Evaluation of Genetic Diversity Among '*Candidatus Liberibacter asiaticus*' Isolates Collected in Southeast Asia

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ABSTRACT

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The aim of this study was to investigate the genetic diversity and relationships among '*Candidatus Liberibacter asiaticus*' isolates from different hosts and distinct geographical areas in Southeast Asia. Genetic diversity among '*Ca. Liberibacter asiaticus*' was estimated by sequencing four well-characterized DNA fragments: the 16S ribosomal DNA (rDNA) and 16S/23S intergenic spacer regions; the outer membrane protein (*omp*) gene region; the *trmU-tufB-secE-nusG-rplKAJL-rpoB* region (gene cluster region); and the bacteriophage-type DNA polymerase region. The sequences of the 16S rDNA and 16S/23S intergenic spacer regions were identical among all '*Ca. Liberibacter asiaticus*' isolates. In contrast,

nucleotide substitutions were observed in both the *omp* gene and the gene cluster regions. However, extended bacteriophage-type DNA polymerase sequences acquired by thermal asymmetric interlaced polymerase chain reaction provided the most sequence diversity among isolates. Phylogenetic analysis of the bacteriophage-type DNA polymerase sequences revealed three clusters in the Southeast Asian '*Ca. Liberibacter asiaticus*' population. All Indonesian '*Ca. Liberibacter asiaticus*' isolates clustered in one group. The other clusters were not correlated with geographic distribution. The differences in genetic sequences did not reflect differences in the original citrus host (mandarin or pummelo). These results suggest that the bacteriophage-type DNA polymerase region would be useful for molecular differentiation between different Southeast Asian '*Ca. Liberibacter asiaticus*' isolates.

Additional keywords: citrus greening.

Citrus greening (Huanglongbing) is one of the most destructive diseases that affect citrus trees in many parts of the world (Asia, North America, South America, and Africa). Citrus greening is a threat to the citrus production industry (9). The causal agents of this disease are '*Candidatus Liberibacter spp.*', which are gram-negative, phloem-limited bacteria. The first cultivation of some isolates of these bacteria have been just reported (27). The pathogens are transmitted by the psyllids *Trioza erytreae* in Africa (2) and *Diaphorina citri* in Asia, North America, and South America (13). Compared with *D. citri*, *T. erytreae* is found in cooler areas and at higher altitudes. *D. citri* is more widely spread in warmer lowlands in tropical and subtropical areas. These pathogens are also transmitted by contaminated plant materials used for propagation of nursery plants. Three '*Ca. Liberibacter spp.*' that act as pathogens have been identified: '*Ca. Liberibacter asiaticus*', '*Ca. Liberibacter africanus*' (16), and '*Ca. Liberibacter americanus*' (8).

Infected citrus trees show various symptoms, mainly on their leaves and fruit. However, none of the symptoms are specific to citrus greening (6). In Japan, this disease was found for the first time in the southern-most islands in 1988 (23). Since then, the disease has spread northward to the main production areas where it poses a great threat to domestic citrus cultivation. Citrus greening is still limited to relatively few orchards and backyard gardens in Japan, and it is managed by the removal of infected trees at the early stages of infection.

Assessment of genetic diversity provides a framework for understanding the taxonomy, population structure, and dynamics of phytobacteria. It also provides a key for devising sensitive, specific, and rapid methods for detecting the pathogen, diagnosing plant disease, and managing disease risk (22). Methods to distinguish '*Ca. Liberibacter asiaticus*' isolates are fundamental for ecologic and epidemiologic studies. In epidemiology, the knowledge of genetic variation in the pathogen is important for determining the source of the infection. Molecular techniques are considered to be the most accurate means of fingerprinting microorganisms on a species and subspecies level (22). The comparison of ribosomal DNA (rDNA) sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaeobacteria, and eukaryotic organisms (32). The best-characterized regions of '*Ca. Liberibacter spp.*' are the 16S rDNA and 16S/23S intergenic spacer regions (15,16,24,29). For several Japanese, Philippine, and Indonesian isolates, the nucleotide sequences of these regions are identical. The sequences of these isolates are similar to those from India and China and are distinct from those from Africa (24,29). Thus, comparison of '*Ca.*

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The nucleotide sequence data reported in this article have been deposited in the DDBJ, EMBL, and GenBank databases with the following accession numbers: AB473569-AB473597 and AB480072-AB480161.

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a supplemental table showing the variable nucleotides in the *omp* gene region, the *trmU-tufB-secE-nusG-rplKAJL-rpoB* region, and the bacteriophage-type DNA polymerase region among '*Candidatus Liberibacter asiaticus*' isolates.

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Liberibacter asiaticus' isolates using these genomic sequences has shown little variation among isolates.

A potential gene locus for evaluation is the gene that codes for the outer membrane protein (*omp*), which has a few nucleotide differences among the isolates (3). The strains isolated from pummelo plants in China were highly homogeneous (10). A third candidate for discriminating between strains of '*Ca. Liberibacter asiaticus*' is the *nusG-rplKAJL-rpoB* gene cluster (31). These cluster sequences were extended by Okuda and colleagues (24) and Lin and colleagues (19). However, little is known about the genetic sequence diversity of these gene clusters among '*Ca. Liberibacter asiaticus*' isolates. Okuda and colleagues (24) showed that there were a few nucleotide differences between the Japanese and Indonesian isolates. These previous reports indicated some genetic variation among '*Ca. Liberibacter asiaticus*' isolates but these differences were insufficient to discuss intra-species genetic diversity. The other potential gene locus for evaluation is the bacteriophage-type DNA polymerase region. It became increasingly clear that prophage DNA has played an important role in the evolution of bacterial pathogenicity (7). This region had been characterized as the DNA polymerase of *Bacillus subtilis* bacteriophage SPO2 (31) and not characterized largely thereafter. Also, this bacteriophage belongs to *Siphoviridae*, an unclassified Lambda-like virus.

Knowledge about the genetic diversity of '*Ca. Liberibacter asiaticus*' from different geographical areas and citrus cultivars is indispensable for the management of disease risk. However, there have been few reports about this diversity. Of the isolates examined in the present study, five isolates from Taiwan were carefully examined for pathogenicity in citrus cultivars (30). These isolates had remarkably different pathogenicity among the cultivars. It is unknown whether these biological differences reflect genomic variation. Thus, these isolates from Taiwan were included in this study.

The objectives of this study were to determine the genetic diversity among '*Ca. Liberibacter asiaticus*' isolates using several gene regions. The relation between genetic diversity and the geographic origin of the isolates and citrus cultivars was investigated. The results suggest that the polymorphic bacteriophage-type DNA polymerase region may be useful for molecular differentiation among the Southeast Asian '*Ca. Liberibacter asiaticus*' isolates.

MATERIALS AND METHODS

Isolation of genomic DNA from '*Ca. Liberibacter asiaticus*'.

Except for sources of isolates from Japan (OK901 and KIN1) and Taiwan (TW1, 2, 3, 5, and 6) that were maintained on rough lemon or sweet orange trees in a greenhouse, field plants from Vietnam, Thailand, Indonesia, and Japan were the sources of the genomic DNA from '*Ca. Liberibacter asiaticus*'. Leaf samples were collected from infected citrus trees (mandarin and pummelo) in different groves in subtropical and tropical regions of Southeast Asia (Table 1). Total DNA was extracted from ≈ 0.2 g of the leaf midrib from the infected citrus tree using a DNeasy Plant Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The extracted DNA was suspended in 20 to 200 μ l of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Thermal asymmetric interlaced polymerase chain reaction.

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was performed to determine the nucleotide sequences of the regions adjacent to the *tufB-secE-nusG-rplKAJL-rpoB* gene cluster and the bacteriophage-type DNA polymerase region. The isolate IDN03-5 was used for the TAIL-PCR experiments. Specific primers for the regions upstream and downstream of the known sequences were used in combination with the short arbitrary degenerate (AD) primers reported by Liu and Whittier (21). The primary TAIL-PCR was carried out in a 20- μ l reaction

mixture volumes containing 2 μ l of DNA template, 0.2 μ M specific primer, 5 μ M AD primer, 250 μ M dNTP mixture, 1 $\times$ PCR buffer, and 1 unit of *Ex Taq* DNA polymerase Hot Start Version (TaKaRa, Japan). The resulting mixture was diluted 50 times and subjected to secondary PCR. Tertiary PCR was carried out using the secondary PCR template diluted 50 times. These rounds of TAIL-PCR cycling were performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA) with the condition reported previously (21,24). The secondary and tertiary PCR products (7 of 20 μ l) were separated by electrophoresis in an agarose gel. The specificity of the PCR products is confirmed by the expected overlapping of sequences between the known region and the TAIL-PCR product.

PCR amplification and sequencing. Amplification of the 16S-specific fragment from '*Ca. Liberibacter asiaticus*' was carried out using the primer pair OI1 and OI2c (Table 2) (29). The spacer intergenic region between 16S and 23S rDNA was amplified using the primer pair OI2 and 23S1-rev (29). The reaction mixtures and thermal cycling conditions have been described (29). Amplification of a specific fragment from the *omp* region was performed using the primer pair OMP5F and OMP5R, which correspond to nucleotides 169 to 188 and 3081 to 3100, respectively (GenBank accession no. AY642159) (3). Amplification of the *trmU-tufB-secE-nusG-rplKAJL-rpoB* region (gene cluster region), including newly obtained sequences by use of TAIL-PCR, was carried out using five primer pairs (Table 2). The resulting DNA fragment (GenBank accession no. M94320) contains the putative bacteriophage-type DNA polymerase region and is 988 nucleotides long. Based on this sequence, the primer pair GODNPFW1 and GODNPRV1 was designed (Table 2). Amplification of longer DNA polymerase regions was carried out to produce fragments 1 and 2 (Table 2; Fig. 1) using two primer pairs that were designed after the sequences of the flanking regions were determined by TAIL-PCR. The PCR reactions were performed using an iCycler thermal cycler (Bio-Rad Laboratories Hercules, CA) in 20- μ l reaction mixture volumes containing 2 μ l of DNA template, 1 μ M each primer, 200 μ M dNTP mixture, 1 $\times$ PCR buffer, and 0.5 units of *Ex Taq* DNA polymerase Hot Start Version (TaKaRa). The thermal cycling conditions were as follows: initial denaturing at 94°C for 2 min; 40 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min per kilobase of expected PCR product size; and a final extension step at 72°C for 5 min. The amplified PCR products were separated by electrophoresis in an agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Each purified PCR product was sequenced by primer walking in both directions using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an Applied Biosystems Genetic Analyzer DNA Model 310. Sequence data were assembled using BioEdit version 5.0.9 (14).

Sequence analyses. The open reading frames (ORFs) of the bacteriophage-type DNA polymerase region were searched using GENETYX-WIN computer program (version 3.2.0; Hitachi, Japan). Each ORF sequence was compared with the EMBL and GenBank entries by use of BlastN and BlastX (1). The sequences of the 27 '*Ca. Liberibacter asiaticus*' isolates were first aligned using Clustal X (17). The phylogenetic relationships of the sequences were determined by use of the neighbor-joining algorithm of PHYLIP (version 3.5) (12). For neighbor-joining analysis, a distance matrix was calculated by DNADIST with the Kimura two-parameter option (18). The tree was constructed from this matrix by use of the neighbor-joining method (26). A bootstrap value for each internal node of the neighbor-joining tree was calculated using 1,000 random resamplings with SEQBOOT (11). The calculated tree was displayed by use of TREEVIEW (25).

PCR amplification of fragments of the bacteriophage-type DNA polymerase region. After the sequence of the bacterio-

phage-type DNA polymerase region was determined, five primer sets were designed to amplify various parts of this region. The sequences and positions of the primers are indicated in Table 2 and Figure 1.

RESULTS

Sequences of the 16S rDNA and 16S/23S intergenic spacer regions. Up to 31 isolates of '*Ca. Liberibacter asiaticus*' were collected throughout Southeast Asian countries (Table 1). The reference isolates reported previously were also listed in Table 1. Sequences of the 16S rDNA and 16S/23S intergenic spacer regions of 31 '*Ca. Liberibacter asiaticus*' isolates were determined and compared. The nucleotide sequences of these regions were compared with those previously reported for the isolates OK901, KIN1 (29), and Y02-57, as well as three Indonesian isolates (IDN03-2, IDN03-5, and IDN03-7) (24). All sequences

except that from the India-Poona isolate were identical. Sequence differences observed in the India-Poona isolate consisted of 23 nucleotide changes, as reported by Subandiyah and colleagues (29).

Sequences of the *omp* gene region. The sequences of the *omp* gene region were determined. The sequences obtained from all 31 isolates were 2,832 nucleotides in length. When the *omp* gene region sequences were compared among the 31 isolates sequenced in this study, two nucleotide differences (positions 857 and 2415) were found (supplemental table). At nucleotide position 857, four Japanese isolates (OK901, KIN1, Y02-57, and Ishi1) and one Vietnamese isolate (V61) had adenine at this location whereas the other isolates had thymine at this location. This difference caused a nonsynonymous amino acid substitution from serine to threonine. Nucleotide change at position 2,415 was specific to four Japanese isolates (OK901, KIN1, Y02-57, and Ishi1). The Japanese isolates had an adenine while the other

TABLE 1. Isolates and field sources of '*Candidatus Liberibacter asiaticus*' used in this study

Isolates, field sources	Location, year of collection	Host	Ref.	Sequenced region ^a			
				ITS	omp	Cluster	DNA poly
Indonesian field sources							
IDN03-2	Seberkat, Tebes, Sambas, West Kalimantan, 2003	Siem Pontianak (<i>Citrus reticulata</i>)	24	AB480087	AB480117	AY342001	AB473582
IDN03-5	Sindhuarjo, Ngaglik, Sleman, Yogyakarta, 2003	Siem Mandarin (<i>C. reticulata</i>)	24	AB480088	AB480118	AB480147	AB473583
IDN03-6	Sindhuarjo, Ngaglik, Sleman, Yogyakarta, 2003	Siem Mandarin (<i>C. reticulata</i>)	...	AB480089	AB480119	AB480148	AB473584
IDN03-7	Karangduwur, Kemili, Purworejo, Central Java, 2003	Siem Mandarin (<i>C. reticulata</i>)	24	AB480090	AB480120	AB480149	AB473585
IDN03-17	Tesbatan, Kupang, Kupang, West Timor, 2003	Soe mandarin (<i>C. reticulata</i>)	...	AB480091	AB480121	AB480150	AB473586
B3T1	Buleleng, Bari, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480092	AB480122	AB480151	AB473587
B3T2	Buleleng, Bari, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480093	AB480123	AB480152	AB473588
B3T3	Buleleng, Bari, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480094	AB480124	AB480153	AB473589
B3T4	Buleleng, Bari, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480095	AB480125	AB480154	AB473590
B3T5	Buleleng, Bari, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480096	AB480126	AB480155	AB473591
FC	Purworejo, Central Java, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480097	AB480127	AB480156	AB473592
FD	Purworejo, Central Java, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480098	AB480128	AB480157	AB473593
EJ5	East Java, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480099	AB480129	AB480158	AB473594
K1T2	Kintamani, Bangli, Bari, 2007	Siem Mandarin (<i>C. reticulata</i>)	...	AB480100	AB480130	AB480159	AB473595
PM13	East Java, 2007	Pomelo (<i>C. grandis</i>)	...	AB480101	AB480131	AB480160	AB473596
PM18	East Java, 2007	Pomelo (<i>C. grandis</i>)	...	AB480102	AB480132	AB480161	AB473597
Thailand field source							
Thai1	Thailand, 1997	Som-Keowan (<i>C. reticulata</i>)	...	AB480086	AB480116	AB480146	AB473581
Vietnamese field sources							
V1	Vinh Long, 2004	King Mandarin (<i>C. nobilis</i>)	...	AB480081	AB480111	AB480141	AB473576
V2	Vinh Long, 2004	King Mandarin (<i>C. nobilis</i>)	...	AB480082	AB480112	AB480142	AB473577
VN50	Khanh Hoa, 2004	King Mandarin (<i>C. nobilis</i>)	...	AB480083	AB480113	AB480143	AB473578
V61	Ha Giang, 2004	King Mandarin (<i>C. nobilis</i>)	...	AB480084	AB480114	AB480144	AB473579
V62	Ha Giang, 2004	King Mandarin (<i>C. nobilis</i>)	...	AB480085	AB480115	AB480145	AB473580
Taiwanese isolates							
TW1	Chiayi, Taiwan	Sweet orange (<i>C. sinensis</i>)	30	AB480076	AB480106	AB480136	AB473571
TW2	Hsinchu, Taiwan	Ponkan (<i>C. reticulata</i>)	30	AB480077	AB480107	AB480137	AB473572
TW3	Chiayi-Hualien, Taiwan	Pomelo (<i>C. grandis</i>)	30	AB480078	AB480108	AB480138	AB473573
TW5	Chiayi-Hualien, Taiwan	Pomelo (<i>C. grandis</i>)	30	AB480079	AB480109	AB480139	AB473574
TW6	Miaoli, Taiwan	Ponkan (<i>C. reticulata</i>)	30	AB480080	AB480110	AB480140	AB473575
Japanese isolates, field sources							
OK901	Iriomote, Okinawa, 1988	Rough lemon (<i>C. jambhiri</i>)	23,24,29	AB480072	AB473569	AB473570	...
KIN1	Kin, Okinawa, 1994	Rough lemon (<i>C. jambhiri</i>)	24,29	AB480073	AB480103	AB480133	...
Y02-57	Yoron, Kagoshima, 2002	Tankan (<i>C. tankan</i>)	24	AB480074	AB480104	AB480134	...
Ishi1	Ishigaki, Okinawa, 2005	Unknown local citrus	...	AB480075	AB480105	AB480135	...
Reference isolates							
India-Poona	Poona, India	Not known	3,15,16,31	L22532 U61359	AY642159	...	M94320
China-Behai	Behai, China	Not known	3	...	AY842429	...	...
Nepal-Pokhara	Pokhara, Nepal	Not known	3	...	AY842430	...	...
Philippines-Lipacity	Lipacity, Philippines	Not known	3	...	AY842431	...	...
Thailand-NakhomPathom	NakhormPathom, Thailand	Not known	3	...	AY842432	...	...
GL-1	Guilin, Guangxi, China	Ponkan No1 (<i>C. reticulata</i>)	18	...	...	EF164805 EF164807	...

^a ITS = 16S and 16S/23S intergenic spacer regions, *omp* = the outer membrane protein gene region, Cluster = the *trmU-tufB-secE-nusG-rplKAL-rpoB* region, and DNA poly = the bacteriophage-type DNA polymerase region.

isolates had cytosine. This difference caused an amino acid substitution, also nonsynonymously, from threonine to asparagine.

The *omp* genes (2,346 nucleotides long) of the 31 isolates were compared with the five reference isolates of India-Poona, China-Behai, Nepal-Pokhara, Philippines-Lipacity, and Thailand-NakhomPathom. Nucleotide differences were observed at 17 sites (supplemental table). Of the 17 nucleotide differences, 7 were specific to the India-Poona isolate, which suggests that this isolate is distinct from other isolates from Southeast Asia.

Sequences of the *trmU-tufB-secE-nusG-rplKAJL-rpoB* region. In an effort to identify additional sites of sequence diversity, we extended the genetic region of the *nusG-rplKAJL-rpoB* gene cluster (24); namely, 1,975 nucleotides from the 5' end and 1,496 nucleotides from the 3' end. The combined sequence was 9,616 nucleotides in length. The sequence consisted of several coding regions, beginning with a partial coding sequence for tRNA (5-methylaminomethyl-2-thiouridylyl)-methyltransferase at the 5' end and ending with a partial gene coding for RNA polymerase β subunit at the 3' end. This sequence formed the *trmU-tufB-secE-nusG-rplKAJL-rpoB* region (gene cluster region). The sequences of the gene cluster region (excluding the primer sequences at both ends), which were 9,575 nucleotides long, were analyzed for each isolate.

The nucleotide sequence of each isolate, except for the isolate IDN03-2, was determined. Because there was not enough DNA from IDN03-2 to amplify the gene cluster region, the sequence of the newly extended region could not be determined. Therefore, a partial sequence of IDN03-2 that had previously been published was used (24). The reference sequences (GenBank accession nos. EF164805 and EF164807) were used for comparison among the '*Ca. Liberibacter asiaticus*' isolates. These sequences were from the Chinese isolate GL-1 and corresponded to nucleotide posi-

tions 1 to 3605 and 6521 to 9575 in the newly sequenced gene cluster regions.

When the sequences were compared among the 31 isolates and reference isolate GL-1, 13 nucleotide differences were found (supplemental table). Of these, eight differences were specific to only one isolate. Of the eight specific differences, six were in the isolate GL-1. The other specific differences were at nucleotide positions 3084 and 4259, which were unique to the isolates TW3 and TW2, respectively. The nucleotide differences in the intergenic region were observed at nucleotide positions 165 and 3084. A previous study (24) reported three nucleotide differences, which correspond to nucleotide positions 3760, 6966, and 8061 in this study. The results showed that polymorphisms at any nucleotide position in this region were not sufficient to determine the geographical origin of the '*Ca. Liberibacter asiaticus*' isolate. Of the 13 nucleotide differences, 6 were nonsynonymous. These amino acid differences were observed in the genes *trmU* (leucine to valine), *tufB* (lysine to arginine and histidine to arginine), *rplK* (proline to serine), and *rpoB* (isoleucine to threonine and cysteine to tyrosine).

Sequences of the bacteriophage-type DNA polymerase region. Previously, Villechanoux and colleagues (31) reported the partial sequence of the India-Poona isolate of the bacteriophage-type DNA polymerase gene, which was deposited in GenBank (accession no. M94320). In the present study, this region among 27 isolates, excluding the four Japanese isolates (OK901, KIN1, Y02-57, and Ishi1), was amplified and sequenced. The comparison of this region revealed that the India-Poona isolate was remarkably different from the other isolates. For example, the nucleotide identity was 91.9% between the India-Poona and IDN03-5 isolates. This region was amplified by use of TAIL-PCR with specific primers for the bacteriophage-type DNA poly-

TABLE 2. Oligonucleotide primers for polymerase chain reaction amplification used in this study

Primer name ^a	Nucleotide sequences (5'–3')	Nucleotide position ^b
16S rDNA		
OI1	GCGCGTATGCAATACGAGCGGCA	39–61
OI2c	GCCTCGCGACTTCGCAACCCAT	1,183–1,204
16S/23S ITS		
OI2	ATGGGTTGCGAAGTCGCGAGGC	1,183–1,204
23S1-rev	CGCCCTTCTCTCGCGCTTGA	...
<i>omp</i>		
Omp5F	GCATTCTTGGCTATGTTTTC	229–248
Omp5R	ATTATCGCAGAAGCCTTACA	3,081–3,100
Cluster		
FW38	GAAGTATAGTCAAGACAGTG	–21 to –1
RV2127	CCCGTAATTTCTTCTTGAGC	2,042–2,063
FW1991	GGAGAGTCTTGGTCTCAGTAC	1,927–1,947
RV4101	ACTCTACTGGTGTGACACG	4,019–4,037
FW3988	GTGTCTCTGATGGTCCGTTTG	3,924–3,944
RV6199	CGCAGAAGCAGAAACACCC	6,117–6,135
FW5978	GGTACGCCACAGACTCAAGTTG	5,914–5,935
RV8120	AGCAGACACAACAGGTTTGG	8,037–8,056
FW8016	GGGTTATTGCGTATGGAGCG	7,952–7,971
RV9659	AGCGAAGTCCACCATTTGAG	9,576–9,595
DNA poly		
DNApoly3735FW28	GAGGTGTTACCTACTCTTCGC	–21 to –1
DNP3735R600	GTATCAAGAGCAGGGTACG	533–551
GODNPFW1	TCCTGAGAATTACACACAAAC	786–806
GODNPRV1	TCTAAGTCTATCCTGTAACCC	1,746–1,766
DNP3735F1632	CTCCGTATGACTGTACTCGTGC	1,583–1,604
DNP3735R2390	CTTACCAGCAATCCATGCC	2,322–2,341
DNP3735R2060	CCGATGATGCTAAGCGGTTT	1,992–2,011
GODNPTAFW1	GGGGAGTACTGCAAACGAGAC	1,633–1,653
DNP3735R2934	CAATCGCCCTTTACATCACTG	2,865–2,885
DNP3735F2534	GGGGCTAAGGTGTTTAAGAC	2,485–2,504
DNApoly3735RV3680	AAGGACAGACGAGGATCAAG	3,611–3,631

^a rDNA = ribosomal DNA; ITS = intergenic spacer regions, with primers reported by Subandiyah and colleagues (28); *omp* = the outer membrane protein gene region; Cluster = the *trmU-tufB-secE-nusG-rplKAJL-rpoB* region; and DNA poly = the bacteriophage-type DNA polymerase region.

^b Nucleotide positions are numbered following the sequences in the GenBank database: AY642159 for the *omp* gene region, AB473570 for the gene cluster region, and AB473571 for the bacteriophage-type DNA polymerase region.

merase, which amplified 807 nucleotides from the 5' end and 1,858 nucleotides from the 3' end of M94320. The combined sequence was 3,653 nucleotides long. Excluding the primer sequences from each end, the 3,610 nucleotide sequences were analyzed for each isolate (Fig. 1). The primers designed to amplify the whole region of the sequence detected a DNA fragment of the expected size from all except four Japanese isolates. This region was not amplified from the four Japanese isolates in spite of repeated attempts using five primer pairs which cover the overall region of the bacteriophage-type DNA polymerase (Fig. 2). Nucleotide sequence alignment showed that the sequences of the 27 isolates could be roughly classified into two sequence types. One type was 3,610 nucleotides long and the other was 3,609 nucleotides long. One nucleotide deletion was observed at nucleotide position 486 (supplemental table).

A phylogenetic tree was constructed based on the nucleotide sequences of the 27 isolates of '*Ca. Liberibacter asiaticus*' for which the bacteriophage-type DNA polymerase region could be amplified. The relationships among the isolates were investigated by neighbor-joining. The resultant tree was separated into three groups (Fig. 3, groups A, B, and C). All of the 16 Indonesian isolates belonged to group A. Although the isolates V62 (Vietnamese) and TW1 (Taiwanese) were in group A, most Vietnamese and Taiwanese isolates were in group B. Group C appears to be a minor group, consisting of only the isolates of Thai (Thailand) and VN50 (Vietnamese).

Four putative ORFs encoding proteins longer than 100 amino acids were identified from the obtained nucleotide sequences (Table 3). Of the four ORFs, the functions of ORF1, 2, and 4 were unknown. However, ORF3 was predicted to code for a DNA polymerase. In a previous study, Villechanoux and colleagues (31) suggested that the part of the bacteriophage-type DNA polymerase sequence was a SPO2-like DNA polymerase. The present

study revealed complete amino acid sequence of this putative protein. ORF3 and ORF4 of the bacteriophage-type DNA polymerase region were similar to *Bordetella* phage BPP-1 coding proteins (20).

DISCUSSION

In this study, new sequences of the *trmU-tufB-secE-nusG-rp/KAJL-rpoB* region and the bacteriophage-type DNA polymerase region of '*Ca. Liberibacter asiaticus*' were characterized, and genetic diversity among the '*Ca. Liberibacter asiaticus*' isolates, including these regions, was determined. The degree of genetic diversity was different in each analyzed region, and the most genetic diversity was found in the bacteriophage-type DNA polymerase region. The nucleotide sequence of this region allowed the '*Ca. Liberibacter asiaticus*' isolates to be separated into distinct groups, suggesting that these differences could be used to differentiate between the Southeast Asian '*Ca. Liberibacter asiaticus*' isolates.

TAIL-PCR is the one of the most efficient methods used to obtain unknown sequences adjacent to known sequences (21). Two regions (the gene cluster region and the bacteriophage-type DNA polymerase region) were extended by an additional 9.6 and 3.6 kbp, respectively. Recently, additional sequences from the gene cluster region have been reported (19). The sequences from the present study were included in the analysis, and the gene composition was the same. Genome walking applications are also useful to obtain unknown sequences adjacent to known sequences (19). By using these methods, more information about '*Ca. Liberibacter asiaticus*' will continue to accumulate.

Genetic diversity in bacteria can be assessed by examining specific restriction enzyme sites, repetitive elements, amplicons produced by random primers, or genome sequences (22). There

Fig. 1. Schematic representation of gene organization of the bacteriophage-type DNA polymerase region and positions of primers used for polymerase chain reaction amplification. The amplified DNA fragments (fragments 1 and 2) for sequencing are shown in gray boxes. The amplified DNA fragments (fragments A to E) for verifying the absence of bacteriophage-type DNA polymerase region are shown in hatched boxes. For sequences of primers, see Table 2. For putative products of open reading frames (ORFs), see Table 4.

have only been a few reports of the genetic diversity among '*Ca. Liberibacter asiaticus*' isolates. In these reports, only one DNA fragment has been investigated in several isolates from a limited geographical area (3,10,24,29). It has been reported that the 16S rDNA and the 16S/23S intergenic spacer regions are highly conserved among isolates in Southeast Asia (29). Among the 31 isolates sequenced in the present study, no nucleotide differences were observed, which confirms the previous results. The highly conserved nucleotide sequence among the '*Ca. Liberibacter asiaticus*' isolates in the 16S rDNA and 16S/23S intergenic spacer region can be useful for developing detection methods. For example, the conservation of nucleotide sequences was indispensable when gene diagnosis was performed using the PCR and LAMP methods, as reported by Okuda and colleagues (24).

The isolates used in this study originated from Southeast Asian countries, including Indonesia, Thailand, Vietnam, Taiwan, and Japan. The nucleotide sequences of all four regions' fragments analyzed suggest that Southeast Asian isolates of '*Ca. Liberibacter asiaticus*' are remarkably different from the India-Poona isolates. The differences in the bacteriophage-type DNA polymerase region are especially striking. Among the Southeast Asian isolates, the nucleotide sequences in the 5' region of the bacteriophage-type DNA polymerase region (positions 1 to 600) can be classified into two groups (A and B). The nucleotide sequences of the group C isolates Thai1 and VN50 have chimera-like nucleotide sequences, which were possibly produced as a result of recombination. However, no evidence of recombination was observed in this study, because this region did not have adequate polymorphisms to allow statistical analysis of recombination (data not shown). One nucleotide deletion at position 486 was common among the isolates in group B, which included most of the Vietnamese and Taiwanese isolates. The nucleotide differ-

ences unique to one isolate observed in the 3' region of the bacteriophage-type DNA polymerase region reflected the intra-group relationships analyzed in this study (Fig. 3). This is the first evidence of nucleotide differences unique to one isolate in this gene region of '*Ca. Liberibacter asiaticus*'. These differences may be useful for monitoring the movements of '*Ca. Liberibacter asiaticus*' in the field. Some isolates could not be differentiated from the others, and further investigation is needed to explore more genetic markers. This is especially true for the Japanese isolates, which apparently lack the bacteriophage-type DNA polymerase region. In the expanded bacteriophage-type DNA polymerase sequences, ORF3, which was completely sequenced in this study, was predicted to code for a phage-type DNA polymerase. The bacteriophage-derived genes have been found in the genome of one of the citrus pathogenic bacteria, *Xylella fastidiosa* (4,5). Moreover, a comparative analysis of the complete genomes of *X. fastidiosa* strains revealed that some strains lack bacteriophage-related genes. This is considered direct evidence of phage-mediated horizontal gene transfer (28). Existence of a homologous gene suggests that a similar gene transfer occurred in the evolutionary history of '*Ca. Liberibacter asiaticus*'.

On the basis of sequence differences in the four regions determined in this study, '*Ca. Liberibacter asiaticus*' can be classified into five groups. The first group, group A, consists of mainly Indonesian isolates. Group B consists of mainly Vietnamese and Taiwanese isolates. These two groups include the majority of '*Ca. Liberibacter asiaticus*' isolates from Southeast Asia. The third group, group C, included isolates Thai1 and VN50. The fourth group most likely includes the India-Poona isolate. Using the sequence information from the India-Poona isolate, the partial sequence of the bacteriophage-type DNA

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction (PCR) products amplified from the bacteriophage-type DNA polymerase region using the five primer sets. A to E, Results of PCR amplification for fragments A to E, respectively. The positions of these regions are shown in Figure 1. Lanes 1 to 5 are TW6 (positive control), OK901, KIN1, Y02-57, and Ishi1 isolates from Japan, respectively; lane 6 is the negative control of healthy rough lemon; lane M indicates 100-bp DNA size markers. DNA bands of expected size are indicated by arrows.

Fig. 3. Neighbor-joining tree calculated from 27 isolates of the bacteriophage-type DNA polymerase region sequences of '*Candidatus Liberibacter asiaticus*'. Numbers at each node indicate the percentage of supporting bootstrap samples (only values >60 are shown). Branch length is drawn to scale with the bar indicating 0.01 nucleotide replacements per site.

TABLE 3. Open reading frames (ORFs) predicted from the bacteriophage-type DNA polymerase region sequences obtained from 27 '*Candidatus Liberibacter asiaticus*' isolates

ORF	Start	Stop	No. of codon	Most closely related GenBank entries (GenBank accession no.)	Blastx E value	Putative product	Gene product showing amino acid sequence similarities with ORF found in this study
1	181	480	100	Deep-sea thermophilic <i>Geobacillus</i> bacteriophage GVE2 ORF31 (DQ453159)	0.17	Unknown	N-terminal region (amino acids 10–180) had 36% amino acid similarity with GVE2.
2	502	1,149	216	' <i>Ca. Liberibacter asiaticus</i> ' (M94320)	5e-60	Unknown	Also similar to the P50 of <i>Acyrtosiphon pisum</i> bacteriophage (APSE-1 and 2) (E = 2e-10 and e-10, respectively).
3	1,156	3,180	675	<i>Bordetella</i> phage BPP-1 bpp42 (AY029185)	e-121	DNA polymerase	Also similar to the phage-related DNA polymerase from <i>Xylella fastidiosa</i> strains (E ≤ e-112) and <i>Candidatus Liberibacter asiaticus</i> (partial 206 amino acids; E = e-106).
4	3,180	3,488	103	<i>Bordetella</i> phage BPP-1 bpp43 (AY029185)	5e-11	Unknown	Also similar to the phage-related or VRR_NUC domain from <i>Xylella fastidiosa</i> strains (E ≤ 4e-8).

polymerase region was compared. Remarkable sequence differences were observed in this region. These striking differences were not observed among the Southeast Asian isolates sequenced in this study. The fifth '*Ca. Liberibacter asiaticus*' group included four Japanese isolates, which lack the bacteriophage-type DNA polymerase region, that were unique from the others. The phylogenetic relationships of the Japanese isolates cannot yet be determined because a highly variable DNA fragment, such as the bacteriophage-type DNA polymerase, has not been found. Future research will include a search for a new variable DNA fragment that can be used to distinguish Japanese isolates from each other.

Citrus cultivars show different degrees of susceptibility to citrus greening (9). In general, sweet orange (*Citrus sinensis*) and mandarin (*C. reticulata*) are the most susceptible, and pummelo (*C. grandis*) and rough lemon (*C. jambhiri*) are tolerant. '*Ca. Liberibacter asiaticus*' isolates were collected from several Southeast Asian countries, and this study was performed to examine the correlation between citrus host cultivars and '*Ca. Liberibacter asiaticus*' genome sequences (Fig. 3). In this study, a correlation between Indonesian isolates collected from mandarin and pummelo was investigated. All of these Indonesian '*Ca. Liberibacter asiaticus*' belonged to group A. The result suggested that the population structure of Indonesian '*Ca. Liberibacter asiaticus*' is

homogeneous, regardless of the type of citrus host and location. No genetic evidence for a host determinant was found in '*Ca. Liberibacter asiaticus*' isolates. For the other countries (Vietnam, Thailand, Taiwan, and Japan), there were not sufficient numbers of isolates to allow a conclusion about the relationships between genotype and citrus host cultivar to be made. Further investigation is necessary to explore for genes that are closely associated with the pathogenicity of '*Ca. Liberibacter asiaticus*'.

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