of orange jasmine, psyllid vectors, and '*Candidatus* Liberibacter asiaticus' cultures. Conditions for PCR amplification were as followed: denaturation at 95°C for 3 minutes, followed by 35 cycles of amplification with denaturation at 94°C for 45 seconds, annealing at melting temperature of each primer pairs for 45 seconds (as shown in Table 1), and DNA extension at 72°C for 1 min with a final extension at 72°C for 10 min using a thermocycler (Perkin- Elmer 9600/Applied Biosystem, Bedford, MA). The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer. The gel was stained with ethidium bromide and amplified DNA bands were viewed under UV-transilluminator.

Table 1. Primers and probes used in this study

Name	Sequence (5'-3')	Target genes or flanking region	Source of reference
Conventional PCR primers			
CGO3f	RGGGAAAGATTTTATTGGAG	16S rRNA genes	Zhou et al., 2007
CGO5r	GAAAATAYCATCTCTGATATCGT	16S rRNA genes	Zhou et al., 2007
LJ729	TTGCTAGTCTTATCGGCTTATC	5'flanking region of *hyv*$_{I}$ gene	Zhou et al., 2011
LJ730	TTGCGACTAAAGACAACGAG	3'flanking region of *hyv*$_{I}$ gene	Zhou et al., 2011
Real time PCR primers and probes			
HLBasf	TCGAGCGCGTATGCGAATACG	16S rRNA genes	Li et al., 2006
HLBr	GCGTTATCCCGTAGAAAAAGGTAG	16S rRNA genes	Li et al., 2006
HLBp	*AGACGGGTGAGTAACGCG**	16S rRNA genes	Li et al., 2006

*6-FAM™ at 5' –end; **Iowa Black FQ at 3' –end

Cloning, Sequencing, and Sequence Assembly

PCR products containing hyv_I gene from different isolates were ligated into TOPO TA vector pCR2.1 and transferred to TOPO 10 chemical competent cells as per manufacture's instruction manual. Plasmid DNA was isolated from *E. coli* cultures using the QIAprep Spin Miniprep kit (Qiagen). DNA sequencing was performed at BioDesign Company. Sequences were assembled by ContigExpress of Vector NTI (Invitrogen) and analyzed by Align X in Vector NTI.

Phylogenetic Analysis

Sequencing data of all samples for hyv_I gene were aligned by BioEdit software. Phylogentetic tree of each loci was analyzed by MEGA5 software using UPGMA method.

Results

Variation of hyv_I Gene of '*Ca.* Liberibacter asiaticus' Isolates from Psyllids

Total of 250 psyllids (*Diaphorina citri*) fed on *Citrus* sp. and *Murraya paniculata* were collected from diverse geographical regions in Thailand. Mostly the samples were collected from the Central plain, and some are from the Northern and Eastern area of Thailand. All DNA samples were extracted from '*Ca.* Liberibacter asiaticus' infected psyllids in Thailand in order to evaluate the diversity of hyv_I gene. The isolates confirmed positive results for '*Ca.* Liberibacter asiaticus' with threshold cycle (Ct value) between 17 and 29 by 16S rRNA gene based real time PCR (Table 2). '*Ca.* Liberibacter asiaticus' was detected by conventional PCR in samples from Bangkok, Nakhon Pathom, Samut Songkhram, Rayong, Phichit, and Kamphaeng Phet provinces. The detection of '*Ca.* Liberibacter asiaticus' was based on PCR amplification of an expected 534 bp DNA amplicon with primer set CGO3f/CGO5r (Figure 1). The hyv_I gene from prophage region was indicated with primer set LJ729/LJ730 (Figure 1). The prophage gene was detected only in DNA samples prepared from psyllids infected with '*Ca.* Liberibacter asiaticus'. All negative control DNA extracted from healthy plant and psyllid samples indicated negative results for '*Ca.* Liberibacter asiaticus' by conventional PCR. Psyllids fed on Citrus trees, '*Ca.* Liberibacter asiaticus' was detected in 80 (67.79%) out of 118 psyllid DNA samples and the prophage hyv_I gene was detected in 65/80 (81.25%) from six provinces. Psyllids collected from murraya tree were found positive with '*Ca.* Liberibacter asiaticus' in 22 (16.66%) out of 132 psyllids DNA samples and the prophage hyv_I gene was detected in 20/22 (90.91%). The PCR detection of '*Ca.* Liberibacter asiaticus' and the prophage frequencies are summarized in Table 2.

Table 2 Polymerase chain reaction (PCR) detection of '*Candidatus* Liberibacter asiaticus' from psyllids (*Diphorina citri*) collected from different hosts and locations using 16S rRNA and prophage gene primers.

Geographic origin	Host species	Total sample	Range of Ct value of 16s rRNA gene based on real time PCR	PCR detection (%)[1/]	
				'*Ca.*L. asiaticus' (CGO)	Prophage (hyv_I)
Psyllids fed on citrus spp.					
Bangkok	*D. citri*	20	10	10	8
Nakhon pathom	*D. citri*	10	9	9	8
Samut Songkhram	*D. citri*	14	10	10	10
Kamphaeng Phet	*D. citri*	40	39	39	33
Phichit	*D. citri*	24	7	7	4
Rayong	*D. citri*	10	5	5	2
Total		118	80 (67.79%)	80 (67.79%)	65(81.25%)
Psyllids fed on *M. paniculata*					
Bangkok	*D. citri*	20	6	6	4
Nakhon pathom	*D. citri*	20	5	5	5
Samut Songkhram	*D. citri*	20	3	3	3
Kamphaeng Phet	*D. citri*	12	2	2	2
Phichit	*D. citri*	40	4	6	5
Rayong	*D. citri*	20	2	2	1
Total		132	22 (16.66%)	22(16.66%)	20(90.91%)

[1/] Numbers in parentheses correspond to detection rate. The '*Candidatus* Liberibacter asiaticus' was represented by the 16S rRNA gene, and prophage was represented by hyv_I gene.

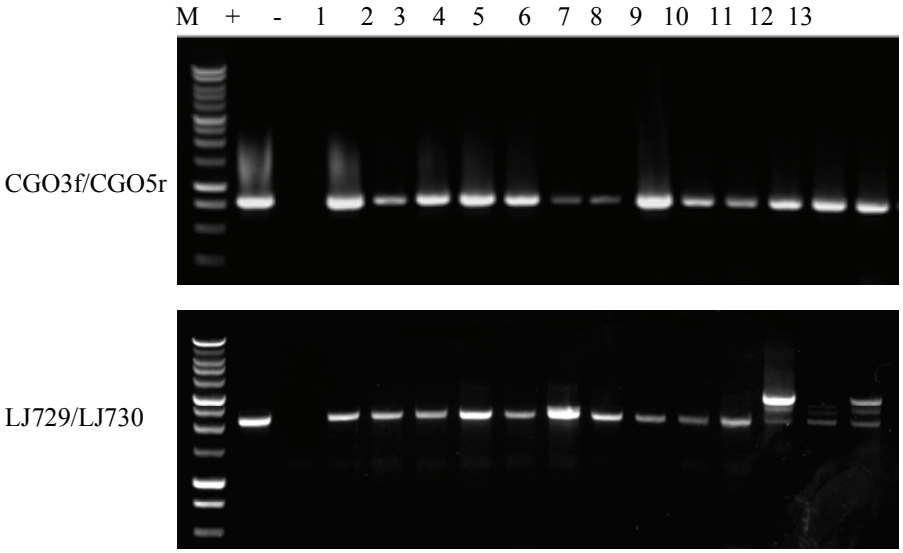

Figure 1 PCR amplification of '*Candidatus* Liberibacter asiaticus' represented by 16S rRNA gene and hypervariable prophage gene (hyv_I) in psyllids samples collected from different hosts and locations in Thailand using two primer sets CGO3f/CGO5r and LJ729/LJ730. Lane M: 1-kb DNA ladder (Promega), +: positive control, -: negative control, lane 1 and 2 DNA from '*Ca.* Liberibacter asiaticus' infected psyllids collected from *Citrus reticulata* in Bangkok province, lane 3 DNA from '*Ca.* Liberibacter asiaticus' infected psyllids collected from *Murraya paniculata* in Bangkok province, lane 4 and 5 DNA from '*Ca.* Liberibacter asiaticus' infected psyllids collected from *Citrus reticulata* in Nakhon Pathom province, lane 6 DNA from '*Ca.* Liberibacter asiaticus' infected psyllids collected from *M. paniculata* in Nakhon Pathom province, lane 7 to 9 DNA from '*Ca.* Liberibacter asiaticus' infected psyllids collected from *Citrus reticulata* in Kamphaeng Phet province, and lane 10 to 13 DNA from '*Ca.* Liberibacter asiaticus' infected psyllids collected from *Citrus aurantifolia* in Phichit province.

The hyv_I gene amplicon showed more variation, and different size amplicons were observed in psyllid from Phichit province. The samples yielded multiple weak bands in addition to the dominant band for the hyv_I region (Figure 1). Fourteen hyv_I amplicons were cloned and sequenced from the '*Ca.* Liberibacter asiaticus' isolates of psyllid collected from different host and locations. Sequence analysis confirmed the presence of different size amplicons belonging to hyv_I, and clone(s) reflecting one to three dominant sizes in each library are listed in Table 3. Further sequence analyses of these amplicons revealed that the amplicons size differences were due to changes in the repeat (full or partial) number and the arrangement of individual units in each hyv_I gene.

The number of full repeat varied from 1 to 7, and the arrangement of full and partial repeats in each hyv_I gene was also highly variable (Table 3). However, all repeats were found to be in frame when the gene was translated into protein by FGENESB software (Zhou et al., 2011). Therefore, deletion or insertion of a full or partial repeat unit did not disrupt the open reading frames in any hyv_I clones.

Diversity of hyv_I Gene in '*Ca.* Liberibacter asiaticus' Isolates from Psyllids

The '*Ca.* Liberibacter asiaticus' isolates were obtained from HLB-infected psyllids collected in six provinces of Thailand (Table 2). These isolates were confirmed to be positive with '*Ca.* Liberibacter asiaticus' by evaluating the Ct value using 16S rRNA gene based real time PCR. The number of '*Ca.* Liberibacter asiaticus' DNA isolated from '*Ca.* Liberibacter asiaticus' infected psyllid samples are listed in Table 3. The DNA fragments of the hyv_I gene were amplified by the primer set LJ729/LJ730 from DNA samples of '*Ca.* Liberibacter asiaticus' infected psyllids in Bangkok, Naknon Pathom, Kamphaeng Phet, and Phichit provinces. Fourteen clone libraries were constructed from PCR amplicons of '*Ca.* Liberibacter asiaticus' DNA by primer targeting the hyv_I region. Sequencing of the hyv_I gene of Las showed various, nearly identical tandem repeats (NITR) number that were similar to those reported by Zhou et al. (2010). The dominant form of hyv_I clone from psyllid collected from Murraya plant in Bangkok, Nakhorn Pathom provinces and psyllid collected from citrus in Kamphaeng Phet and

Table 3 Hypervariable gene hyv_I of '*Ca.* Liberibacter asiaticus' from infected psyllid DNA samples of various geographical regions in Thailand.

Name of '*Ca* Liberibacter asiaticus' Thailand isolate	Source of Psyllid	Location	Ct value of 16s rRNA gene based on real time PCR	hyv_I[1/]	
				Cloning ID	No. of full/partial repeat[2/]
THA-BK-MD7	*Citrus reticulata*	Bangkok	17.19	pHYVI29.2	1/2
THA-BK-Mur9	*Murraya paniculata*	Bangkok	26.5	pHYVI30.15	2/4
THA-NP-Mur3	*Murraya paniculata*	Nakhon Pathom	26.21	pHYVI3.19	2/4
THA-KP-MD5	*Citrus reticulata*	Kamphaeng phet	21.6	pHYVI31.6	2/4
				pHYVI31.10	1/2
				pHYVI31.15	2/4
				pHYVI31.24	1/2
THA-KP-MD11	*Citrus reticulata*	Kamphaeng phet	26.61	pHYVI34.1	2/4
				pHYVI34.8	2/3
				pHYVI34.12	3/5
				pHYVI34.14	4/3
THA-PCH-LM11	*Citrus aurantiifolia*	Phichit	15.59	pHYVI21.1	7/2
THA-PCH-LM16	*Citrus aurantiifolia*	Phichit	18.51	pHYVI25.7	0/1
				pHYVI25.15	2/4

[1/] The hyv_I gene was cloned from LJ729/LJ730 PCR product.
[2/] The number of full and partial repeats in hyv_I gene with the dominant size in each clone library.

Phichit provinces were the same size and contained two nearly identical tandem repeats (NITR) and four partial repeat (Figure 2C; Table 3). There were five additional sizes of the hyv_I gene: psyllid sample collected from *Citrus reticulata* in Bangkok and Kamphaeng Phet provinces containing 1 and 2 full and partial repeats, psyllid samples collected from *Citrus reticulata* in Kamphaeng Phet samples containing 2 and 3 (i), 3 and 5 (ii), and 4 and 3 (iii) full and partial repeat respectively, and psyllid samples collected from *Citrus aurantifolia* in Phichit containing 7 and 2 (i), and 0 and 1 (ii) full and partial repeat respectively. The hyv_I upstream and downstream sequences flanking the repeat region share 93% to 100% similarity among all isolates and the hyv_I gene from Thailand was different in terms of both numbers of NITR and downstream sequences flanking the repeat region (Zhou et al., 2011).

Phylogenetic Analysis

Based on the variable nucleotide sequences from '*Ca.* Liberibacter asiaticus' isolates of psyllid collected from different locations in Thailand, amino acid sequences deduced from this hyv_I gene sequence were aligned and integrated into phylogenetic analyses. The results of these phylogenetic analyses for hyv_I gene demonstrated that gene bifurcate into clades on the basis of sampling locations (Figure 3). The psyllid samples from all provinces in Thailand formed a well-supported sister clades to the sample set for most of the psyllid samples from Florida, China, and Taiwan, although without bootstrap support (Figure 3).

Discussion

Diversity studies of '*Ca.* Liberibacter asiaticus' have been reported utilizing the prophage gene sequences (Tomimura et al., 2009 and Villechanoux et al., 1983). Our study described the analyses of the sequence variation of a phage DNA polymerase gene from different sources in Thaialand. With the hyv_I gene of prophage, we noted that the HLB-infected psyllid collected from citrus and *M. paniculata* strains are highly similar or identical. Therefore, these strains shared a common recent origin. In contrast, the Florida strain is much further apart because its genetic distance is longer than those of the China strains. Characterization of the variations of prophage in the HLB bacteria may provide insight into their evolution and adaptation approaches. The hyv_I gene was identified in prophage regions of the '*Ca.* Liberibacter asiaticus' Psy62 genome. This gene

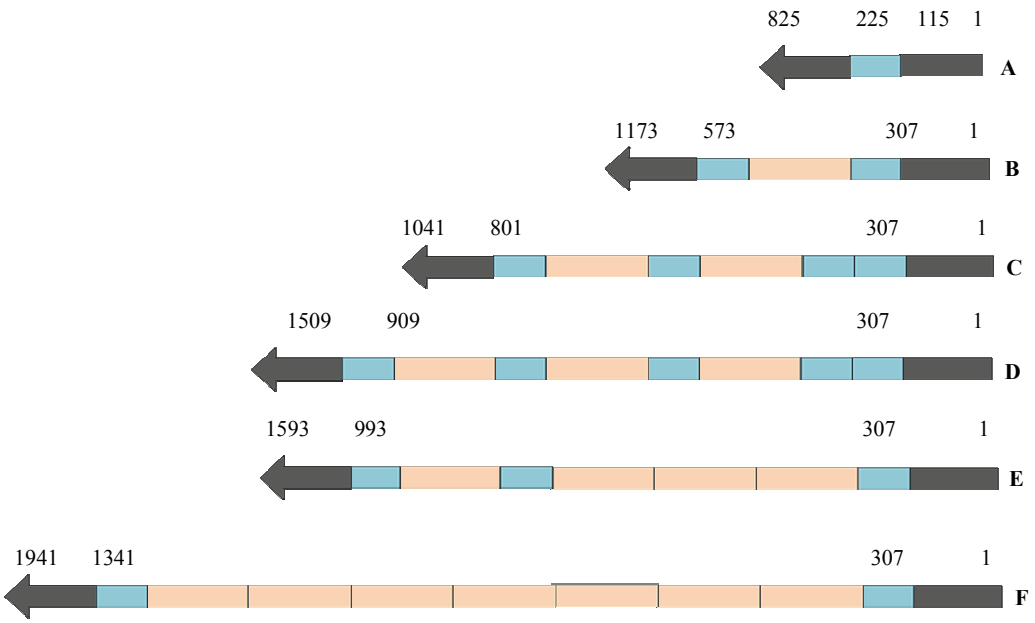

Figure 2 General feature of *hyv*$_{I}$ (scaled) in the genome of '*Candidatus* Liberibacter asiaticus' in HLB-infected psyllids samples in Thailand. The black arrow boxes in A to F gene patterns represent the open reading frames of *hyv*$_{I}$ gene with different numbers of full or partial repeat units in the middle. Each pink box in the middle of the gene represents full tandem repeat unit, and blue box stands for partial repeat unit. The numbers above the *hyv*$_{I}$ gene indicate the gene sizes and the end positions of repeat regions in the *hyv*$_{I}$ gene.

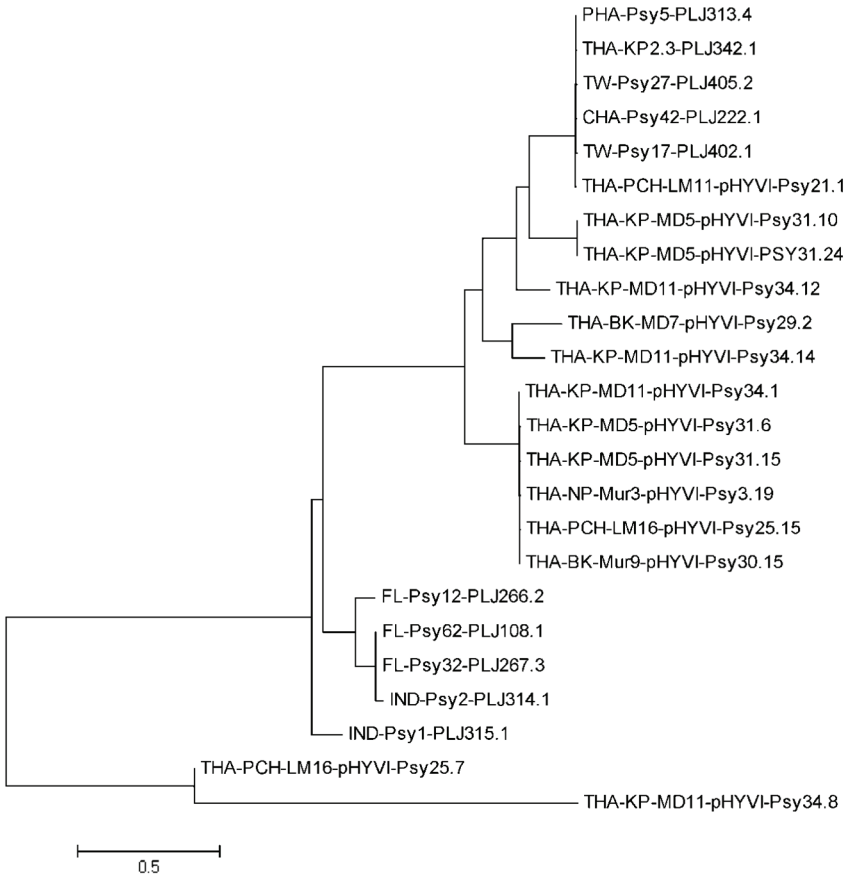

Figure 3 Phylogenetic tree represents *hyv*$_{I}$ gene from '*Candidatus* Liberibacter asiaticus' isolates from HLB-infected psyllids collected from Citrus and murraya plants in Thailand compared with *hyv*$_{I}$ gene reported in GenBank. Each branch label reflects the identification listed in Table 3 and indicates the geographic origin of the samples. The tree was constructed using the UPGMA method.

was found not only in samples of distinct geographical origins but also from a single origin and even from a single '*Ca.* Liberibacter asiaticus' infected samples, as indicated by the hyv_I gene in citrus and periwinkle (Zhou et al., 2011). The variation of repeat number of hyv_I gene in the same isolate indicates co-infection by different '*Ca.* Liberibacter asiaticus' population in a single HLB infected sample. However, the variation caused by different number of tandem repeats provides the opportunity for functional diversity and alterations in phenotypes (Verstrepen et al., 2005). Detection of sequencing of Las in psyllid from citrus was higher (67%) than murraya (16%) indicating that murraya is in a low potential source of inoculum of HLB comparing to citrus (Jantasorn et al, in submitted). Further study of population dynamics in these co-infected samples may reveal their potential roles in HLB disease development and epidemiology. Zhou et al. (2011) reported that the function of hyv_I gene is unknown, and the structure of tandem repeats present in hyv_I gene is similar to those in *pth*A, the pathogenicity gene of citrus canker bacteria (*Xanthomonas citri* pv. *citri*) (Boch et al., 2009).

The high copy number of NITR in the hyv_I gene provides excellent target for the development of more sensitive methods for the detection of '*Ca.* Liberibacter asiaticus'. The protocol reported herein is a very important method for study the HLB bacterial pathogens because they are fastidious and often exist in low titer in plant hosts and insect vectors. This method is also useful for seed transmission studies where '*Ca.* Liberibacter asiaticus' population is in low titer seedling. The variation tandem repeat of hyv_I gene provides resolution of genetic diversity, which is useful for identification of the origin and development of '*Ca.* Liberibacter asiaticus' in Thailand. This method is also useful for plant quarantine since the source of inoculum from other countries can easily be proved.

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