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Diversity and Plasticity of the Intracellular Plant Pathogen and Insect Symbiont "*Candidatus* Liberibacter asiaticus" as Revealed by Hypervariable Prophage Genes with Intragenic Tandem Repeats▽†

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"*Candidatus* Liberibacter asiaticus" is a psyllid-transmitted, phloem-limited alphaproteobacterium and the most prevalent species of "*Ca*. Liberibacter" associated with a devastating worldwide citrus disease known as huanglongbing (HLB). Two related and hypervariable genes (*hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$) were identified in the prophage regions of the Psy62 "*Ca*. Liberibacter asiaticus" genome. Sequence analyses of the *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ genes in 35 "*Ca*. Liberibacter asiaticus" DNA isolates collected globally revealed that the *hyv*$_{\mathrm{I}}$ gene contains up to 12 nearly identical tandem repeats (NITRs, 132 bp) and 4 partial repeats, while *hyv*$_{\mathrm{II}}$ contains up to 2 NITRs and 4 partial repeats and shares homology with *hyv*$_{\mathrm{I}}$. Frequent deletions or insertions of these repeats within the *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ genes were observed, none of which disrupted the open reading frames. Sequence conservation within the individual repeats but an extensive variation in repeat numbers, rearrangement, and the sequences flanking the repeat region indicate the diversity and plasticity of "*Ca*. Liberibacter asiaticus" bacterial populations in the world. These differences were found not only in samples of distinct geographical origins but also in samples from a single origin and even from a single "*Ca*. Liberibacter asiaticus"-infected sample. This is the first evidence of different "*Ca*. Liberibacter asiaticus" populations coexisting in a single HLB-affected sample. The Florida "*Ca*. Liberibacter asiaticus" isolates contain both *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$, while all other global "*Ca*. Liberibacter asiaticus" isolates contain either one or the other. Interclade assignments of the putative Hyv$_{\mathrm{I}}$ and Hyv$_{\mathrm{II}}$ proteins from Florida isolates with other global isolates in phylogenetic trees imply multiple "*Ca*. Liberibacter asiaticus" populations in the world and a multisource introduction of the "*Ca*. Liberibacter asiaticus" bacterium into Florida.

Citrus huanglongbing (HLB), also known as citrus greening, is one of the most destructive diseases of citrus. Occurring in most of the global citrus-producing regions, HLB affects all citrus cultivars by causing rapid decline and shortening the life span of infected trees (6, 18, 22). From the first documentation in the early 20th century in China (55) to the recent finding in Sao Paulo (44) and Florida (8), this century-old disease has been a major problem for citrus production, especially in the top-producing countries: China, Brazil, and the United States (19). The causal agents of the disease are believed to be three species of *Alphaproteobacteria* in the genus of "*Candidatus* Liberibacter," namely, "*Ca.* Liberibacter asiaticus," "*Ca.* Liberibacter americanus," and "*Ca.* Liberibacter africanus." These fastidious bacteria reside in the phloem of the plant hosts (25) and are vectored by the citrus psyllids *Diaphorina citri* and/or *Trioza erytreae* (6, 22). Of the three bacterial species, "*Ca.* Liberibacter asiaticus" is the most prevalent and draws the most research attention worldwide.

As an obligate plant pathogen and insect symbiont, "*Ca.* Liberibacter asiaticus" has a significantly reduced genome with 1.23 Mb (16). However, "*Ca.* Liberibacter asiaticus" bacteria have evolved into two distinct ecological niches, plant and insect. This small-genome bacterium not only multiplies in most if not all citrus species, citrus hybrids in the genus of *Citrus*, and many other members of the *Rutaceae* family as well as in periwinkle of the *Apocynaceae* family (22) and tomato in the *Solanaceae* family (15) but also multiplies in psyllids, reaching 10^{10} bacteria per individual insect (16). Disease symptoms vary with bacterial titers in HLB-affected citrus and periwinkle plants both in the field and in the greenhouse (57). These differences were noted within the same variety of citrus and periwinkle plants, suggesting that the genetic variations of the pathogen may play important roles in these host-microbe interactions (57).

Annotation of the "*Ca.* Liberibacter asiaticus" Psy62 genome did not reveal any known transposon or insertion sequence (IS) elements (16). 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 bacterium's genome

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and are major contributors to genetic variability, including virulence factors and pathogenicity islands between individuals within species (10). Consequently, prophage DNA can play an important role in the evolution of bacterial pathogenicity (7). *In silico* analyses revealed multiple regions of the "*Ca.* Liberibacter asiaticus" Psy62 genome contained 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 in 2011 by Zhang et al. (54). In these prophage regions, two candidate genes were identified and tentatively named hyv_{I} and hyv_{II}.

Little was known about the genetic diversity of the HLB bacterial pathogens since the bacteria were not cultivable *in vitro* and limited sequence data were available before the recent publication of the complete sequence of the "*Ca.* Liberibacter asiaticus" genome (16). Prior to the availability of the "*Ca.* Liberibacter asiaticus" genome, diversity studies were restricted to 16S/23S rRNA genes, the *omp* gene region, or the *rplKAJL-rpoBC* operon sequence (3, 12, 17, 26, 38, 42, 49, 57). The genetic variation in these housekeeping genes was limited, and most consisted of single-nucleotide polymorphisms (SNPs). However, since the publication of the "*Ca.* Liberibacter asiaticus" genome sequences, a number of loci including simple repeat sequences (SSRs) and prophage genes have been demonstrated to be the better markers for differentiating the "*Ca.* Liberibacter asiaticus" isolates from different geographic origins, including Southeast Asia (47), China versus Florida (11), Japan, Taiwan, Indonesia (28), and two citrus-growing provinces in China (33).

Here we present characteristics and phylogenetics of two closely related prophage genes (hyv_{I} and hyv_{II}) cloned from "*Ca.* Liberibacter asiaticus" isolates in different hosts and geographic origins. Hypervariations in these two genes among "*Ca.* Liberibacter asiaticus" isolates or within a single isolate imply a potentially important mechanism of adaptation available to "*Ca.* Liberibacter asiaticus," although the functions of these genes remain to be determined.

MATERIALS AND METHODS

Bacterial strains, plasmid, and media. TOPO TA vector pCR2.1 (Invitrogen, Carlsbad, CA) was used for cloning PCR products containing the hyv_{I} and hyv_{II} genes. *Escherichia coli* Top10 cells (Invitrogen) were used to maintain the TOPO cloning plasmid. Individual clones of *E. coli* were grown in Luria-Bertani (LB) medium supplemented with 50 mg/ml ampicillin or 50 mg/ml kanamycin at 37°C (40).

"*Ca.* Liberibacter asiaticus"-infected samples and DNA extraction. A total of 266 DNA extracts from "*Ca.* Liberibacter asiaticus"-infected hosts (112 citrus, 36 periwinkle, 20 dodder, and 98 psyllid samples) from Florida were used to characterize the hyv_{I} and hyv_{II} genes. Representative samples with cloning and sequencing analysis are listed below (see Table 2). DNA samples from citrus and psyllids were prepared either by our collaborators or in our laboratory. "*Ca.* Liberibacter asiaticus"-infected citrus samples were collected from citrus groves in different countries and across the state of Florida, including from the United States Horticulture Research Laboratory farm (Ft. Pierce, FL). Some "*Ca.* Liberibacter asiaticus"-infected source plants were propagated by grafting and maintained in the USHRL insect-proof greenhouse (Ft. Pierce, FL). The "*Ca.* Liberibacter asiaticus"-infected periwinkle plants were obtained by dodder (*Cuscuta campestri*) transmission from "*Ca.* Liberibacter asiaticus"-infected citrus, and the repeated propagation of "*Ca.* Liberibacter asiaticus"-infected periwinkles was obtained either by dodder transmission (56) or by graft transmission from "*Ca.* Liberibacter asiaticus"-infected periwinkle to healthy periwinkle (53). The "*Ca.* Liberibacter asiaticus"-infected psyllid DNA used for the identification of hyv_{I} and hyv_{II} genes was the same as that for Psy62 genome sequencing (16). All the other psyllids (*Diaphorina citri*) collected from "*Ca.* Liberibacter asiaticus"-infected citrus plants were subjected to DNA extraction or preserved in 70% ethanol for later extraction.

Total DNA was extracted from dodder haustoria or midribs of citrus and periwinkle as described in 2006 by Irey et al. (24) with some modifications. Briefly, 0.1 g dodder or periwinkle tissue or 0.2 g citrus tissue was cut and transferred to an autoclaved 2-ml screw-cap tube containing 1 silicone-carbide sharp particle (4 mm in diameter) and 3 chrome-steel beads (2.3 mm in diameter) in 800 μl of extraction buffer (100 mM Tris base, 50 mM EDTA, 500 mM NaCl, 2.5% polyvinylpyrrolidone, and 10 mM β-mercaptoethanol); homogenized by a Fast Prep-24 homogenizer (MP Bio., Solon, OH) at speed 4.0 for 60 s; and incubated at 65°C for 30 min after addition of 1.5% SDS (vol/vol). After adding 1/3 volume of 5 M potassium acetate, the tube was incubated on ice for 20 min and centrifuged at 14,000 × *g* at 4°C for 5 min to remove plant debris. The supernatant was centrifuged for another 10 min at the same speed, and 800 μl of supernatant was transferred to a new 1.5-ml tube containing 2/3 volume of cold isopropanol. The resulting DNA pellet was washed once with 70% ethanol and then resuspended in 100 μl of sterile water. DNA from individual psyllids was extracted as described previously (16).

Real-time PCR. TaqMan real-time PCR amplifications were performed in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA), using primers HLBasf and HLBr and probe HLBp targeting 16S rRNA genes of "*Ca.* Liberibacter asiaticus" as listed in Table 1 (31). PCR mixtures with a total volume of 15 μl contained 7.5 μl of TaqMan PCR master mix (Applied Biosystems), 250 nM each primer, 150 nM probe, and 100 ng of template DNA. Primer LJ900fr and probe LJ900p (Table 1) targeting the tandem repeats of hyv_{I} and hyv_{II} genes were employed in the same real-time system, following the protocol described elsewhere (J. K. Morgan et al., submitted for publication). Real-time PCR data were analyzed with Applied Biosystems 7500 system SDS version 1.2 software.

Conventional PCR. All the primers used in this study are listed in Table 1. Primers targeting hyv_{I} and hyv_{II} or their flanking regions were designed using primer analysis software Oligo 7.23 (Molecular Biology Insights, Inc., Cascade, CO). LJ729/LJ730 were used to amplify a 3,513-bp fragment including the hyv_{I} full gene and its flanking region; LJ729/LJ788 were designed for the conserved but partial hyv_{I} region of all "*Ca.* Liberibacter asiaticus" isolates; LJ776/LJ730 or LJ812/LJ1089 were used to amplify hyv_{II} gene and its flanking region. To reduce the error rate in sequencing the hyv_{I} and hyv_{II} genes, high-fidelity platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) was used. One unit of the polymerase was added to a 20-μl reaction mixture containing 1× high-fidelity PCR buffer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 2.0 mM $MgSO_4$, 250 nM forward/reverse primer, and 1.5 μl template DNA. The PCR cycles were started with denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 54°C for 30 s, 68°C for 3.5 min, and a final extension at 68°C for 10 min. For regular PCR testing, 10 μl of 2× buffer D (Epicentre Biotechnologies, Madison, WI), 250 nM forward/reverse primer, 1.25 U *Taq* DNA polymerase (New England BioLabs Inc., Ipswich, MA), and 1 to 2 μl template DNA were added to a 20-μl reaction mixture. The PCR cycles were started with 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50 to 54°C for 30 s, 72°C for 2.0 to 3.5 min for different primer sets, and a final extension at 72°C for 10 min.

Cloning, sequencing, and sequence assembly. PCR products containing the hyv_{I} or hyv_{II} gene from different isolates were ligated into TOPO TA vector pCR2.1 and transferred to TOPO 10 chemical competent cells following the manufacturer's protocol. Plasmid DNA was isolated from *E. coli* cultures by using the QIAprep Spin Miniprep kit (Qiagen). DNA sequencing was performed in the U.S. Horticulture Research Laboratory Core Genomics Facility using BigDye Terminator version 3.1 and the 3730xl DNA analyzer (Applied Biosystems). Sequences were assembled by ContigExpress of Vector NTI (Invitrogen) and analyzed by Align X in Vector NTI.

Bioinformatics and phylogenetic analysis. Tandem repeats finder version 4.04 (4) was used to find repeats in hyv_{I} and hyv_{II} sequences. Bacterial gene predictions were carried out using FGENESB software (SoftBerry Inc., Mount Kisco, NY). BLASTP of WU-BLAST v 2.0 in TIGR CMR server (http://blast.jcvi.org/cmr-blast/) or PSI-BLAST in NCBI (2) was used to predict the potential function of hyv_{I} and hyv_{II} genes.

Phylogenetic relationships for protein sequences were inferred using parsimony and maximum likelihood (ML) methods. For all representative proteins, a multiple sequence alignment was produced in Mesquite version 2.73 using ClustalW version 2.0.12 with default settings (34, 46), followed by manual adjustments. These data were analyzed in ProtTest version 2.4 with the Akaike Information Criterion (AIC), resulting in a best-fit substitution model JTT+G+F (1, 13, 21, 27) for amino acid replacement for both Hyv_{I} and Hyv_{II} proteins. Using maximum parsimony, a heuristic search with random stepwise addition and tree bisection-reconnection (TBR) was implemented in PAUP version 4.0b10 (43). Support was assessed using neighbor-joining (NJ) bootstrapping (1,000 repli-

TABLE 1. Primers and probes used in this study

Name	Sequence (5′–3′)	Target gene or flanking region	Source or reference
Conventional PCR primers			
LJ729	TTGCGACTAAAGACAACGAG	5′ flanking region of hyv_{I} gene	This study
LJ730	TTGCTAGTCTTATCGGCTTATC	3′ flanking region of hyv_{I} and hyv_{II} genes	This study
LJ788	GCCGTTTTCTGAAGGATAAGC	hyv_{I} gene	This study
LJ776	TGAAGTTAAATATCCTGATGGCAAC	hyv_{II} gene	This study
LJ812	CCACGGAATACATCAAAGCTC	5′ flanking region of hyv_{II} gene	This study
LJ1089	TTAGTCATCAAAATTAATAAC	hyv_{II} gene	This study
Real-time PCR primers and probes			
LJ900f	GCCGTTTTAACACAAAAGATGAATATC	hyv_{I} and hyv_{II}	This study
LJ900r	ATAAATCAATTTGTTCTAGTTTACGAC	hyv_{I} and hyv_{II}	This study
LJ900p[a]	ACATCTTTCGTTTGAGTAGCTAGATCATTGA	hyv_{I} and hyv_{II}	This study
HLBasf[b]	TCGAGCGCGTATGC**G**AATACG	16S rRNA genes	31
HLBr	GCGTTATCCCGTAGAAAAAGGTAG	16S rRNA genes	31
HLBp[a]	AGACGGGTGAGTAACGCG	16S rRNA genes	31

[a] 6-Carboxyfluorescein (FAM) at 5′ end; Iowa Black FQ at 3′ end.

[b] Additional "G" (boldface underlined) was added in the HLBas primer sequence (31) based on 16S rRNA gene sequence in the "*Ca*. Liberibacter asiaticus" Psy62 genome, and this modified primer was named HLBasf.

cates). ML inference was obtained using RAxML v7.2.6 (41) on the CIPRES teragrid portal with default settings and JTT, followed by 800 (1,000 for Hyv$_{\text{II}}$) bootstrap pseudoreplicates (37). These trees were then used to construct a majority rule consensus tree in PAUP (43).

Nucleotide sequence accession numbers. The nucleotide sequences of hyv_{I} and hyv_{II} genes and their flanking regions of the global isolates from various geographic origins have been deposited in GenBank under accession numbers YP_003084345.1 and HQ263675 to 263719, as listed in Table 2.

RESULTS

Identification of hyv_{I} and hyv_{II} genes in the "*Ca.* Liberibacter asiaticus" Psy62 genome. While closing gaps for the "*Ca.* Liberibacter asiaticus" Psy2 genome, we found two prophage regions containing a large number of phage-related genes (16, 54). Based on cloning and sequencing of a 3,513-bp DNA fragment (cloning ID pLJ108) from one of the prophage regions, one gene containing multiple nearly identical tandem repeats (NITRs) was identified and named as hyv_{I}. The hyv_{I} gene is 2,760 bp long and putatively encodes a 919-amino-acid (aa) acidic protein (pI = 4.54) with a molecular mass of 103.5 kDa. Using Tandem Repeats Finder (4) and manual arrangements, the intragenic tandem repeat region was identified in the hyv_{I} gene. This region includes 12 full NITRs and 4 partial tandem repeats. Each full repeat is of 132 bp, with three partial repeats of 48 bp sitting between full repeats 6 and 7, 8 and 9, and 10 and 11. There is an additional 33-bp partial repeat at the 3′ end of the entire tandem repeat region (Fig. 1A1). The partial repeat sequences are nearly identical to the first 48 or 33 bp of the 132-bp full repeat. The similarity among 12 full repeats within the hyv_{I} gene was 93 to 100% at the nucleic acid level (see Fig. S1A in the supplemental material) and 82 to 100% at the putative protein level (see Fig. S1B in the supplemental material).

Based on the hyv_{I} gene sequence, the hyv_{II} gene was identified from another prophage region ("*Ca.* Liberibacter asiaticus" Psy62-FP2, 38,551 bp long, GenBank accession number: JF773396) of the "*Ca.* Liberibacter asiaticus" Psy62 genome. The hyv_{II} gene is 1,026 bp long and putatively encodes a 341-aa acidic protein (pI = 5.1) with a molecular mass of 38.9 kDa. In the "*Ca.* Liberibacter asiaticus" Psy62 genome, hyv_{II} contained only one partial repeat unit and shared 92% identity with hyv_{I} on downstream sequence outside 3′ end of the repeat unit (Fig. 1B1).

hyv_{I} and hyv_{II} localized to homologous gene clusters within each prophage region (Fig. 1A1 and B1), and all the genes in these two clusters encoded hypothetical proteins of unknown function. Two genes from corresponding loci flanking 5′ or 3′ of the hyv_{I} and hyv_{II} genes were either identical or closely related at the protein level. A BLAST search of the protein database from 723 prokaryote genomes and the protein database in NCBI yielded little information on the Hyv$_{\text{I}}$ and Hyv$_{\text{II}}$ proteins. In the Hyv$_{\text{I}}$ repeat region, 586 aa shared 25% identity to the leucine-rich repeat protein of *Colwellia psychrerythraea* 34H (GenBank accession number: AAZ26055), and 560 aa exhibited 22% identity with cell wall-associated biofilm protein of *Staphylococcus epidermidis* (ZP_06614153). The N-terminal (133-aa) and C-terminal (195-aa) sequences outside of the repeat region did not share a significant homology with any other protein (E-value better than the threshold) of known function in the databases.

Variations of hyv_{I} and hyv_{II} genes among Florida "*Ca.* Liberibacter asiaticus" isolates from different hosts. A total of 265 DNA isolates were selected from different "*Ca.* Liberibacter asiaticus"-infected host plants and vector psyllids in Florida to evaluate the diversity of hyv_{I} and hyv_{II} genes. The isolates, confirmed "*Ca.* Liberibacter asiaticus" positive with threshold cycle (C_T) values of between 17 and 29 by 16S rRNA gene-based real-time PCR (Table 2), were equivalent to "*Ca.* Liberibacter asiaticus" populations ranging between 2.95×10^8 and 1.08×10^5 bacterial cells/μl DNA extracts based on the grand universal regression equation $Y = 13.82 - 0.2866X$, where X is the mean C_T value and Y is the log concentration of the target DNA copies (32). All the Florida "*Ca.* Liberibacter asiaticus"-positive samples tested positive for both hyv_{I} and hyv_{II} genes by conventional PCR using hyv_{I}- and hyv_{II}-specific primer sets LJ729/LJ788 and LJ812/LJ1089, respectively. All negative control DNA extracted from healthy plant and psyllid

TABLE 2. hyv_{I} and hyv_{II} genes in isolates of "*Ca.* Liberibacter asiaticus" from infected plant or psyllid DNA samples of various geographic origins

Name of "*Ca.* Liberibacter asiaticus" isolate	Source	Yr of collection	Location	C_T value of 16S rRNA gene-based real-time PCR	hyv_{I} gene[a]			hyv_{II} gene[b]		
					Cloning ID	Accession no.	No. of full/partial repeats[c]	Cloning ID	Accession no.	No. of full/partial repeats
Isolates from Florida										
FL-Psy62(MDA)	*Diaphorina citri*	2007	Picos Farm, Ft. Pierce	19.44	pLJ108.1	YP_003084345.1	12/4		HQ263703	0/1
FL-Psy12	*Diaphorina citri*	2007	Picos Farm, Ft. Pierce	19.71	pLJ266.2	HQ263675	2/1	pLJ391.1	HQ263704	0/1
FL-Psy32	*Diaphorina citri*	2007	Picos Farm, Ft. Pierce	19.41	pLJ267.3	HQ263676	11/3	pLJ394.1	HQ263705	0/1
FL-14879	*Citrus* sp.	2008	Hardee	28.92	pLJ148.9	HQ263677	1/6	NC	NC	
FL-15704	*Citrus aurantiifolia*	2008	Osceola	21.26	pLJ150.9	HQ263678	10/4	NC	NC	
					pLJ150.3	HQ263679	0/1			
FL-R8T1	*Citrus maxima*	2007	Picos Farm, Ft. Pierce	27.54	pLJ144.2	HQ263680	6/5	NC	NC	
FL-CG3MV	*Citrus sinensis*	2008	Greenhouse, Ft. Pierce	23.95	pLJ153.1	HQ263681	1/1	NC	NC	
FL-CG8-S	*Citrus limon*	2008	Greenhouse, Ft. Pierce	21.36	pLJ169.2	HQ263682	12/4	pLJ399	HQ263706	0/1
FL-CG8-R	*Poncirus trifoliata*	2008	Greenhouse, Ft. Pierce	18.85	pLJ171.6	HQ263683	5/3	pLJ400	HQ263707	0/1
FL-P1-B1	*Catharanthus roseus*	2008	Greenhouse, Ft. Pierce	19.05	pLJ158.14	HQ263684	12/4	NC	NC	
FL-P1-B2	*Catharanthus roseus*	2009	Greenhouse, Ft. Pierce	19.12	pLJ163.1	HQ263685	2/1	pLJ233	HQ263708	0/1
FL-PP11-B1	*Catharanthus roseus*	2009	Greenhouse, Ft. Pierce	18.94	pLJ168.9	HQ263686	10/3	pLJ401.1	HQ263709	2/4
								pLJ401.3	HQ263710	0/1
FL-PP11-B2	*Catharanthus roseus*	2009	Greenhouse, Ft. Pierce	19.23	NC	NC		pLJ392	HQ263711	0/1
FL-PG15	*Catharanthus roseus*	2008	Greenhouse, Ft. Pierce	18.86	pLJ157.1	HQ263687	12/4	NC	NC	
					pLJ157.3	HQ263688	0/1			
FL-Dod-citrus	*Cuscuta campestri*	2008	Greenhouse, Ft. Pierce	20.86	NC	NC		NC	NC	
FL-Dod-periwinkle	*Cuscuta campestri*	2008	Greenhouse, Ft. Pierce	18.37	NC	NC		pLJ395.3	HQ263712	0/1
FL-Dod-tobacco	*Cuscuta campestri*	2007	Greenhouse, Ft. Pierce	17.87	NC	NC		NC	NC	
FL-Dod-potato	*Cuscuta campestri*	2009	Greenhouse, Ft. Pierce	20.53	pLJ161.5	HQ26389	12/4	pLJ396.2	HQ263713	2/4
								pLJ396.14	HQ263714	0/1
Isolates from China										
CHA-Cit4	*Citrus reticulata*	2009	Fujian	25.56	pLJ341.1	HQ263690	4/4	–	–	
CHA-Cit5	*Citrus* sp.	2009	Fujian	25.49	–	–		pLJ393.1	HQ263715	1/0
CHA-Cit18	*Citrus reticulata*	2009	Fujian	26.65	pLJ316.1	HQ263691	7/2	–	–	
CHA-Psy7	*Diaphorina citri*	2009	Fujian	22.36	–	–		pLJ225	NC	1/0
CHA-Psy42	*Diaphorina citri*	2009	Fujian	24.24	pLJ222.1	HQ263692	7/2	–	–	
TW-Psy17	*Diaphorina citri*	2009	Taiwan	27.07	pLJ402.1	HQ263701	7/2	–	–	
TW-Psy27	*Diaphorina citri*	2009	Taiwan	25.87	pLJ405.2	HQ263702	10/2	–	–	
Isolates from Thailand										
THA-Cit2	*Citrus* sp.	2009	Kamphaeng Phet	26.02	–	–		pLJ232.1[d]	HQ263716	3/3
								pLJ232.2[d]	HQ263717	1/13
THA-TF	*Poncirus trifoliata*	2010	Chanthaburi	25.76	–	–		pLJ397.1	HQ263718	1/0
THA-KP2.3	*Citrus reticulata*	2010	Chanthaburi	24.16	pLJ342.1	HQ263693	7/2	–	–	
THA-Psy25	*Diaphorina citri*	2010	Kamphaeng Phet	28.44	pLJ343.1	HQ263694	5/2	–	–	
THA-Psy22	*Diaphorina citri*	2010	Kamphaeng Phet	26.30	–	–		pLJ398.4	HQ263719	1/0
Isolates from Philippines										
PHA-Psy5	*Diaphorina citri*	2009	Unknown	18.67	pLJ313.4	HQ263695	7/2	–	–	
					pLJ313.5	HQ263696	5/2			
					pLJ313.6	HQ263697	1/2			
Isolates from India										
IND-citrus	*Citrus* sp.	2009	Unknown	22.01	–	–		–	–	
IND-Psy1	*Diaphorina citri*	2009	Unknown	26.63	pLJ315.1	HQ263698	1/1	–	–	
IND-Psy2	*Diaphorina citri*	2009	Unknown	24.66	pLJ314.1	HQ263699	4/1	pLJ286.1		0/0
Isolate from Brazil										
Bra-Cit1	*Citrus* sp.	2007	São Paulo	23.96	pLJ236.1	HQ263700	7/2	–	–	

[a] The hyv_{I} gene was cloned from the LJ729/LJ730 PCR product.

[b] The hyv_{II} gene was cloned from the LJ812/LJ1089 PCR product.

[c] The number of full and partial repeats in hyv_{I} and hyv_{II} genes with the dominant size in each clone library.

[d] Cloning from LJ776/730 PCR product: NC, PCR positive but not cloned; −, PCR negative by all hyv_{I} and hyv_{II} primers.

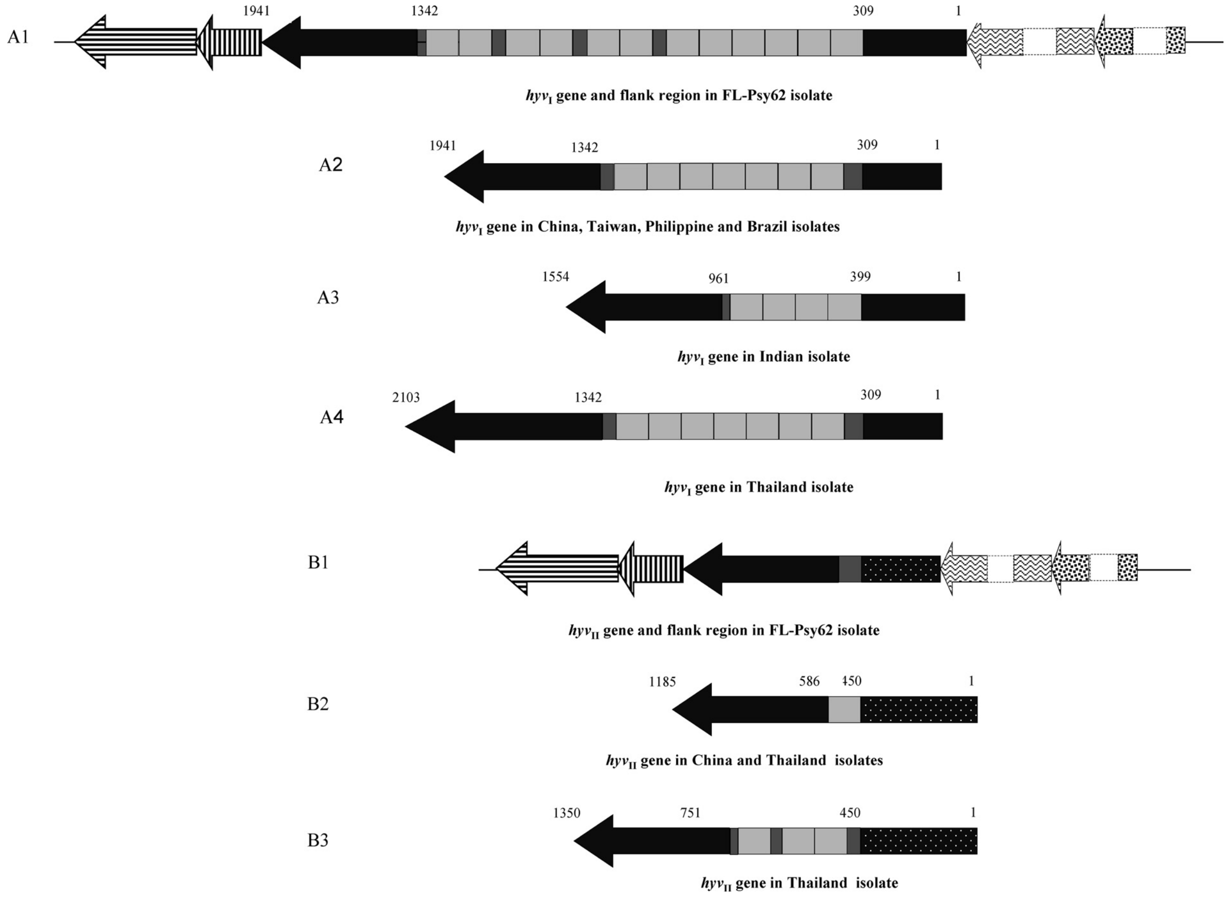

FIG. 1. General features of hyv_I and hyv_{II} genes (scaled) in the genome of "*Candidatus* Liberibacter asiaticus" from various geographic isolates. The black arrow boxes in A1 to A4 and B1 to B3 represent the open reading frames of hyv_I and hyv_{II} genes with different numbers of full or partial repeat units in the middle. Each light grey box in the middle of the gene represents a 132-bp full tandem repeat unit, and each deep grey box stands for partial repeat unit. The numbers above the hyv_I and hyv_{II} genes indicate the gene size and the start and end positions of repeat regions in the hyv_I and hyv_{II} genes. A1 and B1 are hyv_I and hyv_{II} genes with two flanking genes on both 5′ and 3′ ends in the FL-Psy62 genome. Dark horizotal- and vertical-arrow boxes in A1 and B1 represent two hypothetical genes neighboring the 3′ end of the hyv_I (A1) and hyv_{II} (B1) genes; zigzag and large-confetti arrow boxes with dashed outline boxes represent two other hypothetical genes neighboring the 5′ ends of the hyv_I and hyv_{II} genes (not to scale). The spotted box or arrow in B1 to B3 represents the sequence variations (similarity of less than 80%) compared to the sequence in same region within the hyv_I gene or the flanking genes.

samples tested negative for "*Ca.* Liberibacter asiaticus" by conventional PCR.

According to the hyv_I gene sequence from the Psy62 genome (GenBank accession number CP001677), LJ729/LJ788 should amplify a 2,867-bp product. However, different sizes and/or multiple sizes of PCR products were observed in most of the Florida "*Ca.* Liberibacter asiaticus"-infected DNA samples. The hyv_I amplicons showed more variation than those of hyv_{II} (Fig. 2A and B). In addition, variation of the hyv_I amplicons was greater in citrus than in periwinkle, dodder, or psyllid (Fig. 2A). Furthermore, the differences in the hyv_I amplicons were observed not only among different host plants or vector psyllids but also between citrus scions and rootstocks (Fig. 2A, lanes 25 and 26) and among different periwinkle branches with the same HLB inoculum (Fig. 2A, lanes 30 and 31). Most of the samples yielded multiple weak bands in addition to the dominant band for the hyv_I region (Fig. 2A). Eighteen hyv_I and nine hyv_{II} amplicons were cloned and sequenced from the "*Ca.* Liberibacter asiaticus" isolates of different sources. Sequence analyses confirming the presence of different sizes of amplicons belonging to hyv_I or hyv_{II}, and the clone(s) reflecting one to three dominant sizes in each library, are listed in Table 2. Further sequence analyses of these amplicons revealed that the amplicon size differences were due to changes in the repeat (full or partial) numbers and the arrangement of individual units in each hyv_I gene.

The number of full repeats varied from 1 (pLJ153) to 12 (pLJ108), and the arrangement of full and partial repeats in each hyv_I gene was also highly variable (Table 2). However, all repeats were found to be in frame when the gene was translated to protein by FGENESB software. Therefore, deletion or insertion of a full or partial repeat unit did not disrupt their open reading frames in any hyv_I or hyv_{II} clone.

In comparison to hyv_I amplification, hyv_{II} PCR products were more uniform (Fig. 2B). All PCR products from HLB-affected citrus showed one single band. More than one amplicon was observed only in a periwinkle DNA sample (Fig. 2B, lane 30) and a dodder DNA sample (Fig. 2B, lane 37). The

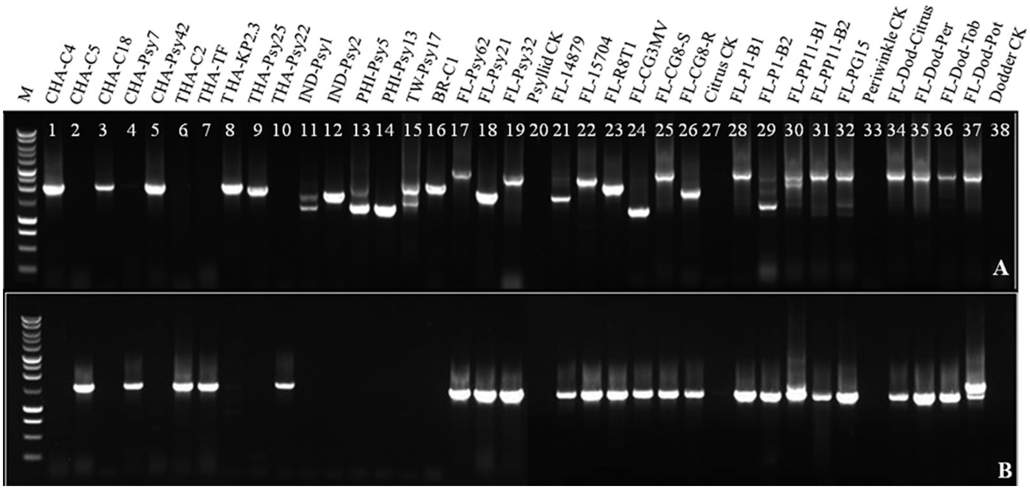

FIG. 2. PCR amplification of the hyv_{I} and hyv_{II} gene target regions in isolates of "*Candidatus* Liberibacter asiaticus" from Florida and other global origins, using primer sets LJ788/LJ729 for hyv_{I} (A) and LJ812/LJ1089 for hyv_{II} (B). Lane M: 1-kb DNA ladder (Promega); lanes 1 to 16, DNA from "*Ca.* Liberibacter asiaticus"-infected plants or psyllids of global origins outside the United States; lanes 17 to 38, DNA from "*Ca.* Liberibacter asiaticus"-infected different hosts in Florida; lanes 17 to 19, DNA from "*Ca.* Liberibacter asiaticus"-positive psyllid from field; lane 20, DNA from "*Ca.* Liberibacter asiaticus"-free psyllid control from greenhouse; lanes 20 to 23, DNA from "*Ca.* Liberibacter asiaticus"-positive citrus from field; lanes 24 to 26, DNA from "*Ca.* Liberibacter asiaticus"-positive citrus from greenhouse, CG8-S/CG8-R leaf samples collected from scion/rootstock of grafted citrus CG8; lane 27, "*Ca.* Liberibacter asiaticus"-free citrus control from greenhouse; lanes 28 to 32, "*Ca.* Liberibacter asiaticus"-positive periwinkle from greening, B1 and B2 from branch 1 and branch 2 in dodder-transmitted periwinkle plants P1 (lanes 28 and 29) and PP11 (lanes 30 and 31), PG from grafted-transmitted periwinkle plant; lane 33, "*Ca.* Liberibacter asiaticus"-free periwinkle control; lanes 34 to 37, "*Ca.* Liberibacter asiaticus"-positive dodder collected from citrus, periwinkle, tobacco, and potato; lane 38, "*Ca.* Liberibacter asiaticus"-free dodder control.

difference in hyv_{II} amplicon sizes was also due to variation in the number of repeat units. The largest amplicon of hyv_{II} was cloned from periwinkle FL-PP11-B1 and contained 2 full repeats and 4 partial repeats. Interestingly, periwinkle DNA sample FL-PP11-B2, isolated from a different branch of the same periwinkle plant as FL-PP11-B1, only had one additional amplicon containing one partial repeat. Similarly, multiple amplicons were observed only in one out of four dodder samples used to transmit "*Ca.* Liberibacter asiaticus" from HLB-affected citrus to the experimental host, potato.

Diversity of hyv_{I} or hyv_{II} genes in "*Ca.* Liberibacter asiaticus" isolates of other global origins. "*Ca.* Liberibacter asiaticus" isolates were kindly provided by our collaborators from HLB-affected citrus plants and psyllids collected in China, Thailand, Philippines, India, and Brazil (Table 2). All these isolates were confirmed "*Ca.* Liberibacter asiaticus" positive with C_T values between 18 and 29 using 16S rRNA gene-based real-time PCR (Table 2), indicating "*Ca.* Liberibacter asiaticus" populations between 1.53×10^{11} and 1.08×10^{8} cells/gram of fresh plant tissue. Unlike the Floridian isolates evaluated, only one gene, either hyv_{I} or hyv_{II}, was detected in these international "*Ca.* Liberibacter asiaticus" isolates (Fig. 2A and B, lanes 1 through 16) regardless of citrus or psyllid origins (Table 2). The numbers of "*Ca.* Liberibacter asiaticus" DNA isolates from "*Ca.* Liberibacter asiaticus"-infected citrus and psyllid samples of global origin are listed in Table S1 in the supplemental material. DNA fragments of the hyv_{I} gene were amplified by the primer set LJ729/LJ730 or LJ729/LJ788 from isolates of "*Ca.* Liberibacter asiaticus"-infected citrus or psyllids in China, Taiwan, Thailand, India, Philippines, and Brazil, while hyv_{II} fragments were obtained by the primer set LJ776/LJ730 or LJ812/LJ1089 only from isolates collected in China and Thailand (Table 2; also see Table S1 in the supplemental material). However, using the same primer sets, neither the hyv_{I} nor the hyv_{II} gene was amplified from eight Indian citrus DNA samples and six Philippine psyllid DNA samples, although all these DNA samples were from HLB-symptomatic tissue and tested "*Ca.* Liberibacter asiaticus" positive with C_T values from 17.7 to 27.8 (Table 3) by 16S rRNA gene-based real-time PCR. To further evaluate the presence or absence of the hyv_{I} and hyv_{II} genes in these samples, a newly developed real-time PCR assay with hyv_{I} repeat-based primers and probe

TABLE 3. Results of 16S rRNA gene- and hyv_{I}- and hyv_{II}-based TaqMan real-time PCR for DNA extracts from HLB-infected Indian citrus and Philippine psyllid samples testing negative by hyv_{I}- and hyv_{II}-based conventional PCR

Name of DNA extract	Source	Real-time PCR C_T value by: 16S rRNA gene-based HLBasfpr	Real-time PCR C_T value by: hyv_{I}- and hyv_{II}-based LJ900fpr
IND-C1-1	Citrus	27.80	33.35
IND-C1-11	Citrus	23.52	27.30
IND-C1-15	Citrus	22.98	32.66
INDC1-23	Citrus	23.31	31.12
IND-C2-15	Citrus	22.01	33.38
IND-C2-16	Citrus	24.66	31.10
IND-C2-17	Citrus	23.05	32.27
IND-C2-18	Citrus	23.24	32.86
PHA-Psy4	Psyllid	17.70	17.15
PHA-Psy7	Psyllid	23.18	23.17
PHA-Psy11	Psyllid	17.74	16.56
PHA-Psy14	Psyllid	19.30	18.18
PHA-Psy16	Psyllid	27.07	26.74
PHA-Psy17	Psyllid	19.83	17.86

LJ900fpr was performed, and this confirmed the presence of the NITR in both the Indian citrus samples and the Philippine psyllid samples (Table 3).

Sixteen clone libraries were made from the PCR amplicons of international "*Ca.* Liberibacter asiaticus" DNA by primers targeting the hyv_{I} and hyv_{II} regions. Sequencing of the hyv_{I} and hyv_{II} genes in international "*Ca.* Liberibacter asiaticus" isolates showed various NITR numbers that were similar to those from the Florida isolates (Fig. 1A2 to A4, B2, and B3; Table 2). The dominant form of hyv_{I} clones from China, Philippines, Taiwan, and Brazil were of the same size and contained seven NITRs and two partial repeats (Fig. 1A2; Table 2). There were three additional sizes of the hyv_{I} gene: a Taiwanese psyllid sample containing 10 and 2 full and partial repeats, respectively, and two Philippine psyllid samples containing 5 and 2 (i) and 1 and 2 (ii) full and partial repeats, respectively. The hyv_{I} upstream and downstream sequences flanking the repeat region shared 93% to 100% similarity among all isolates (see Table S2 in the supplemental material). The hyv_{I} genes from Indian and Thailand were different in terms of both numbers of NITR and downstream sequences flanking the repeat region (Fig. 1A3 and A4; see Table S2 in the supplemental material). The hyv_{II} genes obtained from the Florida, China, and Thailand isolates shared 91% to 100% sequence similarity outside of the repeat region (see Table S3 in the supplemental material). The hyv_{II} gene sequences amplified by LJ776/730 and LJ812/1089 were identical among the Florida and Chinese isolates. However, two variants of the hyv_{II} gene were amplified from a Thai citrus DNA (THA-C2) by two sets of primers targeting the hyv_{II} gene. The primer set LJ812/LJ1089 amplified a sequence identical to the hyv_{II} sequence from China (Fig. 1B2; Fig. 2, lane 6), while LJ776/LJ730 amplified two products with 3 and 3 (i) and 1 and 11 (ii) full and partial repeats, respectively (Table 2). The sequence flanking the repeat region was more closely related to the same region in hyv_{II} from Florida.

Phylogenetic analysis of hyv_{I} and hyv_{II} protein sequences. Based on the variable nucleotide sequences from "*Ca.* Liberibacter asiaticus" isolates of global origins, amino acid sequences deduced from these hyv_{I} and hyv_{II} gene sequences were aligned and integrated into phylogenetic analyses using parsimony and maximum likelihood methods. The leucine-rich repeat protein homolog from *Colwellia psychrerythraea* was employed as an outgroup for both Hyv proteins. The results of these phylogenetic analyses for $\mathrm{Hyv_I}$ and $\mathrm{Hyv_{II}}$ demonstrated that both genes bifurcate into clades on the basis of sampling locations (Fig. 3 and 4). $\mathrm{Hyv_I}$ phylogenetic inferences, containing 30 taxa and 948 informative characters, resulted in a tree with two well-supported clades that reflect the geographic origins of the sample sets (Fig. 3). The samples from Indian psyllids form a well-supported sister clade (100% bootstrap) to the sample set for most of the Florida isolates. The Florida samples were not well resolved by either method and are illustrated in the parsimonious tree as an unresolved polytomy. Two samples from Thailand formed a well-supported sister clade to a psyllid isolate from Philippines, although without bootstrap support.

Although the hyv_{II} gene did not display as much size variability as the hyv_{I} gene, these sequences were also different in "*Ca.* Liberibacter asiaticus" isolates based on geographic origins (Fig. 4). $\mathrm{Hyv_{II}}$ phylogenetic inferences, containing 18 taxa and 584 informative characters, resulted in a tree with well-supported clades whose segregation was based on geographic site of origin. One citrus sample from China and two psyllid samples from Thailand were grouped into one well-supported sister clade to those of the Florida samples. The Florida "*Ca.* Liberibacter asiaticus" isolates clustered into two clades, although without support at deep internodes. Only the clade consisting of periwinkle and dodder samples demonstrated strong branch support (100% bootstrap).

DISCUSSION

The prophage/phage accounts for approximately 1/15 of the significantly reduced "*Ca.* Liberibacter asiaticus" genome (16, 54). Characterization of the variations of these prophages/phages in the HLB bacteria may provide insight into their evolution and adaptation to host plants and insects. Through genomic approaches, two genes (hyv_{I} and hyv_{II}) were identified in two prophage regions of the "*Ca.* Liberibacter asiaticus" Psy62 genome. Although these two genes are closely related and share a homologous repetitive sequence unit, the numbers of repeats and sequences flanking the repeat region are significantly different. These differences were found not only in samples of distinct geographical origins but also from a single origin and even from a single "*Ca.* Liberibacter asiaticus"-infected sample, as indicated by the hyv_{I} gene in citrus FL-15704 and periwinkle FL-PG15. The variation of repeat numbers and sequences flanking the repeat region of hyv_{I} in the same isolate indicates coinfection by different "*Ca.* Liberibacter asiaticus" populations in a single HLB-affected sample. Therefore, HLB disease may be caused by coinfection of either different species of *Ca.* Liberibacter (45) or different stains/isolates of a *Ca.* Liberibacter species. Further investigation of population dynamics in these coinfected samples may reveal their potential roles in HLB disease development and epidemiology. Hypervariation of sequences, especially in the repeat number, were observed in both the hyv_{I} and hyv_{II} genes of the "*Ca.* Liberibacter asiaticus" isolates from Florida, Brazil, China, Thailand, India, and Philippines. The repeat numbers of these two genes varied from 1 partial to 12 full and 4 partials, and no apparent correlations were found between repeat number and bacterial titers, disease symptoms, or different hosts, reflecting the plasticity of the bacterial genome (Table 2 and Fig. 2). Potential mechanisms for deletion or insertion of the tandem repeats include slipped-strand mispairing, unequal crossover, rolling-circle or circle excision, and reinsertion occurring during bacterial replication (30, 39). This size variation caused by different numbers of tandem repeats provides the opportunity for functional diversity and alterations in phenotypes (20, 29, 48). Although the functions of hyv_{I} and hyv_{II} genes are unknown, BLASTP analysis using position-specific iterative basic local alignment search tool (PSI-BLAST) indicated that the repeat region of $\mathrm{Hyv_I}$ protein shares homology with a leucine-rich repeat protein, cell wall-associated biofilm protein, and a cell surface protein. These results suggest that the variations in the intragenic tandem repeats of hyv_{I} gene may be associated with cell surface diversity (36). It is worthy to note that the structure of tandem repeats present in hyv_{I} and hyv_{II} is similar to those in *pthA*, the pathogenicity gene (transcription activator-like effector) of the

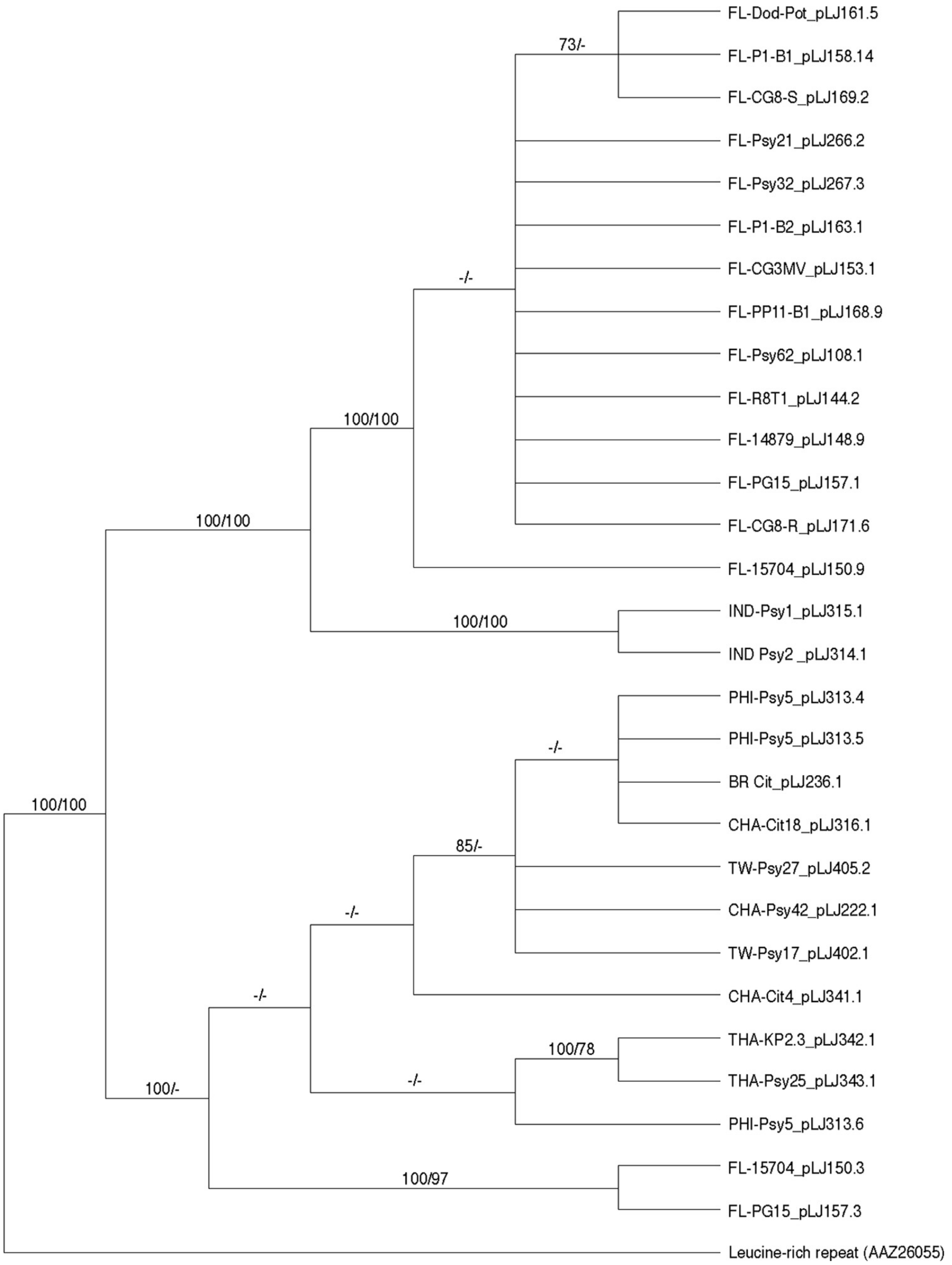

FIG. 3. Majority rule consensus tree based on maximum parsimony analyses of 29 amino acid sequences representing hyv_{I} gene from global isolates of "*Candidatus* Liberibacter asiaticus." The leucine-rich repeat protein sequence from *Colwellia psychrerythraea* is used as the outgroup. Branch support values represent maximum-parsimony bootstrap (≥70%) before the slash and RAxML (≥70%) bootstrap values after the slash. Each branch label reflects the identification listed in Table 2 and indicates the geographic origin of the sample.

citrus canker bacterium (*Xanthomonas citri* pv. citri) (5, 51). *pthA* contains 17.5 copies of a nearly identical tandem repeat unit that encodes 34 amino acids and is responsible for pathogenicity and host specificity (50). It undergoes intragenic recombination resulting in rapid adaptations to new hosts (9, 52), while the expression of *pthA* within plant cells elicits division, enlargement, and cell death, mimicking canker (14). Genes that contain coding tandem repeats often encode proteins with diverse functions, and the variable numbers of tandem repeats affect the pathogenicity or antigenicity in several human/animal pathogens, such as *Treponema denticola* and *Streptococcus agalactiae* (23, 35, 36). Experiments to reveal the functions of the hyv_{I} and hyv_{II} genes are under way.

It is interesting to note that all the Florida isolates in this study contained both the hyv_{I} and hyv_{II} genes, while the isolates from other geographic origins only contained one or the other. The presence or absence of hyv_{I} and/or hyv_{II} genes varied with the origins of the "*Ca.* Liberibacter asiaticus"-infected samples. Nevertheless, at least one of them was always associated with the presence of a "*Ca.* Liberibacter asiaticus" bacterium.

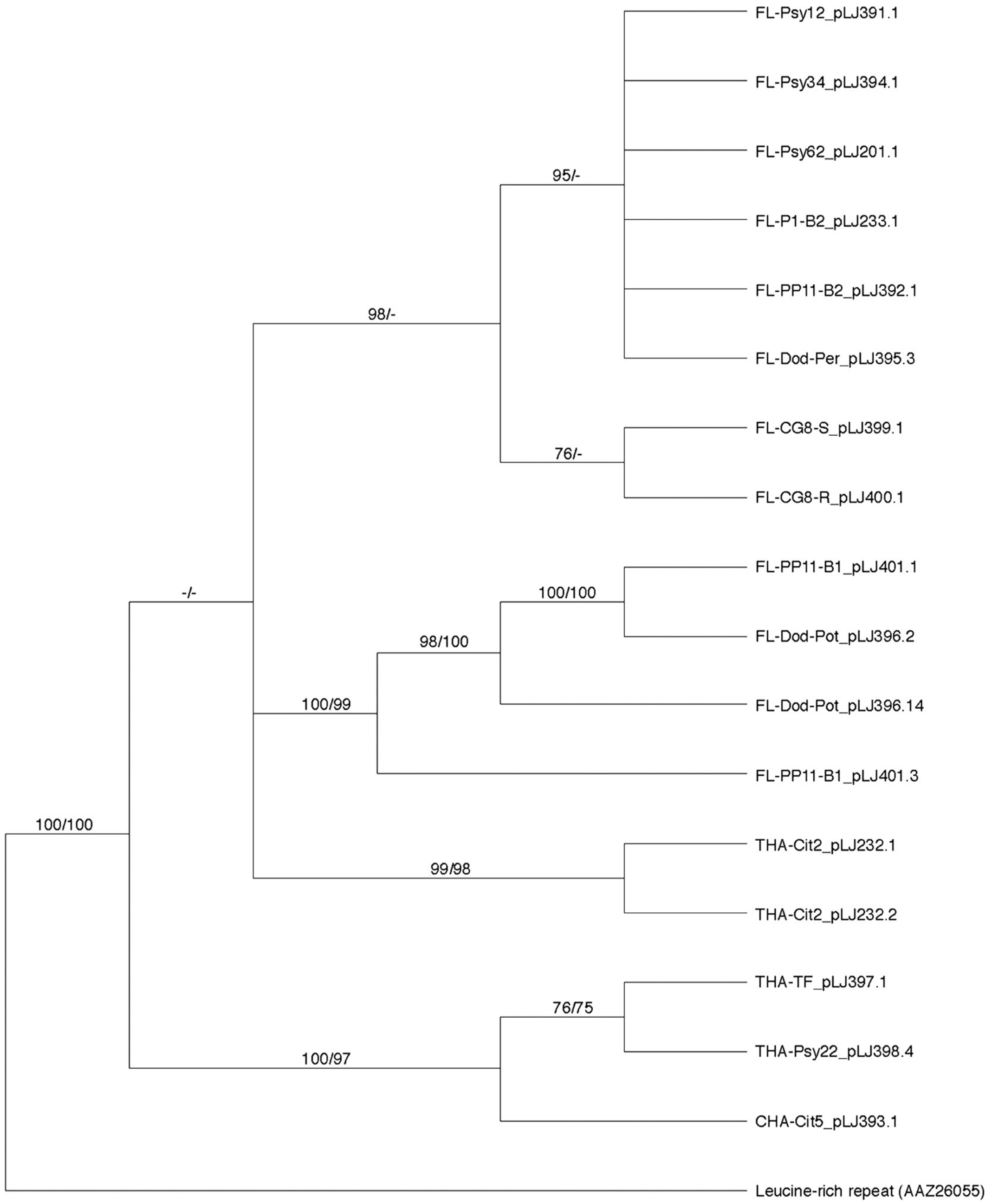

FIG. 4. Majority rule consensus tree based on maximum parsimony analyses of 17-amino-acid sequences representing the *hyv*$_{\mathrm{II}}$ gene from global isolates of "*Candidatus* Liberibacter asiaticus." The leucine-rich repeat protein sequence from *Colwellia psychrerythraea* is used as the outgroup. Branch support values represent maximum-parsimony bootstrap (≥70%) before the slash and RAxML (≥70%) bootstrap values after the slash. Each branch label reflects the identification listed in Table 2 and indicates the geographic origin of the sample.

Results from real-time PCR using the *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ repeat-based primers and probe indicate the presence of NITR in eight Indian citrus and six Philippine psyllid DNA samples that previously tested negative for *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ genes by conventional PCR using *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ gene-based primers. This result implies that more distinct variations of *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ might exist in these isolates. Recent findings revealed two circular phage genomes, SC1 and SC2, which are present in the Florida "*Ca.* Liberibacter asiaticus" bacterial genome (54). The forms of the phage/prophage genomes, circular, linear, or integrated into the "*Ca.* Liberibacter asiaticus" chromosome, varied with their different hosts (54). Since *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ were localized in two different prophage regions of the "*Ca.* Liberibacter asiaticus" genome or the two circular phage genomes, SC1 and SC2, it is hypothesized that the "*Ca.* Liberibacter asiaticus" isolates from the other six countries or regions may contain only one of the two prophage/phages or other unknown prophage/phages with sequences divergent from those of SC1 and SC2. This hypothesis was supported by PCR analyses of different loci, which indicated the presence of only one prophage/phage in these international samples (data not shown).

Phylogenetic placements were inferred for *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ by using two distinct methods. The most parsimonious, yielding the least number of steps on the trees, found several well-supported clades with clear demarcations based on the geographic origin of the sample. Maximum likelihood, utilizing a predicted model of evolution for both Hyv proteins, supported the resulting clades based on sample locations. Interestingly, different hosts—insect, citrus, dodder, or periwinkle—did not influence phylogenetic placements. The clustering of the Florida samples based on the full/partial repeat numbers, syntenic arrangement, or sequences flanking repeat region provides strong evidence of multiple introductions of HLB disease into Florida. Based on the phylogenetic tree established on the basis of the *hyv*$_{\mathrm{I}}$ gene variations, an early introduction of the disease agent (containing only the *hyv*$_{\mathrm{I}}$ gene) may have occurred in India, and the introduction into the majority of the Florida "*Ca.* Liberibacter asiaticus" isolates occurred later. More-recent introductions of the HLB disease into Florida may be evident, as two of the Florida "*Ca.* Liberibacter asiaticus" isolates were placed with good support in the Hyv$_{\mathrm{I}}$ clade with samples from Brazil, Philippines, Thailand, and China, including Taiwan (Fig. 3). In addition, the full *hyv*$_{\mathrm{I}}$ genes from these international "*Ca.* Liberibacter asiaticus" isolates were identical, indicating a high possibility of the same source of "*Ca.* Liberibacter asiaticus" introduction into these four regions. The Hyv$_{\mathrm{II}}$ phylogenetic tree suggests that another major introduction of the HLB disease may have occurred from Thailand to Florida.

The high copy numbers of NITR (132 bp each) in the *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ genes provided excellent targets for the development of more sensitive diagnostic methods. This is especially important for the HLB bacterial pathogens because they are fastidious and often exist at extremely low titers in host plants and insect vectors. Protocols targeting this repeat unit were developed using SYBR green 1 (LJ900fr) and TaqMan (LJ900fpr) methods. Compared to the 16S rRNA gene-based real-time PCR method, an increase in *Ca.* Liberibacter detection likelihood at a range of 100- to 2,000-fold was demonstrated. This highly sensitive method consequently reduces false-negative results frequently associated with other detection methods (Morgan et al., submitted). This method is especially useful in seed transmission studies where "*Ca.* Liberibacter asiaticus" populations are usually at extremely low titer in the seedlings (data not shown). The conservation and variations observed in the sequences flanking the repeats in the *hyv*$_{\mathrm{I}}$ and *hyv*$_{\mathrm{II}}$ genes (Fig. 1; also see Tables S2 and S3 in the supplemental material) provide an excellent resolution of genetic diversity, which is useful for identifying the origins of individual isolates and developing the phylogenetics of "*Ca.* Liberibacter asiaticus" around the world.

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