academicJournals

Vol. 8(30), pp. 2861-2873, 23 July, 2014
DOI: 10.5897/AJMR2014.6706
Article Number: 246ED7D46365
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparative analysis of 16S ribosomal RNA of '*Candidatus* Liberibacter asiaticus' associated with Huanglongbing disease of Persian lime and Mexican lime reveals a major haplotype with worldwide distribution

Moreno-Enríquez A.[1], Minero-García Y.[1], Ramírez-Prado J. H.[2], Loeza-Kuk E.[3], Uc-Varguez A.[4] and Moreno-Valenzuela O. A.[1]*

[1]Centro de Investigación Científica de Yucatán, A.C (CICY), Unidad de Bioquímica y Biología Molecular de Plantas, Yucatán, México.
[2]Centro de Investigación Científica de Yucatán, A.C (CICY), Unidad de Biotecnología, Yucatán, México.
[3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) Campo Experimental Mocochá, Yucatán, México.
[4]Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C.(CIATEJ) Unidad Sureste, México.

Received 11 February, 2014; Accepted 13 June, 2014

Huanglongbing (HLB) is considered as one of the most destructive and devastating disease of citrus trees worldwide. In the present study, we analyzed the genetic diversity of the 16S rDNA gene sequence of '*Candidatus* Liberibacter asiaticus' ('*Ca.*L. asiaticus') strains from symptomatic trees of *Citrus latifolia* and *Citrus aurantifolia* with HLB and performed comparative analysis with sequences in the GenBank database. Two hundred and fourteen (214) samples of trees collected in the Yucatán Peninsula were analyzed using PCR amplification and real-timeq PCR. We detected twenty-five positive trees by PCR, although the positive trees were increased to seventy by real-time q PCR. Blast searches of 75 sequences of 1167 bp in size obtained by PCR-positive trees revealed 99 to 100% similarity with sequences of '*Ca.*L. asiaticus'. Multiple alignment showed 2 clusters of sequences, wherein the first group were 100% identical, and the second group was characterized by the presence of polymorphic sites distributed across the gene sequence. The consensus sequence of the first group was named H36PENINSULAR. Analysis of the 16S rDNA sequences from GenBank revealed the same behavior, wherein one group exhibited 100% similarity (within the group). Alignment of the first groups from each of the two analyses above revealed a complete match between them (100% similarity). Accordingly, we suggest that sequence H36PENINSULAR be considered the principal haplotype of the 16S rRNA gene of '*Ca.*L. asiaticus' because it exhibits worldwide distribution and dominance.

Key words: Huanglongbing, 16s rRNA gene, '*Candidatus* Liberibacter asiaticus', Mexican lime, Persian lime.

INTRODUCTION

Huanglongbing (HLB) or citrus greening, is considered the most destructive and devastating disease of citrus

trees worldwide (Gottwald et al., 2007). The disease affects almost all major citrus fruit trees, with sweet oranges, mandarins and mandarin hybrids being most affected (Bové, 2006). HLB has spread throughout the majority of citrus-producing countries with millions of dollars lost for growers. The disease is feared worldwide because citrus trees, once infected, will irrevocably deteriorate. In the course of many years, no effective treatments for the disease existed and successful control involves preventing trees from becoming infected (Teixeira et al., 2008). HLB disease management involves three principal components, control of the insect vector *Diaphorinacitri* by chemical and biological methods, planting pathogen-free nursery stock and removing the inoculum by destroying infected trees (Grafton-Cardwell et al., 2013). Currently, the use of controlled heat treatments to cure HLB caused by '*Candidatus* Liberibacter asiaticus' ('*Ca.*L. asiaticus') using continuous thermal exposure to 40 to 42°C for a minimum of 48 h was sufficient to significantly reduce titer or eliminate *'Ca.* L. asiaticus' entirely in HLB-affected citrus seedlings (Hoffman et al., 2013).

The HLB disease caused by a phloem-limited bacterium was originally described by Garnier et al. (1984) using electron microscopy as an intracellular pathogen, and was included in the α-*Proteobacteria* subdivision. Three identified species are the causative agents, '*Candidatus* Liberibacter africanus' ('*Ca.* L.africanus'), '*Candidatus* Liberibacter americanus' ('*Ca*. L. americanus'), and '*Ca.L*. asiaticus' (McClean and Oberholzer, 1965; Capoor et al., 1967; Bové, 2006). Nonetheless, given that axenic cultures of these bacteria have been difficult to obtain because it is an obligate pathogen, molecular techniques are essential tools for identifying and analyzing the phylogeny and taxonomy, by means of amplification of 16S rDNA. Diagnosis of HLB is made by means of PCR in leaves of diseased trees with various symptoms such as blotchy mottling, yellowing veins and green islands, wherein the bacterial titer is generally high (Teixeira et al., 2008). Real-time quantitative PCR (qPCR) is another method that has been used for detection and quantification of the pathogen. In plants and insect vectors, positive amplification was achieved with as few as 10 cells per PCR reaction and the presence is detected even at low levels of the pathogen (Li et al., 2006, 2007, 2008; Wang et al., 2006; Teixeira et al., 2008).

Before the complete genome sequence of '*Ca.* L. asiaticus' (ASM2376V1) was reported, information on the genetic diversity of HLB pathogens was scarce (Duan et al., 2009). Diversity studies were restricted to the analysis of sequences from 16S/23S genes, the *omp* gene region, or the rplKAJL-rpoB operon (Villechanoux et al., 1992; Planet et al., 1995; Jagoueix et al., 1997; Subandiyah et al., 2000; Bastaniel et al., 2005). Particularly, analysis of the 16SrDNA region has been used to estimate the genetic diversity among worldwide strains with many Asian strain having identical 16SrRNA sequences, e.g., sequences from Japan, Taiwan, Indonesia, Philippines, Vietnam, and Thailand (Jagoueix et al., 1994; Subandiyah et al., 2000; Tomimura et al., 2009). Furthermore, numerous single nucleotide polymorphisms (SNPs) identified using restriction fragment length polymorphism (RFLP) have been reported in one Chinese and two Indian strains collected in Karnataka in the southwest of India (Adkar-Purushothama et al., 2009).

In México, the first HLB-infected tree was detected in 2009 in the municipality of Tizimin, Yucatán and subsequently in the states of Quintana Roo, Nayarit, and Jalisco. Afterward, the occurrence of HLB was confirmed in different localities of Campeche, Colima, Sinaloa and Michoacán (Senasica-Sagarpa, 2010). We initiated a study with the aim to detect the pathogen '*Ca.* L. asiaticus' in citrus trees on the Yucatán Peninsula with the classical symptoms of the HLB disease. Here we reported the genetic diversity of the16S rRNA gene of '*Ca.* L. asiaticus' strains from symptomatic citrus plants of the species *Citrus latifolia* and *Citrus aurantifolia*. Analyzing the sequences from Mexico and other countries, we identified a universal haplotype (H36PENINSULAR) with worldwide distribution and detected in both the citrus species and the insect vector.

MATERIALS AND METHODS

Plant samples

The plant samples were collected during the years 2010 and 2011 in plantation fields and backyard trees of *C. latifolia* Tanaka (Persian lime) and *C. aurantifolia* Christmann (Mexican lime) located in Yucatán, Quintana Roo, and Campeche States, México. Leaves were sampled from the citrus plants with the characteristic HLB symptoms such as blotchy mottling, yellowing veins and green islands. Vegetal material was stored at 4°C and transported to the laboratory for DNA extraction. The leaves of five healthy citrus plants were used as a control.

Total genomic DNA extraction

Leaves were rinsed twice with sterile distilled water and twice with 95%v(v/v) ethanol and midribs were cut with a sterile scalpel. One-tenth of a gram of midribs was macerated with a pestle in a mortar containing liquid nitrogen. DNA was extracted using the CTAB method (Murray and Thompson, 1980). To eliminate impurities from the DNA preparation, samples were processed twice with one volume of phenol-chloroform (1:1) and once with chloroform. Precipitation of the DNA was performed with 3 M sodium acetate at pH 5.0 ($^1/_{10}$ of volume) plus 1 volume of 2- propanol.

*Corresponding author. E-mail: oamv@cicy.mx. Tel: +52-9999428330. Ext: 286.

The resulting DNA solution was treated with RNAse H and stored at -70°C.

Cloning and sequencing of the 16S rRNA gene

PCR amplification of the 16S rRNA gene was carried out using REDTaq polymerase (Sigma-Aldrich) and oligonucleotides OI1/OI2C (Jagoueix et al., 1994). The reaction was performed in a total volume of 25 µl containing 1X PCR buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 5 ng of template DNA, and 1 unit of DNA polymerase. The PCR conditions were as follows: an initial denaturation step of 94°C for 2 min; 40 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; finally, 72°C for 10 min. Amplified fragments of 1167 bp were purified using the Qiaex II Gel Extraction Kit (Qiagen) and then cloned into the pGEM T-Easy Vector (Promega). Recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing of the plasmids was performed on both DNA strands.

Escherichia coli cells were grown in the Luria-Bertani medium and standard procedures for the growth and transformation of the cells were used (Sambrook and Rusell, 2000). Ampicillin was added at a final concentration of 100 µg/mL.

Analysis of 16S rRNA sequences of 'Ca.L. asiaticus' and phylogenetic tree construction

The sequences were assembled and trimmed using the Sequencher software, version 5.0 (Gene Codes Corporation, Ann Arbor, MI USA [http://genecodes.com]). Edited sequences were analyzed for similarity using the BlastN program, and 16S rDNA gene sequences were retrieved from the non redundant NCBI database (http://blast.ncbi.nlm.nih.gov/).Selected sequences were aligned using the ClustalW software configured for highest accuracy (Larkin et al., 2007). The phylogenetic relationships were determined using the Neighbor-Joining algorithm in the Mega 4 software (Tamura et al., 2004), and the Kimura 2-parameter statistical model was applied (Kimura, 1980). The confidence of the grouping was verified using bootstrap analysis (1000 replications). *Sinorhizobium meliloti* RFP1 (EU271786), *Rhizobium etli* CFN42 (NR029184), and *Escherichia coli* (J01859) were used as outgroup.

Ninety-three sequences of the '*Ca.*L. asiaticus' 16SrRNA gene, at least 1000 bp in size were retrieved from the GenBank sequence database (Benson et al., 2005). Sequences were edited and phylogenetic analysis was performed as described above.

Identification of single nucleotide polymorphic sites and haplotype designation

All the DNA sequences of 16S rRNA gene were aligned using Sequencher software version 5.0. The identical sequences were separated and the group was realigned to confirm the percentage of identity. A representative sequence was used for realignment with the sequences with an identity value below 99.5%. Single nucleotide polymorphisms (SNPs) were identified visually (den Dunnen and Antonarakis, 2001). A sequence was considered a haplotype when 2 or more samples had a mutation in the same position (Arias et al., 2010). The SNPs and haplotypes was confirmed by means of the DnaSP software version 5.0 (Librado and Rosas, 2009). Definition of SNPs for the 16S ribosomal gene sequences of '*Ca*. L. asiaticus' obtained from the GenBank database was performed with a multiple alignment by using the Mexican sequence as reference to identify the positions of the SNP's.

Real-time qPCR

TaqMan amplification reactions were performed on a Real-Time PCR StepOnethermocycler (Applied Biosystems, Foster City, California, USA). PCR amplifications were performed with EXPRESS qPCRSupermix Universal (Invitrogen) in a 20 µl reaction containing 10 µl 2X qPCRSuperMix, 25 µM ROX reference dye, 250 nM of each target primer (HLBas and HLBr), and 150 nM of target probe (HLBp). For positive internal control, 300 nM (each) internal control primers (COXf and COXr), 150 nM internal control probe (COXp) were used (Li et al., 2006).The cycling amplification conditions were 95°C for 2 min followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. To exclude false-positive results some control reactions with genomic DNA sample from a PCR-positive HLB-diseased plant, DNA from five healthy citrus plants and distillated water were done. A second real-time PCR assay was carried out with positive reactions.

RESULTS AND DISCUSSION

Detection of '*Ca*.L. asiaticus'

PCR amplification of the 16S rRNA gene of '*Ca.*L. asiaticus' was carried out for 214 genomic DNA samples purified from leaf samples collected from HLB-symptomatic and asymptomatic citrus trees from Campeche, Yucatán, and Quintana Roo. '*Ca.* L. asiaticus' were detected in 25 trees; 6 of the leaf samples were collected from citrus plants in Campeche, 7 in Quintana Roo, and 12 in Yucatán. The presence of '*Ca.*L.asiaticus' in HLB-symptomatic citric plants was detected mostly in *C. latifolia* trees. The HLB-positive samples were confirmed by means of real-time PCR and C_T values are shown in Table 1. This analysis, however, showed an unexpectedly low number of PCR-positive samples from HLB-symptomatic citrus plants. To increase the reliability of the results, real-time qPCR was carried out for all the 214 samples. The results showed an increase in the positive samples from 25 to 70 (35 from Campeche, 15 from Quintana Roo and 20 from Yucatán). The real-time qPCR test improved the detec-tion of diseased plants by identifying false negative samples. The above results are suggestive of a low titer of the bacteria in the phloem tissue of the leaves and uneven distribution in the host (Hung et al., 1999; Li et al., 2008).

In México, the presence of '*Ca.*L. asiaticus' on citrus plantations was first reported in Yucatán, particularly in the municipality of Tizimin (Senasica-Sagarpa, 2009). Initially, PCR was the method of choice to detect the pathogen, but the low percentage of detection, even in HLB-symptomatic trees made it necessary to use real-time qPCR method. Nowadays, although the HLB vector (*Diaphorina citri)* has been collected in the 23 citricultural states of México, the disease has not yet been reported at all. In this regard, it is worth mentioning that Mexican authorities established Official Mexican Guidelines (NOM-EM-047-FITO-2009, http://www.senasica.gob.mx/?Idioma=2&doc=9366), which specifies actions, such as total destruction of trees,

Table 1. Detection of '*Candidatus* Liberibacter asiaticus' in leaf samples from Huanglongbing-symptomatic citrus trees.

Sample ID	Year of collection	Host	PCR[a]	C_T value[b]
QUINTANA ROO[¥]				
H002Q	2010	*Citrus latifolia*	+	19.63
H64AQ	2010	*Citrus latifolia*	+	21.3
H64BQ	2010	*Citrus latifolia*	+	20.51
H229Q	2010	*Citrus latifolia*	+	20.43
H232Q	2010	*Citrus latifolia*	+	20.32
H238Q	2010	*Citrus latifolia*	+	21.46
H243Q	2010	*Citrus latifolia*	+	20.68
YUCATÁN[§]				
H88Y	2010	*Citrus latifolia*	+	19.86
H89Y	2010	*Citrus latifolia*	+	21.95
H92Y	2010	*Citrus latifolia*	+	32.87
H93Y	2010	*Citrus latifolia*	+	20.63
H117Y	2010	*Citrus aurantifolia*	+	20.84
H143Y	2010	*Citrus latifolia*	+	20.2
H147Y	2010	*Citrus latifolia*	+	20.31
H163Y	2010	*Citrus latifolia*	+	19.09
H166Y	2010	*Citrus aurantifolia*	+	19.3
H259Y	2010	*Citrus aurantifolia*	+	20.62
H261Y	2010	*Citrus latifolia*	+	20.93
H267Y	2010	*Citrus latifolia*	+	21.27
CAMPECHE[†]				
H025C	2011	*Citrus latifolia*	+	26.6
H026C	2011	*Citrus aurantifolia*	+	21.0
H027C	2011	*Citrus aurantifolia*	+	25.2
H028C	2011	*Citrus aurantifolia*	+	22.6
H076C	2011	*Citrus latifolia*	+	19.09
H077C	2011	*Citrus latifolia*	+	21.36

¥: Total samples, 49; positive samples by PCR, 7; positive samples by real time-PCR, 9. §: total samples, 130; positive samples by PCR, 12; positive samples by real time-PCR, 20. †: total samples, 35; positive samples by PCR, 6; positive samples by real time-PCR, 35.[a] Samples used for cloning. [b] C_T values of positive PCR samples.

fruits and derivatives, that must be implemented immediately once the bacteria are detected.

Sequence and phylogenetic analysis

DNA fragments of 1167 bp from the 16S rDNA gene amplified from the 25 conventional PCR-positive samples were cloned, and three independent transformed cell clones harboring the constructs were selected from each positive sample. Sequencing of 75 independent plasmids was performed, and individual sequences were trimmed, edited, and analyzed as mentioned earlier. The multiple alignment showed sequences with similarity of 99 to 100% to sequences of '*Ca.* L. asiaticus', 96% with '*Ca.* L. africanus' and '*Ca.* L. solanacearum', and 94% with '*Ca.* L. americanus'. Phylogenetic tree construction with representative sequences from the Yucatán Peninsula and sequences deposited in the GenBank database is showing Figure 1. As expected, the sequences of our strains clustered with sequences of '*Ca.*L.asiaticus' from different countries. It is clearly a close relation with sequences of '*Ca.*L. africanus' rather than to sequences of '*Ca.*L. americanus'.

The above alignment also showed the clustering of sequences into two groups. The first group contained 26 sequences with an identity of 100%, and the second one contained 43 sequences with 1 to 5-sites of sequence polymorphism. The most important feature of the first subset of sequences was their geographical distribution

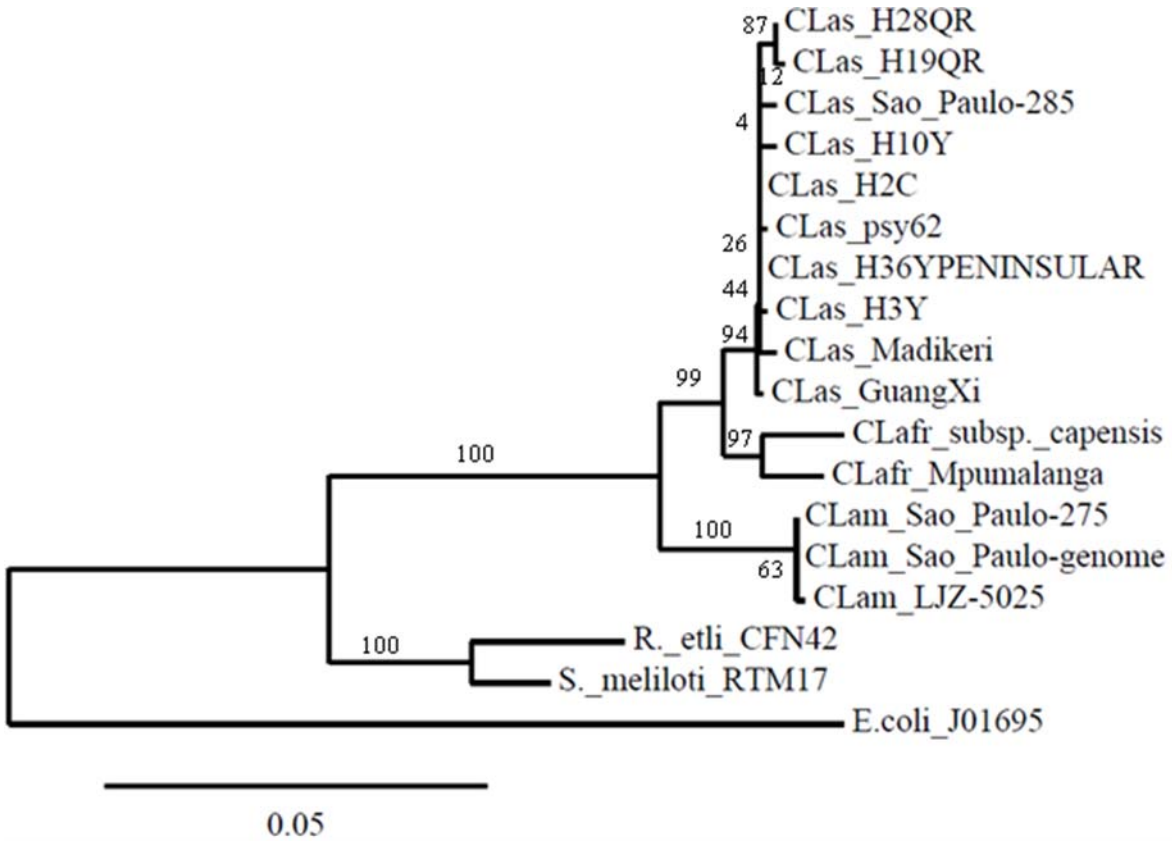

Figure 1. Phylogenetic relationships based on the 16s rRNA gene of '*Ca.* L. asiaticus' strains from the Yucatán Peninsula with closely related HLB species. The tree was constructed with the Neighbor-Joining method. The numbers at the nodes are percentages that indicate the levels of bootstrap support (1000 iterations). The sequences from '*Ca*. L. asiaticus' strains used in the analysis were Sao Paulo 285 (EU921613), Madikeri (FJ827777), Guang Xi-GL-10-CHN (EU921615), and Psy62 (CP00167). The sequences used from '*Ca*. L. africanus' were Capensis (AF137368), and Mpumalanga-UPCRI-06-0071 (EU921621). The sequences used from '*Ca*. L. americanus' were Sao Paulo 275 (EU921623), Sao Paulo 2 (AY742824) and LJZ-5025 (FJ263695). *Sinorhizobium meliloti* RTM17 (EU271786), *Rhizobium etli* CFN42 (NR_029184), and *Escherichia coli* (J01695) were used as the outgroup. Sequences of '*Ca*. L. asiaticus' of the present study were CLas H2C (JQ867437), H3Y (JQ867412), H10Y (JQ867417), H19QR (JQ867449) and H28QR (JQ867452).

into the three Mexican States. For this reason, we considered the sequence as the major haplotype identified on the peninsula, which we named H36PENINSULAR (GenBank accession No. JQ867409).

Worldwide distribution of the haplotype H36PENINSULAR

A similar analysis as previously mentioned was carried out with 93 16S rRNA sequences of'*Ca.*L. asiaticus' retrieved from GenBank sequence database with at least 1000 bp in length (Table 2). Results of the multiple alignment showed the formation of two groups of sequences, wherein the principal group contained 41 sequences with 100% similarity. For simplicity sake, this consensus sequence was named provisionally HLB-CLas, The second group contained sequences with1 to 15-sites (nucleotide) variations in different parts of the gene. Metadata of the members of the first group showed that all sequences originated from samples of different species of citrus trees and from the *D. citri* insect vector collected in countries such as Dominican Republic, Florida (USA), Brazil, Indonesia, Vietnam, Thailand, Taiwan and Japan. Identical sequences of 16S rDNA were obtained in Asiatic strains from Japan, Taiwan, Indonesia, the Philippines, Vietnam and Thailand (Subandiyah et al., 2000; Tomimura et al., 2009). Further studies based on the analysis of the 16S rRNA gene and the *omp* gene region of '*Ca.L*. asiaticus' found the closest relationship of sequences from northeastern India with sequences from Japan, Southeast Asia, USA (Florida) and Brazil, rather than with sequences from other Indian regions. Additionally, the study showed that common Asian strains are distributed in India together with other atypical strains (Miyata et al., 2011). Finally, a sequence comparison of

Table 2. Worldwide 16S rDNA sequences of '*Candidatus* Liberibacter asiaticus' included in the haplotype HLB-CLas and sequences with polymorphic sites.

ID Isolate	Source	Host	Accession	Reference
Haplotype HLB-CLas				
OKA901	Japan	*Citrus jambhiri*	AB480072	Okuda et al., 2005
KIN1	Japan	*Citrus jambhiri*	AB480073	Okuda et al., 2005
Y02-57	Japan	*Citrus tankan*	AB480074	Okuda et al., 2005
ISHI1	Japan	*Citrus*	AB480075	Tomimura et al., 2009
TW1	Taiwan	*Citrus sinensis*	AB480076	Tomimura et al.
TW2	Taiwan	*Citrus reticulata*	AB480077	Tomimura et al.
TW3	Taiwan	*Citrus grandis*	AB480078	Tsai et al., 2008
TW5	Taiwan	*Citrus grandis*	AB480079	Tsai et al.
TW6	Taiwan	*Citrus reticulata*	AB480080	Tsai et al.
V1	Vietnam	*Citrus nobilis*	AB480081	Tsai et al.
V2	Vietnam	*Citrus nobilis*	AB480082	Tsai et al.
VN50	Vietnam	*Citrus nobilis*	AB480083	Tominura et al., 2009
V61	Vietnam	*Citrus nobilis*	AB480084	Tominura et al.
V62	Vietnam	*Citrus nobilis*	AB480085	Tominura et al.
THA1	Thailand	*Citrus reticulata*	AB480086	Tominura et al.
IDN03-2	Indonesia	*Citrus reticulata*	AB480087	Okuda et al., 2005
IDN03-5	Indonesia	*Citrus reticulata*	AB480088	Okuda et al.
IDN03-7	Indonesia	*Citrus reticulata*	AB480090	Okuda et al.
B3T1	Indonesia	*Citrus reticulata*	AB480092	Okuda et al.
B3T2	Indonesia	*Citrus reticulata*	AB480093	Okuda et al.
B2T3	Indonesia	*Citrus reticulata*	AB480094	Okuda et al.
B3T4	Indonesia	*Citrus reticulata*	AB480095	Okuda et al.
B3T5	Indonesia	*Citrus reticulata*	AB480096	Okuda et al.
FC	Indonesia	*Citrus reticulata*	AB480097	Tominura et al., 2009
FD	Indonesia	*Citrus reticulata*	AB480098	Tominura et al.
EJ5	Indonesia	*Citrus reticulata*	AB480099	Tominura et al.
K1T2	Indonesia	*Citrus reticulata*	AB480101	Tominura et al.
PM13	Indonesia	*Citrus grandis*	AB480102	Tominura et al.
PM18	Indonesia	*Citrus grandis*	AB480102	Tominura et al.
IDN03-17	Indonesia	*Citrus reticulata*	AB480091	Tominura et al.
IDN03-6	Indonesia	*Citrus reticulata*	AB480089	Tominura et al.
MDL1398-DR002-12	Domininican Republic	NH[a]	FJ821716	Tominura et al.
MDL1399-DR002-13	Domininican Republic	NH	FJ821717	Tominura et al.
MDL1391-DR002-5	Domininican Republic	NH	FJ821710	N
MDL1396-DR002-10	Domininican Republic	NH	FJ821715	N
MDL1400-DR002-14	Domininican Republic	NH	FJ821718	N
F11Dade	Florida, USA	*Diaphorina citri*	EU130552	N
F16Nassau	Florida, USA	*Diaphorina citri*	EU130553	Manjunath et al., 2008
F17PALMBEACH F	Florida, USA	*Diaphorina citri*	EU130554	Manjunath et al.
18POLK	Brazil	*Diaphorina citri*	EU130555	Manjunath et al.
LSg1	Brazil	*Citrus*	AY919311	N
CLUSTER OF SEQUENCES WITH SNP´s				
A* Guangdong	Guangdong, China	NH	DQ157273	N
A* Guangxi	Guangxi, China	NH	DQ157274	N
B Guizhou	Guizhou, China	NH	DQ157275	N
C Sao Paulo 285	Sao Paulo, Brazil	NH	EU921613	Lin et al., 2009
D Psy62	Florida, USA	*Diaphorina citri*	CP00167	Duan et al., 2009
D Flord1	Florida, USA	Citrus	DQ471900	N

Table 2. Contd.

	NC (Brasil 1)	Sao Paulo, Brazil	Citrus	DQ471901	Texeira et al., 2005
E	Kumquat 1	Taiwan	NH	DQ302750	N
F	Lastm06	Louisiana, USA	Satsuma plant	FJ750459	N
G	Guangxi Gl1	China	NH	DQ778016	Lin et al., 2009
	Guangxi Guilin China	China	NH	FJ914620	N
H	Guangxi Gl7 Chn	Guanxi, China	NH	EU921614	Lin et al., 2008
I	Flirc08		Orange sweet	FJ750456	N
	Flclm09	Florida, USA	*Calamondin citrus*	FJ750457	N
J	Spd53	Sao Paulo, Brazil	NH	EU921622	Lin et al., 2009
K	M20	China	*Diaphorina citri*	GU553033	N
L	Guangxi Gl10 Chn	Guangxi, China	NH	EU921615	Lin et al., 2009
M	Guangxi Gl19 Chn	Guangxi, China	NH	EU921616	Lin et al., 2009
N	Florida 1808	Florida, USA	NH	EU921618	Lin et al., 2009
O	Florida 8	Florida, USA	NH	EU921617	Lin et al., 2009
P	Mpw643	East Timor	*Citrus aurantifolia*	AB555706	Miyata et al., 2011
	Mpw1319a	Papua New Guinea	*Citrus aurantium*	AB555707	Miyata et al., 2011
Q	Satkara Tripura	India	*Citrus macroptera*	GQ369792	Das et al., 2010
	LJZ 4621	Florida, USA	*Citrus spp.*	FJ263698	N
R	LJZ 4622	Florida, USA	*Citrus spp.*	FJ263699	N
	LJZ 575	Florida, USA	*Citrus spp.*	FJ263702	N
S	374 15	California, USA	*Atalantia ceylanica*	GU991651	N
T	373 4	California, USA	*Severina buxifolia* (Poiret)	GU991650	N
U	GFB-T	Malasya	Citrus	EU371106	N
V	LJZ 451	Florida, USA	Citrus	FJ263696	N
W	SIHUI	Florida, USA	NH	EU644449	Deng et al., 2009
X	LAAPC06	Louisiana, USA	*Diaphorina citri*	FJ750458	N
Y	373.1	California, USA	*Severina buxifolia*	GU991649	N
Z	Hunan-NG	Hunan, China	NH	DQ432002	Ding et al., 2009
AA	LJZ 5719	Florida, USA	NH	FJ263703	N
BB	LJZ 4620	Florida, USA	NH	FJ263697	N
CC	CGBNM1	India Nagpur	*Citrus* spp.	EU939452	N
DD	Clona 139	Florida, USA	*Citrus* spp.	EU130556	Manjunath et al., 2008
EE	Chongqing ZG	China Chongqing	NH	DQ432004	Ding et al., 2009
	Karnataka	India Karnataka	*Citrus reticulata*	FJ765088	Adkar-
FF	Madikeri	India Karnataka	Tangerine	FJ827777	Purushothama
	Polibetta 1	India Karnataka	Tangerine	FJ827779	et al., 2012
GG	371	Belize	*Diaphorina citri*	GQ502291	Manjunath et al., 2010
HH	432.26	Belize	*Citrus* spp.	GU061003	Manjunath et al., 2010
II	POONA	Poona India	*Citrus* spp.	L22532	Jagoueix et al., 1994
JJ	LJZ 745	Florida, USA	*Citrus* spp.	FJ236554	N
KK	LJZ4730	Florida, USA	NH	FJ263700	N
	LJZ 5670	Florida, USA	NH	FJ263701	N
LL	GUANGDONG	Guangdong, China	*Citrus* spp.	DQ303210	N
MM	LJZ 5818	Florida, USA	*Citrus* spp.	FJ263704	N
NN	12166	Florida, USA	*Citrus reticulata*	EU265646	N
OO	GFB Pahangkelantan (So)	Pahan Kelatan, Malasya	*Citrus reticulata*	EU224394	N

[a] NH, no host mentioned; [b]N, No reference.

16S rRNA, 23S rRNA and 16S/23S intergenic region of '*Ca L.* asiaticus' associated with yellowing disease of

Kinnow mandarin carried out in India showed lowest identities of 94.7, 95.4 and 95.9 percentage with '*Ca L.* asiaticus' from India (Poona strain), China and, USA and Brazil, respectively (Gupta et al., 2012).

Comparison between the sequences H36PENINSULAR and HLB-Clas was performed using the CLC Workbench software, version 6.1 (data not shown). Alignment of the two sequences showed a perfect match (100% identical), which suggests that the H36PENISULAR sequence can be considered the principal haplotype with worldwide distribution, which includes the Mexican states of Yucatán, Campeche and Quintana Roo. Therefore, we believe that this universal sequence could be used as reference for the analysis of 16S rRNA sequences of '*Ca.* L. asiaticus'.

Genetic diversity of 16s rRNA sequences of 'Ca. L. asiaticus'

To analyze the genetic diversity of the 16S rRNA gene in '*Ca.* L. asiaticus' a nucleotide comparison between the H36PENINSULAR sequence with the two subset of polymorphic sequences described above was carried out. For the Mexican sequences, the SNPs were identified as mentioned in Materials and Methods and the variants names were assigned. Forty-two variants were identified and their sequences were deposited in the GenBank database (Table 3). The table shows the position and type of mutation for each sequence, wherein it is clear, a predominance of sequences with 1or 2 polymorphic sites. The modifications were transitions, transversions, insertions and deletions with dominance of transitions. In the case of the subset of sequences from the GenBank, the variations detected ranged from 1 to 15 SNPs across the gene, thus showing a diversity of single and multiple polymorphic sites (Table 4). Besides, the GenBank sequences showed a higher number of polymorphic sites than the Mexican variants: the predominant nucleotide mutation was transitions (there were also some transversions, insertions, deletions and substitutions (Figure 2).

In relation to the SNPs, our findings show that Mexican strains have a small number of polymorphic sites in the 1167 bp DNA fragment distributed in a random fashion. In contrast, most GenBank sequences show high variability, which is suggestive of misreads during sequencing rather than genuine SNPs. To our knowledge, the '*Ca.*L. asiaticus' variants of Mexican isolates described in this study are the largest set of reported sequences with SNP sites in the 16s rDNA gene; as a result, 43 sequences were identified and added to the GenBank sequence database. Looking for possible phylogenetic relations between the polymorphic sequences, we constructed a tree using the Neighbor-Joining method (Figure 3). It was not possible to distinguish clusters of related polymorphisms, because only 3 well-supported clades were evident: a monophyletic clade that includes all the sequences corresponding to the 16S rRNA gene of '*Ca.*L. asiaticus' irrespective of the geographical region, and 2 independent clades corresponding to '*Ca.*L. africanus' and '*Ca.*L. americanus', as expected. Other studies have analyzed the genetic diversity of the 16S rRNA sequences. Based on the RFLP technique, Adkar-Purushothama et al. (2009) identified 14 genetic lineages by analyzing the SNPs present in the 16S rRNA, which revealed a new lineage on the Indian subcontinent. Recently, a study was conducted as a reanalysis of all '*Ca.*L. asiaticus' 16S rDNA sequences deposited in GenBank database to determine whether the discrepancy in reports of 16S variation can be resolved and whether this variation has a geographic origin (Nelson, 2012). The authors used geographic designations available in the metadata of the deposited sequences. A 302 bp segment common to 175 sequences was used to asses SNPs and the distribution, and the researchers found 118 identical sequences with different accession numbers and another 47 records exhibited 73 SNPs, most of them corresponding to a single accession number. A few SNPs occurred in more than one database record. The authors concluded that the reanalysis does not show sufficient confidence to confirm haplotypes of '*Ca.*L. asiaticus' based on the 16S rDNA sequences because the low percentage of SNPs in the segment studied suggested misreads during sequencing rather than genuine haplotypes. Differing from the above idea is that heterogeneity exists in the 16S rRNA genes detectable in the variable regions of the gene (V1, V2, V3, etc). The location of the differences in the most variable part of the 16S rRNA corroborates that the differences are true differences and not mere sequencing errors (Coenye and Vandamme, 2013).

Our results suggest that the sequence of the haplotype H36PENINSULAR may be a suitable reference sequence for the analysis of the 16s rRNA gene of novel '*Ca*. L. asiaticus' strains instead of the 16s rDNA sequences normally used for the detection of '*Ca*. L. asiaticus', such as the Poona, Karnataka and Madikeri strains. The Poona strain has commonly been used as a reference sequence for phylogenetic analysis, even though its 16S rRNA sequence still contains undetermined nucleotides (Miyata et al., 2011). The amplification and analysis of the 16S rDNA sequences are useful for describing new nucleotide variations across the gene sequence.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGMENTS

M-E A received a scholarship grant from CONACYT No. 165309. FOMIX-Campeche No.Camp-2008-96801 and No. Camp-2008-94224 supported this study. We are grateful to Dr. Zahaed Evangelista-Martínez from CIATEJ A.C. for the critical review and correction of the manuscript.

Table 3. Haplotype designation and SNP´s on the 16S rDNA sequences from Mexico.

ID*	1-200	201-400	401-600	601-800	801-1000	1001-1200	Accession
H1C						1162G>A;1164G>C	JQ867436
H2C	34A>G					1149delG	JQ867437
H3C				704C>T		1076delG	JQ867430
H22C				790A>G			JQ867438
H24C			560A>G				JQ867439
H29C	56-57insT	329T>C					JQ867431
H30C		322delC					JQ867432
H33C	74G>A	256A>G					JQ867433
H35C	34A>G						JQ867434
H1Y				601T>C		1142A>G	JQ867410
H2Y						1110G>A	JQ867411
H3Y				622-623insC		1110G>A	JQ867412
H4Y				622-623insC		1100G>A	JQ867405
H5Y						1065G>A	JQ867413
H6Y					837T>C	1053T>C	JQ867414
H8Y	71A>G	263delG		681G>A	896G>A	1011A>G	JQ867415
H9Y					925G>A	1009T>C	JQ867416
H10Y	45delA			753A>G	1000T>C		JQ867417
H16Y	43G>A				945A>G		JQ867418
H17Y					870G>A;921C>A		JQ867406
H22Y				788T>C			JQ867419
H23Y				700A>G;780A>G			JQ867420
H24Y				622-623insC			JQ867421
H25Y			491-492insC				JQ867407
H26Y		317A>G	464C>T				JQ867422
H27Y			447C>G;448G>T				JQ867423
H29Y		357A>G					JQ867408
H30Y	45delA	286-287insA					JQ867424
H31Y		203A>G; 263delG					JQ867425
H32Y		263G>A					JQ867426
H34Y	45delA						JQ867427
H35Y	34A>G						JQ867428
H7QR							JQ867442
H11QR					998T>C	1021T>C	JQ867443
H12QR			596A>G		995G>A		JQ867444
H13QR					967delG		JQ867445
H14QR				735G>A	967delG		JQ867446
H15QR					876T>C;967delG		JQ867447
H18QR					843G>A		JQ867448
H19QR		230A>G	432T>C		815A>G		JQ867449
H20QR					812A>G		JQ867450
H21QR		230A>G	432T>C				JQ867451
H28QR		230A>G	432T>C				JQ867452

* C, Campeche; Y, Yucatán; QR, Quintana Roo

Table 4. Haplotype designation and SNP's on the 16S rDNA sequences from worldwide sequences.

ID*	1-200	201-400	401-600	601-800	801-1000	1001-1200
A	9G>C					
B	9G>C				840T>C	
C	10C>T; 10insG	315A>T; 372A>C		792A>G	806G>A	
D	10insG					
E	10insG; 49T>C					
F	10insG; 76A>G	254A>G; 281G>A	535T>C;		804delG; 816A>T; 847A>C; 866insC	1126C>T
G	10insG; 88A>G		549T>C; 562T>G; 598G>A	682A>G; 762C>T; 764T>G		
H	10insG; 97G>C			769A>G		1040T>G
I	10insG	205A>T; 398C>T		675C>T		1103insC
J	10insG	274A>C				
K	10insG	283A>G; 358A>G	403G>A			
L	10insG	365T>C	448delA			
M	10insG		572A>G	637A>G		1036T>A
N	10insG				892A>G; 975T>A	1101T>A
O	10insG			787A>T	820G>A; 991insC	
P	17A>T					
Q	35A>G		448A>T; 458T>A; 459A>T		834T>A; 988G>A	
R	73T>C; 175A>G	217A>G		668T>C; 762C>T; 764T>G		1075A>G
S	77A>T; 114A>G; 137T>C	372A>G		752A>G		
T	101T>C; 107G>A; 112A>G	202A>G; 347A>G				
U	168G>A		460G>A; 467C>G; 468G>A; 469T>A: 473G>C; 544G>T; 545A>G; 549G>A; 553delT; 577T>G; 579G>A; 580A>G	602G>A		
V	187T>A			672A>G		
W	187insA; 195G>T; 199insA	213delC; 225insG; 227A>G; 229insC; 234delC; 247delG; 274delA; 283delA; 285C>G				
X	196T>C	315delA		749T>C	803insG; 847A>C; 866insC	

Table 4. Contd.

Y		250A>G; 306T>C		746G>A	827C>T; 885C>G	
Z		327T>C; 350A>T			973A>T	1164G>C
AA		338T>C				
BB		348G>A	454C>T; 562T>C	641G>A	936T>C; 979G>A	
CC		353T>A	429G>T; 442G>T; 448A>T; 459A>T; 461G>C	617C>T; 650G>T; 774G>A		1110T>C
DD		369A>G			817A>G	
EE		374A>G				1024T>C; 1114G>C
FF			404C>T	633G>C		1041T>C
GG			413G>A			
HH			430T>C; 527A>G			
II			463delG; 491delG; 532insC	740G>C; 741C>G;	820delG	1102delG; 1103insC; 1104C>G
JJ			520T>C			
KK				798 A>G		
LL	9G>C				847delA	1149G>A
MM						
NN					944G>C	
OO					988G>A	

*Accession number of the GenBank database are shown in Table 2.

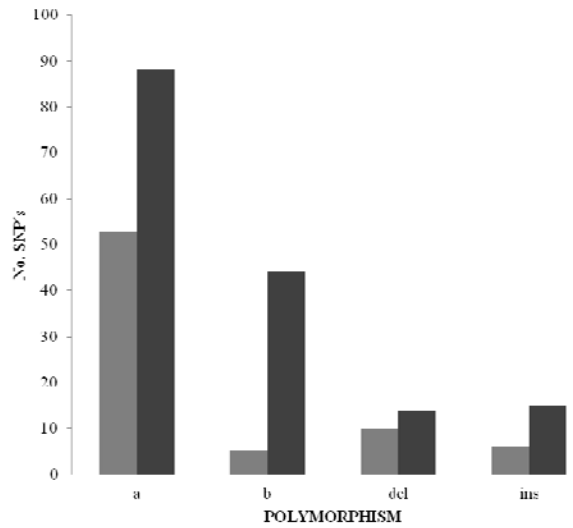

Figure 2. Comparison of polymorphic changes in 16s rRNA sequences of '*Ca*. L. asiaticus' from the Yucatán Peninsula (black bar) and sequences from GenBank database (gray bar). Nucleotide changes: a, transitions; b, transversions; del, deletions; ins, insertions.

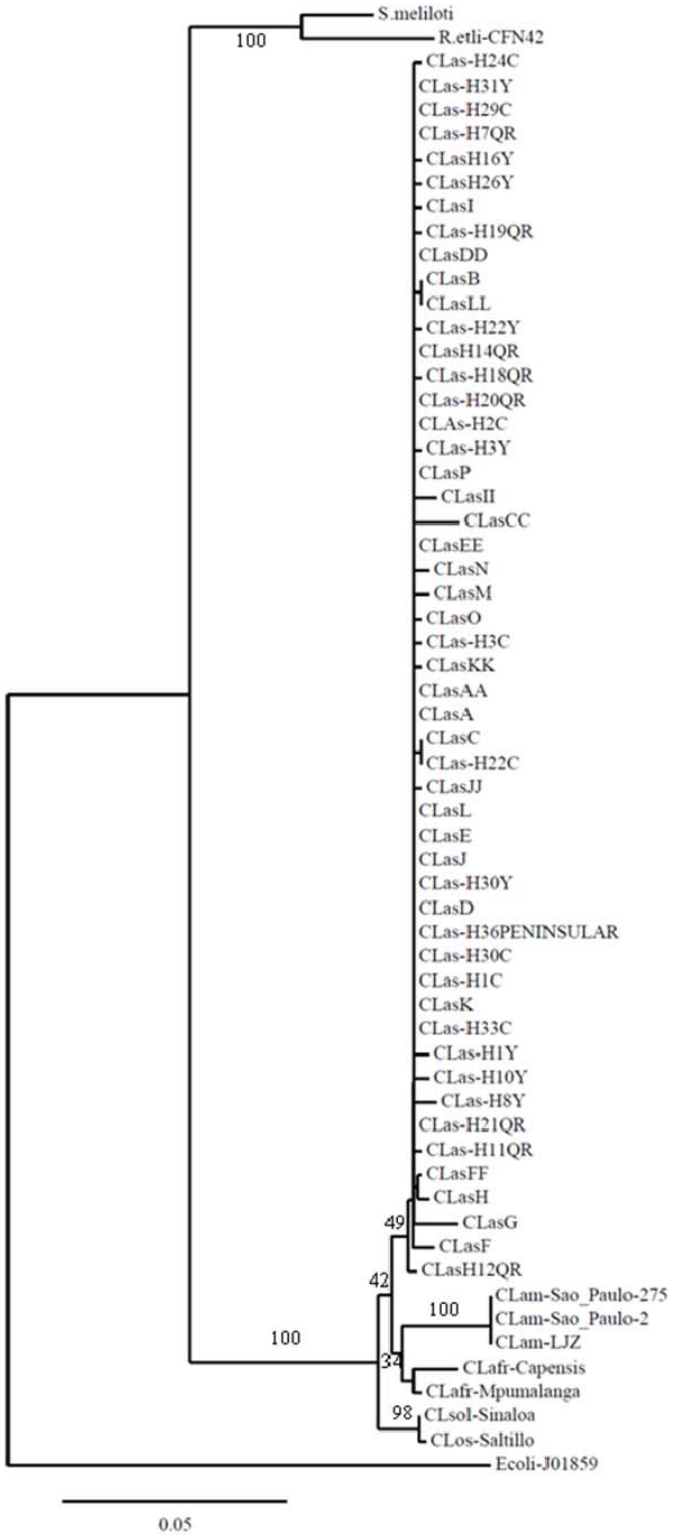

Figure 3. Phylogenetic tree including 16s rRNA sequences from Yucatán Peninsula haplotypes and closely related sequences using the Neighbor-Joining method. The numbers at the nodes are percentages that indicate the levels of bootstrap support (1000 iterations). The sequences used from GenBank were '*Ca.* L. africanus' Mpumalaga UPCRI-05-0232 (FJ914622), Mpumalaga UPCRI-06-0071 (EU921621), Nelspruit (L22533), and subsp. Capensis (AF137368); '*Ca.* L. solanacearum' Sinaloa (FJ957897), NZLS0002 (HM246509), NZ082226 (EU834130), and Saltillo 9 08 (FJ490086); and '*Ca.* L. americanus' LJZ5110 (FJ263689), Sao Paulo 275 (EU921623), Sao Paulo 2 (FJ914621), and Sao Paulo State (AY742824). The rhizobial group consisted of *Agrobacterium tumefaciens* RFP1 (GU902302), *Rhizobium etli* CFN42 (NR029184), and *Shinorizobium meliloti* RTM17 (EU271786). *Escherichia coli* (J01859) and *Pseudomonas* sp. NJ (AY339889) were used as the outgroup.

REFERENCES

Adkar-Purushothama C, Quaglino F, Casati P, Ramanayaka JG, Bianco PA (2009). Genetic diversity among '*Candidatus*Liberibacterasiaticus' isolates based on single nucleotide polymorphisms in 16S rRNA and ribosomal protein genes. Ann. Microbiol. 59:681-689.

Arias CR, Olivares-Fuster O, Goris J (2010). High intragenomic heterogeneity of 16S rRNA genes in a subset of *Vibrio vulnificus* strains from the western Mediterranean coast. Int. Microbiol. 13:179-88.

Bastianel C, Garnier-Samancik M, Renaudin J, Bove JM.Eveillard S (2005). Diversity of "*Candidatus*Liberibacterasiaticus" based on the *omp* gene sequence. Appl. Environ. Microbiol. 71:6473–6478.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2005).GenBank. Nucleic Acids Res. 33:D34-D38.

Bové JM (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol. 88:7-37.

Capoor SP, Rao DG, Viswanath M (1967). *Diaphorinacitri*Kuwayama vector of the greening disease of citrus in India. Indian J. Agric. Sci. 37: 572-576.

Coenye T, Vandamme P (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiol. Lett. 228:45-49.

Den Dunnen JT, Antonarakis SE (2001). Nomenclature for the description of human sequence variations. Hum. Genet. 109:121-124.

Duan Y, Zhou L, Hall D G, Li W, Doddapaneni H, Li H, Liu L, Vahling CM, Gabriel DW, Williams KP, Dickerman A, Sun Y, Gottwald T (2009). Complete genome sequence of citrus Huanglongbing bacterium, '*Candidatus*Liberibacterasiaticus' obtained through metagenomics. MPMI 22:1011-1020.

Garnier M, Danel N, Bové JM (1984). In: Annales de l'Institut Pasteur. Microbiologie, 135, 169-179; Proceedings of 9th Conference, IOCV: 115-124 University of California, Division of Agricultural Sciences, Riverside, CA.

Gottwald TR, da Graça JV, Bassanezi RB (2007).Citrus Huanglongbing: The pathogen and its impact. Plant Health Progress http://www.plantmanagementnetwork.org/pub/php/review/2007/huanglongbing/ doi:10.1094/PHP-2007-0906-01-RV.

Grafton-Cardwell EE, Stelinski LL, Stansly PA (2013). Biology and management of Asian citrus psyllid, vector of the huanglongbing pathogens. Annu. Rev. Entomol. 58:413-432.

Gupta KN, Baranwal VK, Haq QMR (2012). Sequence analysis and comparison of 16S rRNA, 23S rRNA and 16S/23S intergenic spacer region of greening bacterium associated with yellowing disease (huanglongbing) of Kinnow mandarin. Indian J. Microbiol. 52:13-21.

Hoffman MT, Doud MS, Williams L, Zhang MQ, Ding F, Stover E, Hall D, Zhang S, Jones L, Gooch M, Fleites L, Dixon W, Gabriel D, Duan YP (2013). Heat treatment eliminates '*Candidatus* Liberibacterasiaticus' from infected citrus trees under controlled conditions. Phytopathology 103:15-22.

Hung TH, Wu ML, Su HJ (1999).Development of a rapid method for the diagnosis of citrus greening disease using the polymerase chain reaction. J. Phytopathol.147:599-604.

Jagoueix S, Bové JM, Garnier M (1994). The phloem-limited bacterium of greening disease of citrus is a member of the α-subdivision of the Proteobacteria. Int. J. Syst. Bacteriol. 44:379-386.

Jagoueix S, Bove JM, Garnier M (1997). Comparison of the 16S/23S ribosomal intergenic regions of "*Candidatus*Liberobacterasiaticum" and "*Candidatus*Liberobacterafricanum," the two species associated with citrus huanglongbing (greening) disease. Int. J. Syst. Bacteriol. 47:224-227.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol.16:111-120.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948.

Li W, Hartung JS, Levy L (2006). Quantitative real-time PCR for detection and identification of '*Candidatus*Liberibacter' species associated with citrus huanglongbing. J. Microbiol. Methods 66:104-115.

Li W, Hartung J, Levy L (2007). Evaluation of DNA amplification methods for improved detection of '*Candidatus* Liberibacter Species' associated with citrus Huanglongbing. Plant Dis. 91:51-58

Li W, Li D, Twieg E, Hartung JS, Levy L (2008). Optimized quantification of unculturable'*Candidatus*Liberibacterspp'.in host plants using real-time PCR. Plant Dis. 92:854-861.

Librado P, Rozas J (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451-1452.

McClean APD, Oberholzer PCJ (1965). *Citrus psylla*, a vector of the greening disease of sweet orange. S. Afr. J. Agric. Sci. 8:297-298.

Miyata S, Kato H, Davis R, Smith MW, Weinert M, Iwanami T (2011). Asian-common strains of '*Candidatus*Liberibacterasiaticus' are distributed in Northeast India, Papua New Guinea and Timor-Leste. J. Gen. Plant Pathol.77:43-47.

Murray HG, Thompson WF (1980).Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8:4321-4325.

Nelson WR (2012). Reported single nucleotide polymorphisms on the 16S rRNA gene do not support haplotypes of "*Candidatus* Liberibacterasiaticus". Citrus Res. Technol. 33:75-79.

Planet P, Jagoueix S, Bové JM, Garnier M (1995). Detection and characterization of the African citrus greening liberibacter by amplification, cloning and sequencing of the *rplKAJL-rpoBC* operon. Curr. Microbiol. 30:137-141.

Sambrook J, Rusell D (2000). Molecular Cloning: A Laboratory Manual.ThirdEdition. Cold Spring Harbor LaboratoryPress

Servicio Nacional de Sanidad Inocuidad y Calidad Agro-Alimentaria (SENASICA-SAGARPA) (2009). Informe mensual noviembre. http://www.senasica.gob.mx/?doc=20594

Servicio Nacional de Sanidad Inocuidad y Calidad Agro-Alimentaria (SENASICA-SAGARPA) (2010). Informe mensual septiembre. http://www.senasica.gob.mx/?doc=20596

Subandiyah S, Iwanami T, Tsuyumu S, Ieki H (2000). Comparison of 16S rDNA and 16S/23S intergenic region sequences among citrus greening organisms in Asia. Plant Dis. 4:15-18.

Tamura K, Nei M, Kumar S (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci USA 101:11030-11035.

Teixeira DC, Saillard C, Couture C, Martins E, Wulff NA, Eveillard-Jagoueix S, Yamamoto PT, Ayres AJ, Bové JM (2008). Distribution and quantification of '*Candidatus*Liberibacteramericanus', agent of huanglongbing disease of citrus in Sao Paulo State, Brazil, in leaves of an affected sweet orange tree as determined by PCR. Mol. Cell Probes. 22:139-150.

Tomimura K, Miyata S, Furuya N, Kubota K, Okuda M, Subandiyah S, Hung TH, Su HJ, Iwanami T (2009). Evaluation of genetic diversity among '*Candidatus*Liberibacterasiaticus' isolates collected in Southeast Asia. Phytopathology 99:1062-069.