Citrus Huanglongbing Diagnosis Based on Molecular Detection of Associated Liberibacter Species

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International Workshop on Citrus Quarantine Pests,Villahermosa, Tabasco, Mexico, 27-31 July 2009

Major management strategy for HLB control

- To remove infected host plants
- To reduce suspect psyllid populations

Methods to identify the disease or its associated bacteria, currently three known species, '*Ca.* Liberibacter asiaticus', '*Ca.* L. africanus' and '*Ca.* L. americanus'

plants and psyllids

Yellow shoots (China) for HLB finding

Leaf mottle (Philippines) for HLB finding

Vein degeneration (Indonesia) for HLB finding

Dieback (India) for HLB finding

Fruit symptoms (S. Africa) for HLB finding

Traditional methods to detect the HLB-associated bacteria

- Electron microscopy: Lafleche & Bové, 1970.
- ELISA: Garnier et al., 1987.
- Iodine reaction (IR): Schneider H, 1968.
- Fluorescent substance: Schwarz, 1968
- Biological indexing: Schwarz, 1968.

Nucleic acid-based non-PCR methods to detect the HLB-associated bacteria

- Dot-blot hybridization: low sensitivity as electron microscopy (Villechanoux *et al*., 1992).
- Loop-mediated isothermal amplification (LAMP): useful for labs without a PCR machine but vulnerable to contaminations (Okuda *et al*. 2005).
- Cycleave isothermal and chimeric primer-initiated amplification of nucleic acids with probe technology (ICAN): useful for screening labs without PCR machines (Orasaki *et al*., 2008).

PCR methods for detection of HLB-associated bacteria

Kary B. Bullis
California USA

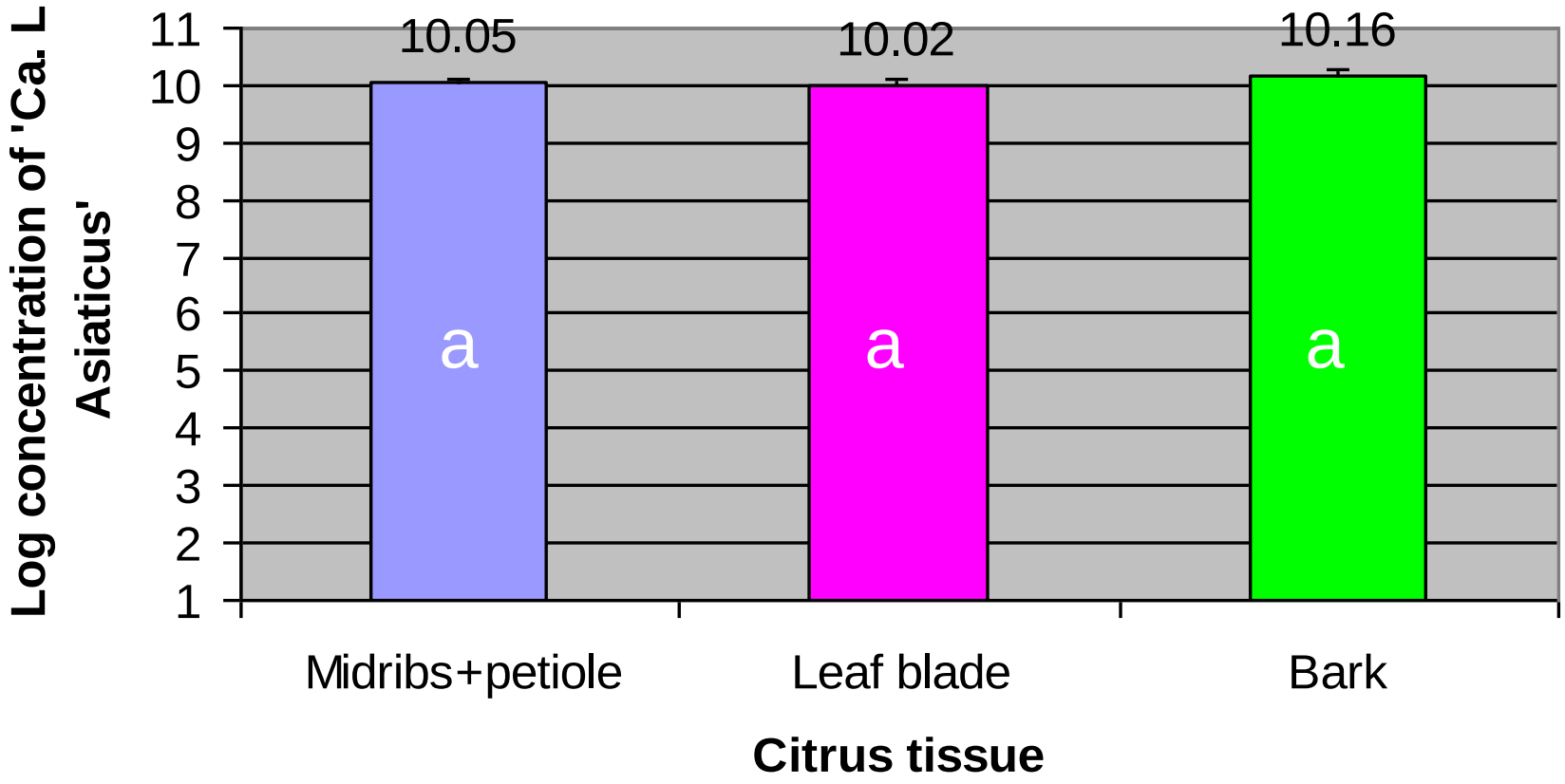
Michael Smith
Vancouver Canada

Since its first introduction in 1983 by the two Nobel prize winners, PCR has become a powerful technique for detection and identification of plant pathogens

Dr. Bové's lab developed first PCR primers for detection and identification of the HLB-associated Liberibacter species (Jagoueix *et al*., 1996).

The main objective of the presentation is to discuss how to use PCR methods for detection, identification and quantification of the HLB bacteria

Quantification of '*Ca*. L. asiaticus' in HLB-affected citrus tissues

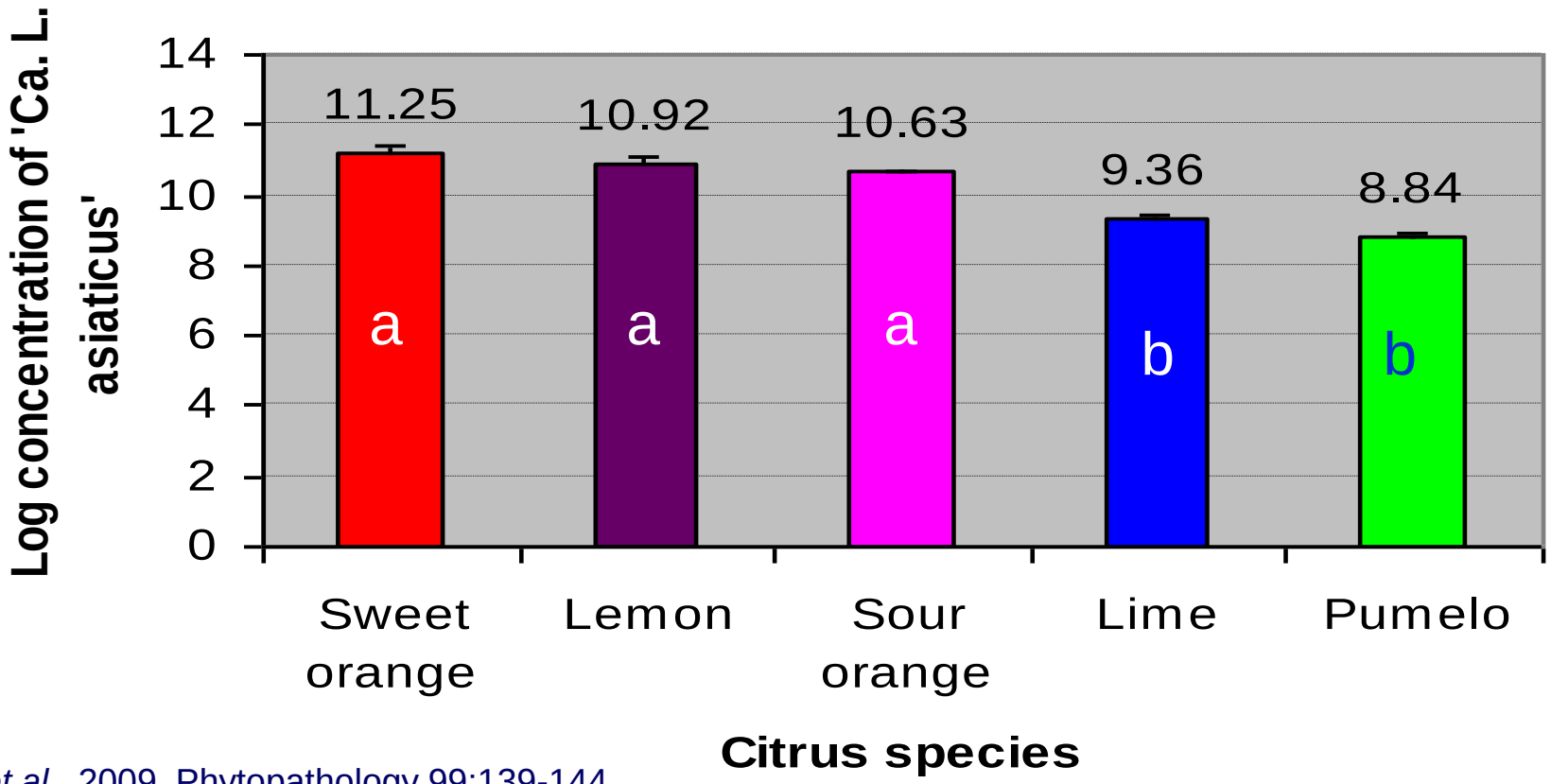


Li *et al*., 2009. Phytopathology 99:139-144

Sampling HLB suspect trees

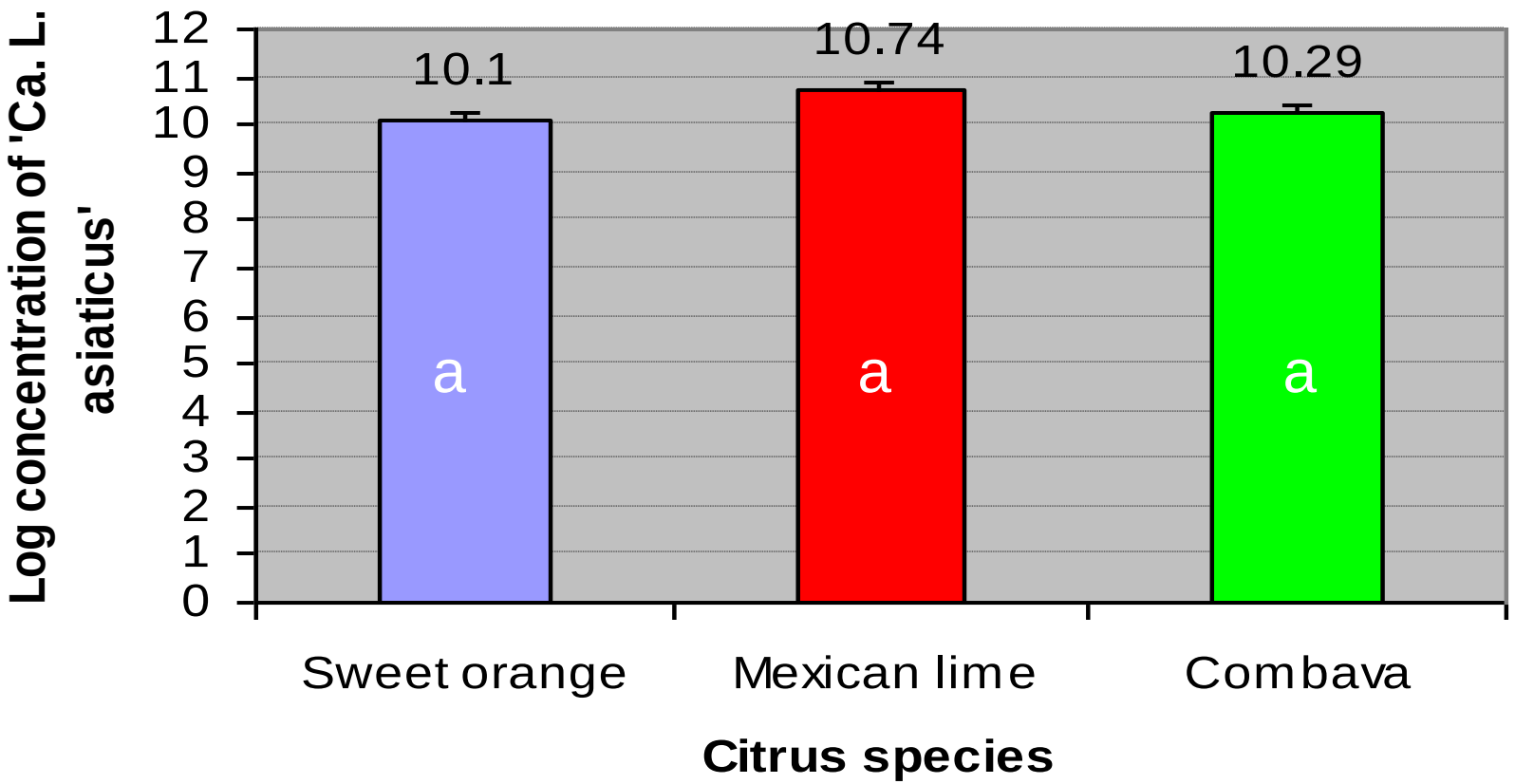
1 to 4 branches (twigs) with symptomatic leaves or fruit from each tree

Quantification of '*Ca.* L. asiaticus' in midribs and petioles of HLB-affected citrus species



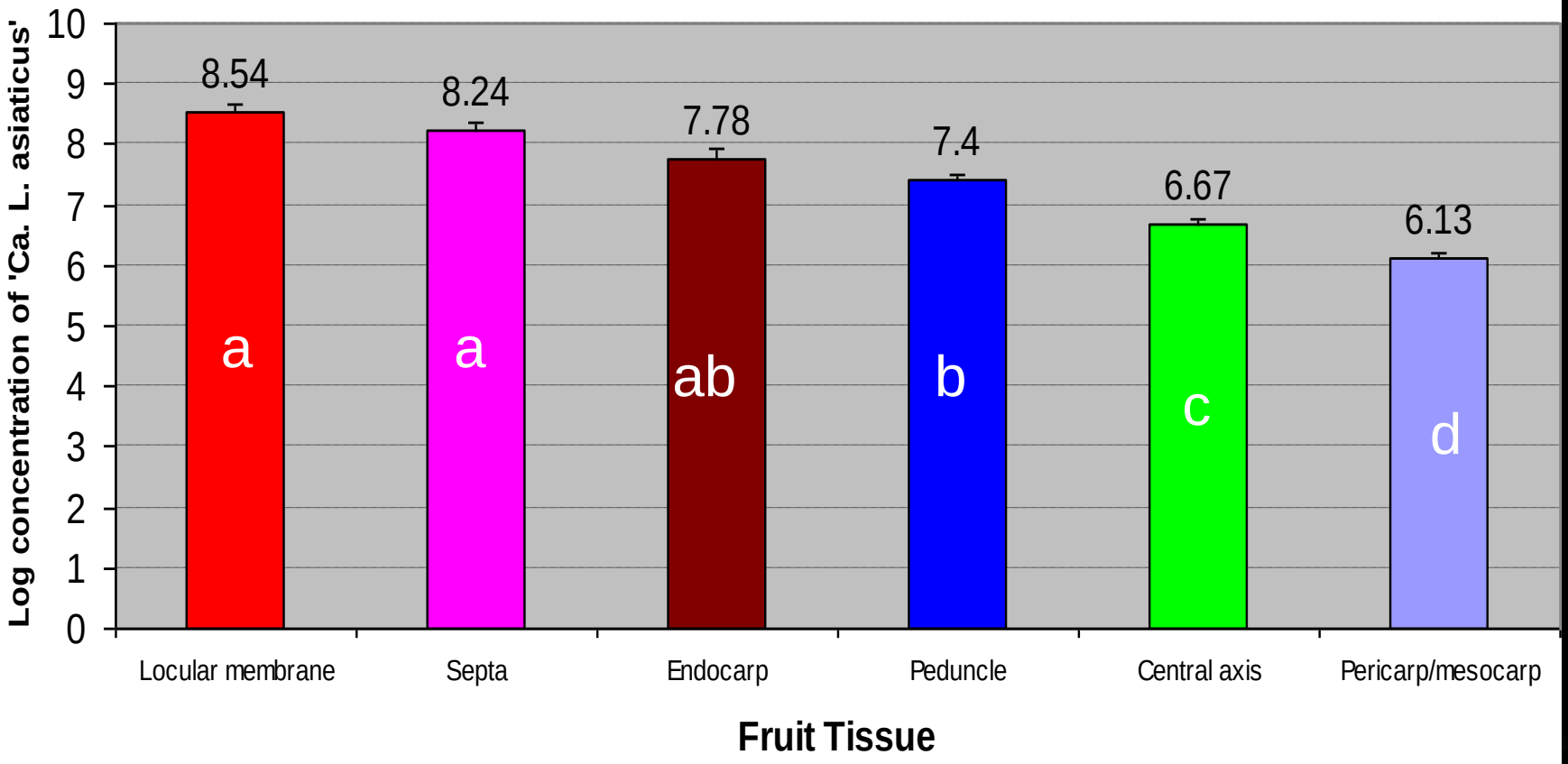
Li *et al*., 2009. Phytopathology 99:139-144

Quantification of '*Ca*. L. asiaticus' in root bark of HLB-affected citrus species



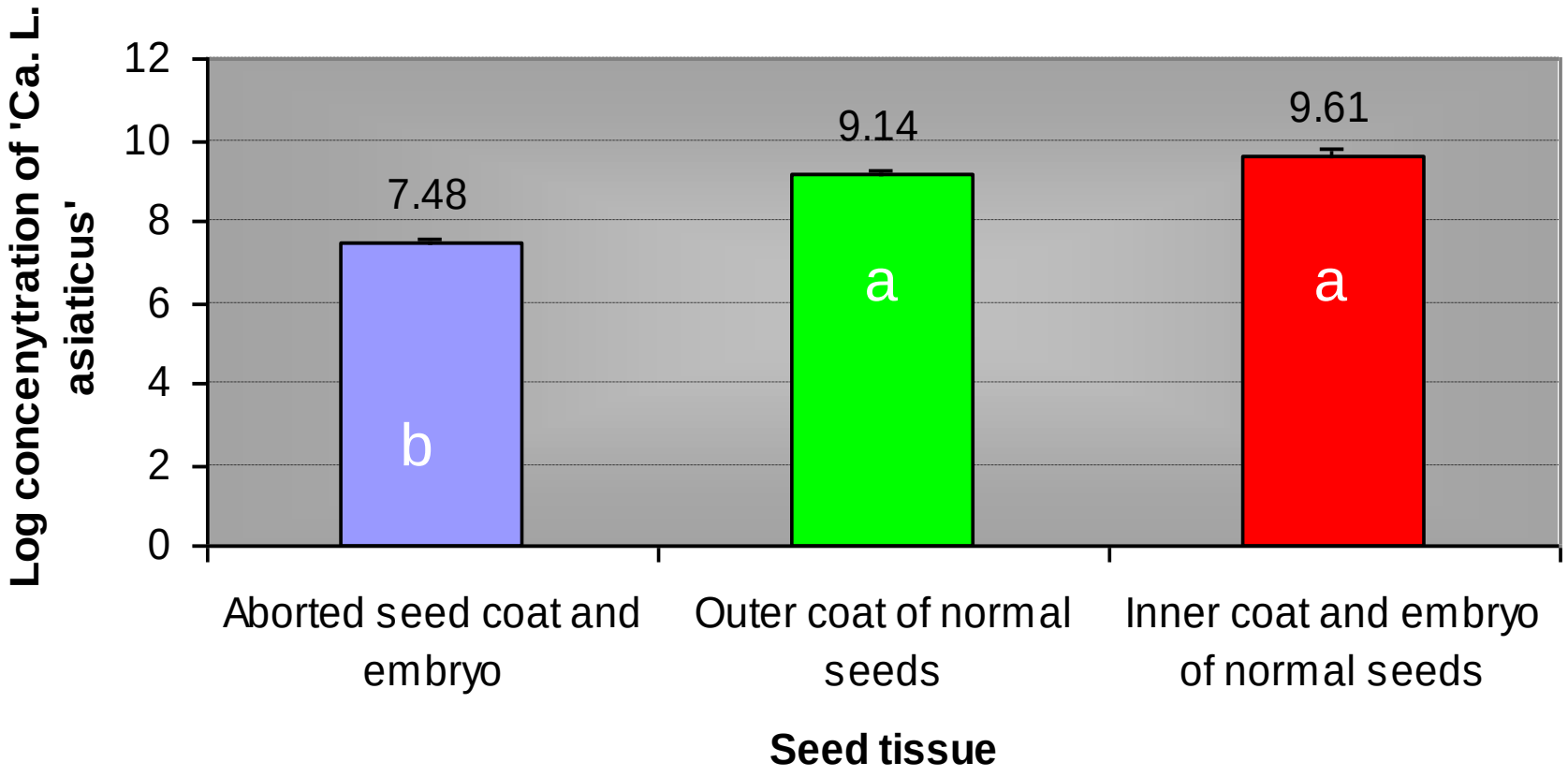
Li *et al*., 2009. Phytopathology 99:139-144

Quantification of '*Ca*. L. asiaticus' in fruit tissues of HLB-affected citrus species



Li *et al*., 2009. Phytopathology 99:139-144

Quantification of '*Ca*. L. asiaticus' in seed tissues of HLB-affected *Citrus limonia* (lime)



Li *2005, unpublished*

Distribution of '*Ca.* L. asiaticus' in fruit tissues of HLB-affected citrus species

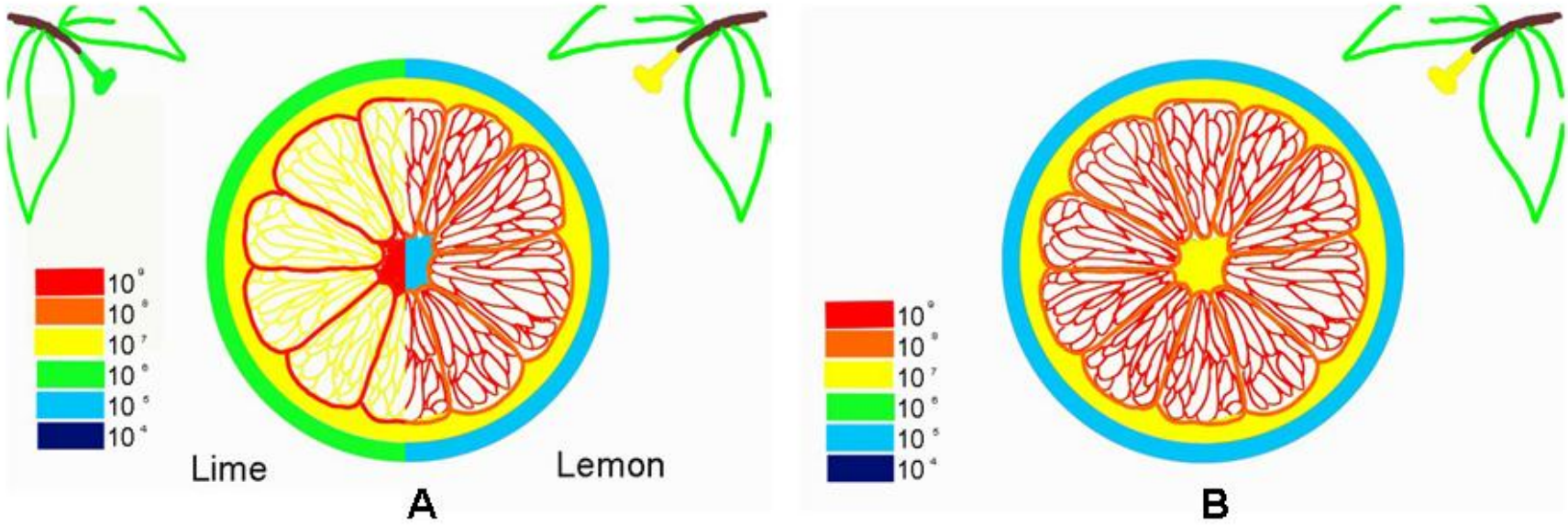


Figure. A. Distribution of *Ca* Las genomes in fruit of lime and lemon. The colors indicate the relative concentration of *Ca* Las genome equivalents per gram of tissue sampled. The peduncles are drawn in the upper corners of the figure. **B.** Distribution of *Ca* Las genomes in fruit of lime and lemon averaged over the two fruits.

Li *et al*., 2009. Phytopathology 99:139-144

Plant sample processing

- clean the samples
- get the midribs and petioles
- cut them into ~2 mm pieces

For Qiagen DNeasy mini kits, 100 to 200 mg per extraction.

For CTAB or modified CTAB, 200 to 500 mg per extraction.

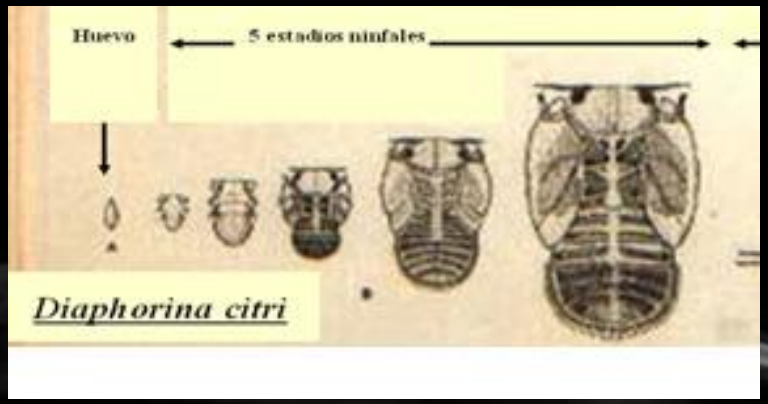
Use a closed homogenization system, *i.e*. Lysing Matrix A tubes by a FastPrep or MiniBeadBeater machine

Li *et al*., 2006. J Mircobiol Methods 66:104-115

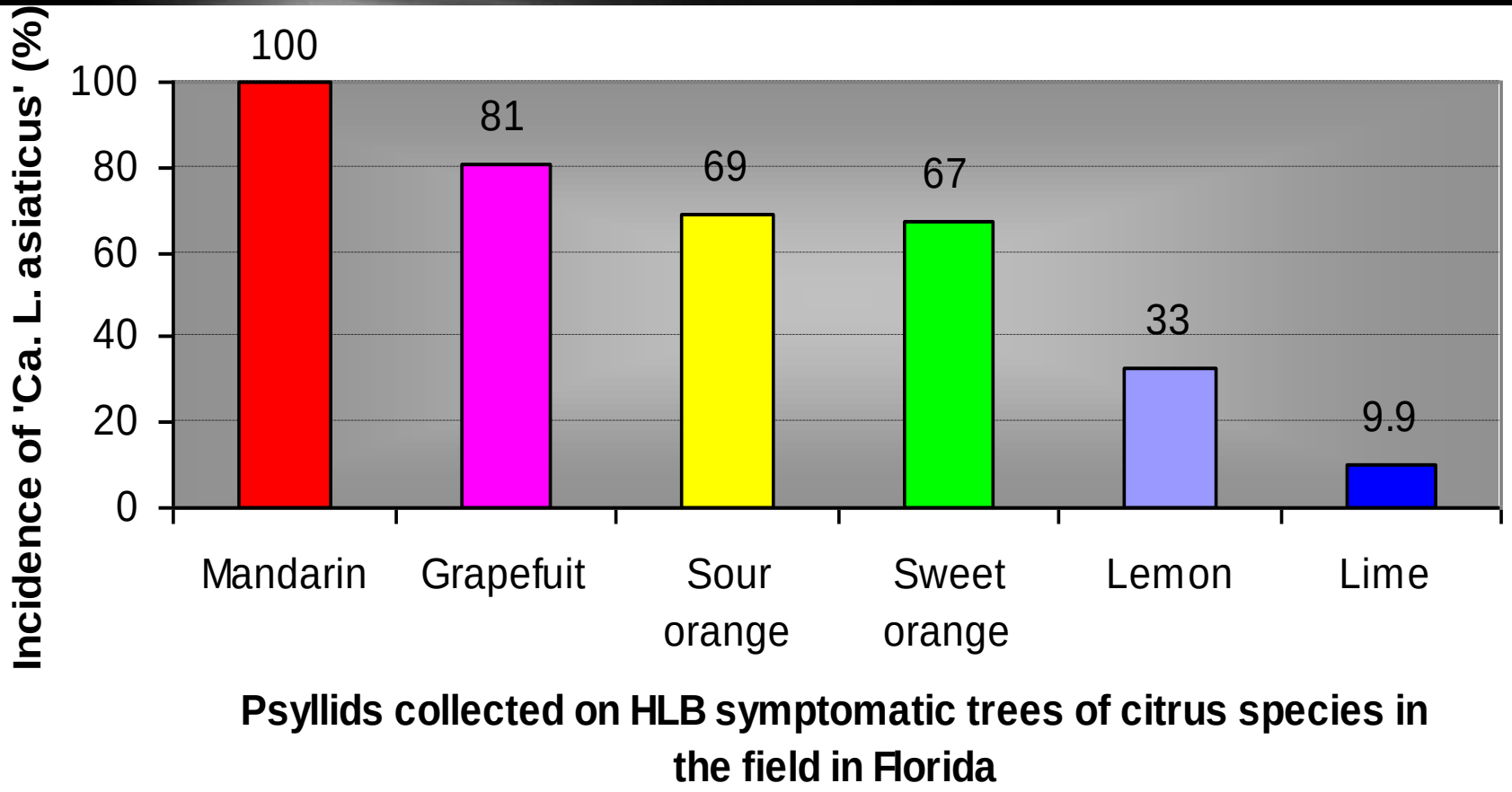
Vector psyllids samples

- Collect nymph and adult psyllids as many as possible on HLB symptomatic or suspect trees.
- Put the psyllids in individual bottles containing 70% ethanol (alcohol)

If necessary, the psyllid samples can be stored at 4ºC for more than one year for DNA extraction and PCR assays.



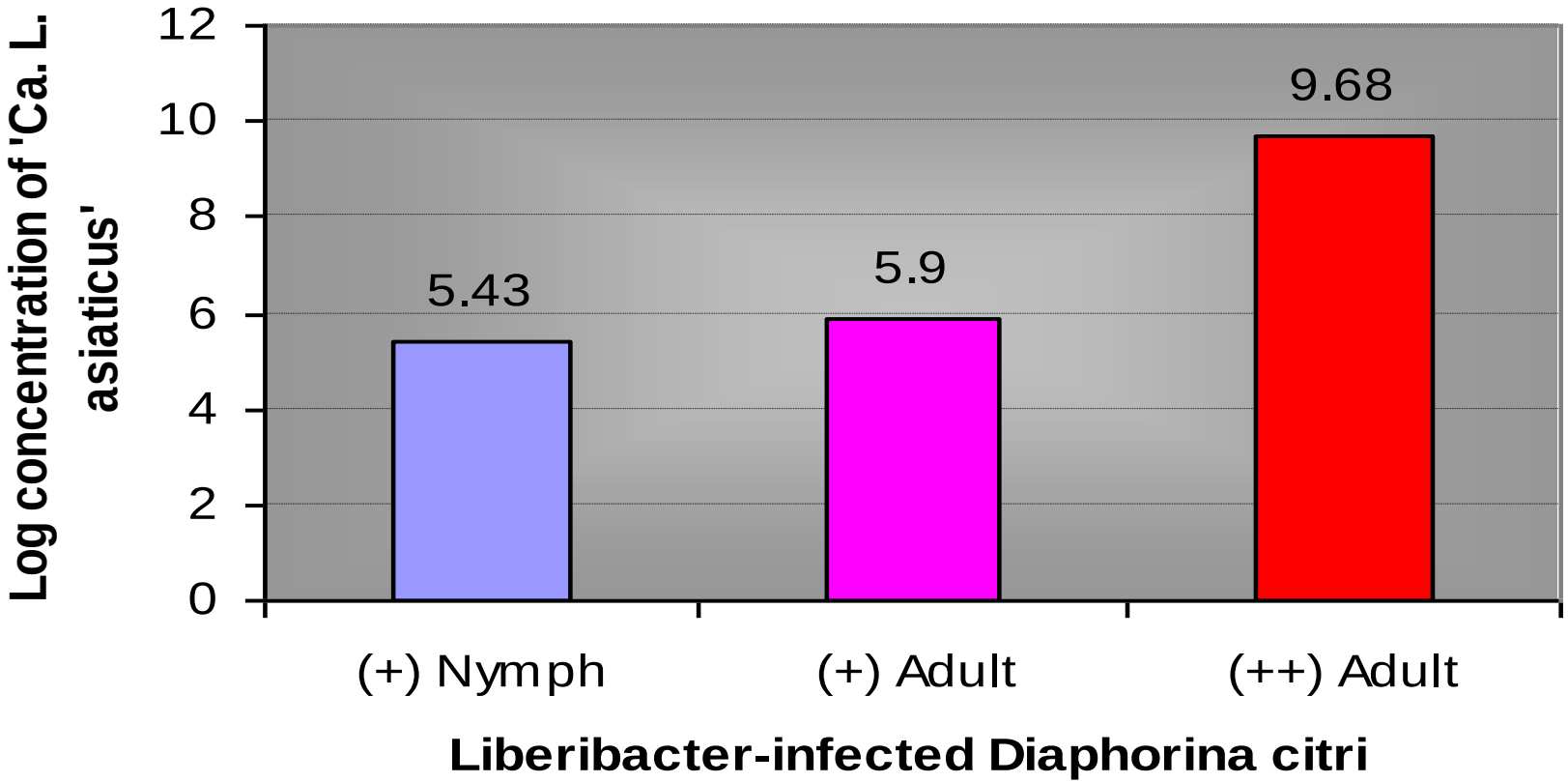
Incidence of '*Ca.* L. asiaticus' in adults of *Diaphorina citri* collected on HLB symptomatic trees of citrus species in the field in Florida , Feb. to Mar. 2007



Psyllids collected on HLB symptomatic trees of citrus species in the field in Florida

Li *et al*., 2008. IRHLB p.231

Population of '*Ca*. L. asiaticus' in adults and nymphs of *Diaphorina citri* collected on HLB symptomatic trees of citrus species in the field in Florida , Feb. to Mar. 2007



Li *et al*., 2008. IRHLB p.231

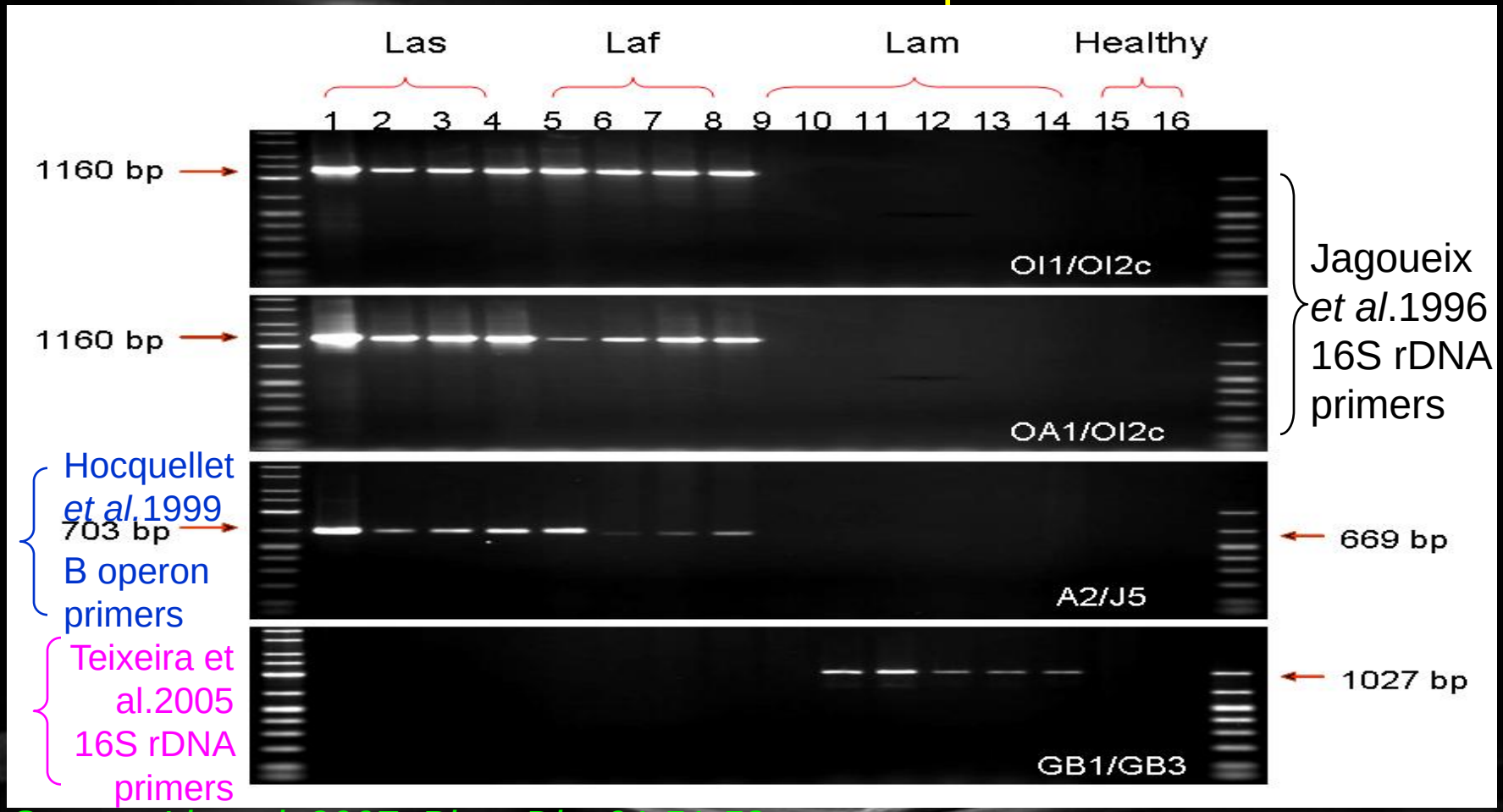
Insect sample processing

- Take psyllids from 70% alcohol on absorbent paper
- air dry the psyllids for 5-10 min
- take them to homogenization tubes or containers

For Qiagen DNeasy mini kits, 1 - 50 nymphs or 1 – 5 adults per extraction (USDA-APHIS).

Use a closed homogenization system, *i.e*. Lysing Matrix A tubes by a FastPrep or MiniBeadBeater machine

Specificity of conventional PCR primers specific to '*Ca*. Liberibacter sp.'



Source: Li et al.,2007. Plant Dis. 91:51-58.

Optimization and Standardization of Conventional PCR Protocol

1. Optimization of reagents.

Chemicals	Volume (µl)	Final concentration
Water	16.05	N/A
10x PCR buffer	2.5	1x
MgCl2 (50 mM)	1.25	2.5 mM
dNTPs (10 mM each)	0.5	240 µM each
Platinum Taq (5U/ (µl)	0.2	1 Unit
primers (2 µM each)	2.5	200 nM
DNA sample	2.0	200 ng – 5 fg
Total	25 µl	

2. Standardization of the PCT protocol using a MJ Research PTC-200.

1 cycle	94º C for 2 min
35 cycles	94º C for 30 S 62º C (OI1/2c and A2J5) or 54º C (GB1/3) for I min 72º C for 1 min
1 cycle	72º C for 10 min

Source: Li et al.,2007. Plant Dis. 91:51-58.

Differentiation of '*Ca*. L. asiaticus' from '*Ca*. L. africanus' by Bové's 16S rDNA primers together with digestion by XbaI

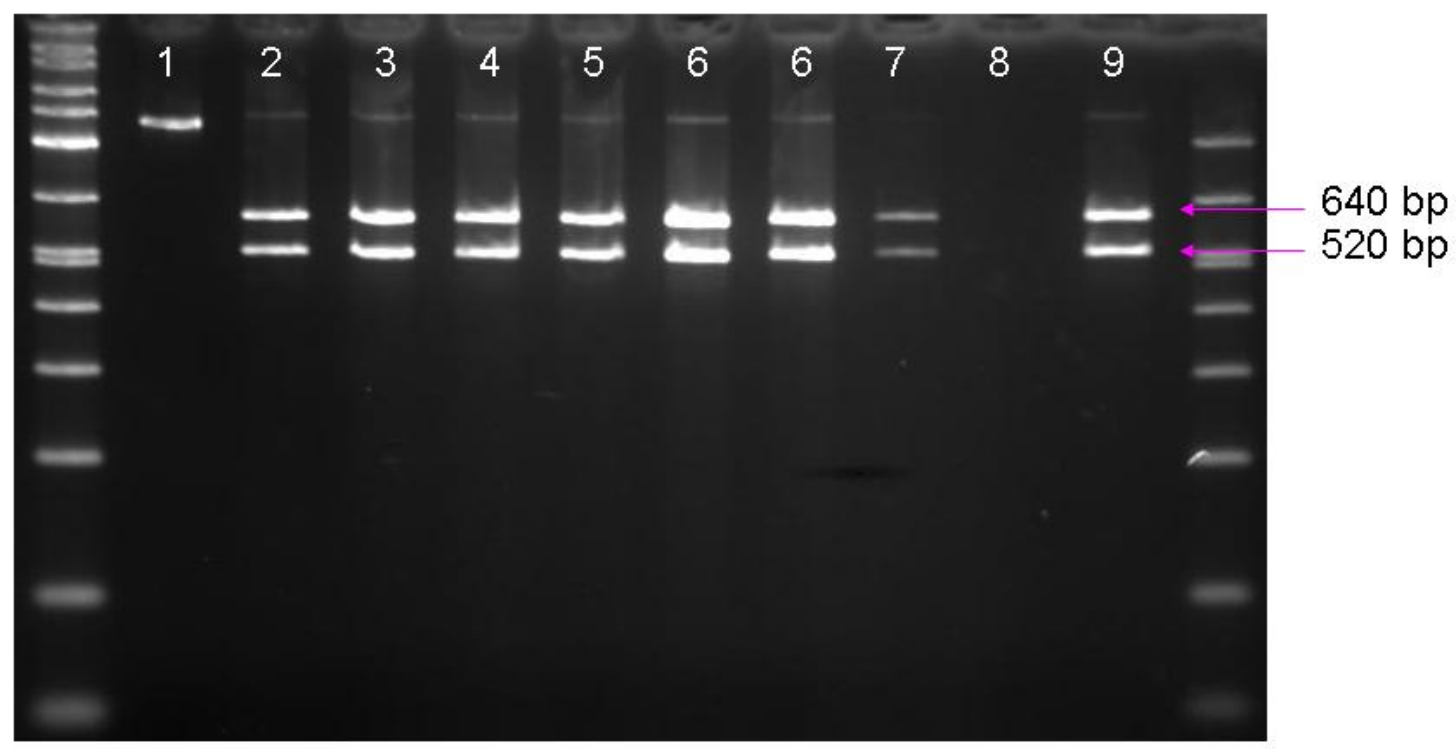
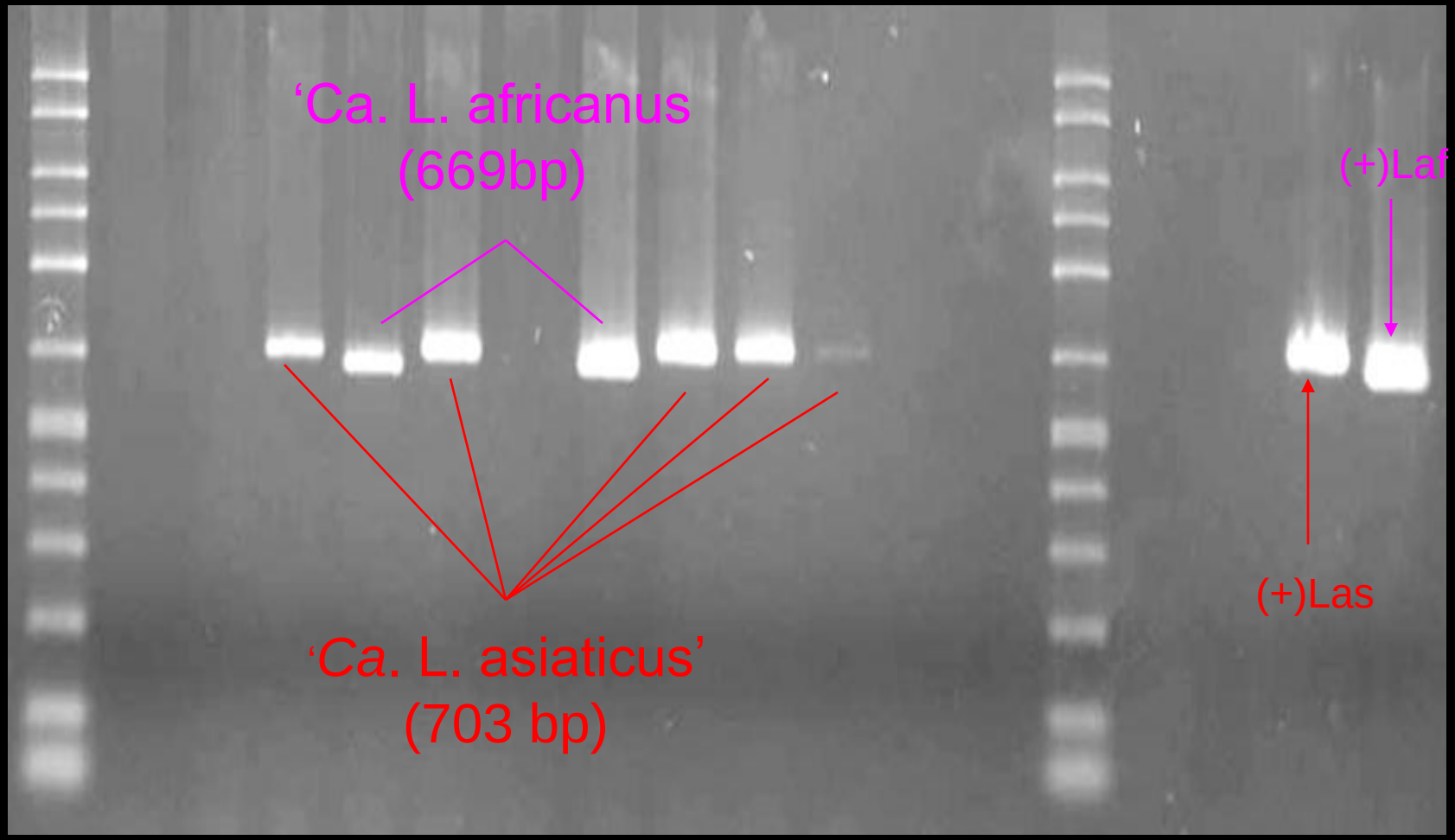


Figure. Digestion (with Xba I) of PCR products obtained with OI1/2c to differentiate Las from Laf. (Las: 640bp and 520bp; Laf: 520bp, 506bp, and 130bp)

Differentiation of '*Ca*. L. asiaticus' (Las) from '*Ca*. L. africanus' (Laf) based on PCR amplicon sizes by Bové's β operon primers



Multiplex Real-time PCR Primer and Probe Design

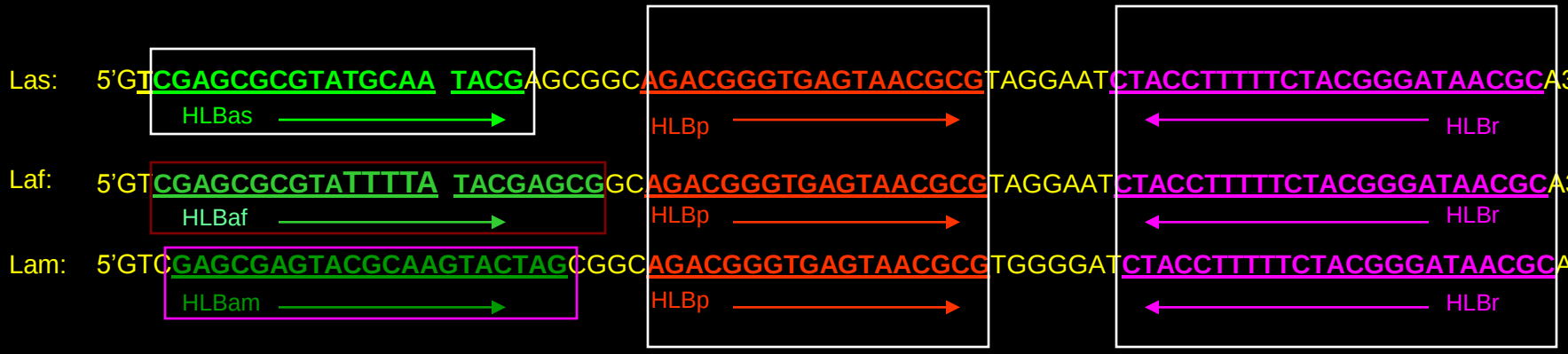
Four HLB primers and one TaqMan probe were designed based on the 16S rDNA sequences:

'*Candidutus* liberibacter asiaticus' (Las) - NCBI GenBank accession # L22532;

'*Candidutus* liberibacter africanus' (Laf) - NCBI GenBank accession # L22533;

'*Candidutus* liberibacter americanus' (Lam) - NCBI GenBank accession # AY742824.

The probe was labeled with 6-carboxy-luorescein (FAM) reporter dye.



Two positive internal control primers and one TaqMan probe were designed on basis of the sequence (NCBI GenBank accesion # AJ297817) of a conserved cytochrome oxidase (COX) gene from citrus . The probe was labeled with tetrachloro-6-carboxy-fluorescein (TET) reporter dye.

Source: Li et al.,2006. J. Microbiol Methods 66:104-115.

Optimization and Standardization of Real-Time PCR Protocol

1. Optimization of reagents.

Chemicals	Volume (µl)	Final concentration
Water	4.7	N/A
10x PCR buffer	2.5	1x
MgCl2 (50 mM)	3.0	6.0 mM
dNTPs (10 mM each)	0.6	240 µM each
Platinum Taq (5U/ (µl)	0.2	1 Unit
HLB primers (2 µM each)	3.0	240 nM
HLB probe (1 µM)	3.0	120 nM
COX primers (2 µM each)	3.0	240 nM
COX probe (1 µM)	3.0	120 nM
DNA sample	2.0	200 ng – 5 fg
Total	25 µl	

2. Standardization of the PCR protocol using a SmartCycler II.

Stage I	95º C for 20 s with optics off
Stage II	40 cycles
	95º C for 1 s with optics off
	58º C for 40 s with optics on

Source: Li et al.,2006. J. Microbiol Methods 66:104-115.

Specificity of TaqMan PCR assays for '*Candidatus* Liberibacter sp.' in plant extracts

Table. FAM Ct values of real-time PCR assays.

Species, strain or isolate		Origin	HLBaspr	HLBampr	HLBasampr
Candidatus liberibacter asiaticus	B239	Taiwan	22.51	0	22.26
	KIN1	Japan	25.26	0	24.89
	IDN5	Indonesia	26.32	0	25.99
	Br892	Brazil	21.61	0	21.05
	Fl136	Florida	20.36	0	24.25
Canduidatus liberibacter americanus	Br974	Brazil	0	23.67	20.12
	Br875	Brazil	0	22.65	23.24
	Br976	Brazil	0	24.11	22.42
	Br977	Brazil	0	24.43	23.98
	Br978	Brazil	0	23.81	24.15
Las + Lam	Br892+974	Brazil	22.21	23.56	21.88
Other citrus pathogens or indophytes	Xac Strain A	Florida	0	0	0
	X. fastidiosa CVC strain	Brazil	0	0	0
	P. Citricola I 22F3	USA	0	0	0
	P. citrophthora I 1E4	USA	0	0	0
	M. Mesophilicum SR1.6/6	Brazil	0	0	0
	C. Flaccumfacies ER1/6	Brazil	0	0	0
	P. Agglomerans ARB18	Brazil	0	0	0
	E. Cloacae PR2/7	Brazil	0	0	0
	Bacillus sp CL16	Brazil	0	0	0
	DNA from plants with CTV T36	Florida	0	0	0
	DNA from plants with citrus blight	Florida	0	0	0
	DNA from plants with citrus sudden death	Brazil	0	0	0

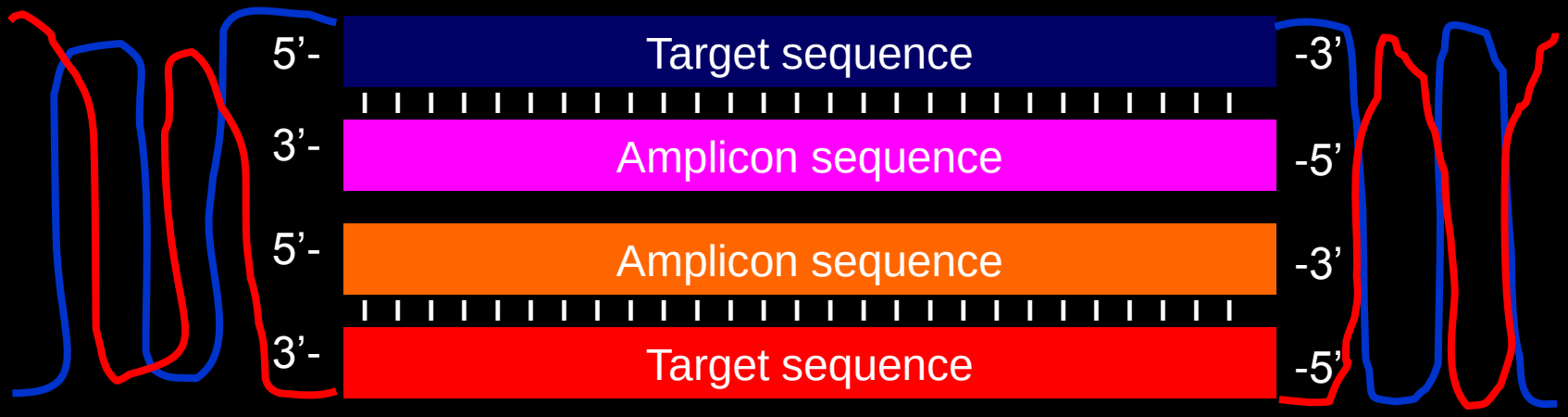
Source: Li et al.,2006. J. Microbiol Methods 66:104-115.

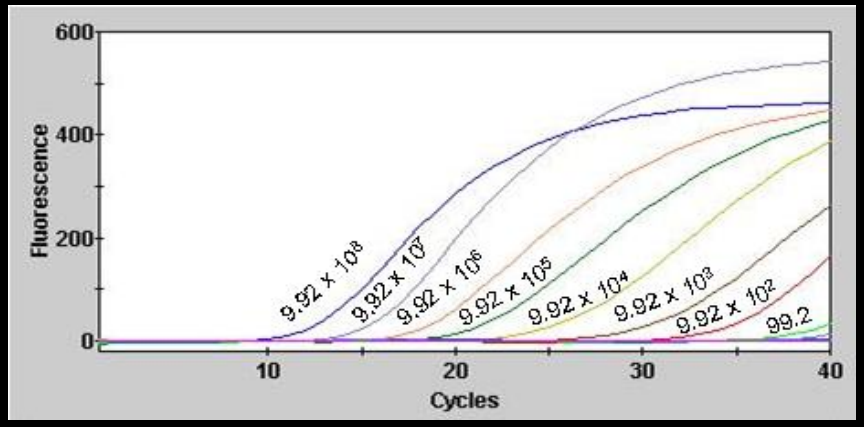
PCR math

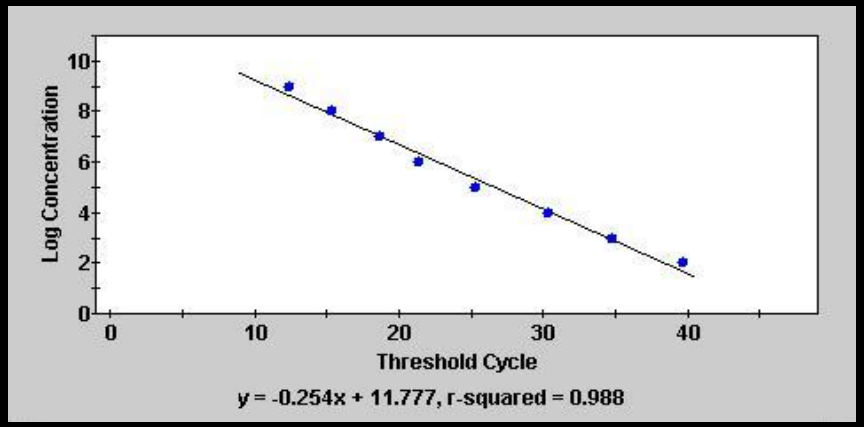
$$ACN = TCN \times 2^{cycle}$$

ACP: amplicon copy number (PCR products)

TCN: target copy number







Real-time PCR standard curve

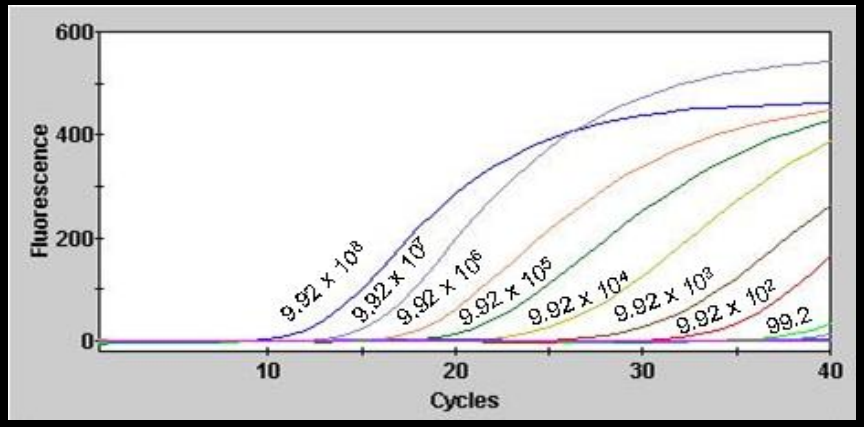
Y= a + bX

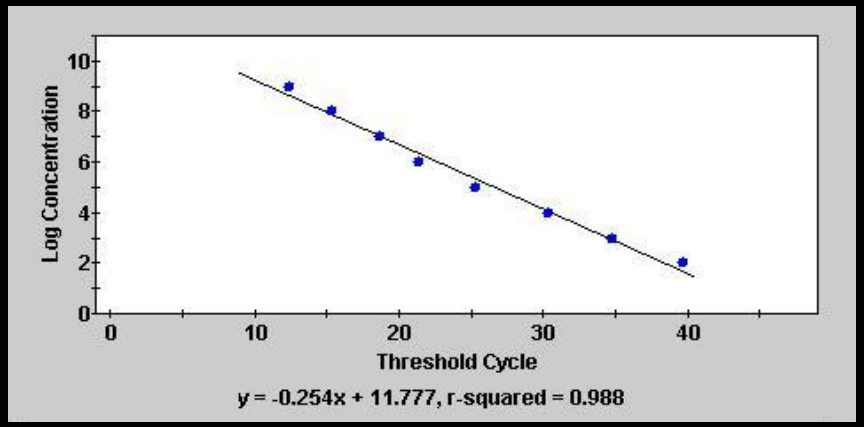
Y: Log concentration of amplicon copy number

X: real-time Ct value (cycles)

a: Y interception

b: slope





Real-time PCR Amplification Efficiency

$$AE = 10^{-slope} - 1$$

slope: b of the real-time standard curve

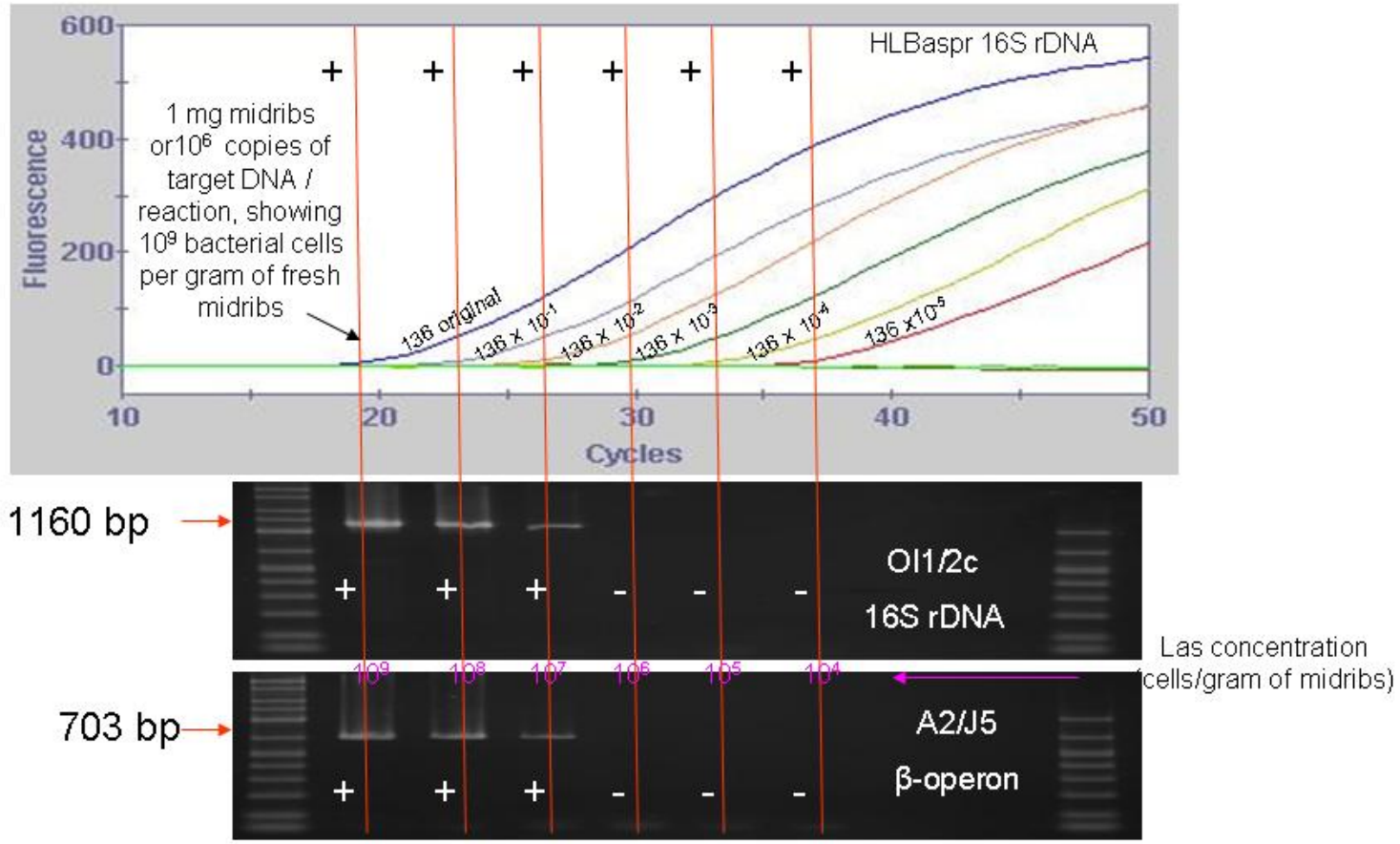


Figure. Sensitivity comparison between real-time and conventional PCRs for a DNA sample (13600a) from a Las-infected tree of grapefruit in Florida.

Source: Li et al.,2007. Plant Dis. 91:51-58.

Table. Real-time and conventional PCR for early detection of '*Ca*. L. asiaticus' in citrus plants experimentally inoculated in a greenhouse.

Plant inoculated	Detection methods	Days post-inoculations			
		30	60	90	120
Common sweet orange	Symptoms	No	No	Yes	Yes
	Ct of qPCR	35.16	32.68	17.68	16.85
	cPCR-OI1/OI2c	-	-	+	+
	cPCR-A2/J5	-	-	+	+
Ridge pineapple sweet orange	Symptoms	No	No	No	Yes
	Ct of qPCR	0	34.24	30.35	19.49
	cPCR-OI1/OI2c	-	-	+	+
	cPCR-A2/J5	-	-	+	+
C. hystrix	Symptoms	No	No	No	Yes
	Ct of qPCR	0	35.06	31.32	20.14
	cPCR-OI1/OI2c	-	-	+	+
	cPCR-A2/J5	-	-	+	+

Source: Wenbin Li et al., 2007. Plant Dis. 91:52-58

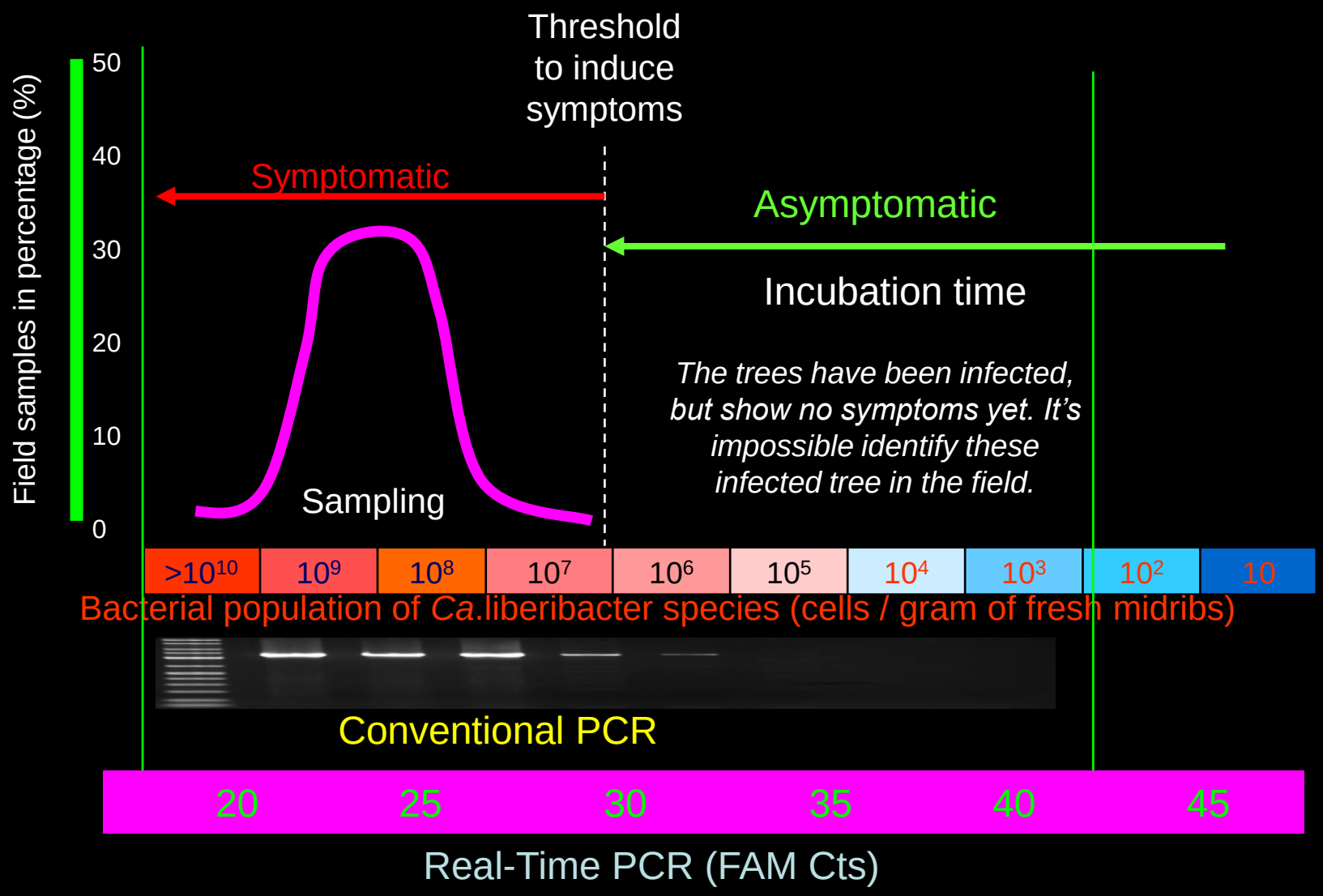
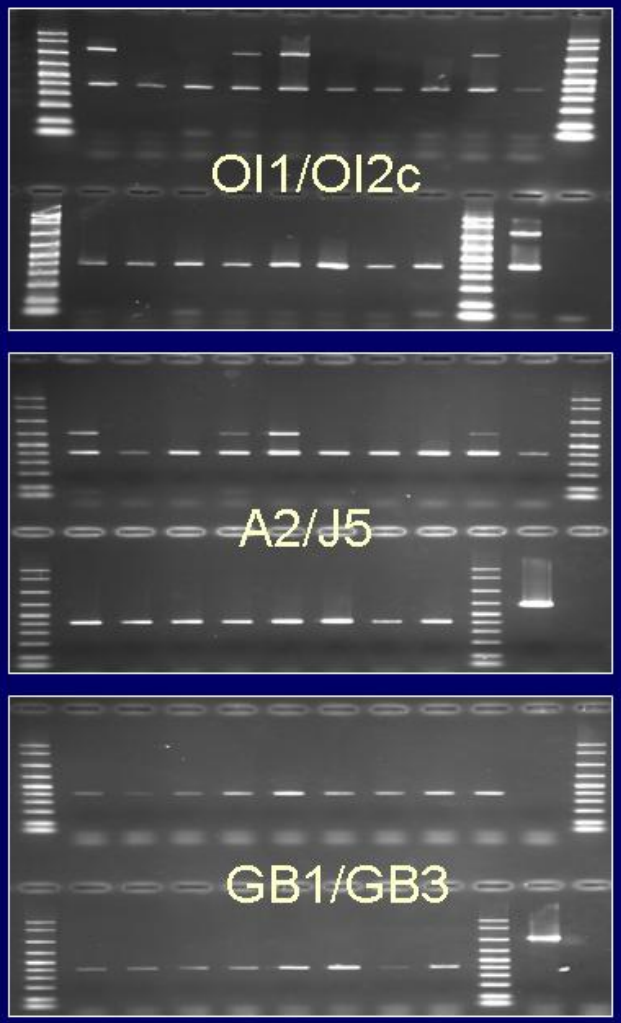


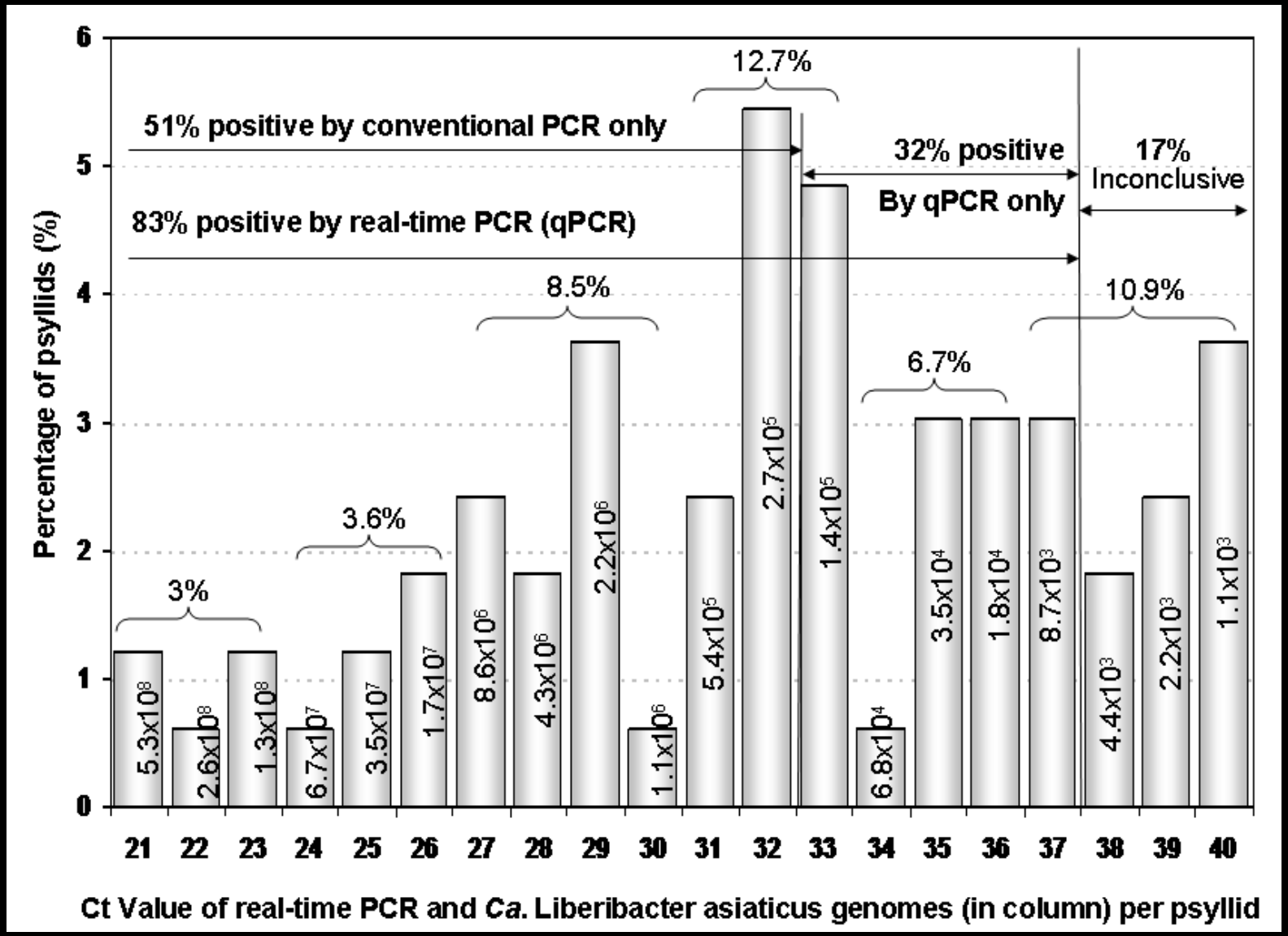
Figure. Comprehension of the Sensitivity of Real-Time PCR.

Table. Comparison of real-time and conventional PCR assays for detection of *Ca*. L. asiaticus Florida strain in single adult psyllids.

Sample	Real-time PCR		Conventional PCR		
	HLBaspr	WGfpr	OI1/2c	A2/J5	GB1/GB3
A1	25.34	27.55	+	+	-
A2	38.05	29.69	-	-	-
A3	0	28.34	-	-	-
A4	28.90	27.69	+	+	-
A5	27.08	27.98	+	+	-
B1	0	28.16	-	-	-
B2	0	28.92	-	-	-
B3	36.37	27.85	-	-	-
B4	31.39	28.13	+	+	-
B5	0	29.17	-	-	-
C1	0	29.08	-	-	-
C2	39.80	28.60	-	-	-
C3	0	29.06	-	-	-
C4	0	28.91	-	-	-
D1	0	28.23	-	-	-
D2	0	26.99	-	-	-
D3	0	28.09	-	-	-
D4	0	25.44	-	-	-
(+) ctrl	24.24	25.37	+	+	+
water	0	0	-	-	-



Source: Wenbin Li et al., 2008, Int Conf HLB, Orlando, FL



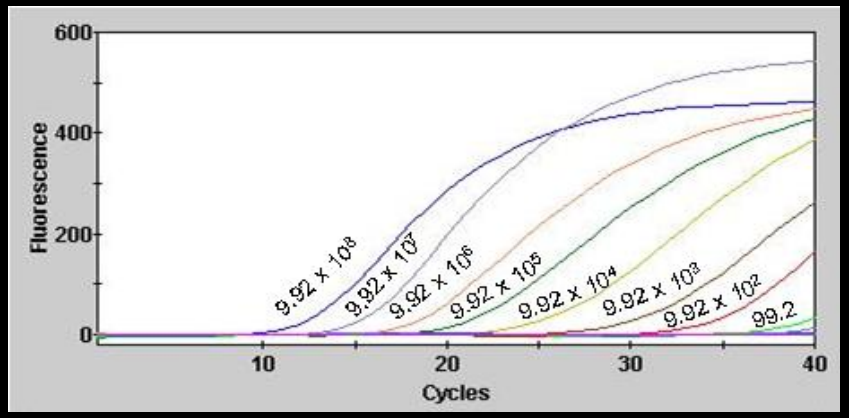
Source: Wenbin Li et al., 2008, Int Conf HLB, Orlando, FL

Low Detection Limit (LDL)

The lowest target concentration (copy number) which can be detected at a probability of 95% and above by a detection method.

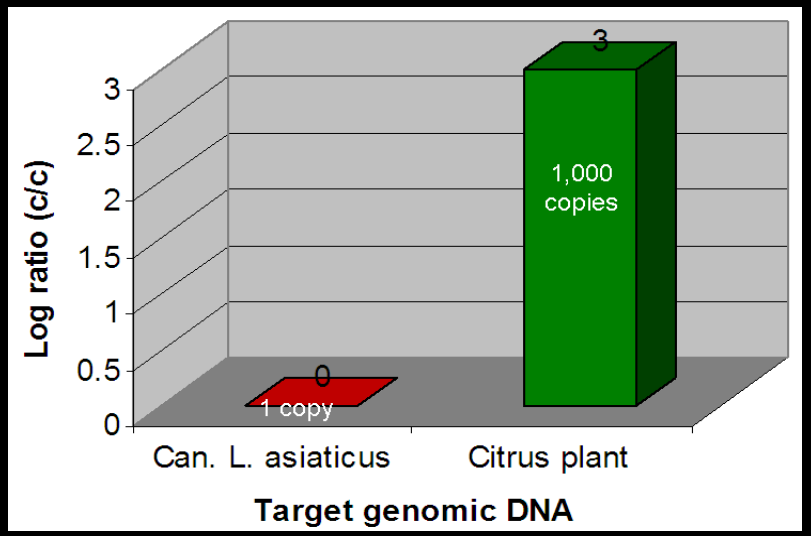
Conventional PCR

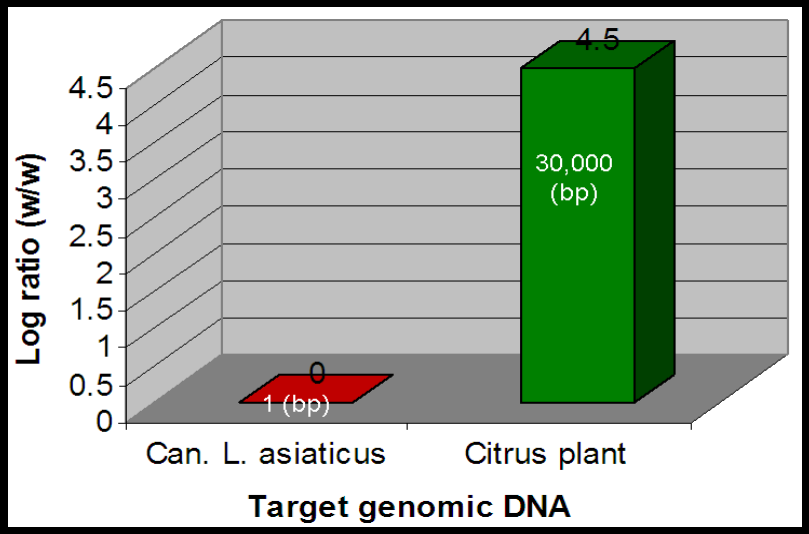
1,000-10,000 target copies per reaction

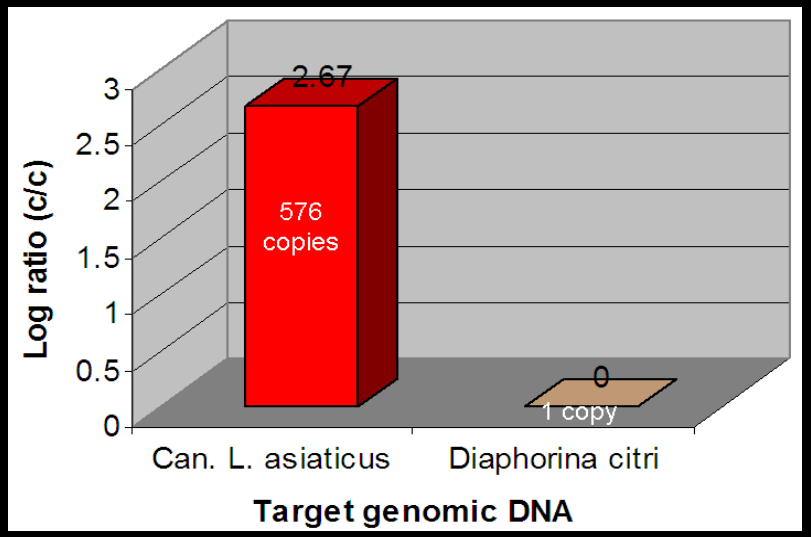


Real-time PCR

10-100 target copies per reaction







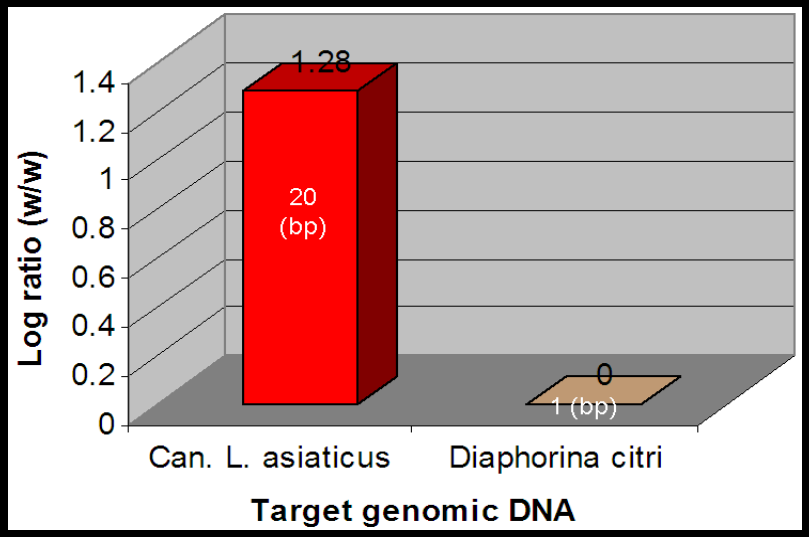


Figure. DNA ratios in extracts from Las-infected citrus and psyllids.

Source: Wenbin Li et al., 2006.JMM 66:104-115; 2008, Int Conf HLB, Orlando, FL

Molecular Plant-Microbe Interactions

22:1011-1020, 2009

MPMI Vol. 22, No. 8, 2009, pp. 1011–1020. doi:10.1094/MPMI-22-8-1011.

e-Xtra*

Complete Genome Sequence of Citrus Huanglongbing Bacterium, '*Candidatus* Liberibacter asiaticus' Obtained Through Metagenomics

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Submitted 27 February 2009. Accepted 15 April 2009.

In Press, 2009

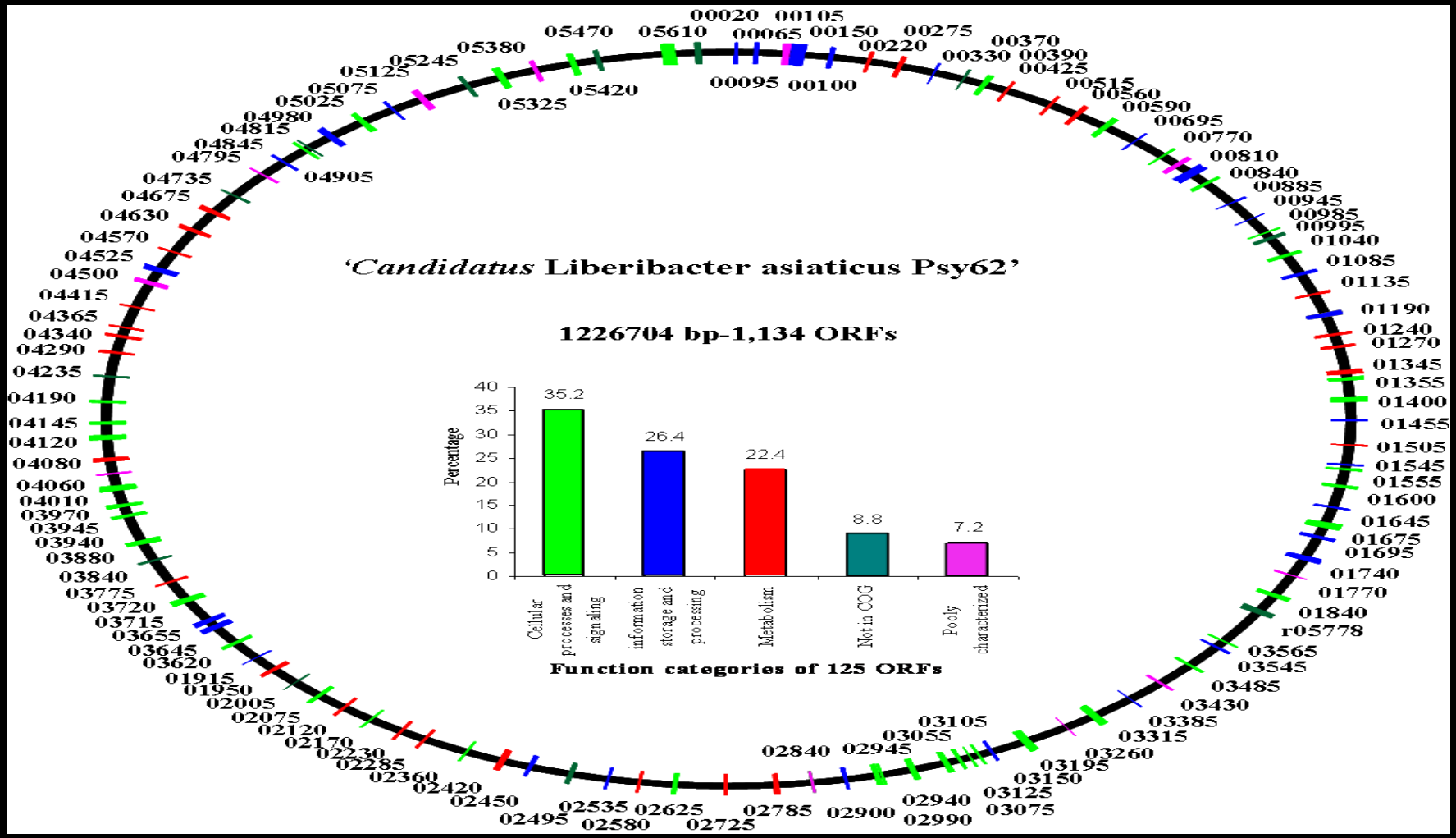
Confirmation of the sequence of '*Candidatus* Liberibacter asiaticus' and assessment of microbial diversity in Hunaglongbing-infected citrus phloem using metagenomic approach

Heather L. Tyler, Luiz W. Roesch, Siddarame Gowda, William O. Dawson and Eric W. Triplett

Dept Microbiology Cell Science, Dept Plant Pathology, CREC, University of Florida

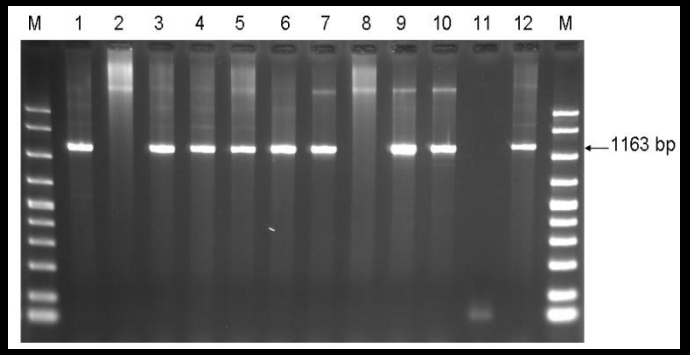
- Used three next generation high-throughput sequencing platforms, 454, Solexa and SOLiD.
- Confirmed 99.99% of the sequence of '*Ca*. L. asiaticus' in HLB-infected citrus phloem.
- A culture independent, PCR-independent analysis of 16S rRNA sequences showed '*Ca*. L. asiaticus' was the only bacterium in HLB-infected citrus phloem.

Liberibacter Genomotyping



Source: Li et al., 2009 (In preparation)

Thank you!



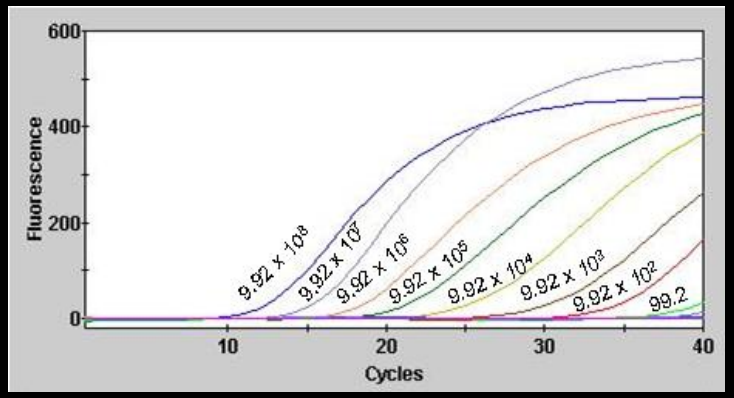


Figure. Zebra chip of potato and detection of the associated '*Ca*. L. solanacearum' by real-time and conventional PCR.

Source: Li et al., 2009, J Microbiol Methods 78:59-65

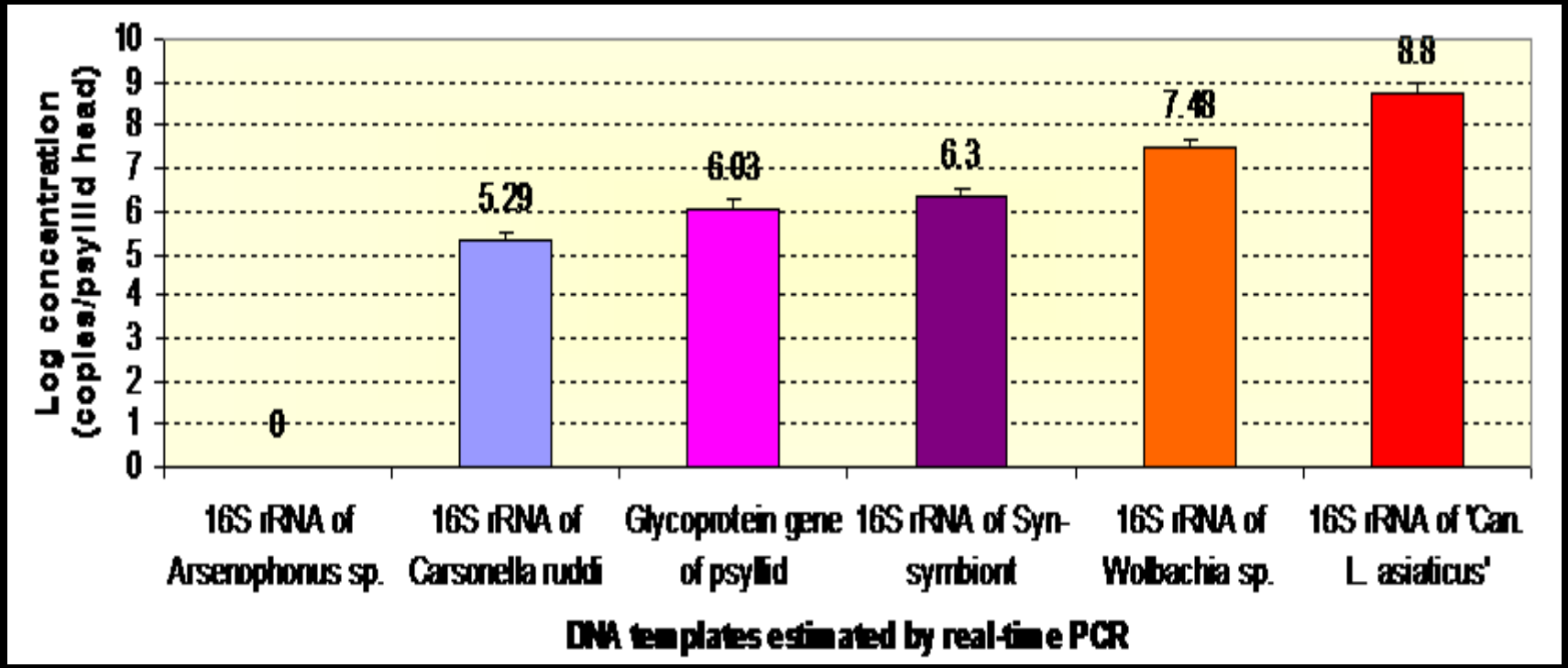


Figure. Symbiont population in an adult of *Diaphorina citri* infected with '*Can*. L. asiaticus' in a citrus orchard in Florida.

Source: Wenbin Li et al., 2008, Int Conf HLB, Orlando, FL

99: 480-485, 2009

Cultivation of '*Candidatus* Liberibacter asiaticus', '*Ca.* L. africanus', and '*Ca.* L. americanus' Associated with Huanglongbing

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Accepted for publication 13 December 2008.