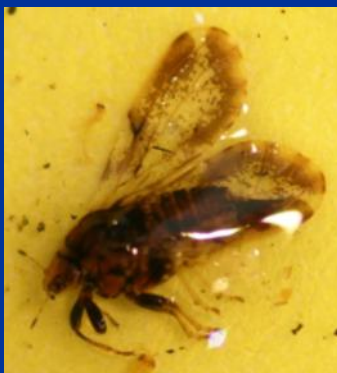


# Early Detection of Huanglongbing-associated Bacteria in Psyllids



Keremane Manjunath,  
Chandrika Ramadugu, Susan Halbert\* and Richard Lee

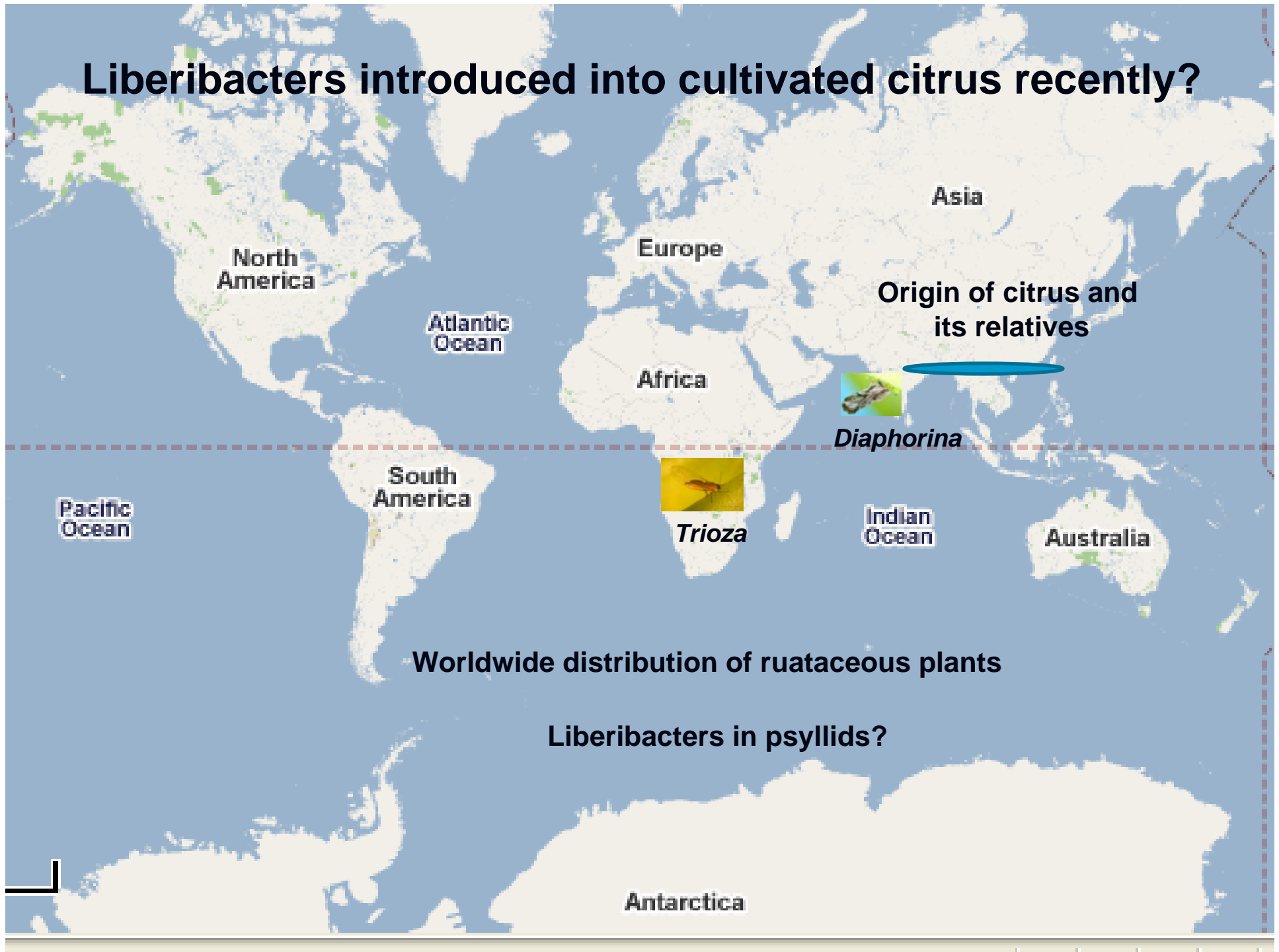
USDA-ARS, National Clonal Germplasm Repository for  
Citrus and Dates, Riverside, CA and

\*Division of Plant Industry, FDACS, Gainesville, FL

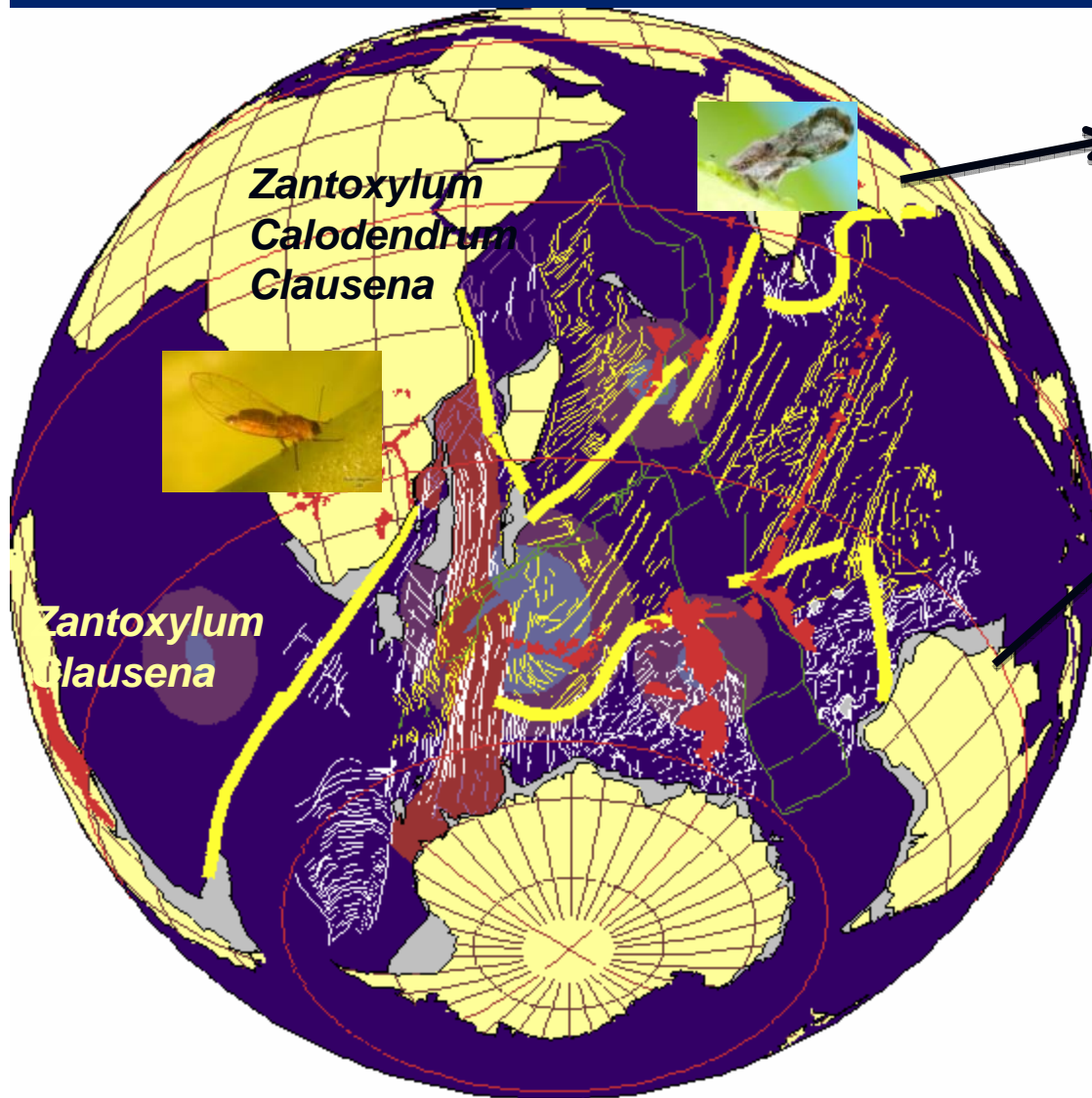
- ❖ Liberibacters may have evolved with psyllids, probably in the family Triozidae
- ❖ Association of HLB and true citrus may be very recent
- ❖ Movement of citrus into new areas has resulted in new diseases-
  - ❖ CVC, leprosis, sudden death. HLB?

Origins of citrus, psyllids and Liberibacters

# Liberibacters introduced into cultivated citrus recently?



# Evolution of plants, psyllids and Liberibacters



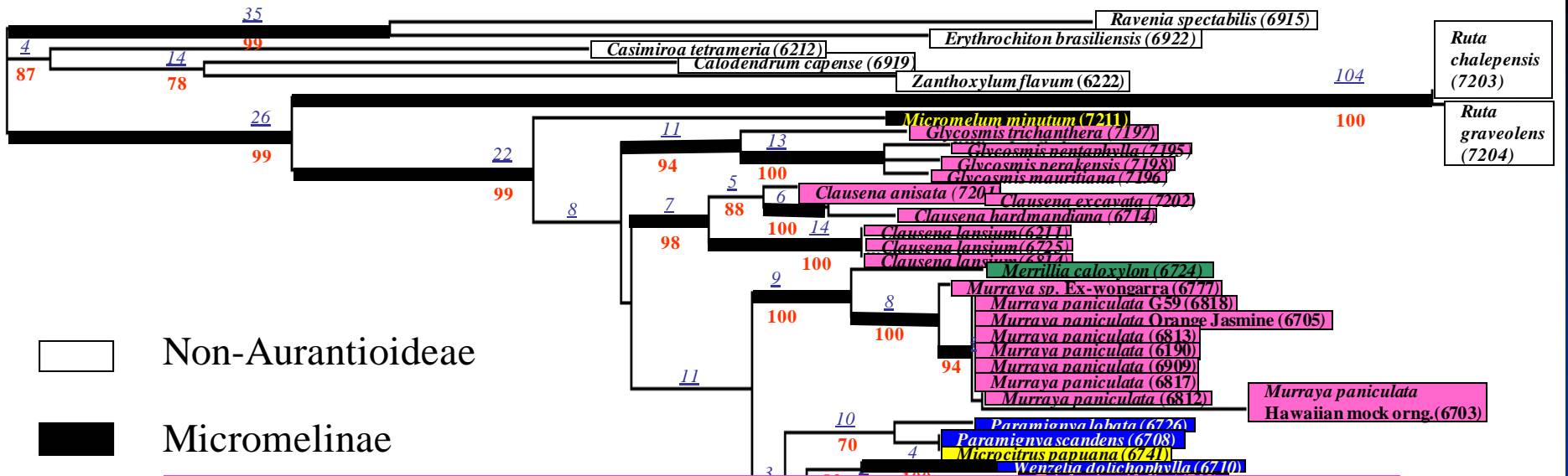
*Citrus ichangensis*, *C. latipes*  
*C. Jambhiri*, *C. limonia*, *C. assamensis*, *C. karna*, *C. limettoides*, *C. aurantium* and progenitors of citron. Lemon and some mandarins

*Microcitrus*, *Eremocitrus*, *Clymenia*, *Oxanthera* in Australia, New Calodenia and Papua New Guinea

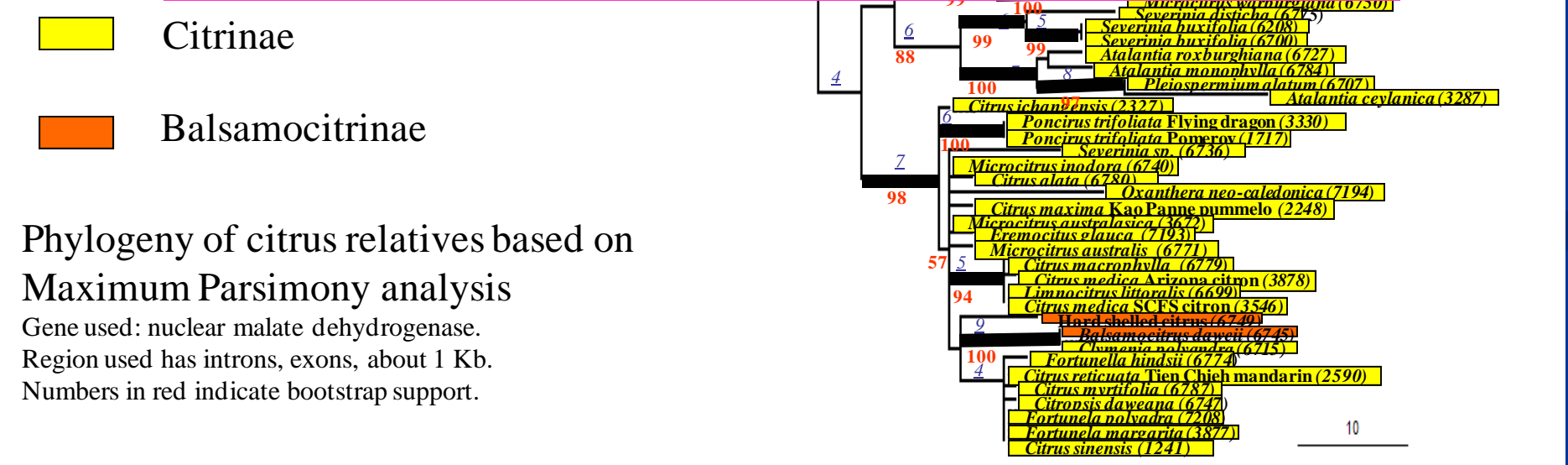
200 Ma (Million years ago)

**30 Ma**

Present



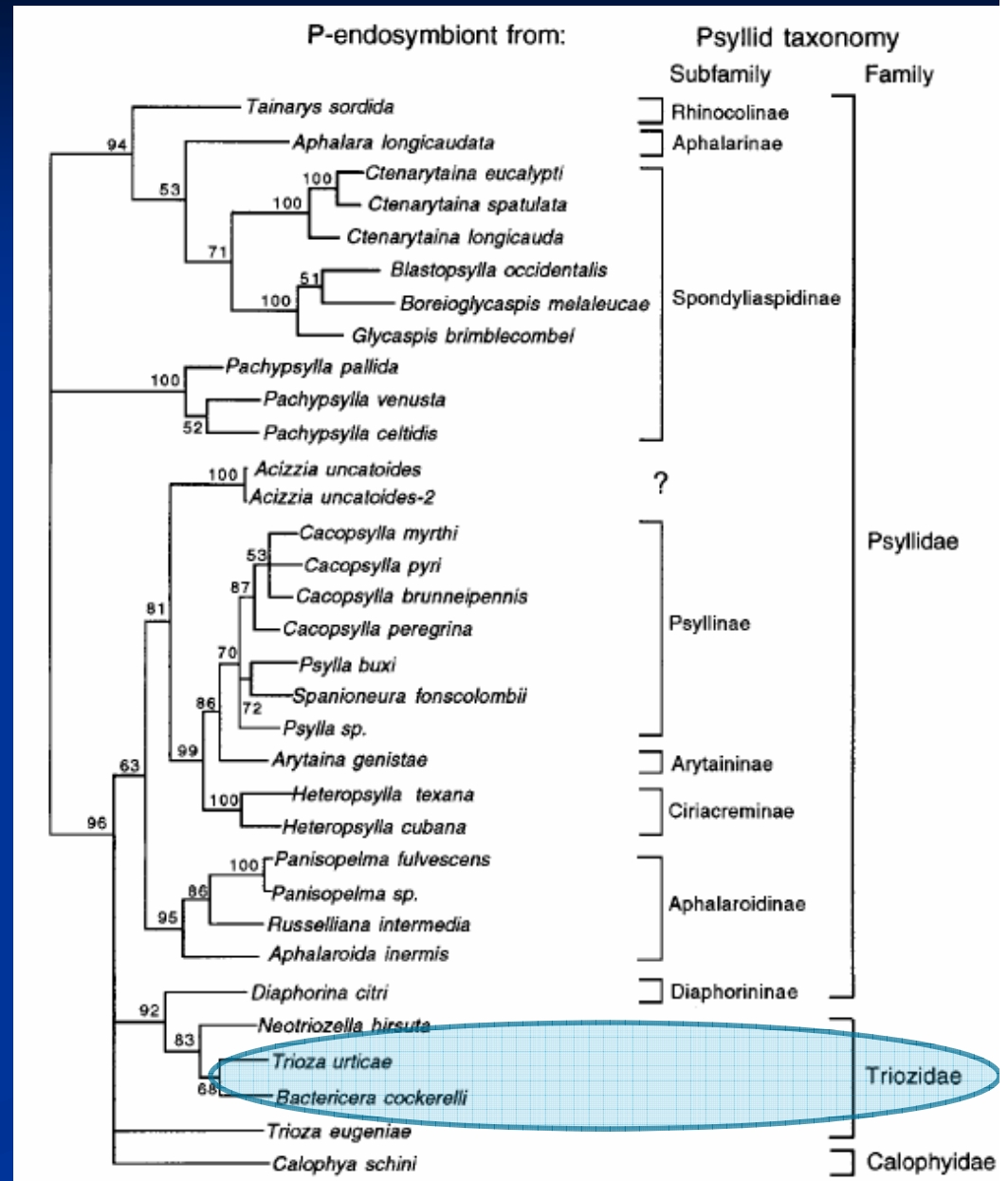
# Phylogenetics for proper identification of host



Phylogeny of citrus relatives based on Maximum Parsimony analysis  
 Gene used: nuclear malate dehydrogenase.  
 Region used has introns, exons, about 1 Kb.  
 Numbers in red indicate bootstrap support.

# Evolution of psyllids, endosymbionts and Liberibacters

- ❖ Psyllids and their primary endosymbionts have coevolved (Thao et al., 2000)
- ❖ Evolution of Liberibacters may not be synchronous with psyllids?
- ❖ Primary evolution of Liberibacters in Triozidae?
  - ❖ Higher titers in *Bactericera*
  - ❖ Transovarial transmission
- ❖ Occasional movement of Liberibacters to other psyllid groups like *Diaphorina*?
  - ❖ Introduced plants and pests
  - ❖ Through host passage via parasitic plants



# HLB

Longer incubation period in plants

A few months to over 2 years

# Prolonged latency of bacteria

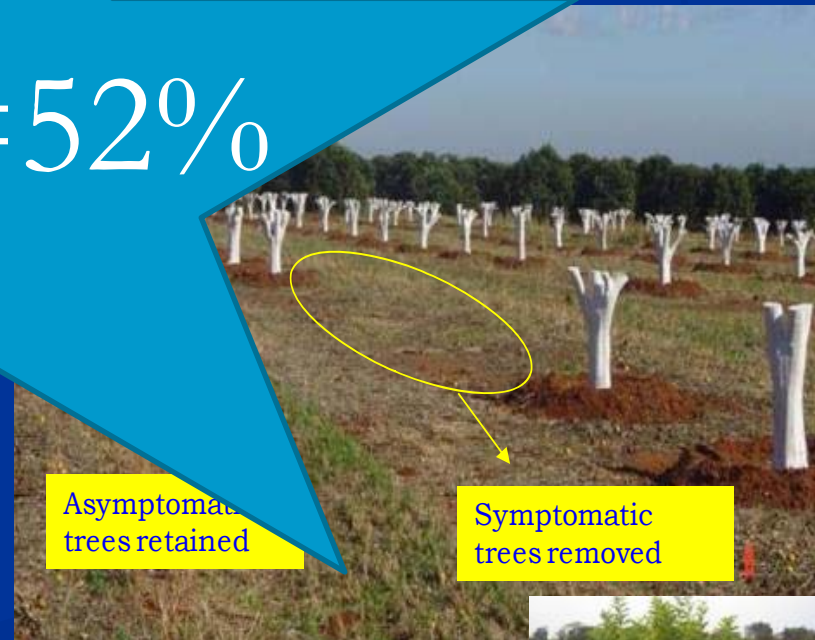


- ❖ When trees were severely pruned, a large number of non-symptomatic trees developed symptoms
- ❖ Longer latency with HLB?

In collaboration with Jose Luiz and Fernando Tersi, Cambuhy Farms, Brazil

No Plants	Greening / Eradicated		
145	27		
118	3	35	11/22/2006
115	8		3/14/2006
107	10		5/20/2006
97	12		9/22/2006
85	1		12/20/2006
84	1	3/2/2007	1%
83	0	11/29/2007	0%
		<b>Total</b>	<b>52%</b>

19% = 52%

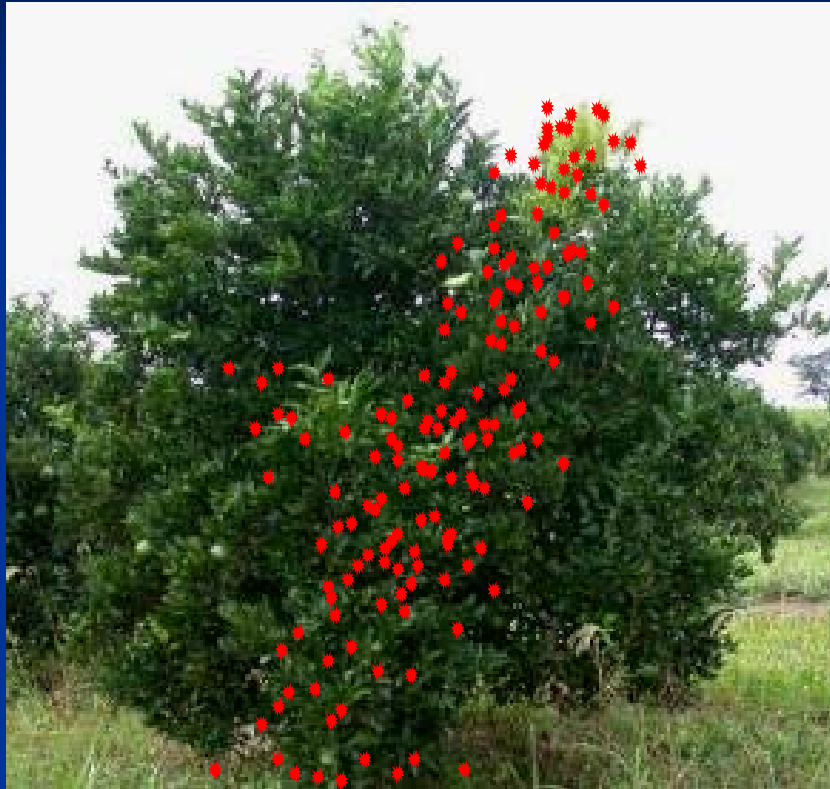


This field trial suggested a latency of up to two years under field conditions





# An early symptom may not mean a recent infection



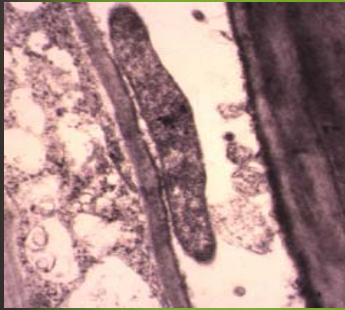
The tree may act as a source of inoculum for psyllid transmission well before the symptoms become visible

Manjunath et al., 2008  
Lopes et al., 2009

# Testing psyllids



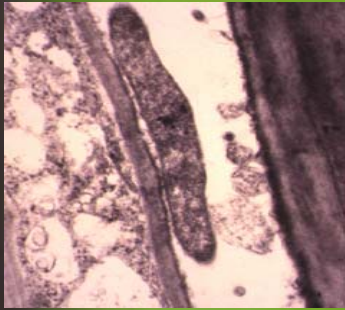
David Zeisk



Garnier



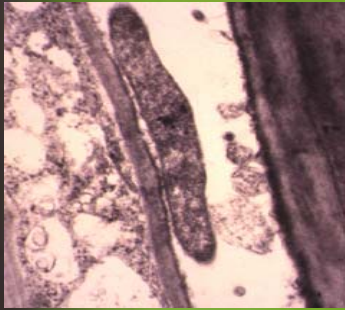
In a low titer situation,  
HLB bacteria may some times  
not be able to survive



Garnier



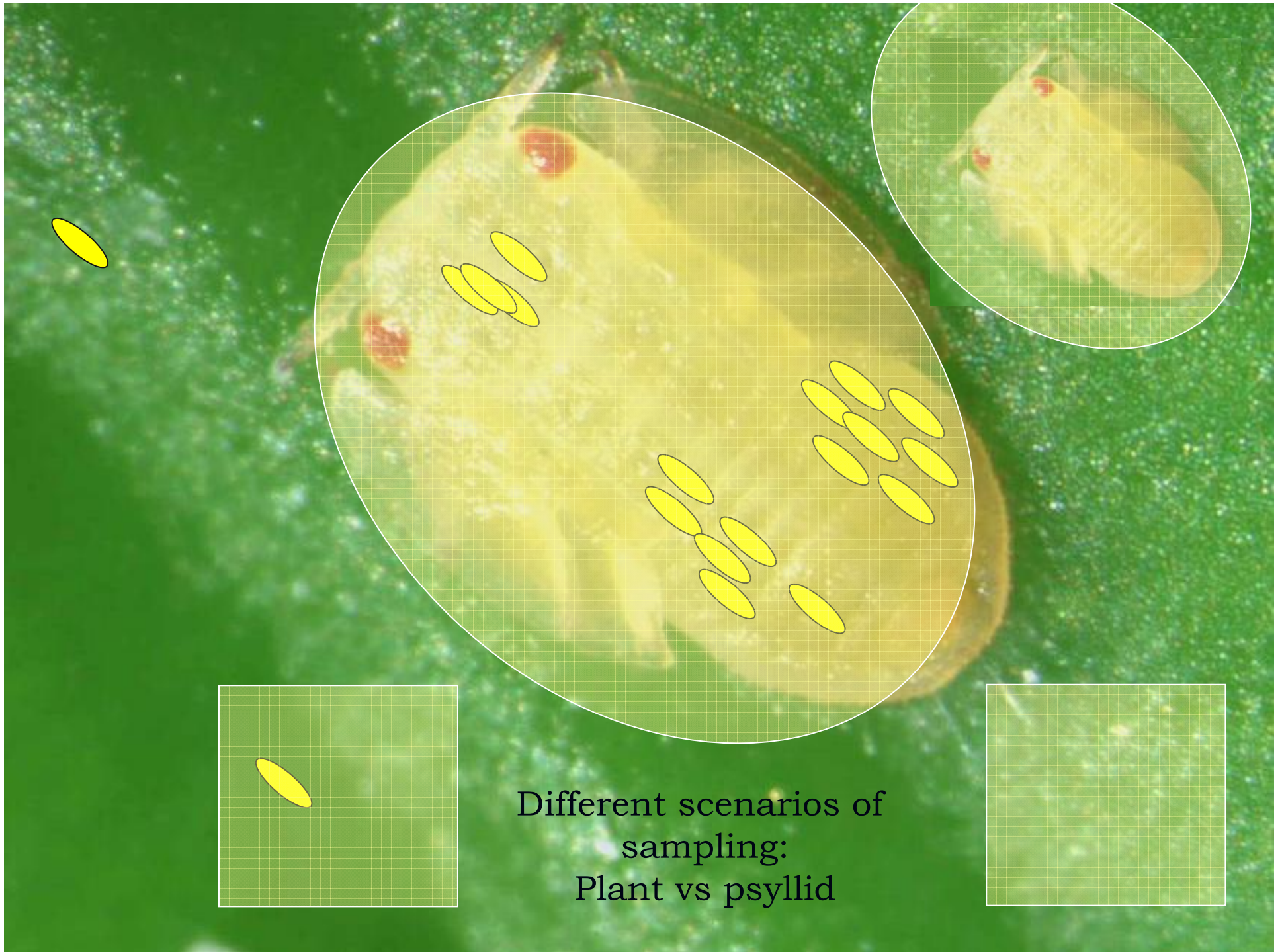
In a low titer situation,  
HLB bacteria may move around  
without being encountered by  
the psyllids



Garnier



In a low titer situation,  
HLB bacteria may be acquired  
by psyllids leading to rapid  
multiplication



Different scenarios of  
sampling:  
Plant vs psyllid

# Sampling for PCR: Plant vs psyllid



—————→  
About 20 days on the same plant  
Sucking continuously.....  
A PCR machine on plant...

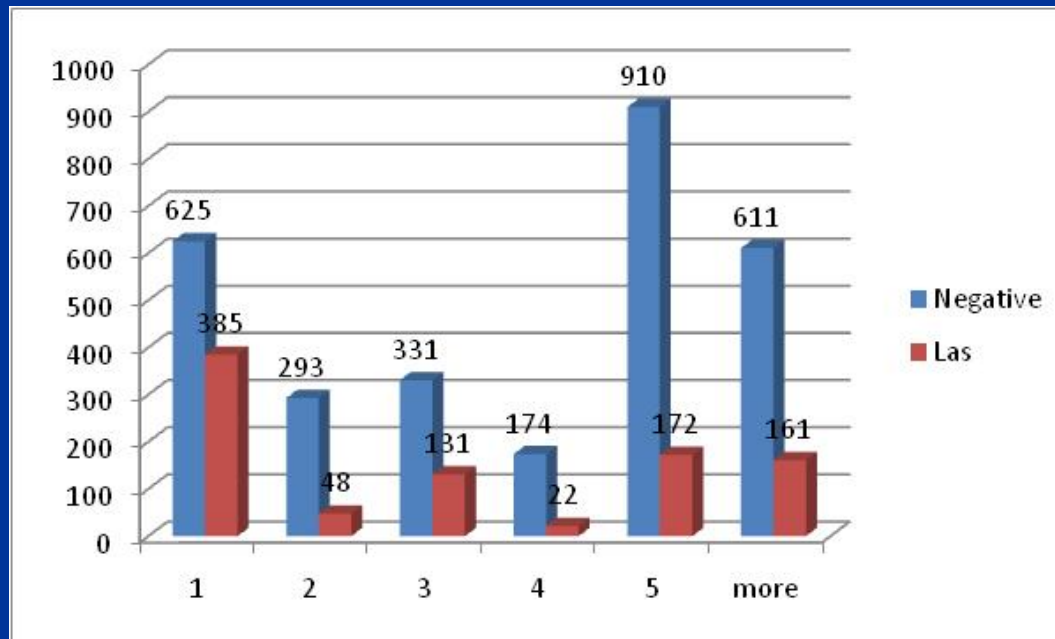
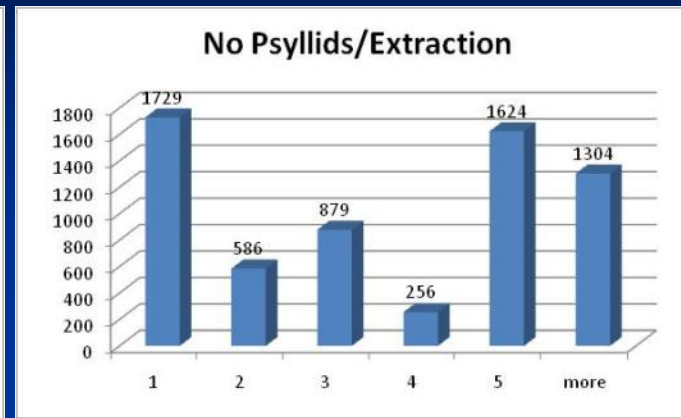
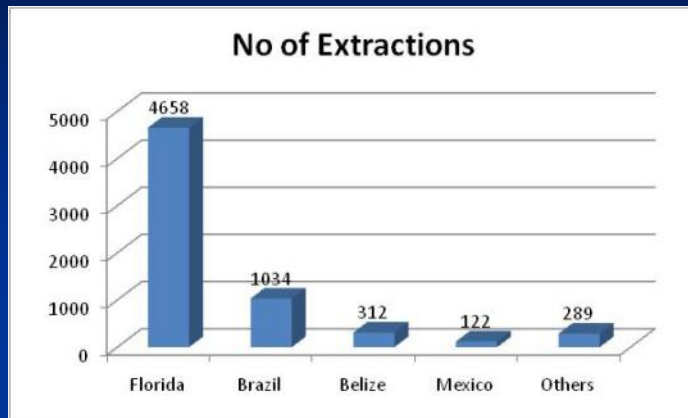
# Sampling methods



1. Hand collection
2. Suction traps
3. Yellow sticky traps (not preferred)

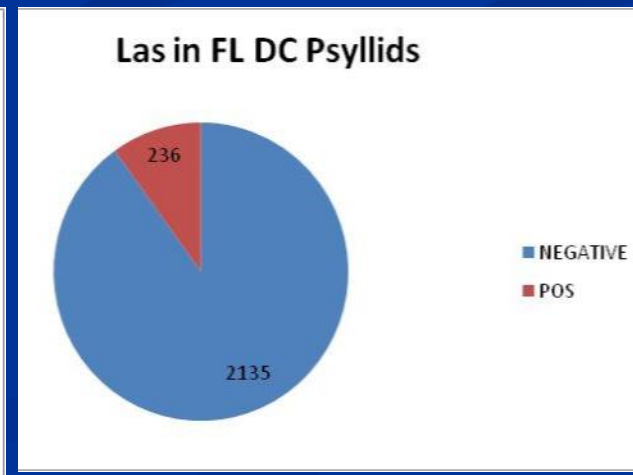
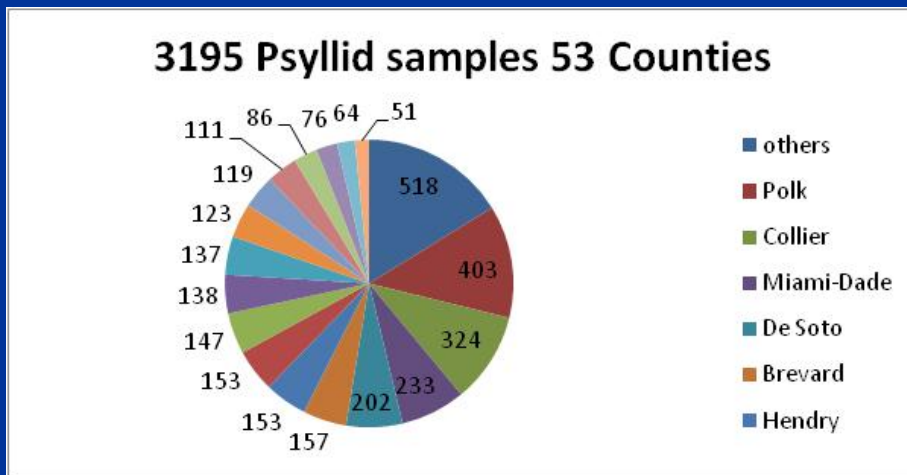
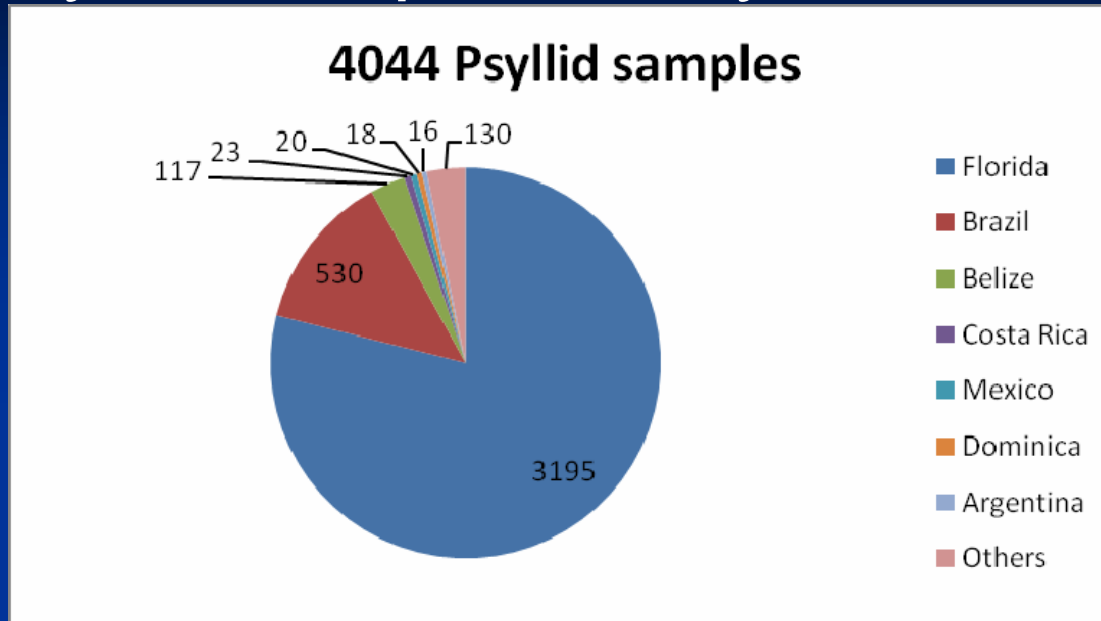


# Psyllid DNA Extractions



Las can be detected from single adult psyllid, or single nymph

# Psyllid samples analyzed in 2007-08

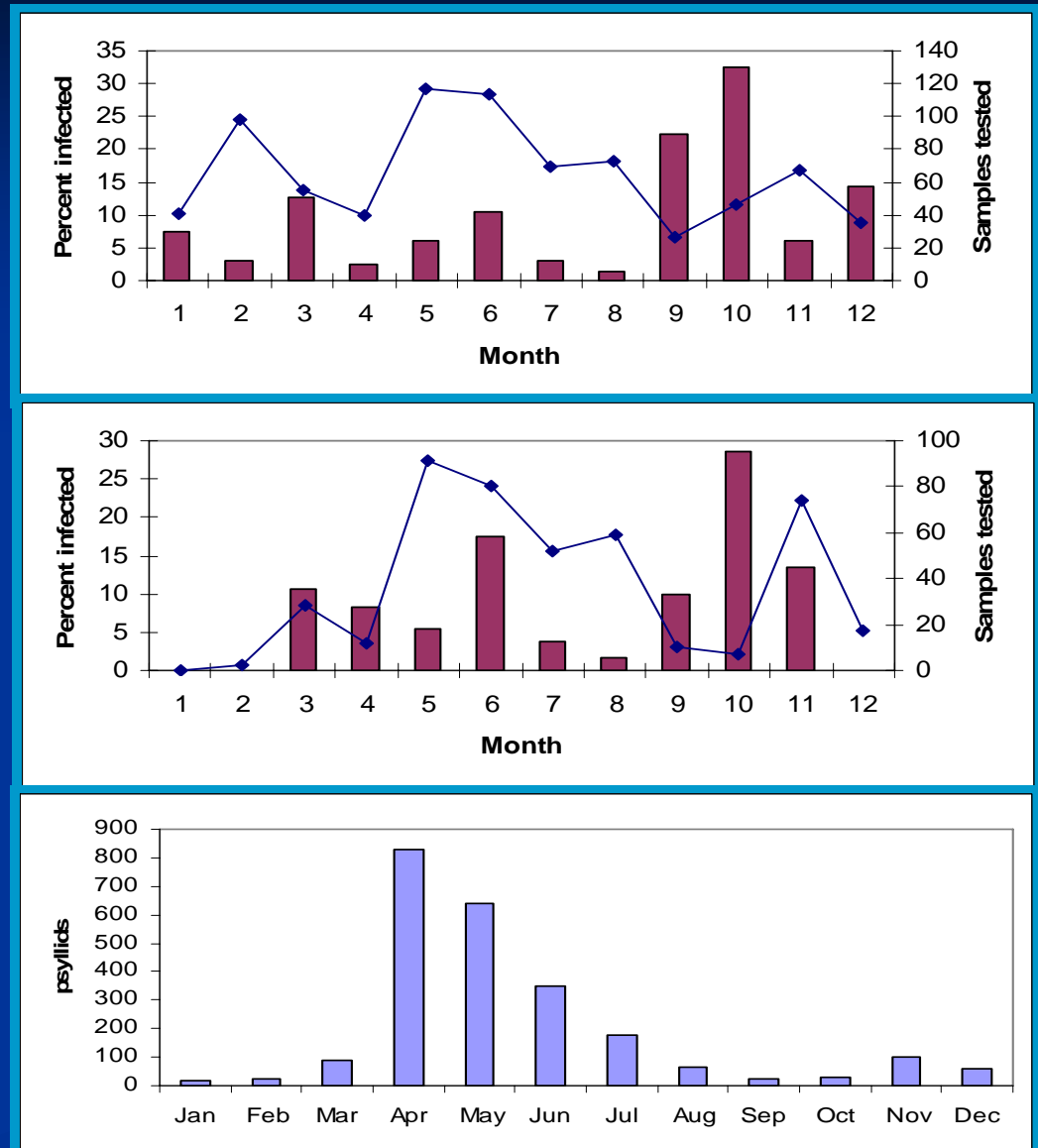


9.95% Positive for Las in FL samples

# Seasonality of psyllid transmission of Las

Detection of Las in psyllid adult (A) and nymph (B) samples collected from Florida in different months. Total number of samples tested each month and percent samples (line graph) with HLB incidence (bar graph) are shown in each graph.

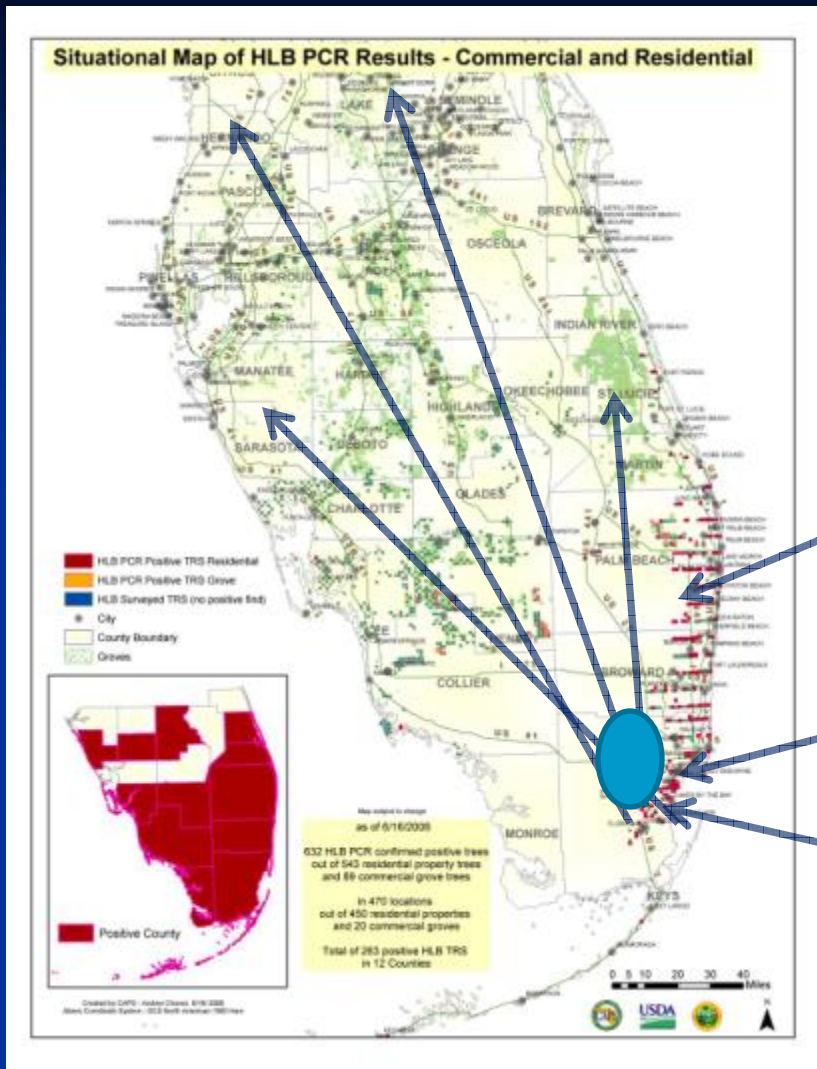
Monthly data on the number of psyllids collected from a suction trap from March 2006 to February 2007, located in a citrus grove in Palm Beach is shown in graph C.



Both psyllids and Las moved around the state and probably to other states on retail nursery plants and on *Murraya* plants

Big box stores provide nearly ideal place for multiplication of Las-infected psyllids

- Friendly temperature conditions
- Ideal young flush for psyllid growth
- Psyllid infestation doesn't attract attention
- Infected plants are not likely to show symptoms while at store



*D. citri* first find  
1998

*D. citri* moved to  
Murraya nursery  
within a year

HLB first find  
Aug 2005

Early detection of Las in psyllids

# Early detection of HLB movement by testing psyllids

County	Psyllid Record <sup>a</sup>	Total	Plant Record <sup>b</sup>
Brevard	5/31/06	10/62	12/7/06
Collier	1/30/06	13/133	11/29/05
Dade	10/08/05	26/139	8/23/05
De Soto	2/13/06	7/75	12/7/05
Hendry	1/28/06	7/104	10/11/05
Highlands	8/2/05	2/12	11/15/05
Marion	6/2/06	13/56	5/17/07
Monroe	10/19/05	2/8	10/15/05
Nassau	9/15/05	1/14	
Palm Beach	10/5/05	6/31	9/28/05
Polk	9/2/05	12/135	9/20/07
St. Lucie	5/26/06	3/31	10/13/05

→ No commercial citrus

In several Counties, orchards, HLB activity was first detected in psyllids well before plant symptoms could be found  
Some first records were in big box stores

Sample No.	Dt Coll.	Host	Source	County	psyllids/extrn	Ct/Las	Ct/DC	Las	Psy DNA
F0136	1.30.2006	<i>C. sinensis</i>	Grove	Collier	50	23.20	29.50	P	P

- An isolated grove with 3-yr old trees
- No HLB in Jan 06
- No HLB found in follow up visits in April, June and August 06
- Nov 06- Found initial symptoms of HLB



F0216	2.13.2006	<i>C. sinensis</i>	Grove	De Soto	50	24.30	26.17	P	P
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- Feb 06- Psyllid samples positive from a large grove
- July 06- Follow up- Two trees with HLB, one PCR positive

F0018n	9.22.2005	<i>C. sinensis</i>	Grove	Polk	5	18.75	29.94	P	P
--------	-----------	--------------------	-------	------	---	-------	-------	---	---

- Positive samples since Sept. 2005
- From four different sites collected several times
- Positive results confirmed by repeat multi and single psyllid extractions
- Confirmed by conventional PCR and sequencing
- Some unique sequences!
- Plant positive finds after two years!



# Psyllids as a tool For management of HLB

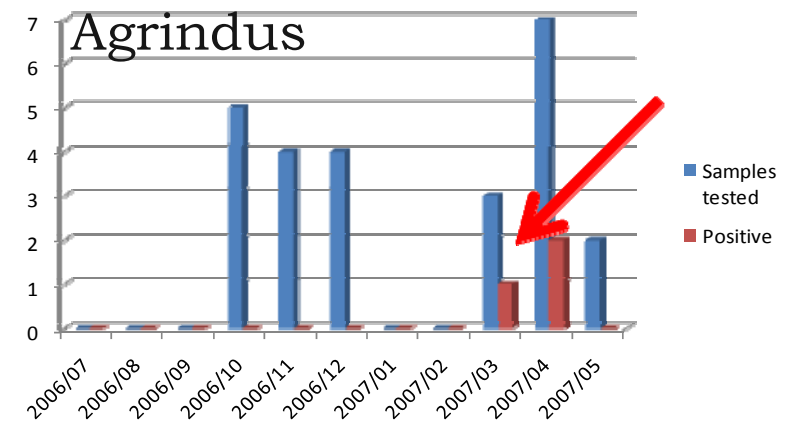
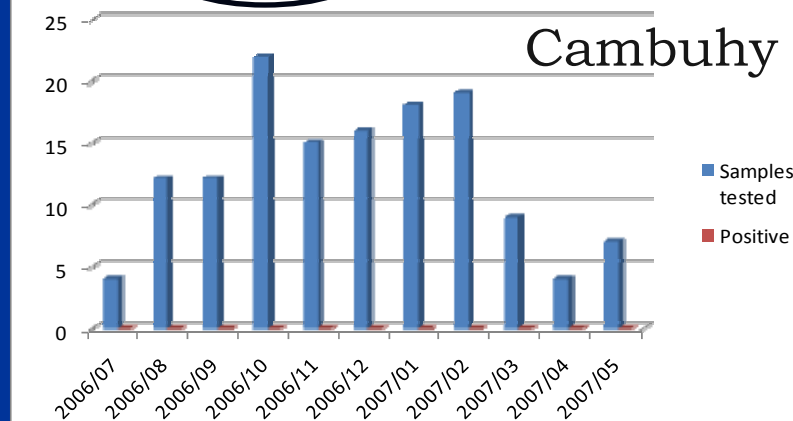
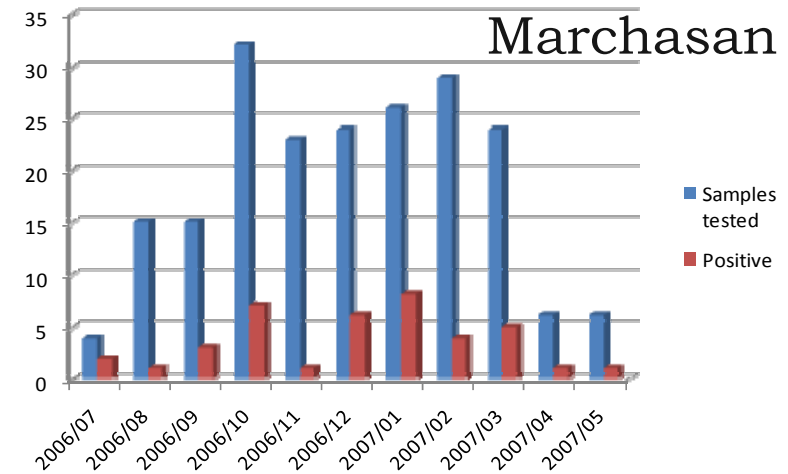
# Procedure

- ❖ Blocks were selected in the three large farms with different management practices in Brazil
- ❖ Psyllid adults and nymphs were collected every week
- ❖ Ricardo Harakava, Pedro Yamamoto and Fernando Tersi organized the psyllid collections and send them to Sao Paulo
- ❖ One extraction per sample, each with 10 adults, or 10 nymphs in Sao Paulo and further extractions in Riverside
- ❖ USDA Riverside: Real time PCR for HLB-Las and Lam



# Summary of psyllid tests for HLB-Las

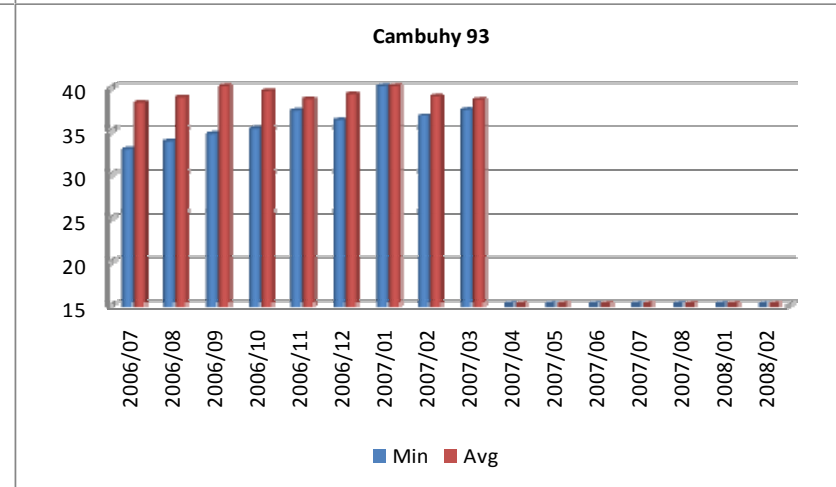
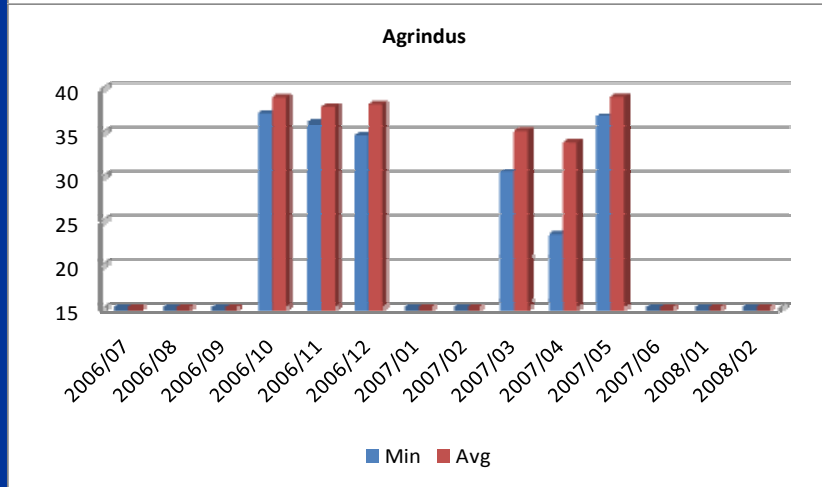
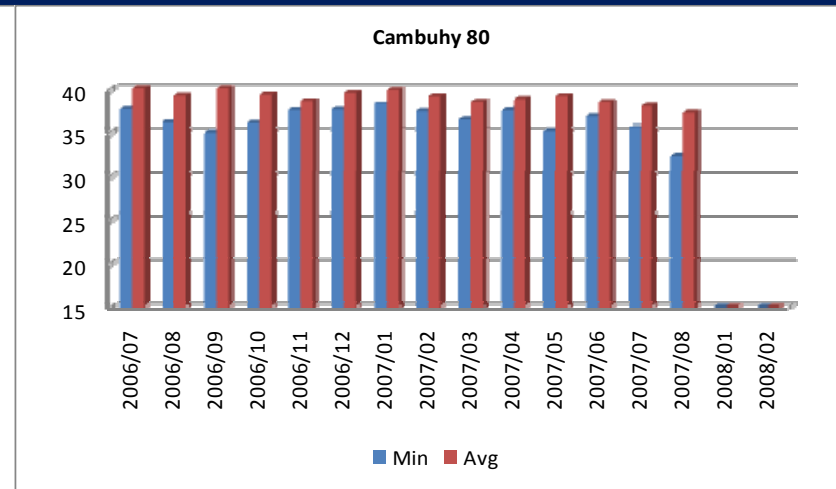
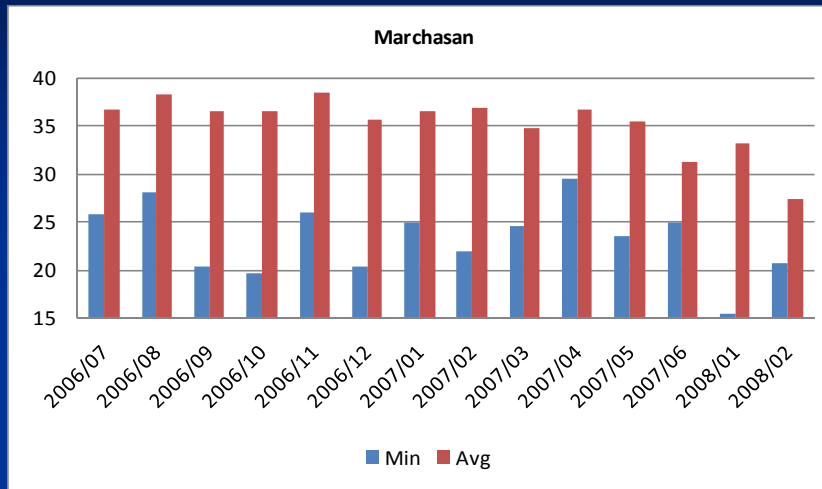
	Marchasan		Cambuhy		Agrindus	
	Samples tested	Positive	Samples tested	Positive	Samples tested	Positive
2006/07	4	2	4	0	0	0
2006/08	15	1	12	0	0	0
2006/09	15	3	12	0	0	0
2006/10	32	7	22	0	5	0
2006/11	23	1	15	0	4	0
2006/12	24	6	16	0	4	0
2007/01	26	8	18	0	0	0
2007/02	29	4	19	0	0	0
2007/03	24	5	9	0	3	1
2007/04	6	1	4	0	7	2
2007/05	6	1	7	0	2	0
<b>Total</b>	<b>204</b>	<b>39</b>	<b>138</b>	<b>0</b>	<b>25</b>	<b>3</b>



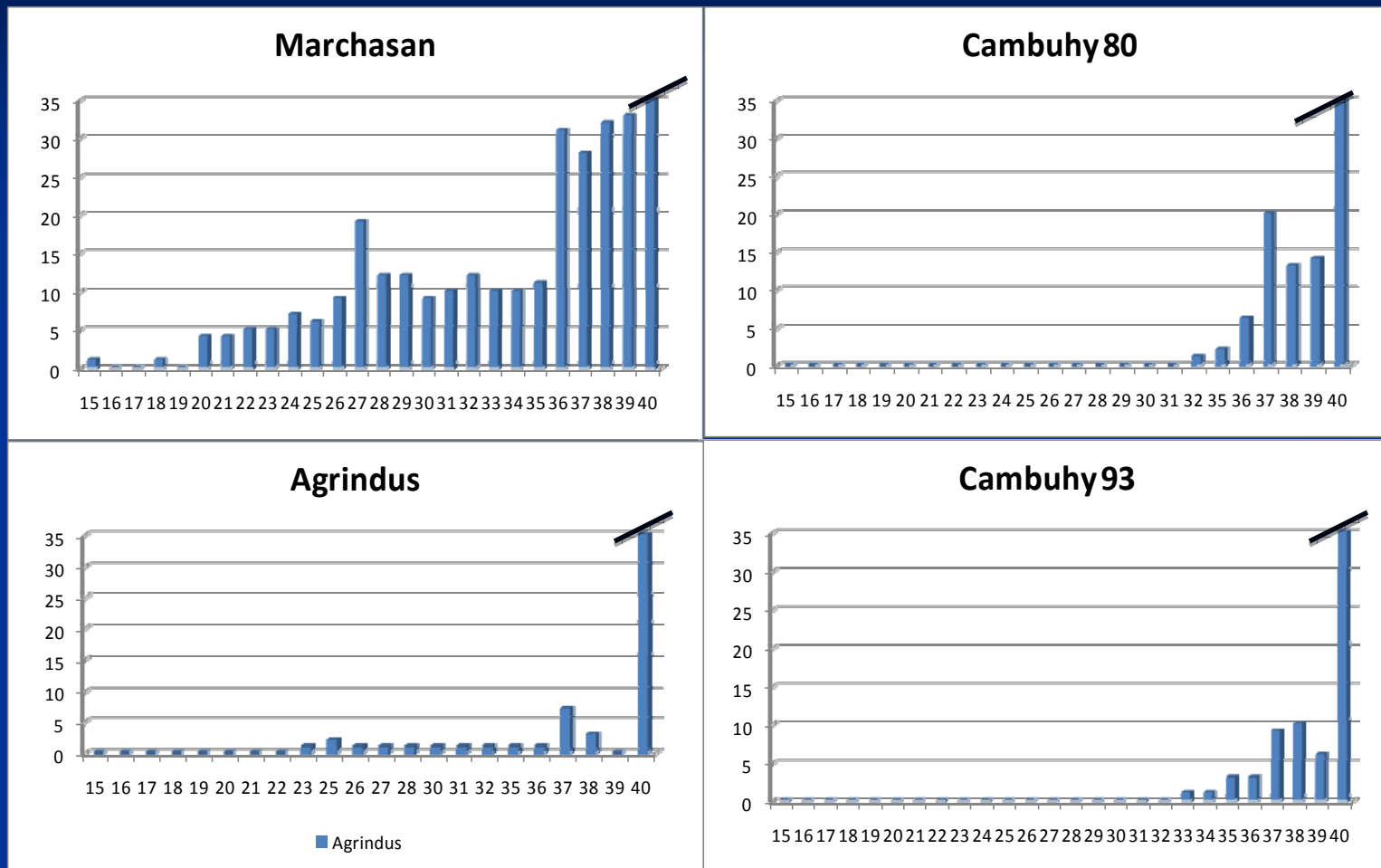
Monthly data of number of samples tested and number of Las positive samples found

# Monthly Average and Minimum Cycle threshold values

Ct values



# Cycle threshold values from qPCR of psyllid samples



Distribution of Ct values tested during one year period starting July 2006 (number of extractions on Y-axis, and Ct values on x-axis; not all samples with Ct value of 40 are shown)

# Two neighbors

2006

2007

2008



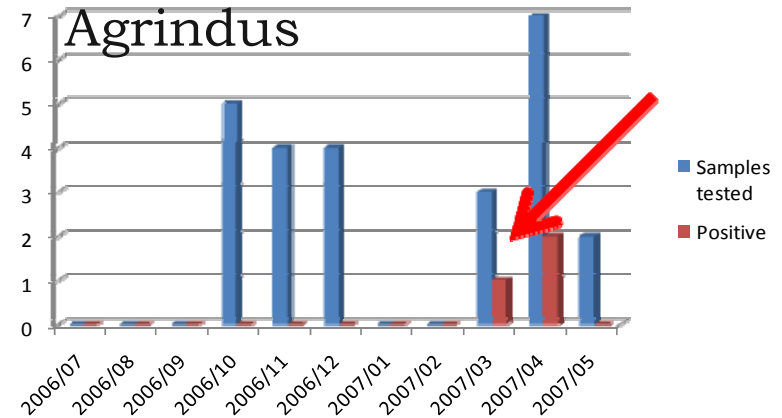
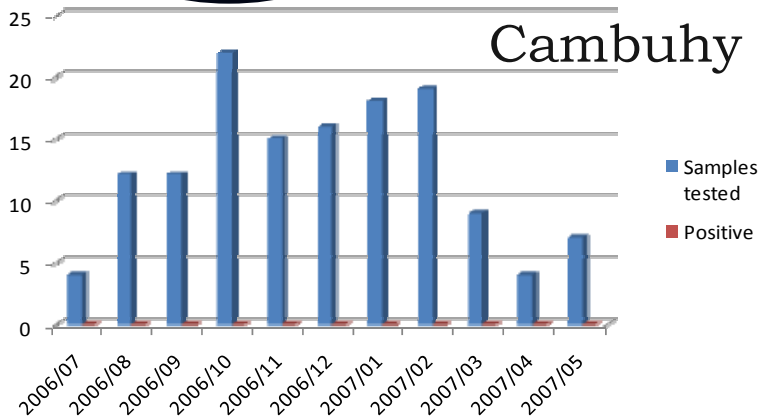
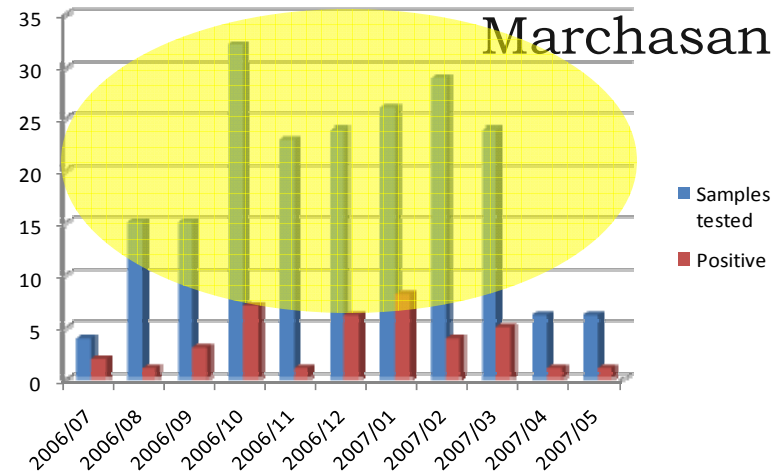
Cambuhy Farm, Brazil

Jose Luiz and Fernando Tersi  
a neighboring farm

Fundecitrus: Silvio Lopes and Pedro Yamamoto  
Instituto Biologico: Ricardo Harakava

# Las-free psyllids

	Marchasan		Cambuhy		Agrindus	
	Samples tested	Positive	Samples tested	Positive	Samples tested	Positive
2006/07	4	2	4	0	0	0
2006/08	15	1	12	0	0	0
2006/09	15	3	12	0	0	0
2006/10	32	7	22	0	5	0
2006/11	23	1	15	0	4	0
2006/12	24	6	16	0	4	0
2007/01	26	8	18	0	0	0
2007/02	29	4	19	0	0	0
2007/03	24	5	9	0	3	1
2007/04	6	1	4	0	7	2
2007/05	6	1	7	0	2	0
<b>Total</b>	<b>204</b>	<b>39</b>	<b>138</b>	<b>0</b>	<b>25</b>	<b>3</b>



# Important considerations

- ❖ Las free plant may not mean it is free from HLB
- ❖ Las free psyllids on a tree may not mean the bacterium is absent
  - ❖ Time of sampling
  - ❖ Number of insectstestedfromasite
  - ❖ Sampling method
  - ❖ There is always a possibility that other known and unknown Liberibacters may be pesent



# Conclusions

- ❖ Infected psyllids were found in orchards well before the appearance of symptoms-MONITOR BOTH PSYLLIDS AND PLANTS
- ❖ Psyllid transmission of HLB bacteria exhibit seasonality with maximum infection in fall followed by spring, summer and winter-MONITOR CONTINUOUSLY
- ❖ Regular monitoring psyllids in addition to scouting and eradication would strengthen HLB management
- ❖ FUTUTE: qPCR may not be the best option for efficient monitoring of psyllids. Need better trapping, faster and cheaper assay

# Acknowledgements

- ❑ Wayne Dixon, Matt Brodie and several members of the Division of Plant Industry, Gainesville, FL
- ❑ Pedro Yamamamoto and Sylvio Lopes, Fundcitrus, Brazil
- ❑ Ricardo Harakava, Biologico Instituto, Sao Paulo, Brazil
- ❑ Jose Luiz, Fernando Tersi, Cambuhy, Brazil
- ❑ Marchasan and Agrindus farms, Brazil

- ❑ Ed Stover, USDA-ARS, Fort Pierce
- ❑ Phil Stansly, J. Qureshi, UF Immokalee
- ❑ R. Brlansky, CREC, UF Lake Alfred

## FUNDING

- ❑ USDA TTAR
- ❑ Citrus Research Board, California
- ❑ FCPRAC, Florida