# "*Candidatus* Liberibacter caribbeanus" (Lca) Analysis of the bacterium associated with citrus huanglongbing in Colombia, S. America

**Manjunath L. Keremane**,
USDA-ARS, National Clonal Germplasm Repository for Citrus and Dates
Riverside, CA, USA.

**Chandrika Ramadugu**, UC Riverside
**Richard Lee**, USDA, ARS, Riverside
**Riverside Lab**: Esteban Rodriguez, Ronak Patel, Hyun Park, Caitlynn Fortner
**Adriana Castañeda** and the lab group,
**Amelio Arévalo**, Epidemiology and phytosanitory surveillance
**Jorge Palacino**, **Amury Jimenez** and the field staff
ICA, Colombia

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PACBIO NextGen sequencing: Genome core, UC Irvine, CA
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University of California, Riverside, CA

# Collaboration with Instituto Colombiano Agropecuario (ICA)

- Collaboration started in 2010. Dr. Chandrika Ramadugu (UCR) visited ICA and helped set up a qPCR assay for detection of Citrus huanglongbing (HLB) -associated Liberibacter.

- Dr. Jorge Angel visited USDA ARS laboratory in Riverside for hands-on training on Citrus HLB related experiments.

- E-mail and telephone conversations with Adriana Castañeda. Testing psyllid and plant samples collected in Colombia at Riverside (2010-2014)

- Dr. Manjunath Keremane visited ICA for ten days to demonstrate qPCR, to discuss with ICA scientists, plan strategies, train personnel and to conduct experiments (May 2014; sponsored by FAS and ICA).

- September 2014 – two scientists from ICA will visit USDA laboratory for two weeks to obtain hands-on training.

# Real time PCR (qPCR) of psyllid samples from Colombia. First positive psyllid samples, test date Nov 2013

2010-14: About 5000 psyllid extractions and about 80 plant DNA extracts tested

Plant DNA and psyllids shipped in alcohol to Riverside for analysis

**Psyllid samples positive by real time PCR-11/2013**

1. 135403
2. 135404
3. 135406
4. 135408
5. 135410
6. 135415

All samples were collected in Cordoba, Colombia

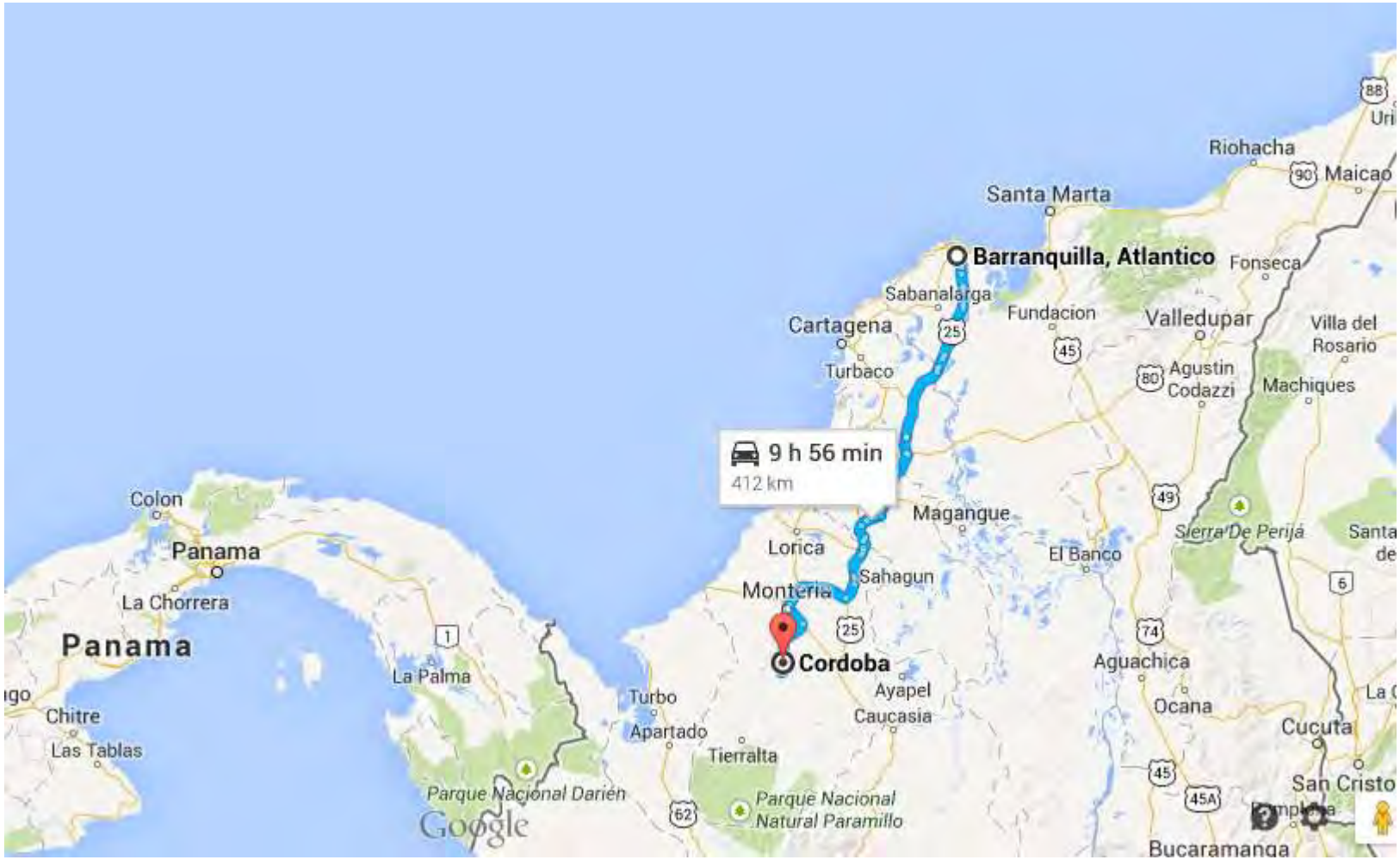
A large number of psyllid samples were collected multiple times, but no more positives until June, 2014

| No | Source | No Psyllids | Psyllid DNA | LAS DNA | comment |
|---|---|---|---|---|---|
| 313 | | | 40 | 15.044 | positive control 1 |
| 312 | | | 40 | 15.711 | positive control 1 |
| 311 | | | 29.718 | 16.770 | positive control 1 |
| 310 | | | 40 | 18.844 | positive control 1 |
| 255 | 135415 | 1 | 23.826 | 19.993 | **LAS infected** |
| 237 | 135410 | 1 | 25.602 | 20.250 | **LAS infected** |
| 236 | 135410 | 1 | 24.962 | 20.601 | **LAS infected** |
| 235 | 135410 | 1 | 25.434 | 21.770 | **LAS infected** |
| 221 | 135408 | 1 | 24.755 | 22.707 | **LAS infected** |
| 220 | 135408 | 5 adults | 23.319 | 22.850 | **LAS infected** |
| 208 | 135404 | 5 adults | 23.145 | 25.958 | **LAS infected** |
| 309 | | | 40 | 26.807 | positive control 2 |
| 308 | | | 40 | 29.337 | positive control 2 |
| 254 | 135415 | 1 | 23.452 | 30.362 | **LAS infected** |
| 307 | | | 40 | 31.513 | positive control 2 |
| 253 | 135415 | 1 | 24.855 | 32.628 | **LAS infected** |
| 212 | 135406 | 5 adults | 22.057 | 33.598 | **LAS suspect** |
| 207 | 135403 | 5 adults | 22.888 | 34.001 | **LAS suspect** |
| 234 | 135410 | 1 | 24.870 | 34.127 | **LAS suspect** |
| 252 | 135415 | 1 | 25.538 | 34.288 | **LAS suspect** |
| 233 | 135410 | 1 | 24.365 | 34.936 | **LAS suspect** |
| 232 | 135410 | 5 adults | 23.089 | 34.981 | **LAS suspect** |
| 306 | | | 40 | 35.030 | positive control 2 |
| 251 | 135415 | 6 adults | 22.990 | 35.321 | **LAS suspect** |
| 231 | 135410 | 1 | 24.389 | 35.512 | **LAS suspect** |
| 250 | 135415 | 1 | 24.104 | 35.871 | **LAS suspect** |
| 249 | 135415 | 1 | 23.848 | 36.075 | **LAS suspect** |
| 230 | 135410 | 1 | 24.888 | 36.079 | **LAS suspect** |
| 206 | 135403 | 1 | 25.968 | 36.746 | **LAS suspect** |
| 229 | 135410 | 1 | 25.210 | 36.846 | **LAS suspect** |
| 205 | 135403 | 1 | 24.505 | 36.982 | **LAS suspect** |
| 219 | 135408 | 1 | 25.798 | 37.002 | negative |
| 218 | 135408 | 1 | 24.739 | 37.344 | negative |
| 228 | 135410 | 1 | 24.989 | 37.642 | negative |
| 248 | 135415 | 1 | 24.880 | 38.054 | negative |
| 247 | 135415 | 1 | 23.979 | 38.093 | negative |
| 217 | 135408 | 1 | 25.374 | 38.721 | negative |
| 246 | 135415 | 1 | 24.297 | 39.083 | negative |
| 227 | 135410 | 1 | 25.635 | 39.227 | negative |
| 216 | 135408 | 1 | 24.893 | 39.549 | negative |
| 226 | 135410 | 1 | 24.302 | 39.772 | negative |
| 1 | 135140 | 4 adults 4 nymphs | 21.520 | 40 | negative |
| 2 | 135140 | 1 adults | 23.967 | 40 | negative |

# First detection of '*Candidatus* Liberibacter' from Colombia
# Tested: Nov 2013; Collected Aug 2013?

The first batch of Liberibacter-positive psyllid samples came from Cordoba, Colombia from backyard citrus. This area has no commercial citrus

# Second detection of '*Candidatus* Liberibacter' from Colombia
Tested: June 2014; collected 2013




1. First positive find site: Cordoba (test date Nov 2013)

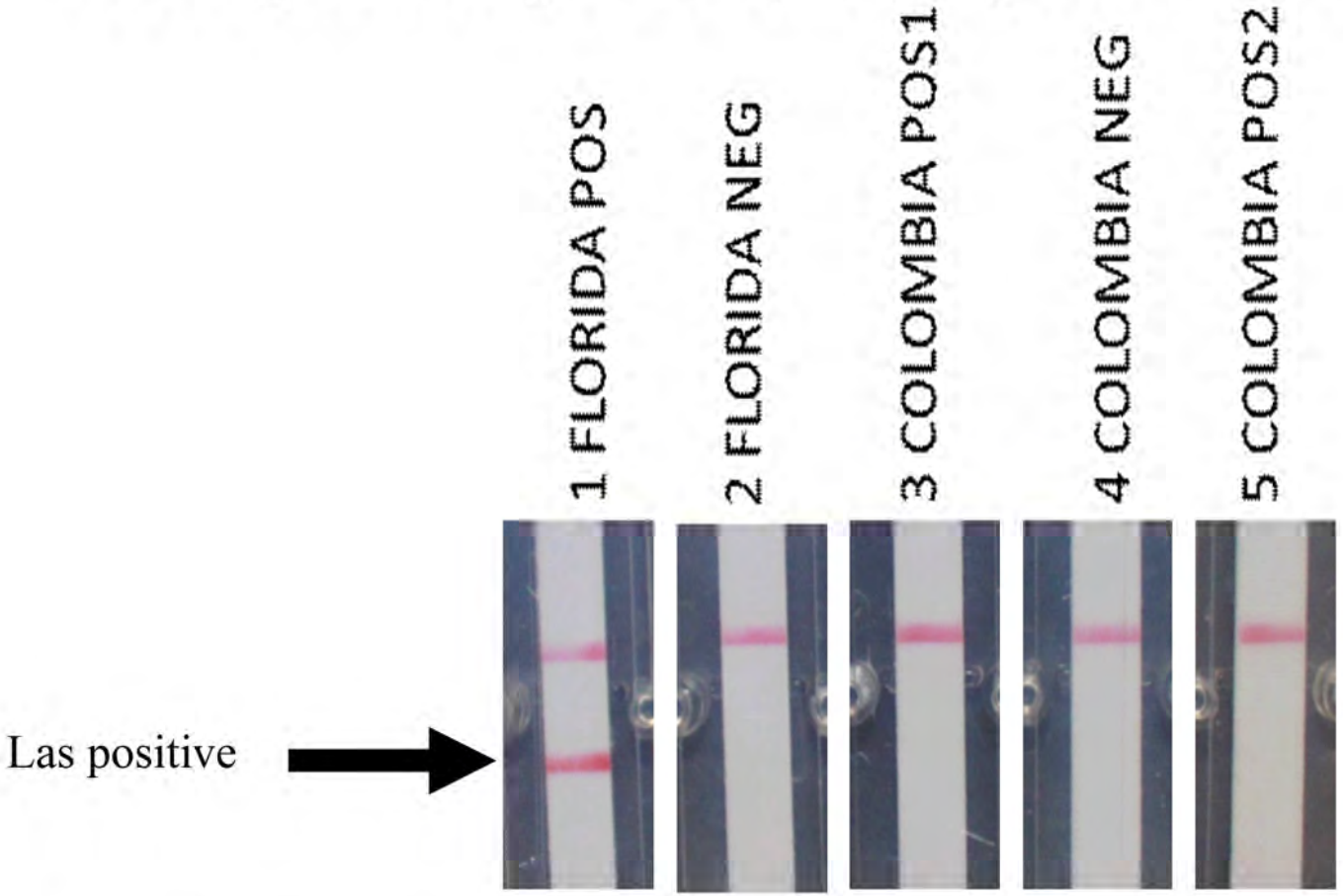
2. Second positive find site: Barranquilla (test date June 2014)

# Real time PCR (qPCR) of psyllid samples from Colombia

## Positive psyllid samples, Test date June 2014

| No | PCR | QMAG | Well | Row | Col | Sample | LAS | DC | Samples | RESULT |
|---|---|---|---|---|---|---|---|---|---|---|
| 3183 | 44 | 475 | 77 | G | 5 | 139179 | 19.932 | 23.543 | Psyllids | POSITIVE |
| 2183 | 31 | 466 | 4 | A | 4 | 1402725 | 20.387 | 22.844 | Psyllids | POSITIVE |
| 2311 | 33 | 469 | 76 | G | 4 | 1402725 | 20.988 | 24.488 | Psyllids | POSITIVE |
| 3178 | 44 | 475 | 17 | B | 5 | 139177 | 28.726 | 24.819 | Psyllids | POSITIVE |
| 3180 | 44 | 475 | 41 | D | 5 | 139178 | 29.002 | 24.471 | Psyllids | POSITIVE |
| 3151 | 44 | 475 | 73 | G | 1 | 1405409 | 30.099 | 23.132 | Psyllids | POSITIVE |
| 3168 | 44 | 475 | 87 | H | 3 | 138202 | 31.845 | 23.729 | Psyllids | POSITIVE |
| 2549 | 37 | 463 | 58 | E | 10 | 1402570 | 31.962 | 29.081 | Psyllids | POSITIVE |
| 2589 | 38 | 464 | 51 | E | 3 | 1402724 | 32.964 | 25.945 | Psyllids | suspect |
| 2928 | 41 | 471 | 93 | H | 9 | 1402849 | 33.702 | 27.430 | Psyllids | suspect |
| 2647 | 38 | 464 | 82 | G | 10 | 1402723 | 34.058 | 26.365 | Psyllids | suspect |
| 1998 | 29 | 461 | 88 | H | 4 | 137325 | 34.160 | 26.216 | Psyllids | suspect |
| 2046 | 29 | 461 | 94 | H | 10 | 138040 | 34.726 | 28.008 | Psyllids | suspect |
| 1846 | 27 | 459 | 93 | H | 9 | 139066 | 34.743 | 26.453 | Psyllids | suspect |
| 2014 | 29 | 461 | 90 | H | 6 | 137329 | 34.783 | 25.074 | Psyllids | suspect |
| 2696 | 39 | 465 | 88 | H | 4 | 1402561 | 34.866 | 25.842 | Psyllids | suspect |
| 2030 | 29 | 461 | 92 | H | 8 | 138039 | 34.960 | 27.979 | Psyllids | suspect |
| 2038 | 29 | 461 | 93 | H | 9 | 138042 | 35.107 | 25.415 | Psyllids | suspect |
| 3150 | 44 | 475 | 61 | F | 1 | 1405363 | 35.133 | 23.165 | Psyllids | suspect |
| 1814 | 27 | 459 | 89 | H | 5 | 139074 | 35.204 | 27.720 | Psyllids | suspect |
| 2006 | 29 | 461 | 89 | H | 5 | 137330 | 35.241 | 25.351 | Psyllids | suspect |
| 2312 | 33 | 469 | 88 | H | 4 | 1402725 | 35.302 | 24.237 | Psyllids | suspect |
| 2648 | 38 | 464 | 94 | H | 10 | 1402723 | 35.366 | 24.989 | Psyllids | suspect |
| 2111 | 30 | 467 | 7 | A | 7 | 1405074 | 35.378 | 21.965 | Psyllids | suspect |
| 3181 | 44 | 475 | 53 | E | 5 | 137176 | 35.440 | 23.719 | Psyllids | suspect |
| 1798 | 27 | 459 | 87 | H | 3 | 139072 | 35.564 | 25.292 | Psyllids | suspect |
| 1822 | 27 | 459 | 90 | H | 6 | 139071 | 35.645 | 25.652 | Psyllids | suspect |
| 2022 | 29 | 461 | 91 | H | 7 | 138039 | 35.729 | 27.638 | Psyllids | suspect |
| 2053 | 29 | 461 | 83 | G | 11 | 138038 | 35.730 | 27.077 | Psyllids | suspect |
| 2288 | 33 | 469 | 85 | H | 1 | 1405377 | 35.759 | 24.021 | Psyllids | suspect |
| 1790 | 27 | 459 | 86 | H | 2 | 139066 | 35.777 | 28.955 | Psyllids | suspect |
| 3204 | 44 | 475 | 44 | D | 8 | 139017 | 35.904 | 25.328 | Psyllids | suspect |
| 1950 | 28 | 460 | 94 | H | 10 | 137334 | 35.925 | 25.410 | Psyllids | suspect |

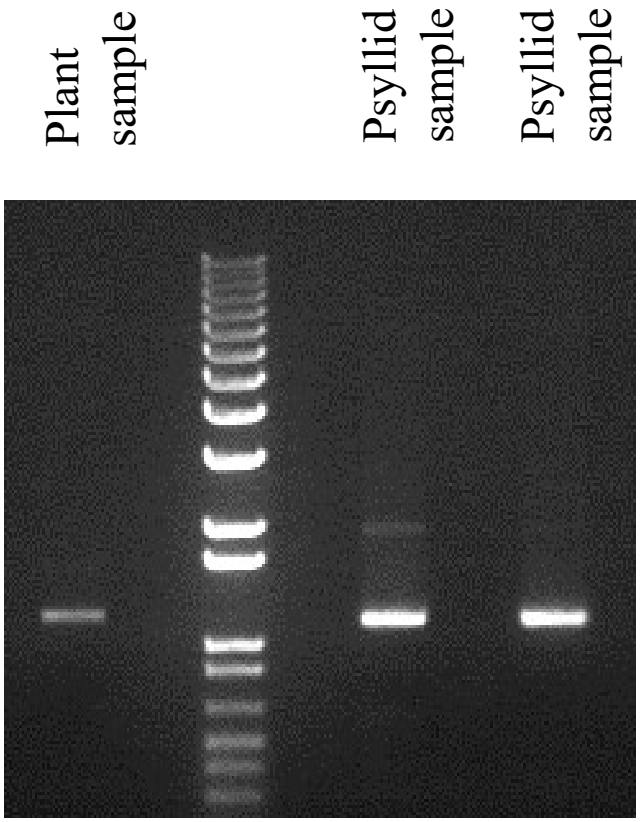
# Testing with Agdia kit (Amplify RP® Acceler 8™)




'*Ca*. L. asiaticus'-positive psyllid extracts from Florida showed a positive band (indicated by an arrow).

'*Ca*. L. caribbeanus'-positive psyllid extracts from Colombia did not show a positive reaction. Agdia kit **does not work** for '*Ca*. L. caribbeanus'.

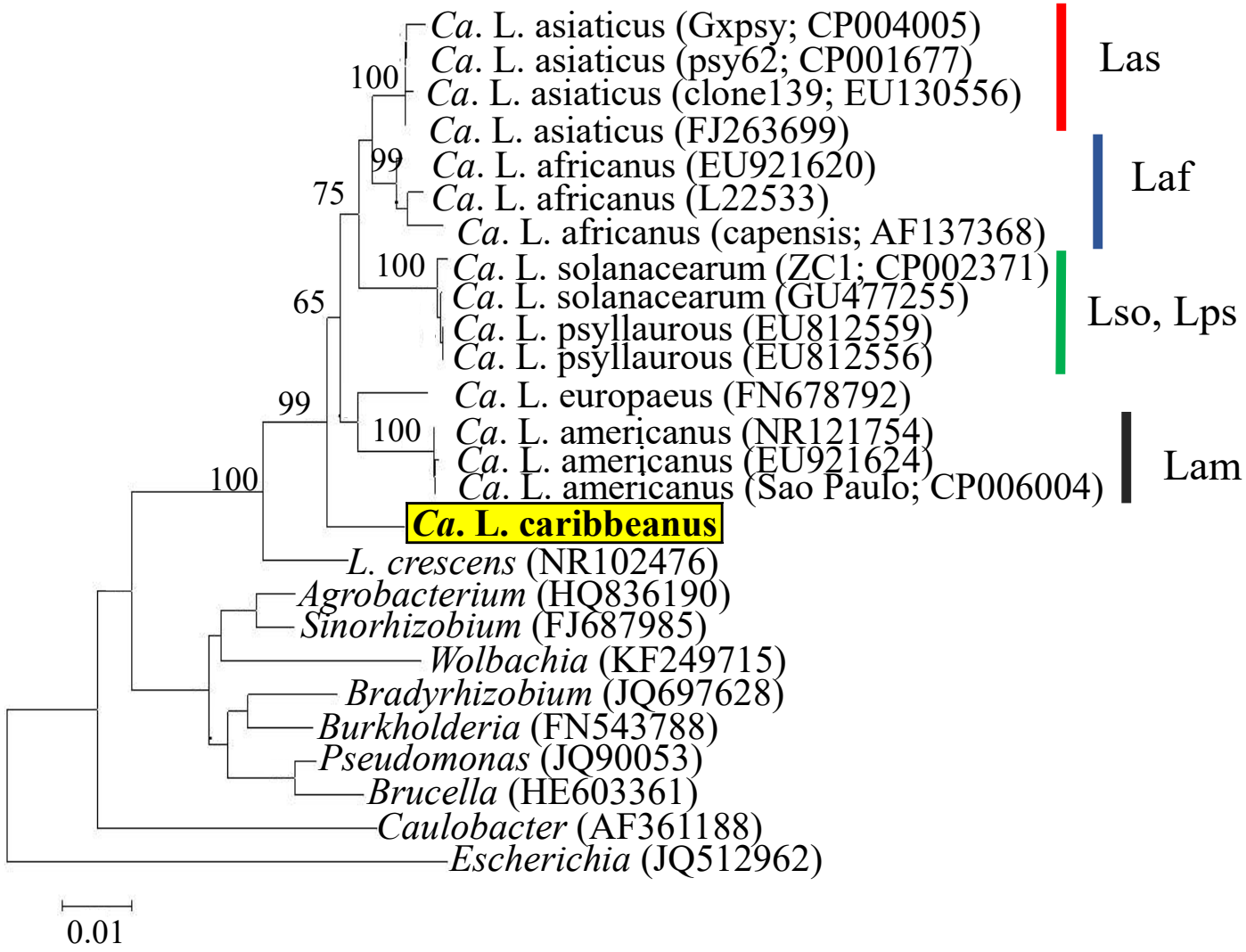
# Conventional PCR, Cloning, Sequencing of 16S rDNA fragment of '*Ca*. L. caribbeanus'




1. Conventional PCR was done with primers OI1, OI2C to amplify 16S rDNA fragment.

2. Cloned in pCR4 Topo vector and sequenced (Sanger method, done at Univ. of California, Riverside, CA).

# 16S rDNA analysis




Neighbor joining tree showing the relationship of different Liberibacters (based on 16S rDNA sequence); 10,000 replications. Bar represents substitutions per nucleotide.

# Distance matrix

| Estimates of evolutionary divergence based on 16S rDNA sequences | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxon | *Ca*. L. caribbeanus | *Ca*. L. asiaticus | *Ca*. L. americanus | *Ca*. L. africanus | *Ca*. L. solanacearum | *Ca*. L. psyllaurous | *Ca*. L. europaeus | *L. crescens* | *Agrobacterium* | *Bradyrhizobium* | *Wolbachia* | *Brucella* |
| *Ca*. L. asiaticus | 0.035 | | | | | | | | | | | |
| *Ca*. L. americanus | 0.043 | 0.038 | | | | | | | | | | |
| *Ca*. L. africanus | 0.035 | 0.014 | 0.034 | | | | | | | | | |
| *Ca*. L. solanacearum | 0.048 | 0.032 | 0.044 | 0.031 | | | | | | | | |
| *Ca*. L. psyllaurous | 0.048 | 0.029 | 0.044 | 0.031 | 0.004 | | | | | | | |
| *Ca*. L. europaeus | 0.045 | 0.036 | 0.034 | 0.033 | 0.051 | 0.050 | | | | | | |
| *Liberibacter crescens* | 0.057 | 0.055 | 0.060 | 0.051 | 0.055 | 0.055 | 0.055 | | | | | |
| *Agrobacterium* | 0.093 | 0.105 | 0.107 | 0.102 | 0.103 | 0.103 | 0.106 | 0.076 | | | | |
| *Bradyrhizobium* | 0.103 | 0.115 | 0.120 | 0.110 | 0.111 | 0.109 | 0.116 | 0.083 | 0.049 | | | |
| *Wolbachia* | 0.120 | 0.131 | 0.140 | 0.128 | 0.133 | 0.133 | 0.136 | 0.106 | 0.062 | 0.089 | | |
| *Brucella* | 0.111 | 0.123 | 0.126 | 0.120 | 0.124 | 0.122 | 0.120 | 0.103 | 0.043 | 0.058 | 0.072 | |
| *Escherichia coli* | 0.190 | 0.192 | 0.204 | 0.195 | 0.202 | 0.199 | 0.188 | 0.192 | 0.182 | 0.180 | 0.196 | 0.173 |

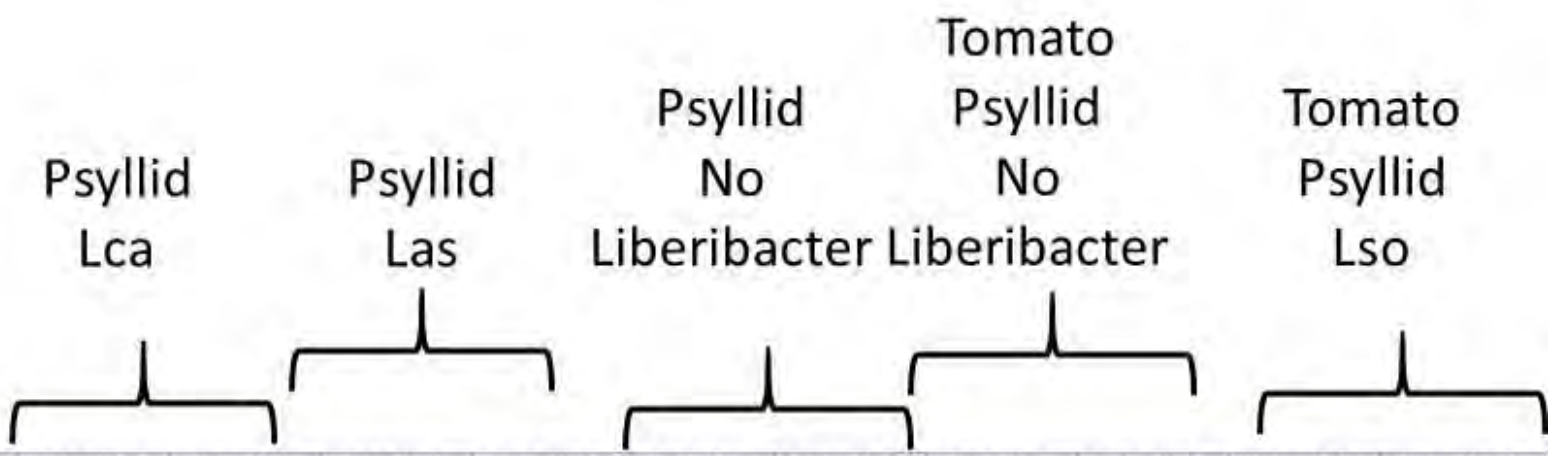
The number of base substitutions per site between sequences are shown. Analyses conducted using the Maximum Composite Likelihood model, conducted in MEGA6. 10,000 iterations.

*Ca*. L. caribbeanus has 92-96% identity with other Liberibacters (based on 16S rDNA sequences)

| Related bacteria | Percent identity of *Ca* . L. caribbeanus |
|---|---|
| *Ca* . L. asiaticus | 95 |
| *Ca* . L. americanus | 96 |
| *Ca* . L. africanus | 95 |
| *Ca* . L. africanus subsp. capensis | 92 |
| *Ca* . L. psyllaurous | 94 |
| *Ca* . L. solanacearum | 94 |
| *Ca* . L. europaeus | 95 |
| L. crescens | 95 |
| *Sinorhizobium* | 92 |

# 16S meta genome analysis

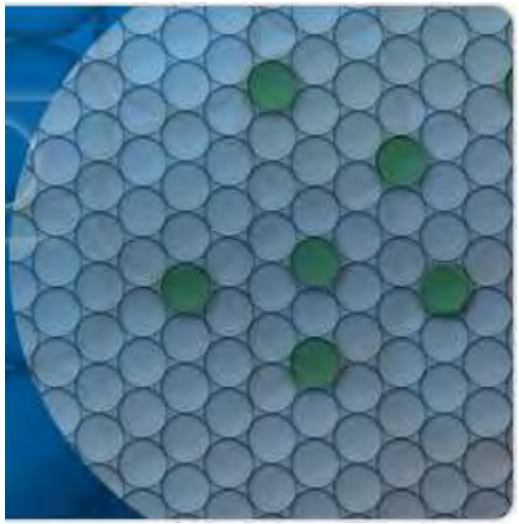
1. The psyllid DNA extracts were analyzed for 16S rDNA sequence by meta-genome analysis (Mr. DNA).
2. We were able to identify Liberibacter like sequences from psyllids from Colombia. These sequences showed about 94% identity with Las
3. Psyllid titer seemed to parallel those found in tomato psyllids (preliminary)

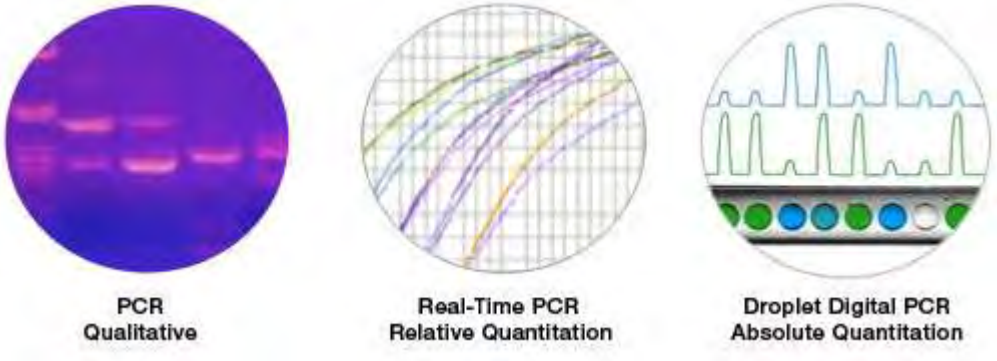



| Bacteria_genus | RIV.MK.1 | RIV.MK.2 | RIV.MK.3 | RIV.MK.4 | RIV.MK.5 | RIV.MK.6 | RIV.MK.7 | RIV.MK.8 | RIV.MK.9 | RIV.MK.10 |
|---|---|---|---|---|---|---|---|---|---|---|
| candidatus liberibacter | 72.385817 | 90.44272 | 0.4659968 | 0.2825154 | 0 | 0 | 0 | 0 | 74.495748 | 62.3431953 |
| wolbachia | 25.500801 | 6.6088544 | 56.414737 | 68.138537 | 17.952603 | 67.512292 | 80.777644 | 67.282135 | 3.5842294 | 33.183432 |
| oxalobacter | 1.8028846 | 2.6015518 | 5.7958352 | 20.81197 | 61.898063 | 12.926673 | 15.25651 | 4.2000609 | 0.0070279 | 0 |
| candidatus_zinderia | 0.3104968 | 0.3103606 | 6.6841415 | 10.557706 | 19.911265 | 19.4969 | 1.4042763 | 0.0202901 | 0 | 0 |

# Digital droplet PCR: LAS, LCA and LAS-California strain.

Similar to real time PCR
Each reaction is fractionated into 20,000 fractions
Positive and negative droplets in each well are read



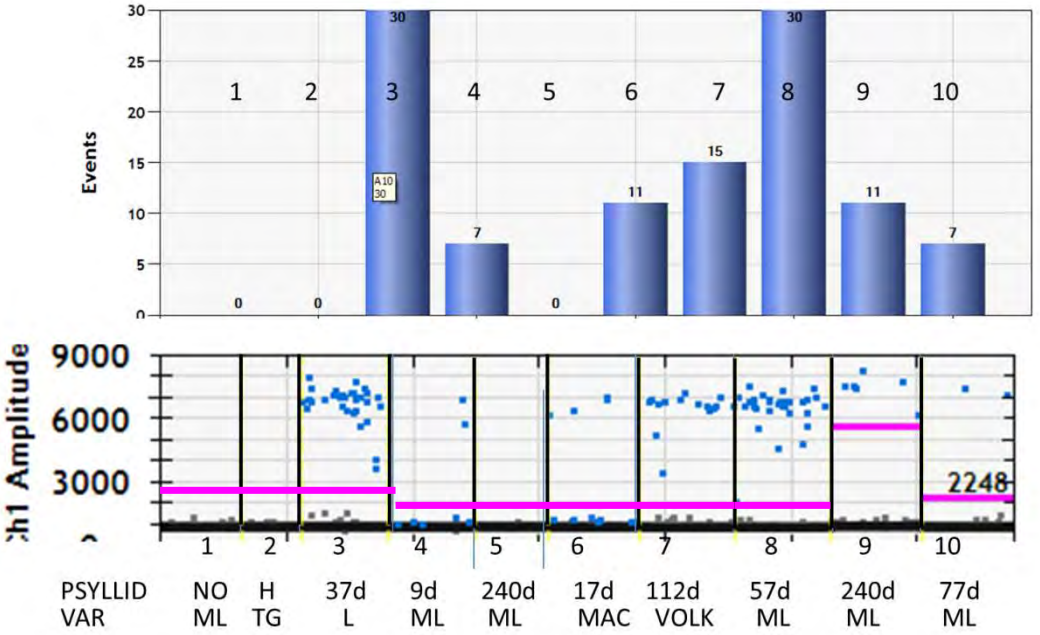



Sensitive, absolute detection of the target. Can differentiate populations of Liberibacters.
Our results indicate a population different from Las. With ddPCR, can detect even minute quantities of the bacterium.
House keeping gene used for plant samples is malate dehydrogenase (single copy gene).
EARLY DETECTION.

# Droplet digital PCR to detect low titers of HLB bacteria

Digital PCR detected Las bacteria in greenhouse trees inoculated as early as 17 days ago

Digital PCR partitions 1 reaction (20 ul solution) used in real time PCR into 20,000 droplets. The reactions are conducted and read separately in 20,000 droplets. Accurate and absolute quantification of bacteria.



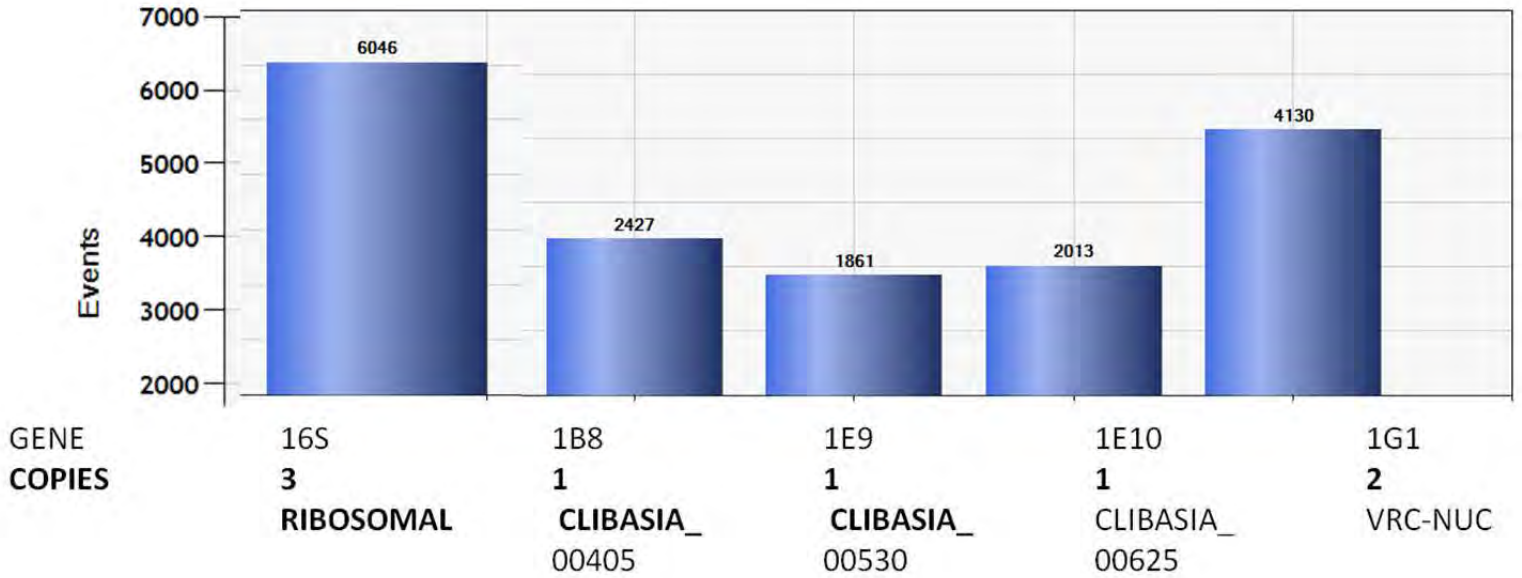

- Ten plants from Hacienda Heights isolate of Las were analyzed
- Two negatives - a Mexican lime seedling and a tangerine seedling exposed to healthy psyllids had no bacteria (as expected; 1 and 2)
- One Mex. lime exposed to psyllids with Las for 9 days tested negative (4)
- Other six plants were positive for Las (3, 6,7,8,9,10)
- Plant exposed to Las for 17 days was Las positive by digital PCR (6)

# Confirmatory tests for Las detection in Droplet digital PCR

- An Las positive sample was analyzed by ddPCR for 16s gene (3 copies per genome) was analyzed.

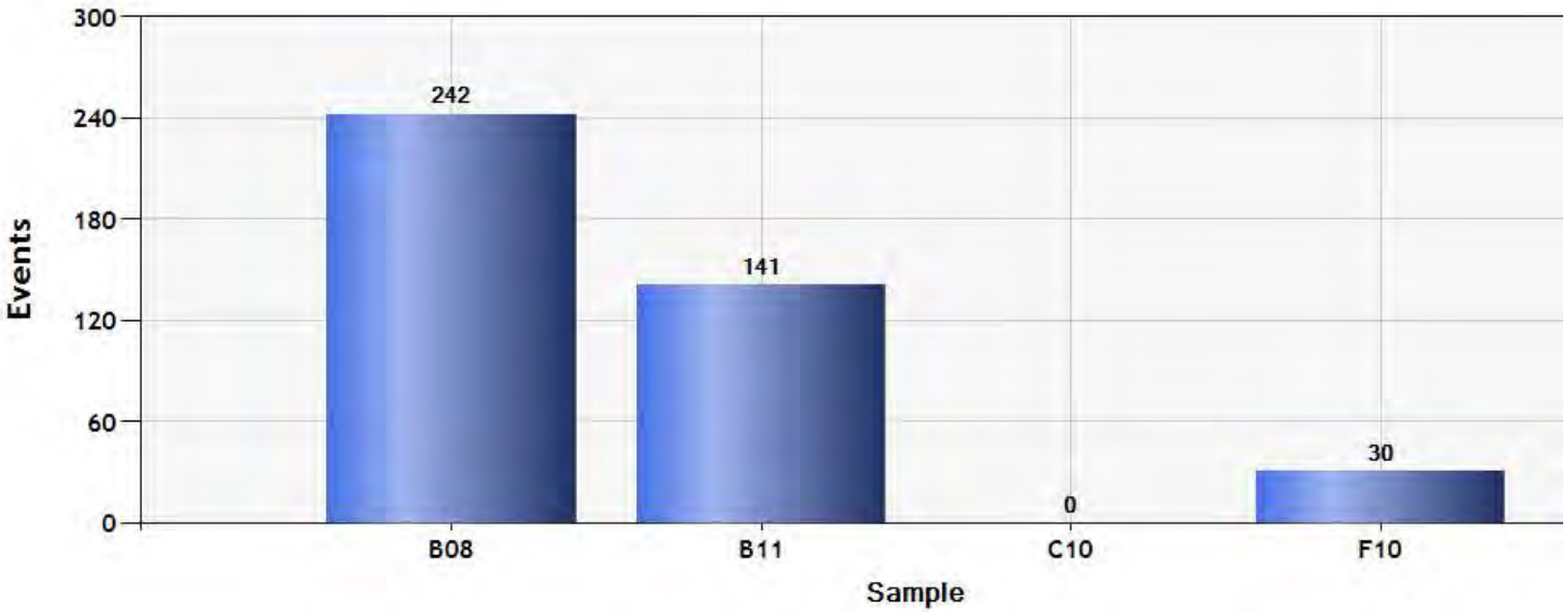
- A set of 12 genes were analyzed; four are shown here.

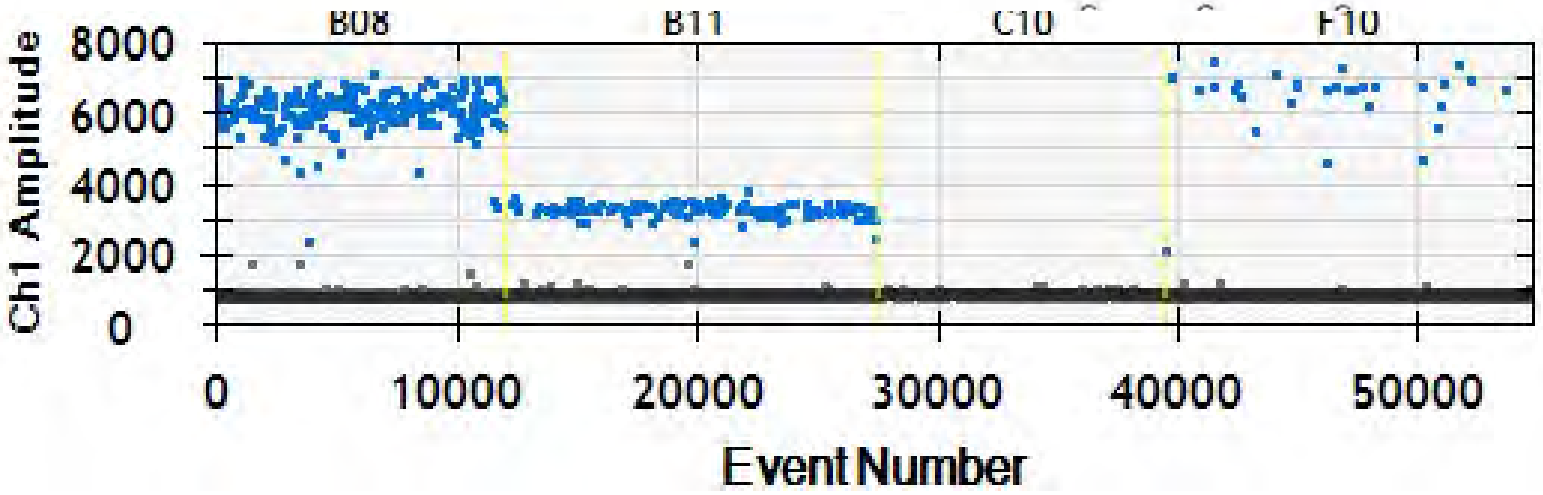
- Out of four genes analyzed, three are single copy genes in Las genome (Florida isolate). The fourth gene is present in 2 copies per genome.



Digital PCR clearly discriminated the Colombian Liberibacter from Las

FLORIDA COLOMBIA NEGATIVE CALIFORNIA

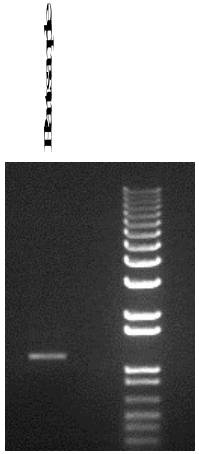


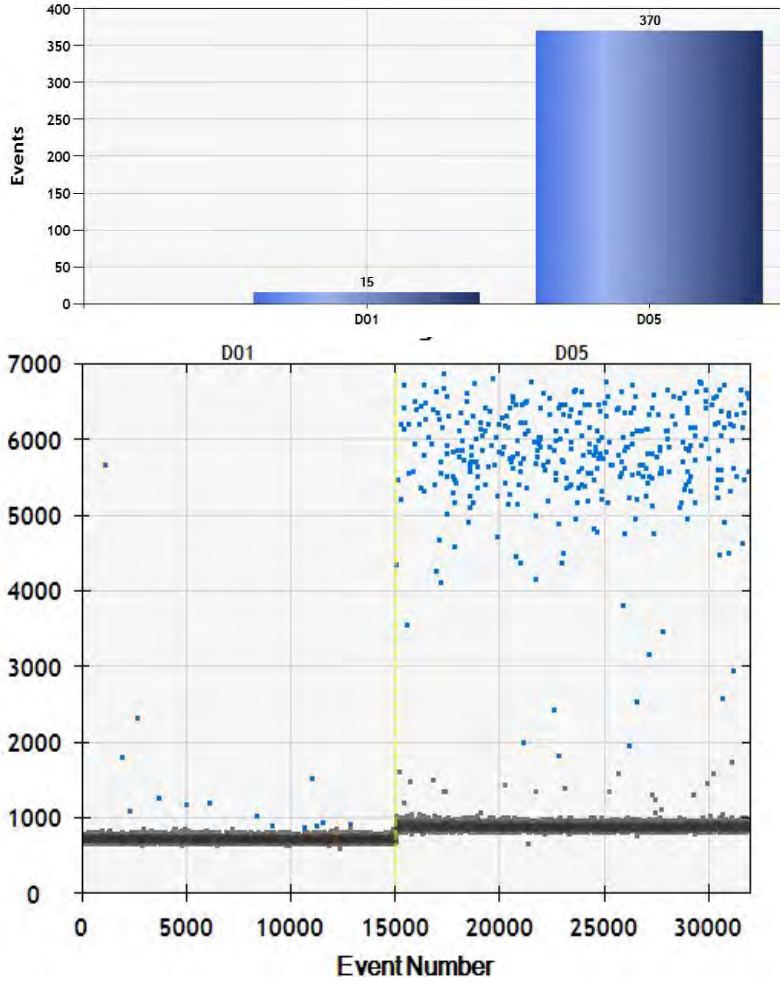
# Digital PCR helped detect Liberibacter positive plant DNAs from Colombia

A plant DNA that was negative by Las primer was analyzed using a newly designed primer which amplified the product better in digital PCR.

Positive droplets can be visualized as dots (bottom) or as a histogram



**LAS primer** **modified primer**



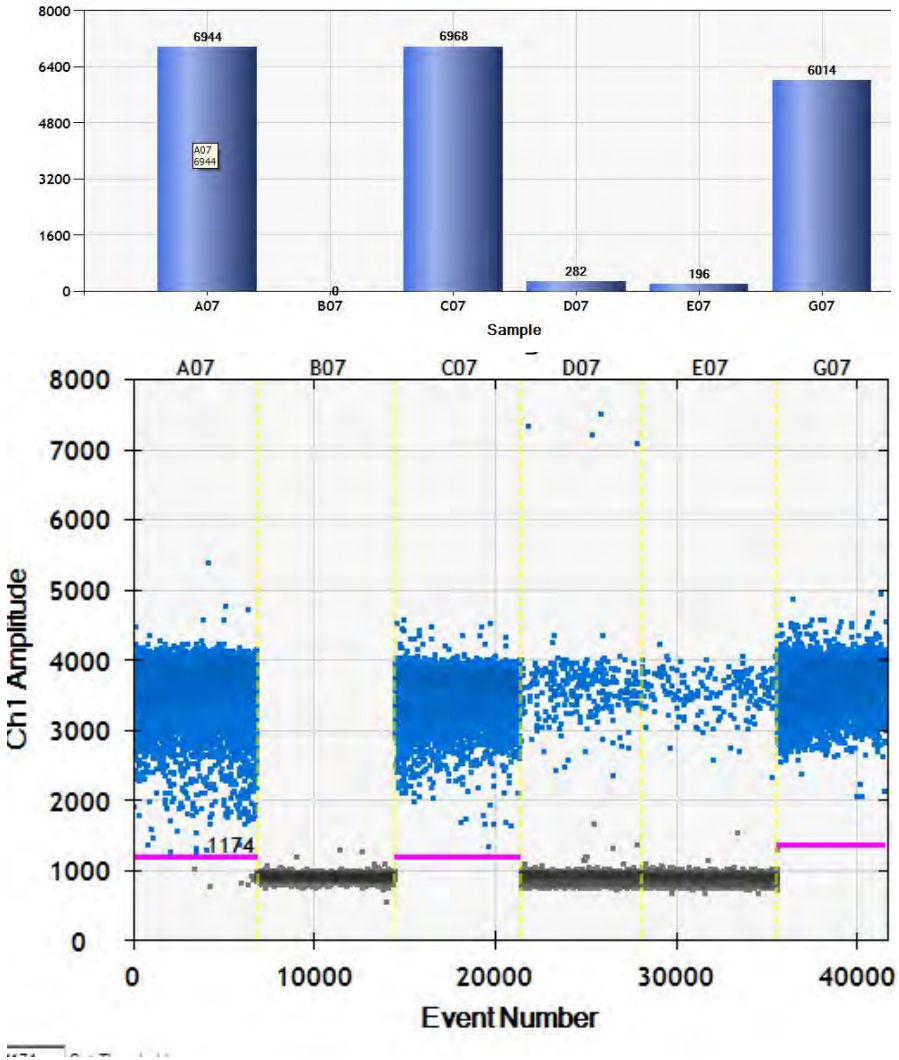
# Digital PCR helped detect Liberibacter positive plant DNAs from Colombia

Two plant samples tested positive for Lca by digital PCR, modified real time PCR, cloning and sequencing came from Cordoba region, one from *Murraya* and another from sweet orange (*Citrus*) tree.

| RecordNo | CA | source | source2 | category | Sample | mo | dt | yr |
|---|---|---|---|---|---|---|---|---|
| 10787 | F9703 | 139111 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10788 | F9704 | 139112 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10789 | F9705 | 139113 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10790 | F9706 | 139114 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10791 | F9707 | 139115 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10792 | F9708 | 139116 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10793 | F9709 | 139117 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10794 | F9710 | 139191-1 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10795 | F9711 | 139191-2 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10796 | F9712 | 139192-1 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10797 | F9713 | 139192-2 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10798 | F9714 | 139193-1 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10799 | F9715 | 139193-2 | DNA | Colombia Plant | DNA | 1 | 13 | 2014 |
| 10800 | F9716 | 13898 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10801 | F9717 | 13899 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10802 | F9718 | 13900 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10803 | F9719 | 13901 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10804 | F9720 | 13902 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10805 | F9721 | 13903 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10806 | F9722 | 13904 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |
| 10807 | F9723 | 13905 | DNA | Colombia Plant | DNA | 12 | 10 | 2013 |

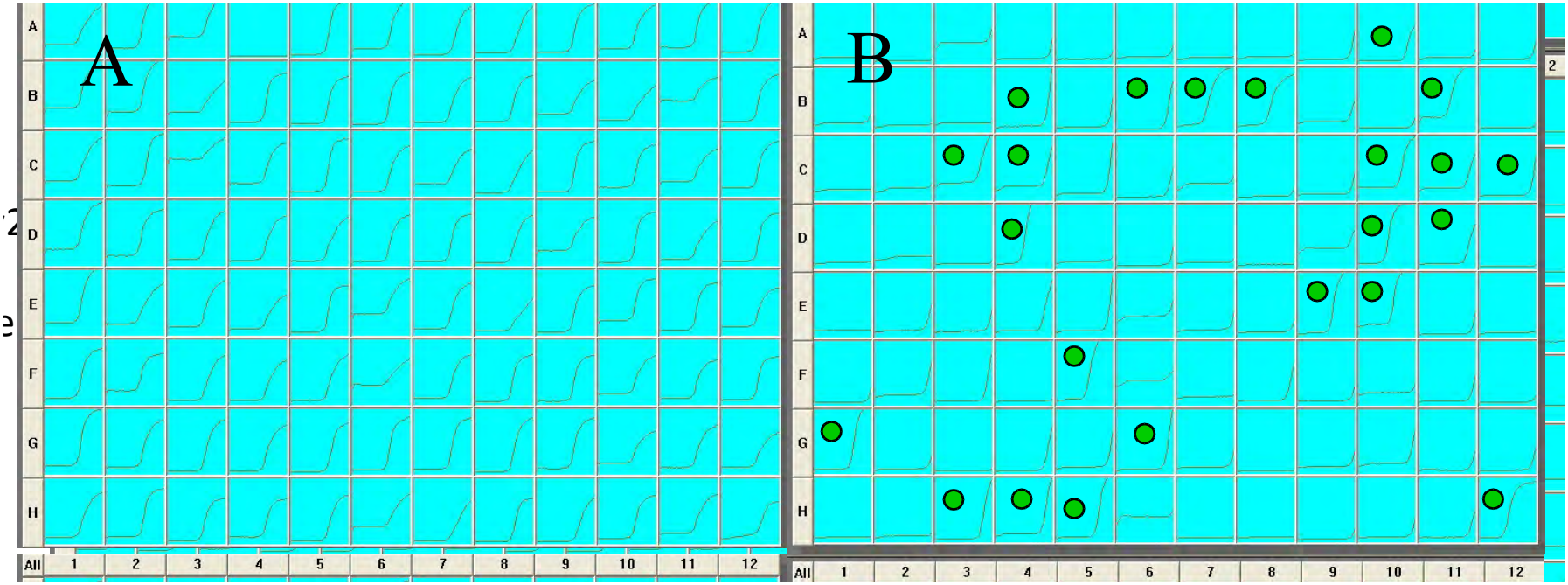
# Digital PCR detection of Lca in psyllids from Colombia

Detection of Lca in psyllids from Colombia by digital PCR using 16s rDNA primers modified for Lca specificity



# LAS 384 array

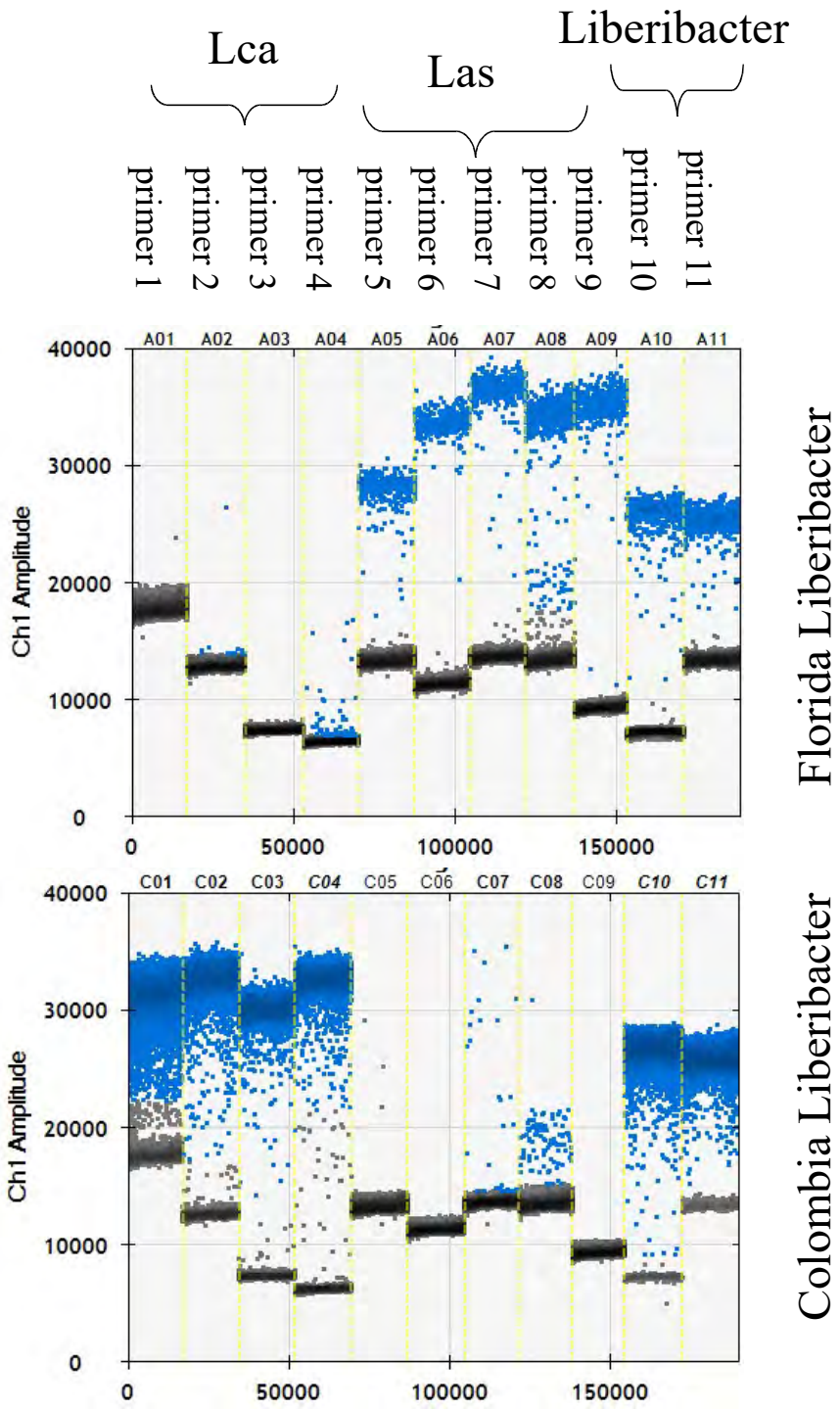
1. Designed 384 primer pairs to Las genomic regions.
2. qPCR with SYBR green for Las sample and for Lps sample.
3. Panel A shows amplification in 95/96 wells.
4. Panel B shows amplification in only some wells (green circles).
5. If the Liberibacter is different from Las, you get a pattern similar to panel B.




Psyllid extract with '*Ca*. L. asiaticus' shows amplification in most wells.

Psyllid extract with '*Ca*. L. psyllaurous' shows amplification in only some wells.

# Analysis of other genomic regions of Liberibacters in psyllids from Florida and Colombia




Psyllid extracts positive for '*Ca*. L. asiaticus' showed positive signal with primers 5, 6, 7, 8, 9,10 and 11. Negative signal for 1, 2, 3, 4.

Psyllid extracts positive for '*Ca*. L. caribbeanus' showed positive signal with primers 1, 2, 3, 4, 10 and 11. Negative signal for 5, 6, 9.

# Next Gen sequencing using PAC-BIO platform

1. Psyllid DNA extract (positive for Lca) is used for amplifying the whole genome of the bacterium.

2. Amplified product assayed by qPCR to get an estimate of amplification.

3. Used for Next Gen Sequencing reactions of the psyllid metagenome using PAC-BIO (Pacific Biosciences) platform. Conducted at University of California Irvine.

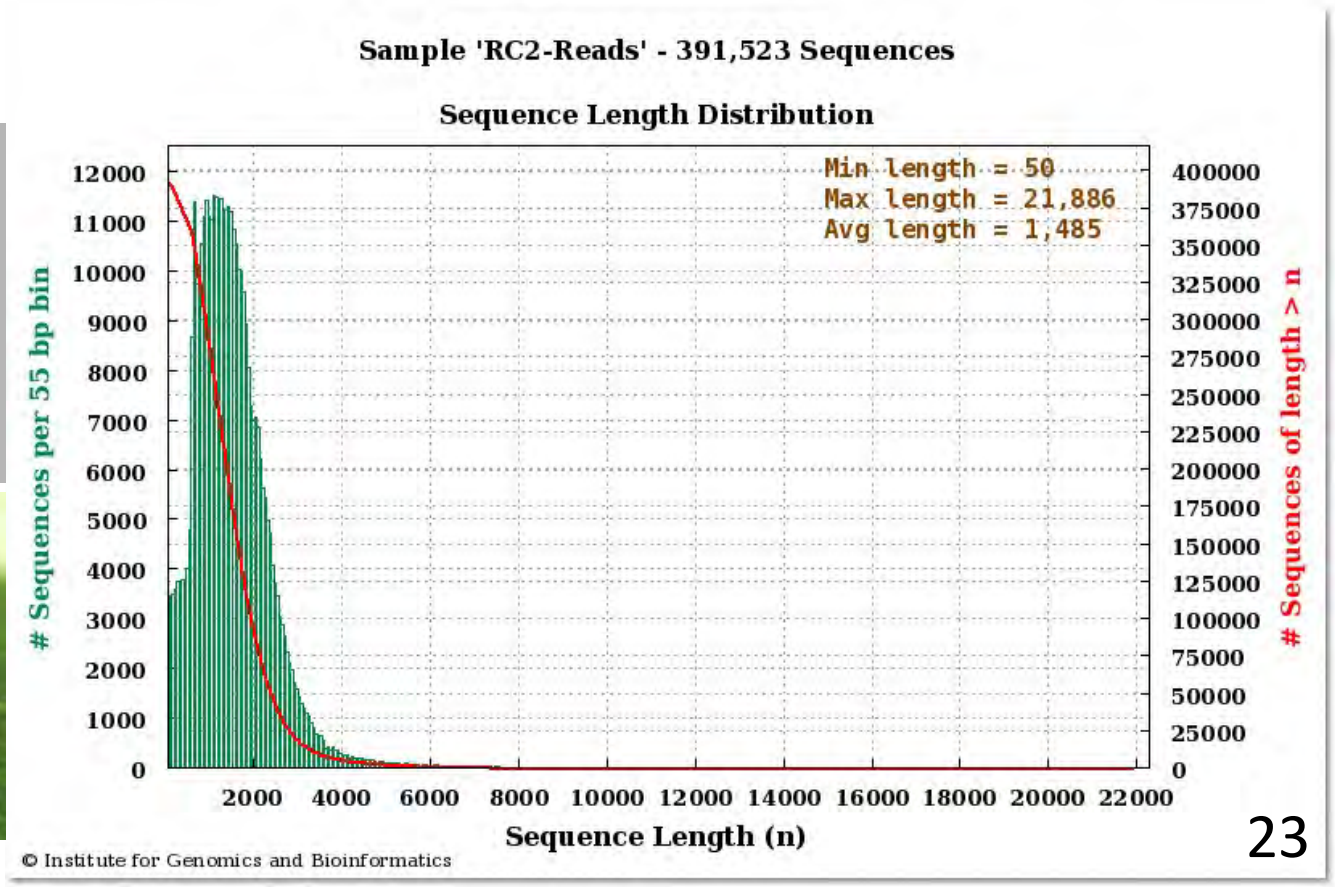
4. Using SMRT (single molecule real time) sequencing technologies, sequences of up to 20 Kb length (Average length 1.5 kb) are generated.

5. We have generated about 250 Kb sequence of '*Ca*. L. caribbeanus', and more bioinformatic analysis is in progress.

7. We are working on completing the genome sequence.

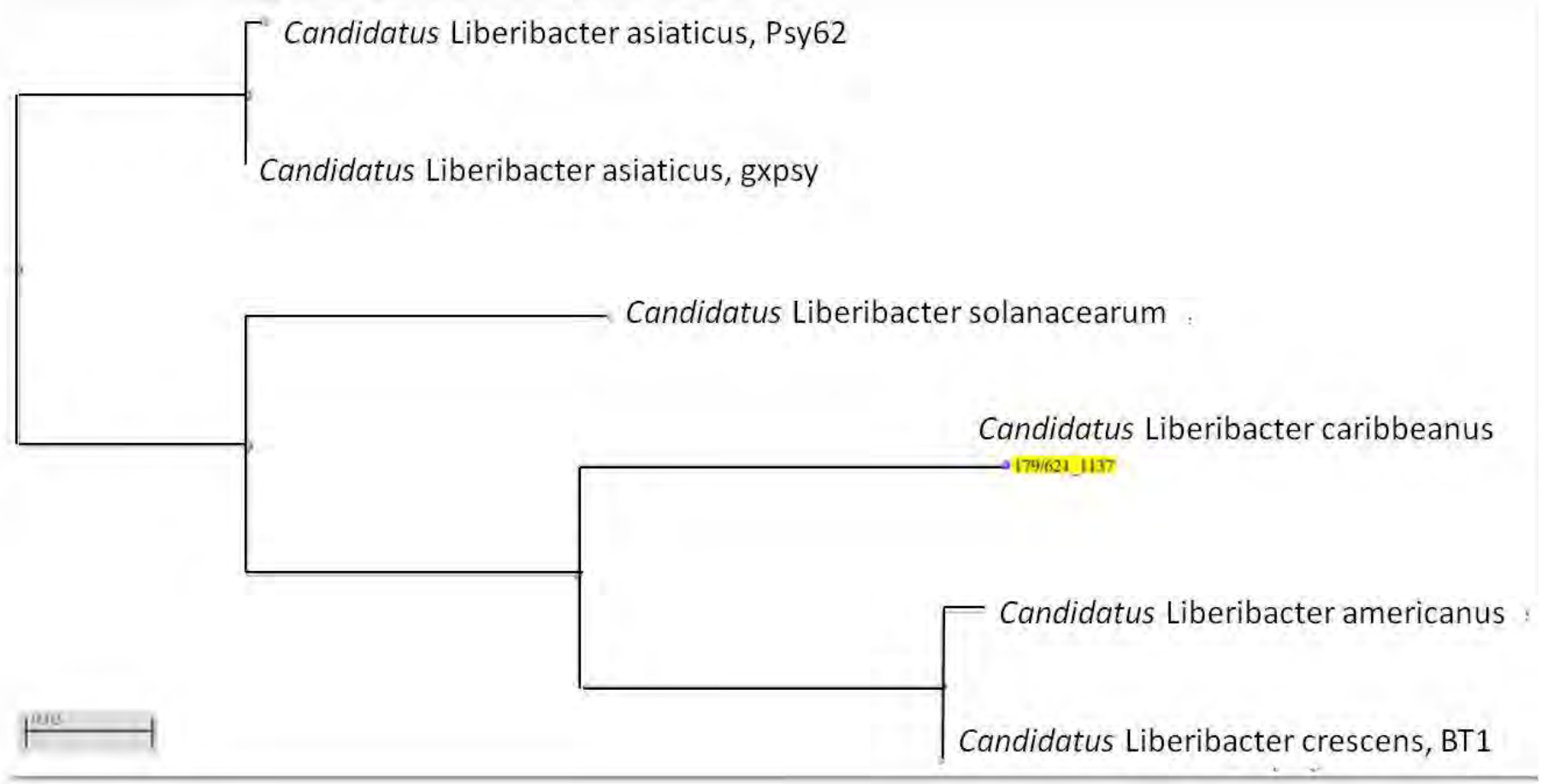
# Next Gen sequencing using PAC-BIO platform

- 15 SMRT cells were used to obtain large amount of sequence
- Each SMRT cell yielded about 400,000 sequences
- Average length of sequecnes was 1500
- 400,000 X15 X 1500 = 9 billion bases
- Bioinformatic analysis has yielded large number of Liberibacter contigs

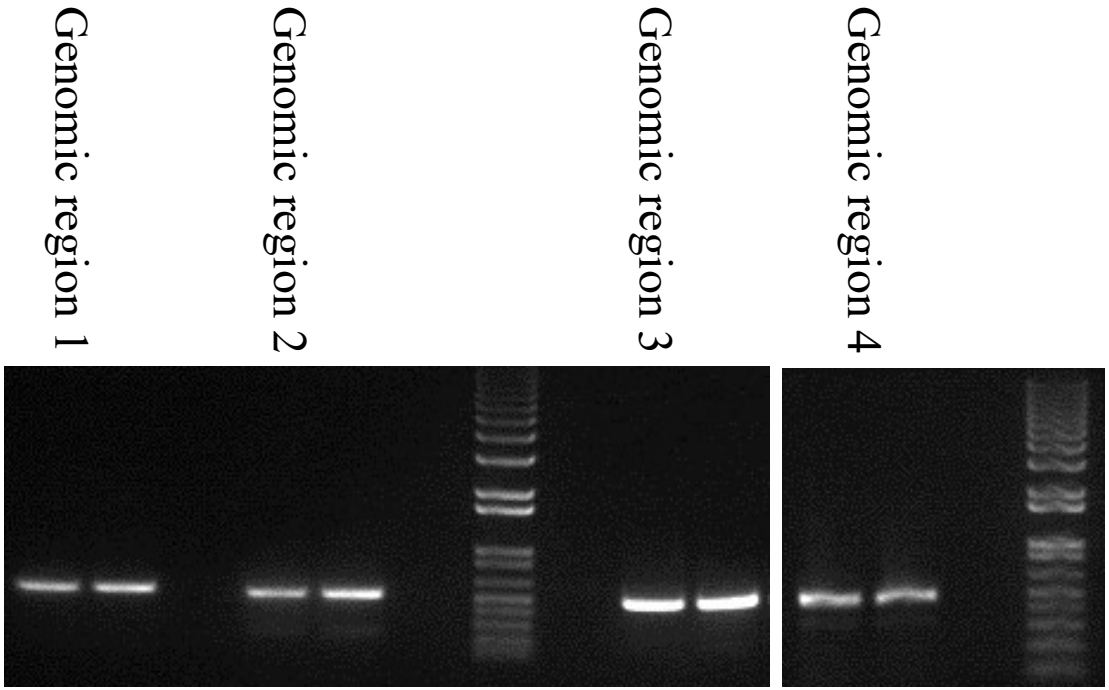
# Next Gen sequencing using PAC-BIO platform

Each contig is then analyzed by Blast for its relationship with other Liberibacters




Full assembly of Lca genome is in progress

# Conventional PCR and sequencing of other genomic regions




1. Conventional PCRs conducted for 4 genomic regions of ‘Ca. L. caribbeanus’.

2. Primer sequence was based on PAC-BIO metagenome analysis.

3. Psyllid extracts positive for ‘*Ca*. L. caribbeanus’ used for PCR amplification, gel fragments cloned and sequenced (Sanger sequencing at UCR core facility).

4. Sequences are about 85-95% similar to known Liberibacters.

# Conclusions

1. The results presented here have resulted from excellent cooperative effort of multiple agencies.

2. A four-year cooperative effort has resulted in identification and characterization of a new species of Liberibacter. We propose the name, '*Candidatus* Liberibacter caribbeanus'.

3. Positive psyllids were found first in November 2013 from Cordoba in the Northeastern part of Colombia close to Caribbean sea.

4. Analysis of about 4000 psyllid extractions resulted in detection of Lca in four more samples (10 extractions). About 30 other extractions were considered suspects.

5. Using digital PCR, we were able to discriminate between Las and Lca and identify Lca in two plant samples (further supported by PCR and sequencing).

6. PACBIO NextGen sequencing was used for sequencing the full genome of '*Candidatus* Liberibacter caribbeanus'.