4.12 Genetic Diversity of *Candidatus* Liberibacter asiaticus and *Ca.* L. americanus Based on Sequence Variations of Their rRNA Operon

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Introduction

Citrus Huanglongbing (HLB), also known as citrus greening, is the most destructive and devastating disease of citrus in the world (Gottwald *et al*., 2007). Since the initial report of the disease in Florida in August of 2005, HLB has spread throughout 32 of the citrus producing counties. The causal agent of this disease is believed to be three species of alpha-Proteobacteria, *Candidatus* Liberibacter asiaticus (Las), *Ca.* L africanus (Laf) and *Ca.* L. americanus (Lam) (Jagoueix, et al., 1994; Teixeira, et al., 2005). Las is the most widely-distributed species and the only species that has been detected in Florida to date. However, different types of symptoms were observed on citrus in the fields, and on HLB-affected periwinkles when the disease was transmitted from Las-infected citrus to periwinkle via dodder. More importantly, these different types of symptoms can be maintained by graft transmission, indicating they are not caused by genetic variation of host plants, but likely by genetic variation of the pathogens. To reveal the variations of the HLB pathogens, we cloned and sequenced the 3.3-3.6 kb rDNA fragments, including 16S rRNA gene, 16S/23S intergenic region and partial 23S rRNA gene from Las and Lam-infected plant DNAs.

Material and methods

Six rDNA libraries of Las were constructed from the different HLB-affected citrus and periwinkle plants with distinct symptoms using PCR-based cloning (Table 1). In addition, a 3.6 kb rDNA library of Lam was also obtained from HLB-affected citrus DNA from Brazil. Two sets of primers were used to amply the targeted DNA fragments: OI1 5'-GCGCGTATGCAATACGAGCGGCA-3' (Jagoueix, et al., 1996) and LJ11f 5'-TCTCACAAGTCCTCCTTCATC-3' for Las rRNA operon; CGO3f 5'-RGGGAAAGATTTTATTGGAG-3' (Zhou, et al., 2007) and LJ18f 5'-TCTCACCCCTCCTATTAACC-3' for Lam rRNA operon. High Fidelity Platinum® Taq DNA Polymerase (Invitrogen Carlsbad, CA) were used for PCR amplification, and the PCR product with expected sized were cloned into the TOPO TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). 60-80 white colonies from each library were randomly selected for PCR-RFLP analysis using restriction enzyme *Msp*I and *Hinc*II, and the clones with different RFLP profiles revealed by the two enzymes were sent for sequencing. Sequence analyses were performed by blasting in NCBI, and multiplex alignment with software Vector NTI Advance 10.3.0, (Invitrogen Carlsbad, CA). Phylogenetic analysis was conducted using *MEGA* version 4 (Tamura, Dudley, Nei, and Kumar 2007), all the sequences used for phylogenetic analysis from this study has been submitted to NCBI.

Result and discussion

Instead of comparing the short sequence of the 16S rDNA or 16S/23S rDNA intergenic regegion (Jagoueix, et. al, 1994 and 1997), a long DNA fragment of 3.3kb (Las) or 3.6kb (Lam)

across 16S rRNA gene, 16S/23S intergenic region and partial 23S rRNA gene were amplified and cloned to generate rDNA libraries. PCR-RFLP results using *Msp*I and *Hinc*II from the six Las rDNA libraries and one Lam rDNA library revealed different RFLP profiles exited in each population at different ratio from 0 to 12% ((Fig.1 and Table 2), indicating the mixed population of the individual species of Las/Lam bacteria within a single HLB-affected plant. It is worth to noting that the library from PG9 had more RFLP profiles and single nucleotide polymorphisms (SNPs) than those of the other 5 Las rDNA libraries, and the HLB-affected periwinkle plant PG9 displayed systemic yellow symptoms in two weeks after grafting, which was two to four weeks earlier than that of the other HLB-infected periwinkle plants. Phylogenetic analysis of the 6 sequences with maximum SNPs from each of Las library based on the 1179bp sequences of their 16S rDNA revealed the sequence R8T1-462 branched out from the rest of Las sequences (Fig. 2). As indicated in the Table 1, R8T1 was the HLB-affected Pummelo citrus that only displayed yellow shoots with vein yellowing and without blotchy mottles on leaves, and contained low titer of Las bacterium.

Currently, there is little information on strain variation of the HLB pathogens. Due to the small genome size (Duan et al., unpublished data) and fastidious nature, the Liberibacter might have limited genetic variations in terms of insertion or deletion of their sequences within single species. However, variation of the mixed population with SNPs revealed in this study may play an important role in the disease development and epidemiology. Further study using other genetic marker(s) may reveal more information regarding the genetic diversity of the HLB pathogens.

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Table 1. rDNA libraries and the HLB-affected source plants and symptoms.

Cloning ID	DNA ID	Source plant	Symptom on source plant
pLJ45	3-3SL	Graft transmitted lemon in green house	No visible symptom
pLJ46	R8T1	Natural infected Pummelo in Fort Pierce, Florida	Yellow shoots with vein yellowing
pLJ47	C3	Natural infected sweet orange removed from field and maintained in green house	Blotchy mottle in the field and turned to nutrition deficiency symptom in greenhouse
pLJ56	PG6	Graft transmitted periwinkle in green house	Small, mottling leaves with vein yellowing
pLJ57	PC11	Dodder transmitted periwinkle in green house	Vein yellowing with wilt tip
pLJ58	PG9	Graft transmitted periwinkle in green house	Rapid development of systemic symptoms of yellow leaves
pLJ50	Lam	Lam-infected citrus DNA from Brazil	No record of symptoms

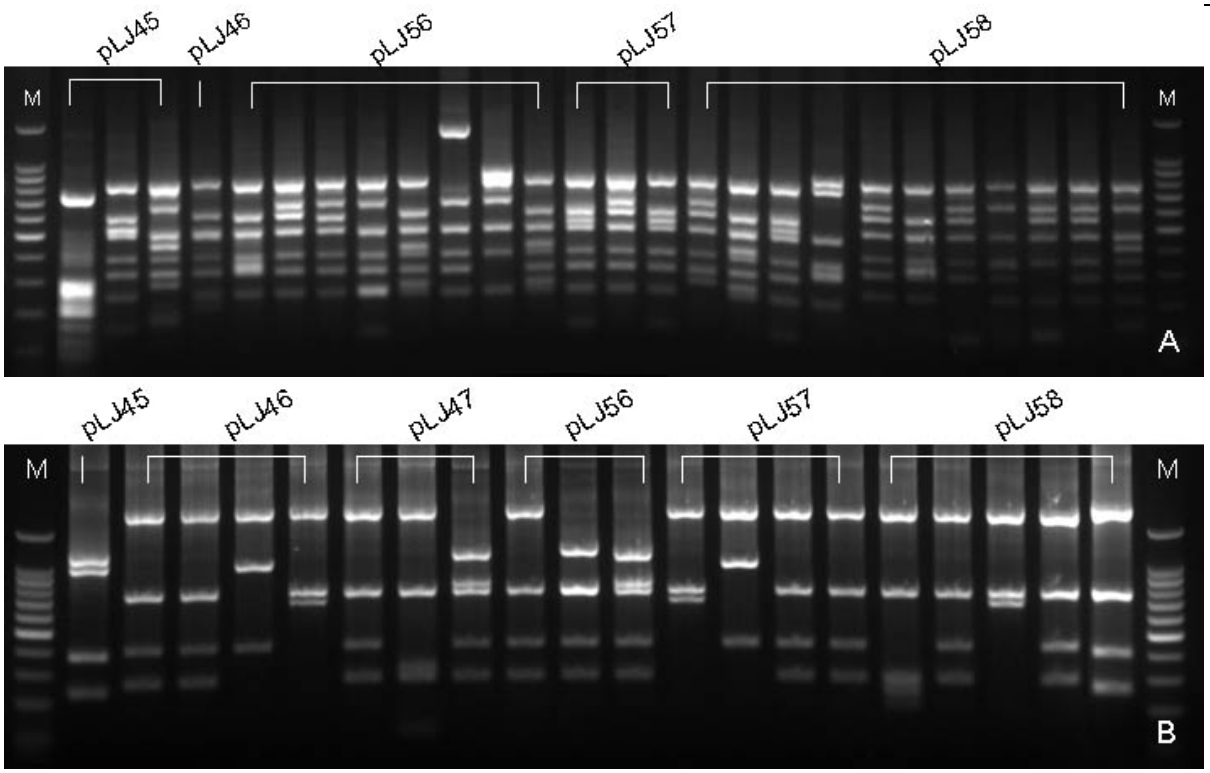

Fig. 1. *Msp*I (A) and *Hinc*II (B) RFLP profile of PCR-amplified rDNA fragment of Las rRNA operon from HLB-infected citrus and periwinkle using primers OI1 and LJ11f.

Table 2. RF LP profiles, mutant ratio in the rDNA libraries and maximum number of single-nucleotide polymorphisms (SNPs) of each Las or Lam rDNA sequence

DNA ID		3-3SL	R8T1	C3	PG6	PC11	PG9	Lam
*Msp*I	Profile number	4	2	1	7	2	8	7
	Percentage of mutants	8.1%	2.9%	0	7.5%	1.7%	8.8%	12%
*Hinc*II	Profile number	2	3	3	3	3	4	2
	Percentage of mutants	2.7%	5.9%	6.7%	2.5%	3.3%	3.8%	2%
Maximum no.of SNPs		7	8	5	4	4	10	7

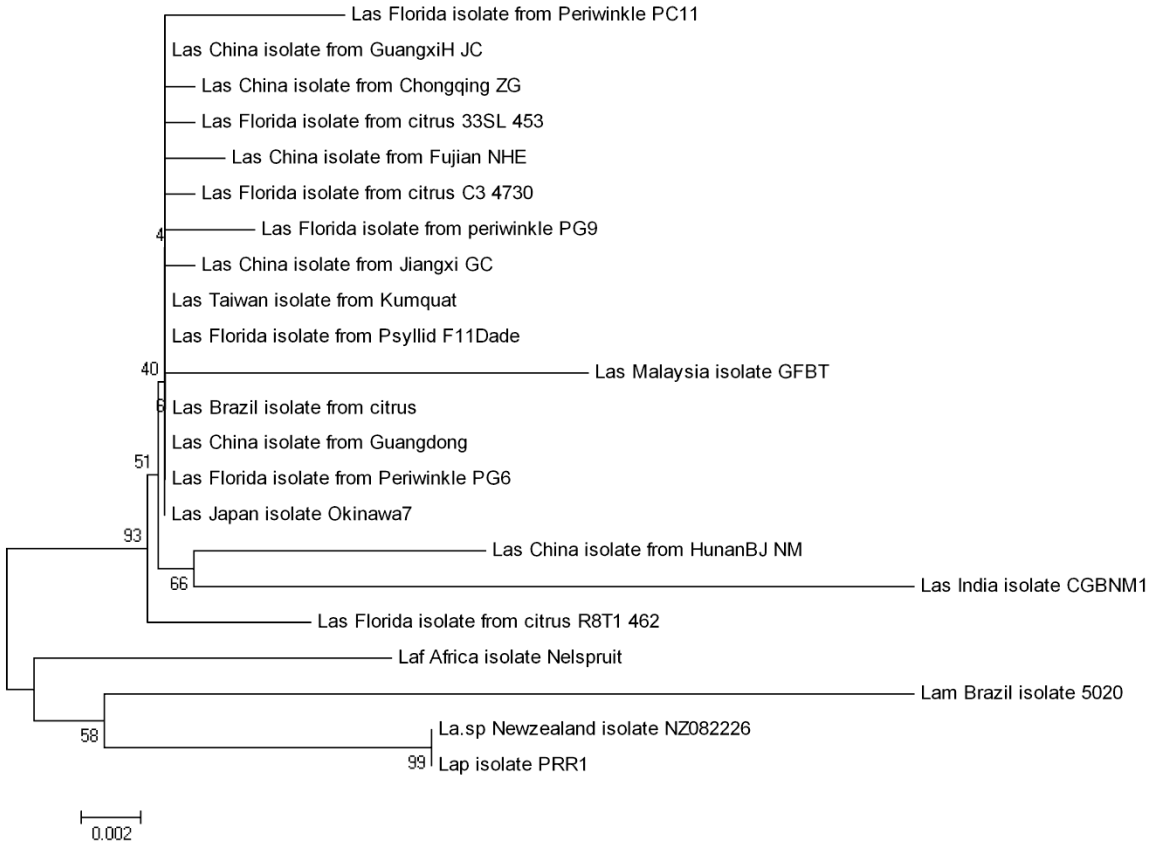

Fig. 2. Neighbor-Joining phylogenetic tree generated from alignment of 1.2kb of 16S rRNA gene sequence of *Ca.* Liberibacter by MEGA4.1 with 1000 bootstrap replication. Bootstrap values are shown at the nodes. Six of Las Florida plant isolates and one of Lam Brazil isolates 16S rRNA gene sequence were obtained in this study; all the other sequences were from GenBank.