Survey for ‘Candidatus Liberibacter’ and ‘Candidatus Phytoplasma’ in Citrus in Chile

Nicolas Quiroga 1,2, Camila Gamboa 1, Gabriela Medina 1, Nicoletta Contaldo 3, Fernando Torres 4, Assunta Bertaccini 3, Alan Zamorano 1 and Nicola Fiore 1,4,*

Abstract: The considerable economic losses in citrus associated with ‘Candidatus Liberibacter’ and ‘Candidatus Phytoplasma’ presence have alerted all producing regions of the world. In Chile, none of these bacteria have been reported in citrus species. During the years 2017 and 2019, 258 samples presenting symptoms similar to those associated with the presence of these bacteria were examined. No detection of ‘Ca. Liberibacter’ associated with “huanglongbing” disease was obtained in the tested samples; therefore, this quarantine pest is maintained as absent in Chile. However, 14 plants resulted positive for phytoplasmas enclosed in subgroups 16SrV-A (12 plants) and 16SrXIII-F (2 plants). Although they have been found in other plant species, this is the first report of these phytoplasmas in citrus worldwide.

Keywords: citrus diseases; phytoplasmas; Liberibacter; nested-PCR/RLFP; sequencing; molecular identification

1. Introduction

Different species of ‘Candidatus Liberibacter’ have been reportedly associated with “huanglongbing” (HLB), the most important disease in citrus worldwide [1,2]. This disease is spread through a large part of citrus-growing areas worldwide [3,4]. In South America, ‘Ca. L. asiaticus’ and ‘Ca. L. americanus’ are present in Brazil [5,6] and Paraguay [7], while only ‘Ca. L. asiaticus’ is reported in Argentina and Colombia [8,9]. Furthermore, Diaphorina citri, the main vector of both bacteria, has been found in all these countries, and also in Uruguay [10], Ecuador [11], and Venezuela [12]. In the American continent, the presence of ‘Ca. L. africanus’ and its vector Trioza erytreae were never reported. Until recently, HLB-associated bacteria and their insect vector(s) have not been detected in Chile; since 2011, monitoring plans have been developed to prevent the entry of the disease’s agents and their insect vectors [13]. Given Chile’s geographic proximity to many countries in which they are present, the risk of the disease spread into its territory is high. Therefore, the Chilean citrus industry is making a great effort in monitoring and verifying the absence of these pathogens and of their insect vectors.

It has been repeatedly observed that citrus plants infected by ‘Candidatus Phytoplasma’ show the typical symptoms observed in HLB infected plants, such as irregular yellowing of the leaves and weakening of the tree. In some cases, it was possible to verify co-infection between the two bacteria [14–16]. For this reason, both pathogens were surveyed to verify...
the local situation [17] with a meticulous inspection followed by specific molecular tests to verify the presence of these bacteria in citrus plants in Chile. The sampling was mainly directed to plants showing symptoms attributable to these bacteria in all the citrus growing areas of the country.

2. Results

All the PCR analyses for the detection of ‘Candidatus Liberibacter’ species in citrus samples were negative. However, 14 samples were positive for phytoplasma presence after amplification on 16S rRNA and Ssu12p genes [18,19]. The amplicons obtained were cloned, and five clones of each sample were sequenced. The sequences resulting from the five clones obtained from each sample were identical and were aligned and compared with the phytoplasma sequences available in GenBank (NCBI).

A 100% nucleotide identity in the 16S rRNA gene was present with samples enclosed in the ‘Ca. P. ulmi’ and ‘Ca. P. hispanicum’ strains. In the case of the Ssu12p gene, the nucleotide identity percentages observed were 99.52% and 100% in comparison with the sequences available in GenBank, respectively. Among the 14 phytoplasma-positive samples, 12 were infected by ‘Ca. P. ulmi’ (elm yellows group, 16Sr-V-A), and two were infected by ‘Ca. P. hispanicum’ strains (Mexican periwinkle virescence group, 16Sr-XIII-A). Maximum parsimony phylogenetic reconstruction using 16S rRNA and Ssu12p gene sequences, including the strains obtained in this study, confirm that the detected phytoplasmas cluster with phytoplasmas enclosed in ribosomal groups 16Sr-V and 16Sr-XIII. In particular, phytoplasma strains from the samples CTC 202, CTC 199, and CTC 192 are closely related to the strain EY1 (GenBank accession number AY197655) (Figure 1A). Strains CTC 170 and CTC 134 grouped with those of the ribosomal subgroup 16Sr-XIII-F, in particular, strain StrCL-1 (GenBank accession number MH939193), from a Chilean strawberry sample. The phylogenetic tree of the Ssu12p gene confirms these phylogenetic relationships (Figure 1B).

![Figure 1. Phylogenetic tree of (A) 16S rRNA gene region (1240 nt) and (B) Ssu12p region (724 nt) enclosing phytoplasmas detected in citrus from Chile, highlighted with filled diamonds, and selected ‘Ca. Phytoplasma’ strains. Information on the phytoplasma strains is reported in Table 1. The tree was constructed using the maximum parsimony algorithm. The numbers in the nodes represent starting values based on 500 pseudo-replications for stability estimation and clade support A. laidlawii is used as outgroup strain.](image-url)
Table 1. List of phytoplasma strains used for the phylogenetic analyses.

| 16Sr Group | Subgroup | Associated Disease ('Ca. Phytoplasma' Species) | Acronym | GenBank Accession Number |
|------------|----------|-----------------------------------------------|---------|-------------------------|
| 16SrI A    | A        | Aster yellows witches’ broom                  | AYWB    | CP000061                |
| B          | B        | Onion yellows mild strain                    | OY-M    | NC_005303               |
| C          | C        | Primrose virescence                          | PRIVA   | MT161512 AY265210       |
| D          | D        | Clover phylloidy                              | KVE     | MT161514 AY265217       |
| E          | E        | Aster yellows from apricot                   | A-AY    | MT161515 AY265211       |
| F          | F        | Peanut witches’ broom                         | PrWB    | AMWZ00000000            |
| 16SrII A   | A        | *Echinacea purpurea* witches’ broom           | E. purpurea | WB | LKAC00000000         |
| B          | B        | Fabric beans phylloidy                        | FBP     | MT161516 EF193354       |
| C          | C        | Tomato big bud                                | TBB     | MT161517 EF193359       |
| 16SrIII A  | A        | Peach x disease ('Ca. P. pruni')              | CX      | LHCF00000000            |
| B          | B        | Italian clover phylloidy                      | ItCPh   | AKIM00000000            |
| C          | C        | Goldenrod yellows                             | GR      | MT161522 FJ76627        |
| D          | D        | Poinsettia branch inducing                    | PoIB    | AKIK00000000            |
| E          | E        | Phytoplasma Vc33                              | Vc33    | LKKG00000000            |
| 16SrV A    | A        | Elm yellows ('Ca. P. ulmi')                   | EY      | MT161527 AY197655       |
| 16SrVI A   | A        | Clover proliferation                           | CP1     | MT161528 HQ589189       |
| 16SrVII A  | A        | Ash yellows ('Ca. P. fraxini')                | ASHY    | MT161529 HQ589190       |
| 16SrIX B   | B        | Almond witches’ broom ('Ca. P. phoenicium')   | SA213   | JPSQ00000000            |
| C          | C        | *Picris echioide yellow'                     | PEY     | MT161530 JQ868441       |
| 16SrX A    | A        | Apple proliferation ('Ca. P. mali')           | AT      | CU469464                |
| B          | B        | European stone fruit yellows ('Ca. P. prunorum') | ESFY | MT161533 AM933142       |
| C          | C        | Pear decline ('Ca. P. pyri')                  | PD      | MT161535 AJ542543       |
| 16SrXII A  | A        | "stolbur" ('Ca. P. solani')                  | STOL-SA | MPBG00000000           |
| B          | B        | Austral. grape. yellows ('Ca. P. australiense') | AUSGY | AM422018                |
| C          | C        | Strawberry lethal yellows                     | CPA     | CP002548                |
| 16SrXIII F | F        | *Fragaria × ananassa* phylloyd                | StrPh-CL | MT161538 MH939191     |
| K          | K        | *Fragaria × ananassa* phylloyd                | StrPh-CL | MT161539 MH939192     |
| 16SrV A    | A        | Citrus × sinensis Lane late yellows           | CTC192  | OL690419 OL677628       |
| 16SrXIII F | F        | Citrus × sinensis Fukumoto witches’ broom     | CTC170  | OL690418 OL677243       |

Furthermore, the sequence of sample CTC 192 (deposited in GenBank under the accession number OL677628) was subjected to RFLP in silico to complete the identification of the phytoplasmas at the ribosomal subgroup level using the enzymes RsaI and BfiI for phytoplasmas enclosed in the 16SrV group [20]. The virtual digestion with the RsaI enzyme showed that the profiles of the strains corresponding to groups 16SrV-A, 16SrV-C, 16SrV-D, and 16SrV-E were identical to the one of this strain. The digestion with the BfiI enzyme generated differentiable profiles with the strains of the mentioned subgroups, identical only to those of the ribosomal subgroup 16SrV-A (Figure 2). The enzymes used for in silico RFLP of the phytoplasma strain CTC170 (deposited in GenBank under the accession number OL672243) were KpnI and RsaI [21]. Both digestion profiles show that the Chilean strains are identical to the strains in the subgroup 16SrXIII-F (Figure 3).

The molecular characterization of the strains from 14 citrus samples indicates that 12 samples were positive for phytoplasmas enclosed in the subgroup 16SrV-A, and two samples were positive for phytoplasmas enclosed in subgroup 16SrXIII-F. The overall percentage of phytoplasma infection in the plants was 5.43%. The citrus samples positive for 16SrV-A phytoplasmas were collected in two orchards, both located in the Metropolitana Region (Table 2). The symptoms showed by these plants were leaf yellowing undistinguishable from those reported as associated with the presence of ‘Ca. Liberibacter’ (Figure 4). The samples positive for 16SrXIII-F phytoplasmas were collected in two orchards located in the O’Higgins Region (Table 2), showing (sample CTC134) symptoms of generalized yellowing of the tree, abortion of fruits, defoliation of shoots, and asynchronous flowering.
compared with non-infected trees. Sample CTC 134 showed threadlike leaves and witches' broom shoots (Figure 5).

**Figure 2.** In silico RFLP profiles of the phytoplasma strains in the 16SrV subgroups. (A) Restriction profiles generated by the *RsaI* enzyme. (B) Restriction profiles generated by the *Bfai* enzyme. CTC 192 is a citrus sample. MW: molecular marker PhiX174 digested with *HaeIII*. Fragment size (nt) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72. Phytoplasmas used for comparison are: 16SrV-A, elm yellows (EY) *P. ulmi* (GenBank accession number: AY197655); 16SrV-B, jujube witches' broom (JWB-G1) *Ca. P. ziziphi* (GenBank accession number: AB052876); 16SrV-C, "flavescence dorée" (FD-C) (GenBank accession number: X76560); 16SrV-D, "flavescence doree" (FD-D) (GenBank accession number: AJ548787); 16SrV-E, rubus stunt (RuS) *Ca. P. rubi* (GenBank accession number: AY197648); 16SrV-G, Korean jujube witches' broom (GenBank accession number: AB052879).

**Figure 3.** In silico RFLP profiles of phytoplasmas in the subgroups of 16SrXIII ribosomal group. (A) Restriction profiles generated by the *KpnI* enzyme. (B) Restriction profiles generated by the *RsaI* enzyme. CTC 192 citrus sample. MW: molecular marker PhiX174 digested with *HaeIII*. Fragment size (nt) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72. Phytoplasmas used for comparison: 16SrXIII-A, Mexican periwinkle virescence (MPV) *Ca. P. hispanicum* (GenBank accession number: AF248960); 16SrXIII-B, strawberry green petal (STRAWB2) (GenBank accession number: U96616); 16SrXIII-C, Chinaberry yellows (CBY1) (GenBank accession number: AF495882); 16SrXIII-D, Mexican potato purple top (SINPV) (GenBank accession number: FJ914647); 16SrXIII-E, papaya apical curl necrosis (PACN) (GenBank accession number: EU719111); 16SrXIII-F strawberry red leaf (GenBank accession number: KJ921641); 16SrXIII-G, Chinaberry yellowing (ChTY) *Ca. P. meliae* (GenBank accession number: KU850940); 16SrXIII-H broccoli stunt phytoplasma (GenBank accession number: JX626329); 16SrXIII-I Mexican periwinkle virescence phytoplasma (GenBank accession number: KT444664).
Table 2. Detail of citrus samples positive to phytoplasmas.

| Sample  | Species and Variety       | Region and Locality            | Phytoplasma Detected (Ribosomal Subgroup) |
|---------|---------------------------|--------------------------------|------------------------------------------|
| CTC 182 | Citrus reticulata Murcott | Metropolitan (Pomaire)         | 16SrV-A                                  |
| CTC 184 | Citrus reticulata Murcott | Metropolitan (Pomaire)         | 16SrV-A                                  |
| CTC 188 | Citrus reticulata Murcott | Metropolitan (Pomaire)         | 16SrV-A                                  |
| CTC 190 | Citrus × limon Fino 49    | Metropolitan (Pomaire)         | 16SrV-A                                  |
| CTC 192 | Citrus × sinensis Lane late | Metropolitan (Pomaire)        | 16SrV-A                                  |
| CTC 193 | Citrus × sinensis Lane late | Metropolitan (Pomaire)        | 16SrV-A                                  |
| CTC 199 | Citrus × limon Eureka     | Metropolitan (Mallarauco)      | 16SrV-A                                  |
| CTC 200 | Citrus reticulata Murcott | Metropolitan (Mallarauco)      | 16SrV-A                                  |
| CTC 202 | Citrus × sinensis Valencia | Metropolitan (Mallarauco)      | 16SrV-A                                  |
| CTC 203 | Citrus × limon Eureka     | Metropolitan (Mallarauco)      | 16SrV-A                                  |
| CTC 207 | Citrus reticulata Murcott | Metropolitan (Mallarauco)      | 16SrV-A                                  |
| CTC 212 | Citrus reticulata Murcott | Metropolitan (Mallarauco)      | 16SrV-A                                  |
| CTC 134 | Citrus reticulata Orri    | L. B. O’Higgins (Peumo)        | 16SrXIII-F                               |
| CTC 170 | Citrus × sinensis Fukumoto | L. B. O’Higgins (Pichidegua)   | 16SrXIII-F                               |

Figure 4. Symptoms of leaf yellowing in the citrus samples resulted positive for 16SrV-A phytoplasmas. (A) Orange CTC 192. (B) Lemon CTC 199. (C) Mandarin CTC 200. (D) Orange CTC 202.
The information provided by this study represents the first report of phytoplasmas in citrus plants in Chile. Furthermore, this is the first detection of the phytoplasmas 16SrV-A
and 16SrXIII-F in citrus plants in the world. Phytoplasmas of the ribosomal group 16SrII have been reported to infect citrus plants in Brazil, China, and Iran [22,30,31]. Furthermore, in Brazil, the phytoplasmas of the ribosomal groups 16SrIII and 16SrIX have been detected [17,28]. In Mexico and China, a ‘Ca. P. asteris’ strain (16SrI) was reported [14,15]. Specific detections of ribosomal subgroups not widely distributed worldwide include the 16SrIX group in lemon and orange trees in Puerto Rico [32] and a phytoplasma of the 16SrXIV-A subgroup in lemon trees in India [33]. The presence of 16SrV-A phytoplasma in Chile was previously identified in vineyards of the Metropolitana Region, associated with reddening and short internodes of the grapevine shoots [34]. Moreover, transmission tests with the leafhopper *Amplicephalus curtulus*, widespread in the central zone of Chile, showed the ability of this insect species to transmit the 16SrV-A phytoplasmas [35]. On the other hand, the phytoplasma 16SrXIII-F was detected for the first time in Chile in strawberry orchards of the Region of Valparaiso, associated with symptoms of phyllody and virescence [36]. Subsequent studies showed that this phytoplasma was present in all the strawberry production regions in Chile, including the localities where positive citrus samples have been detected in this survey [21,37]. In addition, this phytoplasma has been reported in calafate (*Berberis microphylla* G. Forst.), a shrub of the Berberidaceae family native to Chilean and Argentinian Patagonia [38,39]. So far, the vector(s) of this phytoplasma is not known. The 16SrXIII group is widely distributed in the other countries of South America, affecting various crops such as potato (*Solanum tuberosum* L.), broccoli (*Brassica oleracea* L.), and papaya (*Carica papaya* L.), among others [40–43].

Evidence shows that citrus phytoplasma infections are sporadic in isolated orchards, sometimes in remote areas surrounded by spontaneous vegetation. In times with higher-than-average rainfall during spring and autumn, the growth of spontaneous shrubs and weeds is favored, thereby increasing insect-feeding in these plants. Insect vectors can, therefore, sporadically visit crops and transmit phytoplasmas, suggesting that spontaneous vegetation may also be a reservoir for these phytoplasmas [29,44,45]. This situation has been evidenced in epidemiological studies associated with grapevine yellows in Chile [46–48].

The presence of viruses and viroids that infect citrus plants in Chile could lead to confusion among the symptoms. It is essential to consider all these factors in the climate change scenario. Studies carried out in Chile show that the climatic conditions that are projected for the future could influence the appearance of diseases of alien origin [49]. The discovery of phytoplasmas associated with symptoms like those of HLB reinforces the need to continue monitoring and promoting epidemiological studies on citrus. Preventing these diseases is everyone’s job, including governments, nurseries, technical advisers, producers, and researchers. Chile is surrounded by countries in which HLB and its insect vector *D. citri* are present. Fortunately, the country has climatic and geographical barriers that have contributed positively to the present local phytosanitary situation. Chile has great potential as a citrus-growing country and being an HLB-free territory is a commercial advantage that should not be lost.

4. Materials and Methods

From the main citrus-producing areas of Chile, 258 samples were collected in two areas during two periods. From December 2017 to April 2018, the regions of Tarapacá, Coquimbo, and Valparaiso were surveyed. The second sampling period was carried out between November 2018 to April 2019 in the Regions of Libertador Bernardo O’Higgins, Maule, and Metropolitana. The number of samples was established based on the number of hectares planted per region; one sample was collected for every 100 ha (Table 3). The samples were collected mainly from citrus trees that showed symptoms referable to HLB, like yellow shoots, leaves with asymmetric chlorotic spots, and thick and leathery texture [50]. In addition, trees with symptoms associated with phytoplasma presence, such as witches’ broom, flower abnormalities, and general decline, were sampled [15,27,29]. Samples that presented symptoms of water deficiency, nutritional deficiencies, nematode, and insect attacks, and phytotoxicity were not collected. In orchards where no symptoms attributable
to these pathogens were observed, asymptomatic plants were randomly sampled. Each sample corresponds to one tree, from which four 15 cm long shoots with at least 5 leaves were collected from different sides of the tree. The geographic coordinates and the origin of the collected samples were recorded. The samples were transported in thermoregulated containers and stored at 4 °C before nucleic acid extraction.

Table 3. Number of citrus plants collected in the main producing regions of Chile.

| Region                  | Number of Samples |
|-------------------------|-------------------|
| Tarapacá                | 10                |
| Coquimbo                | 60                |
| Valparaíso              | 55                |
| Metropolitana           | 62                |
| Libertador Bernardo O’Higgins | 49      |
| Maule                   | 22                |

DNA was extracted from 1 g of midribs using the CTAB method [48]. The nucleic acids were dissolved in Tris-EDTA buffer pH 8.0 and kept at 4 °C. All samples were analyzed by PCR and nested-PCR. For the detection of ‘Ca. Liberibacter’ species five amplification protocols were used: three universal 16S and 23S gene PCRs [51,52]; a PCR-duplex, for the simultaneous and specific detection of the three HLB-associated ‘Ca. Liberibacter’ species [5,53], and a universal nested PCR for the three species [54,55]. Phytoplasma detection was performed by nested-PCR of the 16S rRNA gene [18] and the gene coding the ribosomal Ssu12p gene [19]. The PCR products obtained for both genes were purified from the agarose gel using the EZNA® Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA). The DNA fragments were ligated into the cloning vector pGEM-T Easy, following the manufacturer’s instructions (Promega Inc., Fitchburg, WI, USA). Putative recombinant clones were analyzed by colony PCR and selected fragments sequenced in both directions at Psomagen Inc. (Rockville, MD, USA). The sequences were aligned with the GenBank database using the BLAST engine for local alignment (Blast version N 2.2.12) and compared with those of phytoplasmas published in the National Center for Biotechnology Information (NCBI) available on the internet (http://www.ncbi.nlm.nih.gov/blast/, accessed on 15 November 2021) [56]. The restriction fragment length polymorphism (RFLP) analysis was performed in silico with the appropriate restriction enzymes according to the ribosomal groups obtained. The in silico RFLPs were generated in the iPhyClassifier online tool (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphycalssifier.cgi, accessed on 15 November 2021) [57].

5. Conclusions

The results presented in this study indicate that Chile is free from the “huanglongbing” associated bacteria, maintaining its status of absence of this quarantine pathogen. In the citrus industry of Chile, government services and researchers maintain a constant interaction to prevent the entry of these pathogens and their insect vectors. In some plants that presented symptoms like those of HLB, phytoplasmas enclosed in 16SrV-A and 16SrXIII-F subgroups were detected. This is the first report of phytoplasmas in citrus in Chile; the identified phytoplasmas were not described in other citrus-growing regions in the world.

Author Contributions: Conceptualization, resources, review, and editing, N.Q., N.F. and A.B.; data curation, formal analysis, and original manuscript draft preparation, N.Q., C.G., G.M. and N.C.; bioinformatics analysis, N.Q. and A.Z.; support in the sampling activity and original manuscript draft preparation, F.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was carried out within the framework of the European Union’s Horizon 2020 research and innovation program under grant agreement no. 727459.

Institutional Review Board Statement: Not applicable for studies not involving humans or animals.
Informed Consent Statement: Not applicable for studies not involving humans.

Data Availability Statement: The GenBank Accession Numbers presented in this study are openly available in the National Center for Biotechnology Information (NCBI) at NCBI nucleotide (https://www.ncbi.nlm.nih.gov/nucleotide/, accessed on 15 November 2021).

Acknowledgments: The authors thank the Cooperativa Agrícola Vitivinícola Loncomilla, who contributed to the survey in the Maule Region, and the Chilean National Commission for Scientific and Technological Research (CONICYT) for PhD Scholarship No. 21171998.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Bové, J.-M. “Huanglongbing”: A destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol. 2006, 88, 7–37.
2. Cevallos-Cevallos, J.M.; Futch, D.B.; Shilts, T.; Polimova, S.Y.; Reyes-De-Corcuera, J.I. GC–MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus “huanglongbing”. Plant Physiol. Biochem. 2012, 53, 69–76. [CrossRef]
3. FAO para América Latina y el Caribe. Available online: https://www.fao.org/americas/prioridades/hlb/es/ (accessed on 16 November 2021).
4. Cardinali, M.C.D.B.; Boas, P.R.V.; Milori, D.M.B.P.; Ferreira, E.J.; e Silva, M.F.; Machado, M.A.; Bellete, B.S.; Silva, M.F.D.G.F.D. Infrared spectroscopy: A potential tool in “huanglongbing” and citrus variegated chlorosis diagnosis. Talanta 2012, 91, 1–6. [CrossRef]
5. Teixeira, D.D.C.; Danet, J.-L.; Eveillard, S.; Martins, E.C.; Junior, W.C.D.J.; Yamamoto, P.T.; Lopes, S.A.; Bassanezi, R.B.; Ayres, A.J.; Saillard, C.; et al. Citrus “huanglongbing” in São Paulo State, Brazil: PCR detection of the ‘Candidatus Liberibacter’ species associated with the disease. Mol. Cell. Probes 2005, 19, 173–179. [CrossRef]
6. Teixeira, D.C.; Ayres, J.; Kitaïma, E.W.; Danet, L.; Jagoueix-Eveillard, S.; Saillard, C.; Bové, J.-M. First report of a “huanglongbing”-like disease of citrus in São Paulo State, Brazil and association of a new liberibacter species, ‘Candidatus Liberibacter americanus’, with the disease. Plant Dis. 2005, 89, 107. [CrossRef]
7. EPPO Global Database. Available online: https://gd.eppo.int/taxon/LIBEAS/distribution/PY (accessed on 15 November 2021).
8. Badaracco, A.; Redes, F.J.; Preussler, C.A.; Agostini, J.P. Citrus “huanglongbing” in Argentina: Detection and phylogenetic studies of ‘Candidatus Liberibacter asiaticus’. Australas. Plant Pathol. 2017, 46, 171–175. [CrossRef]
9. Araque, W.; Arévalo, E. Potencial distribución espacial del vector del HLB de los cítricos Diaphorina citri (Hemiptera: Liviidae) en el departamento del Tolima, Colombia. Rev. Colomb. Cienc. Hort. 2018, 12, 545–560. [CrossRef]
10. INIA Uruguay. Available online: http://www.inia.info.uy/digital/bitstream/item/1427/1/111219240807151515.pdf (accessed on 13 November 2021).
11. Corneo, J.; Chica, E. First record of Diaphorina citri (Hemiptera: Psyllidae) in Ecuador infesting urban citrus and orange jasmine trees. J. Insect Sci. 2014, 14, 298. [CrossRef] [PubMed]
12. Fonseca, O.; Valera, N.; Vásquez, C. Registro de diapaño Diaphorina citri Kuwayama (Hemiptera: Psyllidae) en tres hospederos en el estado Lara, Venezuela. Entomotrop. Rev. Int. Estud. Entomol. 2007, 22, 145–152.
13. Comité de cítricos de Chile. Available online: https://comitedecitricos.cl/en/international-news/568-2014-10-07-18-09-05 (accessed on 15 November 2021).
14. Chen, J.; Pu, X.; Deng, X.; Liu, S.; Li, H.; Civerolo, E. A phytoplasma related to ‘Candidatus Phytoplasma asteris’ detected in citrus showing “huanglongbing” (yellow shoot disease) symptoms in Guangdong, P.R. China. Phytopathology 2009, 99, 236–242. [CrossRef]
15. 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. Occurrence of ‘Candidatus Phytoplasma asteris’ in citrus showing “huanglongbing” symptoms in Mexico. Crop. Prot. 2014, 62, 144–151. [CrossRef]
16. Ghosh, D.K.; Motghare, M.; Kokane, A.; Kokane, S.; Warghane, A.; Bhose, S.; Surwase, D.; Ladaniya, M.S. First report of a ‘Candidatus Phytoplasma cynodontis’-related strain (group 16SrXIV) associated with “huanglongbing” disease on Citrus grandis. Australas. Plant Dis. Notes 2019, 14, 9. [CrossRef]
17. Wulf, N.A.A.; Fassini, C.G.; Marques, V.V.; Martins, E.; Coletti, D.A.B.; Teixeira, D.D.C.; Sanches, M.M.; Bové, J.-M. Molecular characterization and detection of 16SrIII group phytoplasma associated with “huanglongbing” symptoms. Phytopathology 2019, 109, 366–374. [CrossRef]
18. Gundersen, D.E.; Lee, J.-M. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol. Mediterr. 1996, 34, 15–141.
19. Cui, W.; Zamorano, A.; Quiroga, N.; Bertaccini, A.; Fiore, N. Ribosomal protein coding genes SSU12p and LSU36p as molecular markers for phytoplasma detection and differentiation. Phytopathol. Mediterr. 2021, 60, 281–292. [CrossRef]
20. Sinclair, W.A.; Townsend, A.M.; Griffiths, H.M.; Whitlow, T.H. Responses of six Eurasian Ulmus cultivars to a North American elm yellows phytoplasma. Plant Dis. 2000, 84, 1266–1270. [CrossRef] [PubMed]
21. Cui, W.; Quiroga, N.; Curkovic, S.T.; Zamorano, A.; Fiore, N. Detection and identification of 16SrXIII-F and a novel 16SrXIII phytoplasma subgroups associated with strawberry yellows phytoplasma in Chile. Eur. J. Plant Pathol. 2019, 155, 1039–1046. [CrossRef]
22. Torres, F.; SAG, Servicio Agrícola y Ganadero, Santiago, Chile. Personal communication, 2020.

23. Lou, B.; Bai, X.; Bai, Y.; Deng, C.; Roy-Chowdury, M.; Chen, C.; Song, Y. Detection and molecular characterization of a 16Srrl-A* phytoplasma in grapefruit (Citrus paradisi) with “huanglongbing”-like symptoms in China. J. Phytopathol. 2013, 162, 387–395. [CrossRef]

24. Luis-Pantoja, M.; Paredes-Tomás, C.; Uneau, Y.; Myrie, W.; Morillon, R.; Satta, E.; Contaldo, N.; Pacini, F.; Bertaccini, A. Identification of Candidatus Phytoplasma species in “huanglongbing” infected citrus orchards in the Caribbean. Eur. J. Plant Pathol. 2021, 160, 185–198. [CrossRef]

25. Das, A.; Nerkar, S.; Thakre, N.; Kumar, A. First report of Candidatus Phytoplasma trifolii (16SrVI group) in Nagpur mandarin (Citrus reticulata) showing “huanglongbing” symptoms in central India. Neat Dis. Rep. 2016, 34, 15. [CrossRef]

26. Al-Ghaithi, A.G.; Al-Sadi, A.M.; Al-Hammadi, M.S.; Al-Shariqi, R.M.; Al-Yahyai, R.A.; Al-Mahmooli, I.H.; Carvalho, C.M.; Elliot, S.L.; Hogenhout, S.A. Expression of phytoplasma-induced witches’ broom disease symptoms in acid lime (Citrus aurantifolia) trees is affected by climatic conditions. Plant Pathol. 2017, 66, 1380–1388. [CrossRef]

27. Zreik, L.; Carle, P.; Bové, J.-M.; Garnier, M. Characterization of the mycoplasmalike organism associated with witches’-broom disease of lime and proposition of a Candidatus taxon for the organism, Candidatus Phytoplasma aurantifolia*. Int. J. Syst. Bacteriol. 1995, 45, 449–453. [CrossRef]

28. Al-Ghaithi, A.G.; Al-Sadi, A.M.; Al-Hammadi, M.S.; Al-Shariqi, R.M.; Al-Yahyai, R.A.; Al-Mahmooli, I.H.; Carvalho, C.M.; Elliot, S.L.; Hogenhout, S.A. Expression of phytoplasma-induced witches’ broom disease symptoms in acid lime (Citrus aurantifolia) India. Plant Dis. 2017, 101, 831. [CrossRef]

29. Davis, R.I.; Schneider, B.; Gibb, K.S. Detection and differentiation of phytoplasmas in Australia. Aust. J. Agric. Res. 1997, 48, 533–544. [CrossRef]

30. Silva, F.N.; Queiroz, R.B.; de Souza, A.N.; Al-Sadi, A.; Siqueira, D.L.; Elliot, S.L.; Carvalho, C.M. First report of a 16Sr lC phytoplasma associated with asymptomatic acid lime (Citrus aurantifolia) in Brazil. Plant Dis. 2014, 98, 1577. [CrossRef]

31. Faghhi, M.; Bagheri, A.; Seyahooei, M.A.; Pezhman, A.; Faraji, G. First report of a Candidatus Phytoplasma aurantifolia*-related strain associated with witches’ broom disease of limequat in Iran. New Dis. Rep. 2017, 35, 24. [CrossRef]

32. Cai, C.D.; Rivera-Vargas, L.I.; Segarra, A.E.; Davis, R.E. 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. Australas. Plant Dis. Notes 2015, 10, 1–8. [CrossRef]

33. Ghosh, D.K.; Bose, S.; Sharma, P.; Warghane, A.; Mokhare, M.; Ladaños, M.S.; Reddy, M.K.; Thorat, V.; Yadav, A. First report of an 16SrXIV group phytoplasma associated with citrus ‘huanglongbing’ symptoms in India. Plant Dis. 2017, 101, 831. [CrossRef]

34. Fiore, N.; Zamorano, A.; Pino, A.M.; Maria, F.N.Z.P.A. Identification of phytoplasmas belonging to the ribosomal groups 16SrIII and 16SrV in Chilean grapevines. Phytopathogenic Mollicutes 2015, 9, 32. [CrossRef]

35. Arismendi, N.L.; Riegel, R.; Carrillo, R. In vivo transmission of Candidatus Phytoplasma ulmi by Amplicephalus curtulus (Hemiptera: Cicadellidae) and its effect on ryegrass (Lolium multiflorum cv. tama). J. Econ. Entomol. 2014, 107, 83–91. [CrossRef]

36. Quiroga, N.; Cabrera, M.; Valdera, M.; Rodriguez, E.; Coronado, R.; Zamorano, A.; Fiore, N. Molecular characterization of a phytoplasma associated to phyllody and witches’ broom in strawberry (Fragaria x ananassa). In Proceedings of the XXIV Congreso de la Sociedad Chilena de Fitopatología, Viña del mar, Chile, 1–3 December 2015. 13p.

37. Cui, W.; Quiroga, N.; Bertaccini, A.; Zamorano, A.; Fiore, N. Use of 12p and 36p genes as molecular markers in support of subgroup identification of two 16SrrlXIII phytoplasmas associated with strawberry phyllody in Chile. Phytopathogenic Mollicutes 2019, 9, 89. [CrossRef]

38. Varas, B.; Castro, M.H.; Rodriguez, R.; Von Baer, D.; Mardones, C.; Hinrichsen, P. Identification and characterization of microsoutacles from calafate (Berberis microphylla, Berberidaeceae). J. Appl. Plant Sci. 2013, 3, 1200003. [CrossRef] [PubMed]

39. Madariaga, M.; Ramirez, I. Identification of a phytoplasma associated with witches’ broom symptoms in calafate (Berberis microphylla G. Forst.). Chil. J. Agric. Res. 2019, 79, 493–499. [CrossRef]

40. Santos-Cervantes, M.E.; Chávez-Medina, J.A.; Acosta-Pardini, J.; Flores-Zamora, G.L.; Méndez-Lozano, J.; Leyva-López, N.E. Genetic diversity and geographical distribution of phytoplasmas associated with potato purple top disease in Mexico. Plant Dis. 2010, 94, 388–395. [CrossRef] [PubMed]

41. Eckstein, B.; Barbosa, J.C.; Kreyci, P.F.; Canale, M.C.; Brunelli, K.R.; Bedendo, I.P. Broccoli stunt, a new disease in broccoli plants associated with three distinct phytoplasma groups in Brazil. J. Phytopathol. 2013, 161, 424–444. [CrossRef]

42. Melo, L.; Silva, E.; Flóres, D.; Ventura, J.; Costa, H.; Bedendo, I. A phytoplasma representative of a new subgroup, 16SrXIII-E, associated with papaya apical curl necrosis. Eur. J. Plant Pathol. 2013, 137, 445–450. [CrossRef]

43. Fernández, F.D.; Meneguzzi, N.G.; Guzmán, F.A.; Kirschbaum, D.S.; Conci, V.C.; Nome, C.F.; Conci, L.R. Detection and identification of a novel 16SrXII subgroup phytoplasma associated with strawberry red leaf disease in Argentina. Int. J. Syst. Evol. Microbiol. 2015, 65, 2741–2747. [CrossRef] [PubMed]

44. Broadbent, P.; Fraser, L.R.; McGechen, J. Australian citrus dieback. In Proceedings of the Seventh International Organization of Citrus Virologists Conference, Athens, Greece, 29 September–4 October 1975. [CrossRef]

45. Timer, L.W.; Garney, S.M.; Graham, J.H. PART I: Infectious (biotic) diseases. In Compendium of Citrus Diseases, 2nd ed.; The American Phytopathological Society: St. Paul, MN, USA, 2000; pp. 5–69. [CrossRef]
46. Quiroga, N.; Longone, V.; González, X.; Zamorano, A.; Pino, A.M.; Picciau, L.; Alma, A.; Paltrinieri, S.; Contaldo, N.; Bertaccini, A.; et al. Transmission of 16SrIII-J phytoplasmas by the leafhoppers Paratanus exitiousus and Bergallia valdiviana. *Phytopathol. Mediterr.* 2019, 58, 231–237. [CrossRef]

47. Quiroga, N.; Soto, D.; Farah, P.; Pino, A.M.; Zamorano, A.; Alma, A.; Picciau, L.; Fiore, N. New contribution about the epidemiology of grapevine yellows associated phytoplasmas in Chile. *Phytopathogenic Mollicutes* 2019, 9, 189. [CrossRef]

48. Quiroga, N.; Gamboa, C.; Soto, D.; Pino, A.M.; Zamorano, A.; Campodonico, J.; Alma, A.; Bertaccini, A.; Fiore, N. Update and new epidemiological aspects about grapevine yellows in Chile. *Pathogens* 2020, 9, 933. [CrossRef]

49. Quiroga, N.; Ivulic, D.; Lagos, J.; Sandoval-Rodriguez, A.; Infante, R.; Morales, L.; Fiore, N. Risk analysis of the establishment of *Scaphoideus titanus*, vector of “flavescence dorée” phytoplasma in grapevine, under current and estimated climate change conditions in Chile. *Phytopathogenic Mollicutes* 2017, 7, 39. [CrossRef]

50. Gottwald, T.R. Current epidemiological understanding of citrus “huanglongbing”. *Annu. Rev. Phytopathol.* 2010, 48, 119–139. [CrossRef]

51. Angelini, E.; Clair, D.; Borgo, M.; Bertaccini, A.; Boudon-Padieu, E. “Flavescence dorée” in France and Italy—Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. *Vitis* 2001, 40, 79–86. [CrossRef]

52. Fujikawa, T.; Miyata, S.-I.; Iwanami, T. Convenient detection of the citrus greening (“huanglongbing”) bacterium ‘*Candidatus Liberibacter asiaticus*’ by direct PCR from the midrib extract. *PLoS ONE* 2013, 8, e57011. [CrossRef] [PubMed]

53. Zafarullah, A.; Saleem, F. Detection and molecular characterization of ‘*Candidatus Liberibacter* spp.’ causing “huanglongbing” (HLB) in indigenous citrus cultivars in Pakistan. *Pok. J. Bot.* 2016, 48, 2071–2076.

54. Hocquellet, A.; Toorawa, P.; Bové, J.-M.; Garnier, M. Detection and identification of the two ‘*Candidatus Liberibacter*’ species associated with citrus “huanglongbing” by PCR amplification of ribosomal protein genes of the β operon. *Mol. Cell. Probes* 1999, 13, 373–379. [CrossRef]

55. Jagoueix, S.; Bové, J.-M.; Garnier, M. PCR detection of the two ‘*Candidatus Liberibacter*’ species associated with greening disease of citrus. *Mol. Cell. Probes* 1996, 10, 43–50. [CrossRef] [PubMed]

56. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 2007, 24, 1596–1599. [CrossRef] [PubMed]

57. Zhao, Y.; Wei, W.; Lee, I.-M.; Shao, J.; Suo, X.; Davis, R.E. Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *Int. J. Syst. Evol. Microbiol.* 2009, 59, 2582–2593. [CrossRef]