



Identification of ‘*Candidatus Phytoplasma*’ species in “huanglongbing” infected citrus orchards in the Caribbean

Maritza Luis-Pantoja · Camilo Paredes-Tomás · Youri Uneau · Wayne Myrie · Raphael Morillon · Eleonora Satta · Nicoletta Contaldo · Francesco Pacini · Assunta Bertaccini

Accepted: 8 February 2021 / Published online: 19 February 2021
© The Author(s) 2021

Abstract “Huanglongbing” (HLB) is one of the most devastating diseases of citrus orchards worldwide. Samples from 183 citrus plants of different cultivars and rootstock/cultivar combinations, showing HLB symptoms in three Caribbean countries (Cuba, Jamaica, and Guadeloupe-France), were collected to verify the possible co-infection of ‘*Candidatus Phytoplasma*’ and ‘*Candidatus Liberibacter*’ species. The 64% of the samples resulted positive to the ‘*Ca. L. asiaticus*’ and the 27% to diverse ‘*Ca. Phytoplasma*’-related species, moreover about the 14% of the samples infected with ‘*Ca. Liberibacter*’ were also found positive to phytoplasmas, indicating the presence of mixed infection especially in the orchards located in Cuba. Moreover, in one of the samples from Jamaica mixed

phytoplasma infection was detected. Moreover the detection of only phytoplasmas in 11 symptomatic citrus samples collected from Cuba and Guadeloupe without ‘*Ca. Liberibacter*’ detection, confirmed that the symptomatology cannot be the sole criterium to discriminate between the presence of the two pathogens, and molecular detection is necessary to identify single or mixed infections. *Diaphorina citri* insects collected from Cuba and Guadeloupe resulted infected with ‘*Ca. L. asiaticus*’ confirming its active role in the dissemination of the pathogen. Only one insect of the *Cicadidae* family, collected in Guadeloupe, was found positive for phytoplasma presence. Considering that the phytoplasmas belonging to some ‘*Candidatus species*’ were detected in the three countries in different citrus varieties, a relevant role as phytoplasma reservoir can be attribute to citrus orchards.

M. Luis-Pantoja · C. Paredes-Tomás
Research Institute of Tropical Fruit Crops, La Habana, Cuba

Y. Uneau
ASSOFWI, Vieux-Habitants, Guadeloupe, France

W. Myrie
Coconut Industry Board, Kingston, Jamaica

R. Morillon
CIRAD, Station de Roujol, Guadeloupe, Petit Bourg, France

E. Satta · N. Contaldo · F. Pacini · A. Bertaccini (✉)
Department of Agricultural and Food Sciences, Alma Mater Studiorum - University of Bologna, Bologna, Italy
e-mail: assunta.bertaccini@unibo.it

Keywords Citrus greening · Phloematic bacteria · Molecular detection · Mixed infection · Insect vectors

Introduction

“Huanglongbing” (HLB) is one of the most devastating citrus diseases in the world (Bové 2006), mainly present with severe outbreaks in Asian and American continents (CABI 2019). It was detected also in several areas of the Caribbean, and, in particular, Cuba at the end of 2006 (Luis et al. 2009), Jamaica in 2009 (Brown et al. 2011), and Guadeloupe in 2012 (Cellier et al. 2014). Citrus industry is present in 140 countries worldwide covering

an area of 10.3 million hectares, with a production of about 167 million tonnes in 2016 (FAOSTAT n.d.). In the Caribbean countries the impact of HLB on the citrus industry is so relevant that both production and export of Cuba and Guadeloupe economy are severely affected.

Despite the lack of fulfilment of Koch's postulates, HLB occurrence has been primarily associated with '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*' and '*Ca. L. americanus*' (Jagoueix et al. 1994; Teixeira et al. 2005) presence in symptomatic citrus plants. However, phytoplasmas belonging to diverse ribosomal groups were also reported in several citrus species showing HLB-like symptoms with or without '*Ca. Liberibacter*' detection (Martinello-Sanches et al. 2016). Since in Cuba and in the Caribbean only '*Ca. L. asiaticus*' has been reported associated with HLB presence, the aim of the study was to confirm whether phytoplasmas are also associated with HLB-like symptoms in single or mixed infection taking into account preliminary investigations carried out in Cuba, Jamaica and Guadeloupe, (Bertaccini et al. 2019).

Materials and methods

Plant and insect material

A total of 103 symptomatic citrus samples were collected in Cuba (Table 1), six in Jamaica, and 74 in Guadeloupe (Table 2) during extensive surveys carried out in 2017–2019 in diverse growing areas (Fig. 1). The citrus plants surveyed in Cuba enclosed "Valencia" sweet orange, grapefruit, "Eureka" lemon, Mexican lime, Persian lime, and "Nova Tangelo" orange of different ages (Table 1). In Guadeloupe, the majority of the tested citrus was grafted onto diploid and tetraploid rootstocks, and the age of the trees varied between 2 and 6 years, only in a few cases 10 or 20 year-old trees were tested (Table 2). In Jamaica the six samples examined were collected from an industrial orchard of "Valencia" sweet orange five years old located in the area of Bog Walk (Saint Catherine Parish) and from a garden plant of the ornamental variety *Citrus aurantium* in Montego Bay (Fig. 1). Leaf samples were collected mainly based on the presence of asymmetric leaf mottling symptoms (Fig. 2); two additional asymptomatic samples per Country were also collected as negative controls. Insect vectors or putative vectors were collected in the surveyed orchards in Cuba and Guadeloupe and, after gross

morphological identification, tested to verify '*Ca. Liberibacter*' and phytoplasma presence as described below.

DNA extraction

DNA extraction was performed from 1 g of leaf midribs using CTAB (Murray and Thompson 1980) and phenol-chloroform (Prince et al. 1993) based methods. The CTAB method was also applied to extract the DNA from the specimens of insect vectors or potential vectors collected in and near citrus orchards in Cuba (extracted in batches of 5) and Guadeloupe (extracted as single specimen).

PCR, RFLP and sequence analyses

The amplifications for the CTAB extraction method were carried out using 1 µl of the extracted DNA diluted 1:30 in sterile distilled water, and 1 µl of a 20 ng/µl solution of the chloroform/phenol extracted DNA. The PCR amplification for '*Ca. Liberibacter*' species detection was performed with primers OA1/OI2c and OI1/OI2c (Jagoueix et al. 1996), while for the '*Ca. Phytoplasma*' species detection the primers R16mF2/R16mR1 (Gundersen and Lee 1996), U5/U3 (Lorenz et al. 1995), and M1/M2(=758f/1232r) (Gibb et al. 1995) were employed in direct and nested-PCR assays, respectively. Further nested-PCR assays following amplification with primers P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995), 16S503f/LY16Sr (Harrison et al. 1999), and R16(I)F1/R1 (Lee et al. 1994) specific for ribosomal groups 16SrIV and 16SrI, -II and -XII respectively, were also performed. Phytoplasma positive controls from micropropagated collection (Bertaccini 2014) or from the published phytoplasma strain DNAs listed in Fig. 3, were amplified separately to avoid cross contamination of the samples. No positive control was used for the '*Ca. Liberibacter*' detection. Negative controls were samples from asymptomatic citrus plants, devoid of nucleic acid template or containing sterile distilled water instead of the DNA template. The '*Ca. Liberibacter*' and '*Ca. Phytoplasma*' species identification was achieved by direct amplicons sequencing and/or RFLP analyses on 16S rDNA amplicons for the phytoplasma ribosomal group/subgroup identification (Lee et al. 1998). Sequence obtained after alignment were used to verify their clustering with reported '*Candidatus Phytoplasma*' species (Bertaccini and Lee 2018). The evolutionary

Table 1 Results of the survey for the detection of ‘*Ca. Liberibacter*’ and ‘*Ca. Phytoplasma*’ in citrus growing provinces from symptomatic citrus plants in Cuba, in bold samples used also for phytoplasma isolation and results from both plant and colony detection of phytoplasmas

Sample Code	Location (Province)	Scion variety (latin name)	‘ <i>Ca. Liberibacter</i> ’	‘ <i>Ca. Phytoplasma</i> ’
M9	Jagüey Grande, Matanzas	<i>Citrus clementina</i> Hort. ex Tan. x <i>C. paradisi</i>	yes	no
L3	Jagüey Grande, Matanzas	<i>Citrus latifolia</i> Tan.	yes	no
LP1, LP2	Jagüey Grande, Matanzas	<i>Citrus latifolia</i> Tan.	yes	no
LP3	Jagüey Grande, Matanzas	<i>Citrus latifolia</i> Tan.	yes	16SrXII+16SrIV
P4	Jagüey Grande, Matanzas	<i>Citrus paradisi</i> Macf.	yes	16SrIV
PC4	Jagüey Grande, Matanzas	<i>Citrus paradisi</i> Macf.	yes	no
M23	Jagüey Grande, Matanzas	<i>Citrus reticulata</i> Blanco x <i>C. sinensis</i> (L.) Osb.	yes	16SrXII+ 16SrXV
N2	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrXI
NN	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	no	16SrXII
OC2	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrXII+16SrIV
OC5	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrXII+16SrIV
N, N5, OC1, C3, C6	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	no
LP4	Jagüey Grande, Matanzas	<i>Citrus latifolia</i> Tan.	yes	no
Citrus Habana	Miramar, La Habana	<i>Citrus aurantifolia</i> (Christm.) Swing.	no	16SrVII
2619, 2623	Sola, Camaguey	<i>Citrus latifolia</i> Tan.	yes	16SrIV
2616, 2617, 2618, 2622	Sola, Camaguey	<i>Citrus latifolia</i> Tan.	yes	no
2621	Sola, Camaguey	<i>Citrus paradisi</i> Macf.	yes	no
2620	Sola, Camaguey	<i>Citrus sinensis</i> (L.) Osb.	yes	no
2624, 2626, 2627, 2628, 2657, 2660	Ceballos, Ciego de Ávila	<i>Citrus latifolia</i> Tan.	yes	16SrIV
2625	Ceballos, Ciego de Ávila	<i>Citrus latifolia</i> Tan.	yes	16SrXII
2644, 2645, 2658, 2659	Ceballos, Ciego de Ávila	<i>Citrus latifolia</i> Tan.	yes	no
2662	Ceballos, Ciego de Ávila	<i>Citrus paradisi</i> Macf.	yes	16SrIV
2643, 2661, 2663, 2664, 2665	Ceballos, Ciego de Ávila	<i>Citrus paradisi</i> Macf.	yes	no
2629, 2633, 2648, 2649, 2650, 2653, 2654, 2655, 2668	Ceballos, Ciego de Ávila	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrIV
2631, 2632, 2651, 2652, 2667, 2669	Ceballos, Ciego de Ávila	<i>Citrus sinensis</i> (L.) Osb.	yes	no
2636	Ceballos, Ciego de Ávila	<i>Citrus tangerina</i> Hort. ex Tan	yes	no
L5	Jagüey Grande, Matanzas	<i>Citrus latifolia</i> Tan.	yes	16SrII
L4, L1, L2, L3, L6, L7, 2464, 2465, 2466, 2467	Jagüey Grande, Matanzas	<i>Citrus latifolia</i> Tan.	yes	16SrX
2468, 2469, 2470	Jagüey Grande, Matanzas	<i>Citrus limon</i> (L.) Burm. f.	yes	no
N7	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrI
2677	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrIV
2676, 2678, 2679, 2680, 2681; N1, N2, N3, N4, N5, N6, N8, N9, N10, N11, N12; 2461, 2462, 2463	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	no
2474, 2475, 2479, 2480, 2489, 2490	Jagüey Grande, Matanzas	<i>Citrus sinensis</i> (L.) Osb.	yes	no
2484	Arimao, Cienfuegos	<i>Citrus paradisi</i> Macf.	yes	no
2485	Arimao, Cienfuegos	<i>Citrus sinensis</i> (L.) Osb.	yes	16SrVII
	Arimao, Cienfuegos	<i>Citrus sinensis</i> (L.) Osb.	yes	no

Table 2 Results of the survey for the detection of ‘*Ca. Liberibacter*’ and ‘*Ca. Phytoplasma*’ in citrus species in diverse locations of Guadeloupe during 2018–2019, in bold samples used also for phytoplasma isolation and results from both plant and colony detection of phytoplasmas

Sample Codes	Location	Scion variety name / ploidy	Rootstock name / ploidy	Tree age (years)	Symptoms	‘ <i>Ca. Liberibacter</i> ’	‘ <i>Ca. Phytoplasma</i> ’
1/19	Baie Mahault	Sour orange	no	10	yes	no	no
2/19	Baillif	Rode Red Valencia sweet orange / 2x	Citrumelo 4475 / 4x	2	no	no	no
3/19	Baillif	Rode Red Valencia sweet orange / 2x	Carrizo citrange / 2x	2	no	no	no
4, 5/19	Capesterre Belle Eau	Filhor AG1 / 4x	no	20	yes	no	no
6/19	Capesterre Belle Eau	Rode Red Valencia sweet orange / 2x	Filhor AG1 / 4x	2.5	no	no	no
7/19	Capesterre Belle Eau	Rode Red Valencia sweet orange / 2x	Filhor AG1 / 4x	2.5	yes	no	no
28/18	CIRAD Capesterre	Filhor AG1 / 4x	no	12	no	no	no
25/18	CIRAD Capesterre	Persian lime / 3x	Citumelo 4475 / 4x	2	yes	yes	no
26/18	CIRAD Capesterre	Persian lime / 3x	Citumelo 4475 / 2x	2	yes	yes	no
27/18	CIRAD Capesterre	Persian lime / 3x	no	12	yes	no	no
8, 9/19	Morne à l'Eau	Washington Navel sweet orange / 2x	Volkamer lemon / 2x	10	yes	no	no
10/19	Moule	Persian lime / 3x	Volkamer lemon / 2x	1	no	no	16SrII
11/19	Moule	Persian lime / 3x	Volkamer lemon / 2x	1	no	no	no
12/19	Petit Bourg	Volkamer lemon / 2x	Volkamer lemon / 4x	2.5	yes	no	no
13/19	Petit Bourg	Volkamer lemon / 2x	Volkamer lemon / 4x	2.5	no	no	no
14/19	Petit Canal	Persian lime / 3x	Volkamer lemon / 2x	6	yes	no	no
15/19	Saint Claude	Fremont mandarin / 2x	Volkamer lemon / 2x	5	yes	no	no
16/19	Saint Claude	Sour orange / 2x	no	20	no	no	no
17/19	Sainte Rose	Persian lime / 3x	Volkamer lemon / 2x	6	yes	no	16SrIX
G6L1	Trois Rivières	Cara Cara Navel sweet orange / 2x	Citrumelo 4475 / 2x	4	no	no	no
C2L4, C2L9 C4L10	Trois Rivières	Cara Cara Navel sweet orange / 2x	Filhor AG1 / 4x	4	no	no	no
C4L3	Trois Rivières	Cara Cara Navel sweet orange / 2x	Volkamer lemon 2x	4	no	no	no
18/19	Trois Rivières	Cara Cara Navel sweet orange / 2x	Filhor AG1 / 4x	3	no	no	no
22/18	Trois Rivières	Créole Mandarin / 2x	no	6	yes	no	no
19/18	Trois Rivières	Fisher Navel sweet orange / 2x	Citrumelo 4475 / 2x	3	no	no	no
20/19	Trois Rivières	Fisher Navel sweet orange / 2x	Filhor AG1 / 4x	3	no	no	16SrIX, 16SrXI, 16SrVI
C2L5, C2L10	Trois Rivières	Navelate sweet orange / 2x	Filhor AG1 / 4x	4	no	no	no
18/18	Trois Rivières	Navelima sweet orange / 2x	Filhor AG1 / 4x	3	no	no	no
C2L2	Trois Rivières	Navelima sweet orange / 2x	Filhor AG1 / 4x	4	no	no	no
23/18	Trois Rivières	Navelima sweet orange / 2x	Citumelo 4475 / 2x	4	no	no	16SrVII
24/18	Trois Rivières	Persian lime / 3x	Volkamer lemon / 2x	6	yes	no	16SrXII, 16SrIV
19/19	Trois Rivières	Persian lime / 3x	Volkamer lemon / 2x	3	no	no	no
17/18	Trois Rivières	Rode Red Valencia sweet orange / 2x	Volkamer lemon / 4x	3	no	no	no
C2L6, C2L7, C4L13, C4L4	Trois Rivières	Rode Red Valencia sweet orange / 2x	Citrumelo 4475 / 2x	4	no	no	no
C4L7	Trois Rivières	Rode Red Valencia sweet orange / 2x	Citrumelo 4475 / 2x	4	no	no	16SrVI, 16SrXII

Table 2 (continued)

Sample Codes	Location	Scion variety name / ploidy	Rootstock name / ploidy	Tree age (years)	Symptoms	'Ca. Liberibacter'	'Ca. Phytoplasma'
C4L9 , C4L12	Trois Rivières	Tangor Ellendale / 2x	Citrumelo 4475 / 2x	4	no	no	no
C2L1	Trois Rivières	Tangor Ellendale / 2x	Filor AG1 / 4x	4	no	no	no
C4L2	Trois Rivières	Tangor Ellendale / 2x	Filor AG1 4x	4	no	yes	16SrVI
C2L12, C4L6, C4L11, C6L4	Trois Rivières	Tangor Ellendale / 2x	Volkamer lemon / 2x	4	no	no	16SrI
11/18	Vieux Habitants	Cara Cara Navel sweet orange / 2x	Volkamer lemon / 2x	3	yes	no	16SrI
23/19	Vieux Habitants	Cara Cara Navel sweet orange / 2x	Volkamer lemon / 2x	4	no	no	no
7/18	Vieux Habitants	Falگو mandarin / 2x	Citrumelo 4475 / 4x	3	yes	yes	no
14/18	Vieux Habitants	Fisher Navel sweet orange / 2x	Citrumelo 4475 / 2x	3	yes	no	no
4/18	Vieux Habitants	Jackson Orangelo / 2x	Citrumelo 4475 / 4x	3	yes	no	16SrI
10/18	Vieux Habitants	Jackson Orangelo / 2x	Citrumelo 4475 / 2x	3	yes	no	16SrI
1/18	Vieux Habitants	Maltaise sweet orange / 2x	Volkamer lemon / 2x	2	no	no	no
15/18	Vieux Habitants	Mexican lime / 2x	Volkamer lemon / 2x	1	no	no	no
13/18	Vieux Habitants	Navelate sweet Orange / 2x	Filor AG1 / 4x	3	yes	no	16SrI
22/19	Vieux Habitants	Navelina sweet orange / 2x	Filor AG1 / 4x	4	yes	no	16SrIX
8/18	Vieux Habitants	Nova Tangelo / 2x	Filor AG1 / 4x	3	yes	no	16SrIII
16/18	Vieux Habitants	Persian lime / 3x	Volkamer lemon / 2x	1	no	no	no
21/19	Vieux Habitants	Persian lime / 3x	Volkamer lemon / 2x	3	no	no	no
12/18	Vieux Habitants	Tangor Ellendale / 2x	Citrumelo 4475 / 2x	3	yes	no	no
5/18	Vieux Habitants	Temple Tangor / 2x	Citrumelo 4475 / 2x	3	yes	yes	no
6/18	Vieux Habitants	Temple Tangor / 2x	Citrumelo 4475 / 4x	3	yes	yes	16SrI
9/18	Vieux Habitants	Triumph Orangelo / 2x	Citrumelo 4475 / 4x	3	yes	yes	no
2/18	Vieux Habitants	Valencia Late sweet orange / 2x	Volkamer lemon / 2x	2	no	no	16SrVI
3/18	Vieux Habitants	Valencia Late sweet orange / 2x	Volkamer lemon / 2x	2	yes	yes	no
A/19	Vieux Habitants	Nova Tangelo / 2x	Citrumelo / 2x	4	no	no	16SrX
B/19	Vieux Habitants	Jackson Orangelo / 2x	Citrumelo 4475 / 4x	4	yes	yes	no
C/19	Vieux Habitants	Jackson Orangelo / 2x	Citrumelo 4475 / 2x	4	yes	yes	16SrXII
D/19	Vieux Habitants	Nova Tangelo / 2x	Citrumelo 4475 / 4x	4	no	yes	no

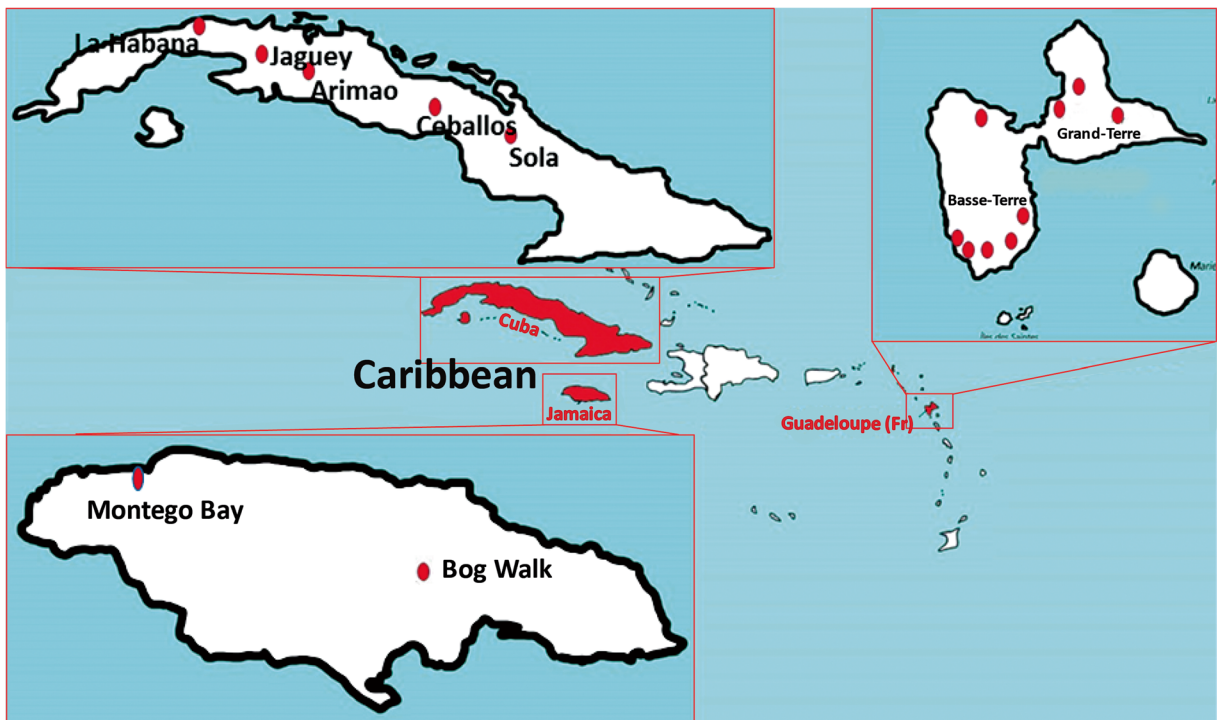


Fig. 1 Map of Cuba, Jamaica and Guadeloupe with locations of the citrus collection

history was inferred using the Neighbor-Joining method (Saitou and Nei 1987) with the Maximum Composite Likelihood method in MEGA6 (Tamura et al. 2004, 2013). ‘*Ca. Liberibacter*’ species sequences were verified by BLAST analyses and sequence identity. For both pathogens the sequences of representative strains were deposited in GenBank.

Phytoplasma isolation, purification, and molecular identification

Randomly selected symptomatic and asymptomatic leaf samples (Tables 1 and 2) plus the six samples from Jamaica were employed for phytoplasma isolation in artificial media, following previously reported protocols (Contaldo et al. 2016, 2019), with slight modifications. Approximately 100 mg of fresh midribs from each sample were placed in sterile mortars and mixed using a sterile pestle with 1 mL of succinate-citrate-phosphate buffer (SCP) (disodium succinate, 1 g/L; trisodium citrate, 1 g/L; K_2HPO_4 , 1.5 g/L; and KH_2PO_4 , 1 g/L; pH 7.0) (Sigma-Aldrich, St Louis, USA) (Minsavage et al. 1994). Immediately afterward, 100 μ L were transferred into 8-mL monovette

urine tubes (Sigma-Aldrich, St Louis, USA) and incubated at 25 ± 1 °C. The tubes were examined daily for an acidification-induced colour change from orange to yellow. Non inoculated tubes were prepared following the same procedure. After the colour change, 100 μ L of medium was inoculated onto plates containing 8 mL of CBs solid medium (Contaldo et al. 2016). Single colonies 36 h-old were transferred with sterile toothpicks into 8-mL monovette containing 2 mL of CBI for the purification steps. A gentle filtration of the liquid culture through 0.8- μ m membrane filters (Whatman, Maidstone, UK), followed by 10–3/10–4 serial dilutions in CBI as described (Contaldo et al. 2019) was then performed. DNA extractions from 48 to 72 h-colonies and from CBI medium after filtration steps were carried out. PCR amplification of 16S rRNA coding gene, using the primer pair R16F2n/R2 (Gundersen and Lee 1996) nested with U5/U3 primers was performed under reported cycling conditions (Contaldo et al. 2012). For each PCR reaction, DDSW (distilled, deionized sterile water) and non-inoculated media were used as negative controls. Phytoplasma identity was verified by RFLP and/or direct sequencing.



Fig. 2 Symptomatology observed in the citrus samples in A, B and C Guadeloupe; in D Cuba in which 1, sour orange; 2, grapefruit; 3, sweet orange; 4, Volkamer lemon; 5, Persian lime and 6, trifoliate orange

Results and discussion

The most common symptoms observed in all the citrus plants from which samples were collected were asymmetric leaf mottle and reduction of the plant growth (Fig. 2). Reduced flowering and fruits displaying a color inversion as they begin to mature before eventually the tree dies were also observed in the majority of the surveyed orchards.

In Cuba, 100 symptomatic samples out of the 103 resulted positive for the '*Ca. L. asiaticus*' presence, and the sequence of sample L3 in Table 1 showing 100% identity with available

sequences of this bacterium was deposited in GenBank under the accession number MW065487. Moreover 39 samples after nested PCR assays or isolation in artificial media were detected as positive for phytoplasmas tentatively assigned to eight ribosomal groups (16SrI, 16SrII, 16SrIV, 16SrVII, 16SrX, 16SrXI, 16SrXII and 16SrXV) as determined by 16S rRNA gene RFLP and/or sequence analyses (Table 1, Fig. 3, and data not shown). After phylogenetic analyses the sequenced citrus phytoplasma strains clustered with seven '*Ca. Phytoplasma*' species and one ribosomal group (Fig. 4). The two samples from

Jagüey Grande, Matanzas and Miramar, la Habana of *C. sinensis* and *C. aurantifolia*, respectively, that were negative to HLB were positive to ‘*Ca. Phytoplasma*’ presence. The varieties in which the mixed infection of ‘*Ca. L. asiaticus*’ and ‘*Ca. Phytoplasma*’ was detected were *C. latifolia*, *C. paradisi*, *C. reticulata* x *C. sinensis* and *C. sinensis*. A total of 105 specimens of *Diaphorina citri* was collected in Cuba in the same citrus orchards surveyed (Fig. 1) and on *Murraya exotica* and *Triphasia trifolia*. All *D. citri* specimens resulted positive to the ‘*Ca. Liberibacter*’ presence and negative for the ‘*Ca. Phytoplasma*’ presence.

In Jamaica, out of six symptomatic samples, five were positive to HLB, while the samples from *C. aurantium* plant were positive in nested PCR to phytoplasmas enclosed in the 16SrI and 16SrXII groups present in mixed infection (Fig. 3, and data not shown).

In Guadeloupe, 11 samples were positive to ‘*Ca. L. asiaticus*’ and 18 were positive to phytoplasmas enclosed in 16SrI, 16SrII, 16SrIII, 16SrIV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI and 16SrXII groups after nested PCR and/or isolation assays (Table 2, Fig. 3, and data not

shown). After phylogenetic analyses the sequenced phytoplasma strains resulted to cluster with nine ‘*Ca. Phytoplasma*’ species and one ribosomal group (Fig. 4). Only in three cases, a 4-year old tree Jackson Orangelo/2x grafted on Citrumelo 4475/2x, a 3-year old tree Temple /Tangor/2x grafted on Citrumelo 4475/4x and a 4-year old Tangor Ellandale/2x grafted on Flhor AG14x, both ‘*Ca. L. asiaticus*’ and ‘*Ca. Phytoplasma*’ were detected in mixed infection (Table 2).

Only seven of the 27 samples processed for isolation resulted phytoplasma positive in the colonies obtained in the artificial medium. In particular, samples 13/18, 24/18, 4/18, C4L2, C4L7, and C4L11 from Guadeloupe, and OC2 and OC5 from Cuba, showed colour changes referable to microorganism growth after approximately 10–12 days from isolation. Afterward from the subsequent plating, colonies morphologically similar with those previously reported for phytoplasmas were obtained from all the isolation tubes but OC5 (Fig. 5) (Contaldo et al. 2012, 2016, 2019). Phytoplasma DNA amplification was performed on a total of 16 DNA aliquots from liquid and/or solid media of these samples, and the results revealed the presence of the same phytoplasmas detected in the corresponding plant materials (16SrI, 16SrIV, 16SrVI and 16SrXII) (Figs. 3

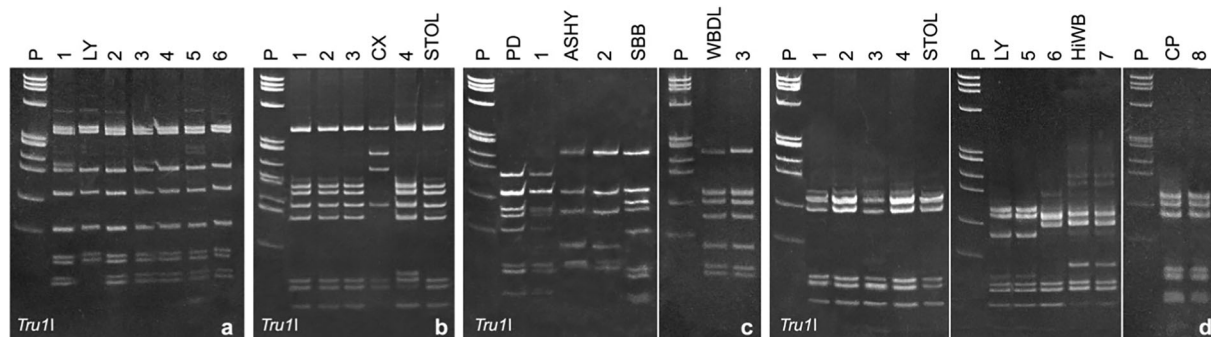


Fig. 3 Phytoplasma RFLP profiles of 16S rDNA in 6.7% polyacrylamide gels of selected amplicons from citrus DNA samples and phytoplasma controls obtained with diverse primer pairs and digested with *Tru*II. In a) Amplicons obtained with primers R16mF2/R16mR1 (about 1400 bp): 1, 24/18 (sample from Guadeloupe Table 2); LY, lethal yellowing strain LY8a from Jamaica (16SrIV-A, Paredes-Tomás et al. 2018); 2, LP3; 3, P4; 4, 2624; 5, 2626, and 6, 2627 (samples from Cuba, Table 1). In b) amplicons obtained with primers R16(I)F1/R1 (1100 bp): 1, 2625; 2, LP3; 3, OC2 (samples from Cuba Table 1); CX, X peach disease (16SrIII-A); 4, C19 (sample from Guadeloupe Table 2); STOL, “stolbur” (16SrXII-A); in c) amplicons obtained with U5/U3 primers: PD, pear decline (16SrX-C); 1, A/19 (sample from Guadeloupe Table 2); ASHY, ash yellows (16SrVII-A); 2, 2484

(sample from Cuba Table 1); SBB, Solanum big bud (16SrIII-F); WBDL, witches’ broom disease of lime (16SrII-B); 3, 10/19 (sample from Guadeloupe Table 2); in d) amplicons obtained with M1/M2 primer pairs: 1, 24/18; 2, C19 (samples from Guadeloupe Table 2); 3, Bog Walk Jamaica sample, 4, Montego Bay Jamaica sample; STOL; LY, lethal yellowing strain LY8a from Jamaica (16SrIV-A, Paredes-Tomás et al. 2018); 5, P4 (sample from Cuba Table 1); 6, NN (sample from Cuba Table 1); HiWB, hibiscus witches’ broom strain HB1 (16SrXV-A, Montano et al. 2011); 7, M23 (sample from Cuba Table 1); CP, clover proliferation (16SrVI-A); 8, C4L7 (sample from Guadeloupe Table 2); P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72

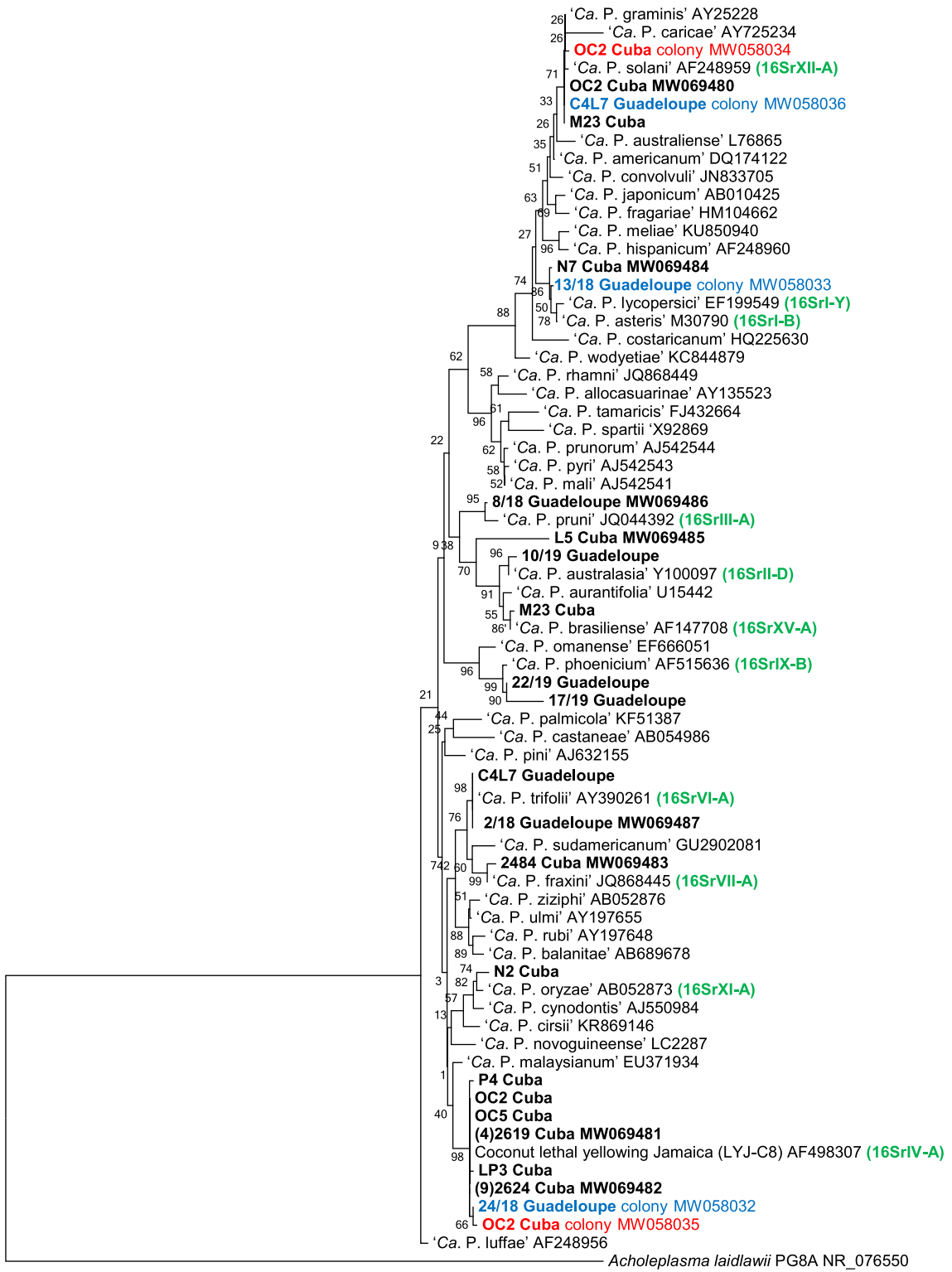
and 4). In particular the phytoplasma isolation from the citrus samples from Guadeloupe was achieved from three symptomatic and three asymptomatic plants of polyploid genotypes grafted on polyploid rootstocks: 24/18-Persian lime 3x grafted on Volkamer lemon 2x, 4/18-Jackson orangelo grafted on 2x Citrumelo 4475/4x, 13/18-Navelate sweet Orange 2x grafted on Flor AG4x, C4L7-Rode Red Valencia sweet orange grafted on 2x Citrumelo 4475/2x, C4L2-Tangor Ellandale 2x grafted on Flhor AG1 4x, and C4L11-Tangor Ellandale 2x grafted on Volkamer lemon/2x, respectively. Four of these samples were negative to the presence of HLB (Table 2). Only one sample from an orange tree collected in Cuba resulted positive to phytoplasma presence in both liquid media and colonies (sample OC2, Table 1). The same sample was positive in plant leaf detection for 16SrXII and 16SrIV phytoplasmas, the same detected in the culture; in the detection from aliquots of liquid medium this sample resulted also positive for phytoplasmas of 16SrX group (data not shown).

In the Guadeloupe surveyed locations beside *D. citri*, specimens of the following insects were collected: *Helochara spp.*, *Lepidosaphes spp.*, *Fiorinia proboscidea*, *Coccus viridis*, *Icerya seychelles*, *Empoasca vitis*, *Zelus lungipe*, *Coccus viridis*, *Cicadidae*, *Muschidae*, and *Psyllidae*. Only one specimen of *D. citri* collected in Vieux Habitants locality resulted positive for the ‘*Ca. L. asiaticus*’ presence, and the sequence was deposited in GenBank under the accession number MW065488; moreover the only unclassified *Cicadidae* collected from Capesterre Belle Eau resulted positive to 16SrXI phytoplasmas (data not shown). Thirty-seven citrus samples all collected from diverse localities in Guadeloupe and belonging to polyploid varieties grafted on diploid and tetraploid rootstocks tested negative to the presence of both pathogens (Table 2). The asymptomatic samples resulted negative in all the PCR assays carried out (data now shown).

Phytoplasmas enclosed in groups 16SrI and 16SrXII were identified in the surveys carried out in all the three islands suggesting a possible primary dissemination with the propagation materials. The 16SrII, 16SrIV, 16SrVII, 16SrX and 16SrXI phytoplasma ribosomal groups were present in citrus in both Guadeloupe and Cuba samplings from experimental and small farms in the first case, and from larger orchards, in the second case. This could indicate that the surrounding cultivated species are influencing the phytoplasma population composition in citrus orchards, as indicated by the relevant presence of the lethal

yellowing disease, that was severely affecting the Cuban palm plantations in the past years (Llauger et al. 2002; Paredes-Tomás et al. 2019). The identification of phytoplasmas enclosed in groups 16SrIII, 16SrVI and 16SrIX in Guadeloupe and in group 16SrXV in Cuba corroborates this hypothesis. In Guadeloupe the 16SrI phytoplasmas were recently detected in pomegranate and mango fruits from orchards (Castañeda-Alvarez et al. 2018) located near to citrus plot where the 16SrI phytoplasma was detected in the present study, which revealed the presence of natural source reservoirs of 16SrI group phytoplasmas in the area. Phytoplasmas of the same ribosomal group were reported from citrus in China and Mexico (Chen et al. 2009; Arratia-Castro et al. 2014). Moreover, the 16SrX phytoplasmas were detected in Guadeloupe in passion fruit trees located besides citrus plots (Satta et al. 2018). The consistent detection of three phytoplasma ribosomal groups in citrus only in Guadeloupe, could also be related to the variety of the diverse species cultivated in the island that are potential phytoplasma reservoirs. These phytoplasmas were already reported in the Caribbean areas and in some cases also in citrus crops (Teixeira et al. 2009; Caicedo et al. 2015; Mauricio-Castillo et al. 2015). In Cuba the presence of phytoplasmas in the 16SrI group was reported in papaya, beans, peppers, cabbage, beets, carrots, cassava, soybeans, strawberry and macadamia, 16SrII group in papaya, radish and medlar, and the 16SrX group in papaya (Arocha et al. 2007; Arocha et al. 2009; Zamora et al. 2012, 2014; Acosta-Pérez et al. 2013; Ferriol-Marchena et al. 2013; Pérez-López et al. 2013). All these species are cultivated as intercrop in citrus fields or are planted closely to citrus orchards.

The results of this work confirm the presence of mixed infection of phytoplasmas enclosed in different ribosomal groups with ‘*Ca. L. asiaticus*’ in almost all the citrus varieties with HLB symptomatology in Cuba, and in only three plants of diverse genotypes (Temple Tangor 2x and Nova tangelo 2x grafted on Citrumelo 4475 4x and Tangor Ellandale 2x grafted on Flhor AG1 4x) in Guadeloupe. This type of mixed infection was reported in other citrus growing areas of the world (Chen et al. 2009; Teixeira et al. 2008; Arratia-Castro et al. 2014). In this survey the phytoplasmas were mainly detected in mixed infection with ‘*Ca. L. asiaticus*’. In 11 samples, two in Cuba and 9 in Guadeloupe, the phytoplasmas were identified in symptomatic citrus plants where ‘*Ca. L. asiaticus*’ was not detected, and this confirms that the symptomatology alone cannot discriminate between the presence of the two



◀ **Fig. 4** Evolutionary relationships of selected phytoplasma strains detected in citrus samples and reported as listed in Tables 1 and 2, using the Neighbor-Joining method. All the ‘*Candidatus* Phytoplasma’ reported species are enclosed in the analyses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis was conducted in MEGA6. *Acholeplasma laidlawii* was used as outroot. The GenBank accession numbers are reported on the right of the ‘*Candidatus* Phytoplasma’ species and of the strains sequenced in the present work (in bold). Ribosomal subgroups are added for phytoplasmas used as reference (green bold)

pathogens, as already reported in Brazil (Martinello-Sanches et al. 2016). Moreover, the only phytoplasma presence in grapefruit was associated with the presence of HLB-like symptoms in China (Lou et al. 2014) and in *Citrus reticulata* in India (Das et al. 2016). The analysis carried out also indicates that ‘*Ca. L. asiaticus*’ has a higher titre than phytoplasmas since the latter were always detected by nested-PCR assays and/or after isolation in artificial media. However, the phytoplasma presence in citrus represents a source of infection for other crops, and made it more difficult to prevent their spreading to the surrounding natural environments or farmland.

In Guadeloupe, molecular tests have been carried out by French authorities (DAAF- SALIM) to identify the presence of ‘*Ca. L. asiaticus*’ in citrus orchards since 2013. A monitoring and information system of *D. citri* in the plots is also conducted as part of the epidemiology-surveillance of the island (BSV Guadeloupe 2020). HLB is present in orchards and its spread is presently not controlled. Several of the plots where *D. citri* is

present are symptomatic and the work carried out clearly indicates the need of surveillance also for ‘*Ca. Phytoplasma*’ species and their possible insect vectors.

In Cuba there is a production system of certified citrus material for the planting new orchards (López-Hernández et al. 2014), however there is a very high source of inoculum in the fields, so the plants are infected very early, and the management of HLB is not yet well established. In most of the commercial orchards there are populations of *D. citri* recorded on symptomatic trees, resulting in low citrus productivity in Cuba and Guadeloupe. These findings of widespread presence of both ‘*Ca. L. asiaticus*’ and various ‘*Ca. Phytoplasma*’ species and ribosomal groups clearly indicate the need for specific surveillance to apply better management tools enclosing those aimed to contain the possible insect vectors presence after their appropriate identification.

Acknowledgements This work was carried out in the frame of EU H2020 R & I programme project “Insect-borne prokaryote-associated diseases in tropical and subtropical perennial crops - TROPICSAFE” GA No 727459.

Author contributions All authors contributed to the study conception, design, material preparation, data collection and analysis. All authors read and approved the final manuscript.

Fundings Open access funding provided by *Alma Mater Studiorum - Università di Bologna* within the CRUI-CARE Agreement. The research leading to these results received funding from EU H2020 R & I programme project “Insect-borne prokaryote-associated diseases in tropical and subtropical perennial crops - TROPICSAFE” under Grant Agreement No 727459. Data availability The datasets generated during the current study are available in the GenBank database.

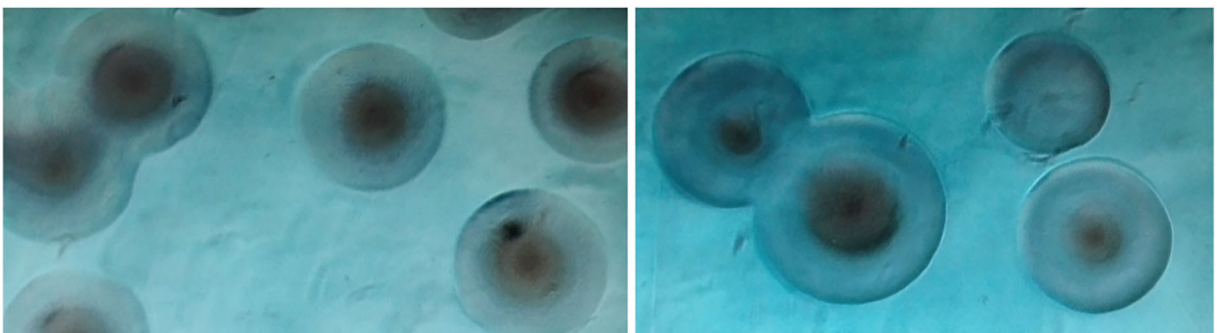


Fig. 5 Phytoplasma colonies from isolation using citrus tissues from sample orange OC2 from Cuba and resulted positive to 16SrXII and 16SrIV phytoplasmas (Table 1 and Fig. 4) (magnification 40 X)

Declarations

Ethical statement The authors declare no potential conflicts of interest. The research is not involving Human Participants and/or Animals. All the authors have read and approved the submitted manuscript text.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Acosta-Pérez, K., Zamora, L., Piñol, B., Fernández, A., Chávez, A., Flores, G., Méndez, J., Santos-Cervantes, M., Leyva-Lopez, N., & Arocha-Rosete, Y. (2013). Identification and molecular characterization of phytoplasmas and rickettsia pathogens associated with “bunchy top symptom” (BTS) and “papaya bunchy top” (PBT) of papaya in Cuba. *Crop Protection*, *45*, 49–56.
- Arocha, Y., Piñol, B., Picornell, B., Almeida, R., & Jones, P. (2007). Broad bean and sweet pepper: Two new hosts associated with ‘*Candidatus* Phytoplasma asteris’ (16SrI phytoplasma group) in Cuba. *Plant Pathology*, *56*, 345.
- Arocha, Y., Piñol, B., Almeida, R., Acosta, K., Quiñones, M., Zayas, T., Varela, M., Marrero, Y., Boa, E., & Lucas, J-A. (2009). First report of phytoplasmas affecting organoponic crops in central and eastern Cuba. *Plant Pathology*, *58*(4), 793–793.
- Arratia-Castro, A. A., Santos-Cervantes, M. E., Fernández-Herrera, E., Chávez-Medina, J. A., Flores-Zamora, G. L., Camacho-Beltrán, E., Méndez-Lozano, J., & Leyva-López, N. E. (2014). Occurrence of ‘*Candidatus* Phytoplasma asteris’ in citrus showing “huanglongbing” symptoms in Mexico. *Crop Protection*, *62*, 144–151.
- Bertaccini, A. (2014). Phytoplasma collection. <http://www.ipwgnat.org/collection>. Accessed 20 Aug 2020.
- Bertaccini, A., & Lee, I.-M. (2018). Phytoplasmas: an update. In G. P. Rao, A. Bertaccini, N. Fiore, & L. Liefting (Eds.), *Phytoplasmas: plant pathogenic bacteria-I. Characterization and epidemiology of phytoplasma-associated diseases* (Chapter 1, pp. 1–29). Singapore: Springer.
- Bertaccini, A., Satta, E., Luis-Pantoja, M., Paredes-Tomás, C., Uneau, Y., & Myrie, W. (2019). ‘*Candidatus* Phytoplasma’ and ‘*Candidatus* Liberibacter’ species detection in citrus. *Phytopathogenic Mollicutes*, *9*(1), 187–188.
- Bové, J.-M. (2006). “Huanglongbing”: a destructive, newly-emerging, century-old dis-ease of citrus. *Journal of Plant Pathology*, *88*, 7–37.
- Brown, S. E., Oberheim, A. P., Barrett, A., & McLaughlin, W. A. (2011). First report of ‘*Candidatus* Liberibacter asiaticus’ associated with “huanglongbing” in the weeds *Cleome rutidosperma*, *Pisonia aculeata* and *Trichostigma octandrum* in Jamaica. *New Disease Reports*, *24*, 25.
- BSV Guadeloupe (2020) Diversification végétale – Arboriculture fruitière, numéro 02 du 31/07/2020.
- CABI. 2019. Liberibacter asiaticus (Asian greening): <http://www.cabi.org/isc/datasheet/16565>.
- Caicedo, J., Vargas, L., Segarra, A., Davis, R. E. (2015). Detection and molecular characterisation of a group 16SrIX phytoplasma infecting citrus (*Citrus sinensis* and *C. limon*), coffee (*Coffea arabica*), periwinkle (*Catharanthus roseus*), and tabebuia (*Tabebuia heterophylla*) in Puerto Rico. *Australasian Plant Disease Notes*, *10*, 28.
- Castañeda-Alvarez, C., Contaldo, N., Satta, E., Uneau, Y., & Bertaccini, A. (2018). Identification of phytoplasmas in mango and pomegranate in Guadeloupe. *Phytopathogenic Mollicutes*, *8*(2), 89–95.
- Cellier, G., Moreau, A., Cassam, N., Hostachy, B., Ryckewaert, P., Aurela, L., Picard, R., Lombion, K., & Rioualec, A. L. (2014). First report of ‘*Candidatus* Liberibacter asiaticus’ associated with huanglongbing on *Citrus latifolia* in Martinique and Guadeloupe, French West Indies. *Plant Disease*, *98*(5), 683–684.
- Chen, J., Pu, X., Deng, X., Liu, S., & Civerolo, E. (2009). A phytoplasma related to ‘*Candidatus* Phytoplasma asteris’ detected in citrus showing “huanglongbing” (yellow shoot disease) symptoms in Guangdong, P. R. China. *Phytopathology*, *99*, 236–342.
- Contaldo, N., Bertaccini, A., Paltrinieri, S., Windsor, H. M., & Windsor, D. G. (2012). Axenic culture of plant pathogenic phytoplasmas. *Phytopathologia Mediterranea*, *51*(3), 607–617.
- Contaldo, N., Satta, E., Zambon, Y., Paltrinieri, S., & Bertaccini, A. (2016). Development and evaluation of different complex media for phytoplasma isolation and growth. *Journal of Microbiological Methods*, *127*, 105–110.
- Contaldo, N., D’Amico, G., Paltrinieri, S., Diallo, H. A., Bertaccini, A., & Arocha-Rosete, Y. (2019). Molecular and biological characterization of phytoplasmas from coconut palms affected by the lethal yellowing disease in Africa. *Microbiological Research*, *223–225*, 51–57.
- Das, A. K., Nerkar, S., Thakre, N., & Kumar, A. (2016). First report of ‘*Candidatus* Phytoplasma trifolii’ (16SrVI group) in Nagpur mandarin (*Citrus reticulata*) showing huanglongbing symptoms in Central India. *New Disease Reports*, *34*, 15.
- Deng, S., & Hiruki, C. (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods*, *14*, 53–61.
- FAOSTAT (n.d.). <http://www.fao.org/faostat/en/#data>

- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*, 783–791.
- Ferriol-Marchena, X., Hernández-Rodríguez, L., Luis-Pantoja, M., García-García, G., Banguela-Castillo, A., & Pérez, J. M. (2013). First report of a '*Candidatus Phytoplasma asteris*' isolate affecting a strawberry nursery in Cuba. *New Disease Reports*, *28*, 19.
- Gibb, K. S., Padovan, A. C., & Mogen, B. D. (1995). Studies on sweet potato little-leaf phytoplasmas detected in sweet potato and other species growing in northern Australia. *Phytopathology*, *85*, 169–174.
- Gundersen, D. E., & Lee, I.-M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, *35*, 144–151.
- Harrison, N., Cordova, I., Richardson, P., & Dibonito, R. (1999). Detection and diagnosis of lethal yellowing. In C. Oropeza, J. L. Verdeil, G. R. Ashburner, R. Cardeña, & J. M. Santamaría (Eds.), *Current advances in coconut biotechnology* (pp. 183–196). The Netherlands: Kluwer Academic.
- Jagoueix, S., Bové, J.-M., & Garnier, M. (1994). The phloem-limited bacterium of greening disease of citrus is a member of the alfa-subdivision of the *Proteobacteria*. *International Journal of Systematic Bacteriology*, *44*(3), 379–386.
- Jagoueix, S., Bové, J.-M., & Garnier, M. (1996). PCR detection of the two '*Candidatus Liberobacter*' species associated with greening disease of citrus. *Molecular and Cellular Probes*, *10*, 43–50.
- Lee, I.-M., Gundersen-Rindal, D. E., Hammond, R. W., & Davis, R. E. (1994). Use of mycoplasma-like organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology*, *84*, 559–566.
- Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., & Bartoszyk, I. M. (1998). Revised classification scheme of phytoplasma based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology*, *48*, 1153–1169.
- Llauger, R., Becker, D., Cueto, J., Peralta, E., González, V., Rodríguez, M., & Rohde, W. (2002). Detection and molecular characterization of phytoplasma associated with lethal yellowing disease of coconut palms in Cuba. *Journal of Phytopathology*, *150*, 390–395.
- López-Hernández, D., Luis-Pantoja, M., Llauger-Riverón, R., González-Fernández, C., Casín-Fernández, J., Peña, I., Batista-LeRiverend, L., Hernández Rodríguez, L., Zamora-Rodríguez, V., & Hernández-Espinosa, D. (2014). Situación de "huanglongbing" de los cítricos en Cuba siete años después de su detección. *Citricifuit*, *31*, 3–9.
- Lorenz, K. H., Schneider, B., Ahrens, U., & Seemüller, E. (1995). Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and non ribosomal DNA. *Phytopathology*, *85*, 771–776.
- Lou, B., Bai, X., Bai, Y., Deng, C., Roy Chowdhury, M., Chen, C., & Song, Y. (2014). Detection and molecular characterization of a 16SrII-A* phytoplasma in grapefruit (*Citrus paradisi*) with "huanglongbing"-like symptoms in China. *Journal of Phytopathology*, *162*, 387–395.
- Luis, M., Collazo, C., Llauger, R., Blanco, E., Peña, I., López, D., González, C., Casín, J. C., Batista, L., Kitajima, E., Tanaka, F. A. O., Salaroli, R. B., Teixeira, D. C., Martins, E. C., & Bové, J.-M. (2009). Occurrence of citrus "huanglongbing" in Cuba and association of the disease with '*Candidatus Liberibacter asiaticus*'. *Journal of Plant Pathology*, *91*, 709–712.
- Martinello-Sanches, M., Wulff, N. A., Ferreira, E. A., Fernandes dos Santos, J., de Oliveria-Angarten, M. B., Carbonari, J. J., de Oliveira, R. P., Nakasone-Ishida, A. K., & Martins, O. M. (2016). Survey for phytoplasmas and '*Candidatus Liberibacter*' sp. from HLB-like symptomatic citrus plants in Brazil. *Citrus Research & Technology*, *37*, 88–93.
- Mauricio-Castillo, J. A., Salas-Muñoz, S., Velásquez-Valle, R., Ambríz-Granados, S., & Reveles-Torres, L. R. (2015). '*Candidatus Phytoplasma trifolii*' (16SrVI) in Mirasol chili pepper (*Capsicum annum* L.) cultivated in Zacatecas, México. *Revista Fitotecnia Mexicana*, *38*(4), 389–396.
- Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leile, R. M. V. B. C., & Stall, R. E. (1994). Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology*, *84*(5), 456–461.
- Montano, H. G., Contaldo, N., David, T. V. A., Silva, I. B., Paltrinieri, S., & Bertaccini, A. (2011). Hibiscus witches' broom disease associated with different phytoplasma taxa in Brazil. *Bulletin of Insectology*, *64*(Supplement), S249–S250.
- Murray, M., & Thompson, W. (1980). Rapid isolation of higher weight DNA. *Nucleic Acids Research*, *8*, 4321–4325.
- Paredes-Tomás, C., Satta, E., Paltrinieri, S., Oropeza Salin, C., Myrie, W., Bertaccini, A., & Luis-Pantoja, M. (2019). '*Candidatus Phytoplasma*' species detection in coconuts in Cuba. *Phytopathogenic Mollicutes*, *9*(1), 191–192.
- Pérez-López, E., Hernández-Rodríguez, L., Pantoja, M. L., & Zaldívar, M. R. H. (2013). First report of a '*Candidatus Phytoplasma asteris*' isolate affecting macadamia nut trees in Cuba. *New Disease Reports*, *28*, 1.
- Prince, J. P., Davis, R. E., Wolf, T. K., Lee, I.-M., Mogen, B. D., Dally, E. L., Bertaccini, A., Credi, R., & Barba, M. (1993). Molecular detection of diverse mycoplasma-like organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. *Phytopathology*, *83*, 1130–1137.
- Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406–425.
- Satta, E., Uneau, Y., Bouriseau, F., & Bertaccini, A. (2018). Detection of phytoplasmas in *Passiflora edulis* in Guadeloupe. *Phytopathogenic Mollicutes*, *8*(1), 8–13.
- Schneider, B., Seemüller, E., Smart, C. D., & Kirkpatrick, B. C. (1995). Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In S. Razin & J. G. Tully (Eds.), *Molecular and diagnostic procedures in mycoplasmaology* (pp. 369–380). San Diego: Academic Press.
- Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining

- method. *Proceedings of the National Academy of Sciences (USA)*, 101, 11030–11035.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30, 2725–2729.
- Teixeira, D. C., Saillard, C., Eveillard, S., Danet, J.-L., da Costa, P. I., Ayres, A. J., & Bové, J.-M. (2005). ‘*Candidatus Liberibacter americanus*’ associated with citrus “huanglongbing” (greening disease) in Sao Paulo state, Brazil. *International Journal of Systematic and Evolutionary Microbiology*, 55, 1857–1862.
- Teixeira, D. C., Wulff, N. A., Martins, E. C., Kitajima, E. W., Bassanezi, R., Ayres, A. J., Eveillard, S., Saillard, C., & Bové, J.-M. (2008). A phytoplasma closely related to the pigeon pea witches’ broom phytoplasma (16SrIX) is associated with citrus “huanglongbing” symptoms in the state of São Paulo, Brazil. *Phytopathology*, 98, 977–984.
- Zamora, L., Acosta, K., & Martínez, Y. (2012). First report of ‘*Candidatus Phytoplasma asteris*’ (16SrI group) affecting common bean in Cuba. *New Disease Reports*, 25, 4.
- Zamora, L., Acosta, K., Piñol, B., Quiñones, M., & Bertaccini, A. (2014). First report of ‘*Candidatus Phytoplasma asteris*’ (16SrI group) causing stunt of tomato in Cuba. *New Disease Reports*, 30, 10.