

## **Presence of *Candidatus Liberibacter solanacearum* in *Bactericera cockerelli* Sulc associated with diseases in tomato, chili and potato**

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### **Abstract**

The “permanent tomato”, “spotted tuber” or “zebra chip” in potato and “chlorotic buds” of chili, are three diseases described in Mexico with coincidental signs of flower abortion, obscuration of vascular tissue at the base of the stem and root of the plants. The association between these diseases and the emerging bacterium *Candidatus Liberibacter solanacearum* (CLs) as well as the psyllid *Bactericera cockerelli* as its vector has been mentioned. These diseases, which were initially located in three states of Mexico, have spread to the main Solanaceae producing regions, both in field conditions and in the greenhouse. The objective of the study was to know the presence of CLs associated with diseases that affect the tomato, para and chili crops in Mexico. The bacterium was identified by PCR of the 16S rDNA gene, cloning and sequencing. The alignment of nucleotide sequences was performed with the Clustal W method and the phylogenetic tree was built with the Neighbor-Joining algorithm from distances calculated with the Tajima-Nei method and a Felsenstein index of 1 000 replicas, using the software MEGA version 5.05. A total of 167 samples were analyzed, of which 86 were positive, from 14 states of Mexico. Five nucleotide sequences from Guanajuato, San Luis Potosí and Sinaloa corresponding to tomato, potato and chili, adults and eggs of *B. cockerelli* were obtained. The analysis of the sequences showed an identity of 99.4% when compared between these and up to 99.8% with GenBank accessions described for CLs in the USA, New Zealand and Canada.

**Keywords:** chlorotic buds, tomato permanent, zebra chip.

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## Introduction

In Mexico, there are three diseases that have caused major changes in the crop program in the country's geography. The "permanent tomato disease" (PT) was first described in the Bajío region of Guanajuato causing damage to tomato production (*Solanum lycopersicum* L.) estimated at 45%, later, in the southern states of Coahuila and Nuevo León mentioned a new disease in the potato crop (*Solanum tuberosum* L.) named "Zebra chip" (ZC) causing 90% damage (Munyanzeza *et al.*, 2009b) and in chili a new disease described as "chlorotic buds" (BC) (Munyanzeza *et al.*, 2009c).

The symptoms of PT are characterized by a curling of the lower leaves in the form of "taco", these leaves are brittle, the apical leaflets are chlorotic with purple margins, the flowers are aborted; nevertheless, it is possible to present an over budding of buds that form thin stems that bloom and reach the production of small fruits. In general, the plants are squashed with yellow leaves and the vascular tissue in the root has a dark brown color (Garzón-Tiznado *et al.*, 2009). In the case of potatoes, the symptoms of the ZC are characterized by purple to chlorotic buds, bulky axillary stems and radial obscuration of the tuber, which loses frying quality and its cooking is nonuniform, these potatoes have a slightly sweet taste, with which they lose their commercial quality.

Finally, in bell-type chili the symptoms of BC are shortening of internodes and small plants (Munyanzeza *et al.*, 2009c). Results of initial investigations suggested that these diseases were caused by the same pathogen, since when grafting tomato plants diseased with PT in healthy potato plants they showed "purple tip" symptoms. Later, it was mentioned that the symptoms in tomato (Garzón-Tiznado *et al.*, 2009) and potatoes were reproduced by the psyllid *B. cockerelli*.

Initially, PT and ZC diseases were associated with phytoplasmas; however, when analyzing the DNA sequence of the 16S-23S region amplified with the pairs of direct primers P1/Tint and P1/P7 and nested R16mF2/r16r2 described for phytoplasmas (Gundersen and Lee, 1996), they presented an identity of 80 to 83% when compared to sequences described for phytoplasmas. In contrast, these sequences revealed a greater association with a non-culturable bacterium transmitted by *B. cockerelli* (Garzon-Tiznado *et al.*, 2009) that was later described with 100% identity with the bacterium *Candidatus Liberibacter solanacearum* in tomato (Munyanzeza *et al.*, 2009a) and in chili cultivation (Munyanzeza *et al.*, 2009c) in the Valley of the Cruz of Elota, Sinaloa.

However, the PT was detected in the early 80's and later the spotting of the potato tuber (Almeyda *et al.*, 2008), characteristic signs of these diseases have been observed in tomato, chili and potato plantations, in the Mexico's main producing regions, so it was considered important to obtain information on the presence of these diseases in Mexico and their relationship with the bacterium *Candidatus Liberibacter solanacearum*.

## Materials and methods

Inoculum of *Candidatus Liberibacter solanacearum*. Under conditions of commercial greenhouse and open field, foliar tissue of tomato plants that had symptoms of “PT”, as well as of potato with symptoms of “ZC” and of chili with “BC” was collected, eggs were also collected, nymphs and *B. cockerelli* adults who colonized tomato plants with PT. The plant samples were placed in plastic bags and stored at -20 °C. The adults of the psyllid were collected with entomological sniffer and the eggs and nymphs were removed from the leaflets with “camel hair” brush and placed in 1.5 mL tubes in 75% ethanol, washed with sterile distilled water and stored at -20 °C.

Extraction of DNA. The extraction of nucleic acids from plants and insects, eggs and nymphs of *B. cockerelli* was carried out with the methodology described by Doyle and Doyle (1990) modified. The 500 mg of foliar tissue was macerated in mortar with 2 mL of extraction solution (100 mM Tris-HCl pH7, 2% CTAB, 20 mM EDTA, 1.4 M NaCl and 0.2%  $\beta$ -mercaptoethanol). In the DNA extractions of the *B. cockerelli* stages, 100 mg of sample was macerated with 200  $\mu$ L of extraction solution. The extracts were incubated at 65 °C for 30 min. After ammonium acetate was added to a final concentration of 0.5 M and incubated on ice for 10 min, a volume of phenol: chloroform: isoamyl alcohol solution (25: 24: 1 v/v/v) was added and stirred in vortex, centrifuged at 13.4 x g for 10 min, the supernatant was recovered and 0.6 volumes of isopropanol were added, the DNA was allowed to precipitate for 1 h at -20 °C. Subsequently, it was centrifuged at 13.4 x g for 10 min. The supernatant was removed and the precipitate was washed in 500  $\mu$ L of 70% ethanol, allowed to dry and suspended in 50  $\mu$ L (plant samples) and 20  $\mu$ L (*B. cockerelli* stage samples) of sterile distilled water. The extracted DNA was stored at 4 °C.

Detection of CLs by PCR. For the detection of CLs, the pair of OA2/OI2c primers was used, which amplify a 1 168 bp fragment of the 16S rDNA gene (Munyanza *et al.*, 2009), in a 25  $\mu$ L reaction volume containing 3 mM of MgCl<sub>2</sub>, 0.2 mM of dNTPs, 0.25  $\mu$ M of each oligonucleotide, 1 U of *Taq* DNA polymerase (Invitrogen) and 50 ng of DNA. The reaction was carried out in a C1000<sup>TM</sup> Thermal Cycler BIO-RAD thermal cycler, with pre-denaturation of 2 min at 94 °C followed by 35 cycles at three temperatures: denaturation at 94 °C for 30 s, alignment at 60 °C for 30 s and extension at 72 °C for 1 min; with a final period of 10 min at 72 °C. The amplicons were analyzed on 1.2% agarose gels stained with gel network (BIOTIUM) and observed in UV light.

DNA cloning and sequencing. The PCR-amplified products were purified from agarose gels using commercial mini-columns (PureLink<sup>TM</sup>) and cloned into the pCR 4-TOPO vector following the supplier's instructions (Invitrogen). Plasmid DNA extraction was performed with the method described by Birnboim and Doly (1979). The transformed bacteria were confirmed by restriction bioassays with the *Eco*RI enzyme following the supplier's instructions (Invitrogen). The sequencing was performed in LANGEBIO, CINVESTAV-IPN- Irapuato in a 3730 XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). The samples were prepared using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems). The DNA identity was obtained by comparing the sequences obtained in this study with those reported in the NCBI database (National Center for Biotechnology Information) using the BLAST algorithm (Basic Local Alignment Search Tool), (Altschul *et al.*, 1990).

Phylogenetic analysis. The alignments of the nucleotide sequences of CLs were made with the Clustal W method and the phylogenetic tree was constructed with the Neighbor-Joining method (Saitou and Nei, 1987) from distances calculated with the Tajima-Nei model (Tajima and Nei, 1984) and a bootstrap of 1 000 replicas (Felsenstein, 1985). Everything analyzes were performed with the MEGA program version 5.05 (Tamura *et al.*, 2011).

## Results and discussion

Detection of CLs by PCR. In 167 samples collected in 14 georeferenced states of the Mexican Republic, 86 samples were amplified with a predicted size for CLs of 1 168 bp. In tomato samples the detection of CLs was 62%, in potatoes 40.3%, 33% in the chili culture, as well as 100% in adults, eggs and nymphs of *B. cockerelli* collected in diseased tomato plants (Table 1). In all three cultures, the common symptom was characterized by flower abortion and brownish darkening at the base of the stem and root of the plant coinciding with the previously described symptoms (Garzón-Tiznado *et al.*, 2009; Munyaneza *et al.*, 2009c).

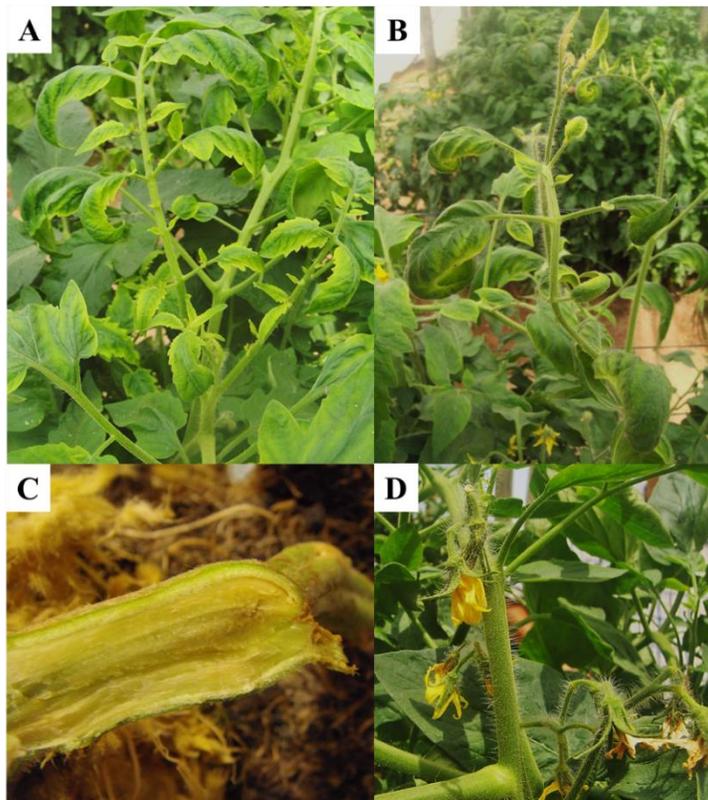
**Table 1. Location of samples of tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.) and chili (*Capsicum annuum* L.) analyzed by PCR, for the detection of *Candidatus Liberibacter solanacearum* in Mexico.**

State	Coordinates	CLs positives				
		<i>S. lycopersicum</i>	<i>S. tuberosum</i>	<i>C. annuum</i>	<i>B. cockerelli</i> *	<i>B. cockerelli</i> **
Aguascalientes	22°05'13.00" N 102°42'25.30" O	3/5	1/5	1/1	1/1	-
Baja California	30°25'54.81" N 115°52'55.81" O	1/10	-	-	-	-
Chihuahua	28°19'1.85" N 106°56'69.00" O	-	1/4	-	-	-
Coahuila	25°19'20.76" N 100°39'56.44" O	-	7/10	-	-	-
Estado de México	19°05'42.46" N 99°34'23.15" O	-	4/8	-	-	-
Guanajuato	20°39'48.17" N 100°45'43.62" O	8/8	-	1/5	4/4	1/1
Guanajuato	21°3'11.66" N 101°42'1.28" O	-	4/8	-	-	-
Jalisco	19°52'35.81" N 103°44'32.83" O	-	1/5	-	-	-
Jalisco	19°45'12.16" N 103°45'36.08" O	5/5	-	-	-	-
Michoacán	19°48'44.88" N 102°22'25.09" O	-	1/5	-	-	-
Michoacán	20°19'23.52" N 102°18'19.23" O	1/2	-	-	-	-
Nayarit	21°45'59.54" N 105°13'58.27" O	1/5	-	-	-	-
Nayarit	21°42'21.61" N 105°16'41.75" O	-	-	0/2	-	-
Nuevo León	24°34'24.14" N 100°16'34.27" O	-	4/7	-	-	-

State	Coordinates	CLs positives				
		<i>S. lycopersicum</i>	<i>S. tuberosum</i>	<i>C. annuum</i>	<i>B. cockerelli</i> *	<i>B. cockerelli</i> **
San Luis Potosí	22°39'56.30" N 100°53'38.50" O	5/5	-	1/1	-	-
Sinaloa	23°57'11.73" N 106°52'18.99" O	6/6	-	5/5	3/3	3/3
Sinaloa	23°54'57.68" N 106°53'3.91" O	-	3/5	-	-	-
Sonora	27°21'40.59" N 110°14'30.93" O	-	2/2	-	-	-
Sonora	26°51'51.40" N 109°32'0.10" O	1/1	-	-	-	-
Zacatecas	23°1'28.37" N 100°39'56.44" O	5/11	1/13	0/10	1/1	-
Total		36/58 (62%)	29/72 (40.27%)	8/24 (33.33%)	9/9 (100%)	4/4 (100%)

\* = adults; \*\* = eggs.

In tomato cultivation, the highest frequency of the pathogen was detected; the symptoms of brittle leaves, chlorotic apical leaflets with purple margins, in addition to the darkening of the roots and abortion of flowers, allowed during the development of the crop the selection of plants where the presence of the pathogen was confirmed (Figure 1).



**Figure 1. Symptoms associated with CLs in tomato plants with PT. A) chlorotic apical leaflets and epinastia; B) on sprouting of buds and chlorotic leaflets with purple margins; C) brown color in vascular tissue of the root; D) flower abortion.**

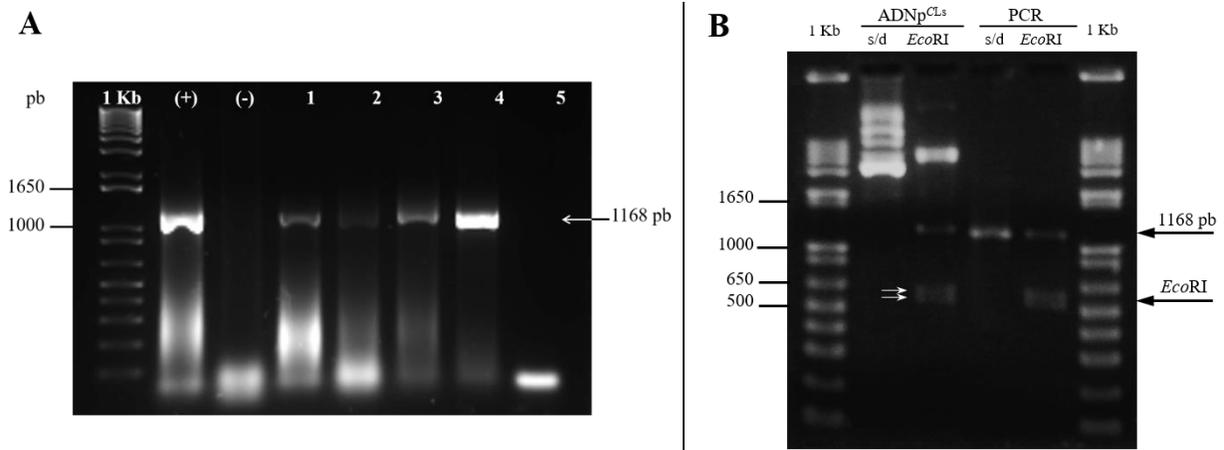
In potato cultivation with a lower frequency, the purple tip syndrome has been described as complex in nature and can also be induced by pathogens other than CLs (Garzón-Tiznado *et al.*, 2008). However, the presence of the pathogen is also associated with the symptom of stem necrosis and zebra chip, which occurs from the intermediate stage of crop development, which made it difficult to detect it in early samples. In the culture of the chili in samplings realized in the states of Nayarit and Zacatecas, the presence of CLs was not detected, in coincidence with the absence of symptoms of chlorotic buds in the plants sampled at random in genotypes of type 'wide' or 'serrano'; however, in the 'bell' or 'habanero' types of samples collected in Guanajuato, Sinaloa and Aguascalientes, the previously described symptoms were observed and the results for the detection of CLs were positive.

The above, suggests that the symptoms can be a first-hand tool for the support of technicians in the field, although the confirmation and certainty of the identity of the bacteria can only be done in the laboratory. In addition, CLs were identified in samples of adults, eggs and nymphs of *B. cockerelli* that hosted tomato plants with symptoms of PT and that were positive for CLs (Figure 2A). The presence of CLs was detected in potato and tomato producing states in Mexico (Table 1). In Sinaloa, Sonora, Nayarit and Jalisco with prevalence of tropical climate and plantings in winter, the damage caused by this pathogen has not yet been as critical as in the other states, where the temperate climate and sowing in summer predominates.

The tomato permanent disease is a disease described in the early 80s in the state of Guanajuato, from there has been observed its spread to other regions of the country. Currently, the morphological characteristics of this disease have been registered in the main potato producing regions in Mexico and those where it is combined simultaneously with the tomato crop. Similar morphological characteristics have also been described in countries such as the United States of America (French-Monar *et al.*, 2010) and New Zealand (Liefing *et al.*, 2009).

DNA cloning and sequencing. Amplicons obtained from DNA extracted from adults and eggs of *B. cockerelli*, as well as from tomato, potato and chili cloned in the vector pCR 4-TOPO, were confirmed by partial restriction with the enzyme *EcoRI*, which allowed the release of two fragments of DNA that added corresponds approximately to the size of the amplicon, which indicated the presence of an intermediate *EcoRI* site (Figure 2B, data shown for psyllids of *B. cockerelli*), which was confirmed by sequencing. The sequences obtained in this study were registered in GenBank with the numbers KF776420 and KF776421 for CLs of adults and eggs of *B. cockerelli* respectively, KF776422 for CLs in tomato with symptoms of PT, KF776423 for CLs in potatoes with symptoms of ZC and KF776424 and for CLs in chili with symptoms of BC.

Analysis of the 16S gene sequences of CLs. Both in the sequences reported in this study, and those recorded in the GenBank used in the analyzes they showed an *EcoRI* site from position 616 to 621 which was observed in the restriction pattern of the recombinant plasmid with *EcoRI* (Figure 2B). To locate the position of the mutations and the *EcoRI* sites, the sequence of the complete 16S rRNA gene of 1507 bp of the *Candidatus Liberibacter solanacearum* genome (NC\_014774) was used as reference.



**Figure 2.** Amplicons of the 16S gene of CLs and restriction of the recombinant plasmid with *EcoRI*. A) detection of CLs by PCR. In lanes 1 and 2 positive and negative controls respectively. Lanes 3, 4 and 5 correspond to eggs, nymphs and adults of *B. cockerelli*. Lane 6, tomato plant with PT and lane 7, negative control of the PCR reaction. B), restriction pattern of plasmid  $p^{CLs}$ . From left to right, plasmid DNA from *B. cockerelli* clone and plasmid digested with *EcoRI*. Then, amplicon, amplicon digested with *EcoRI* and marker of molecular weight of 1 Kb. Electrophoresis in 1.5% agarose gel.

The nucleotide sequences of tomato tissue amplicons with PT, potato with ZC, chili with BC and, eggs and adults of *B. cockerelli* collected in the tissue of diseased tomato, potato and chili plants described in this study presented an identity of 99.4% when comparing each other; that is, a variation of 0.6% was presented. The comparison between sequences previously described for CLs from New Zealand, Texas and Kansas from USA and Sinaloa, Saltillo and Chihuahua from Mexico presented 99.8% identity, 98.5% with *Ca. Liberibacter africanus*, 98.4% with *Ca. Liberibacter asiaticus*, 97.7 with *Ca. Liberibacter americanus*, 97.2% with *Ca. Liberibacter europaeus*, data similar to those previously described by Liefting *et al.* (2009) and 93.1% with *Ca. Phytoplasma*, which showed an even greater difference (Table 2). With the above, the tendency of a greater distance of the PT, ZC, BC with *Ca. Phytoplasma* is evident.

Although in the beginning PT was associated with phytoplasmas, the results of the molecular analysis confirmed a greater association with CLs similar to that described by Munyaneza *et al.* (2009). The adult sequence of *Bactericera cockerelli* showed greater nucleotide variation, among them three mutations were identified: a deletion at position 475 (-/A), a transversion at position 559 (C/G) and a transition at position 822 (A/G). These variations suggest the presence of haplotypes of the bacteria, as has been reported in studies with *Bactericera cockerelli*, potato and tomato samples from the United States of America, New Zealand and Mexico (Warrick *et al.*, 2011).

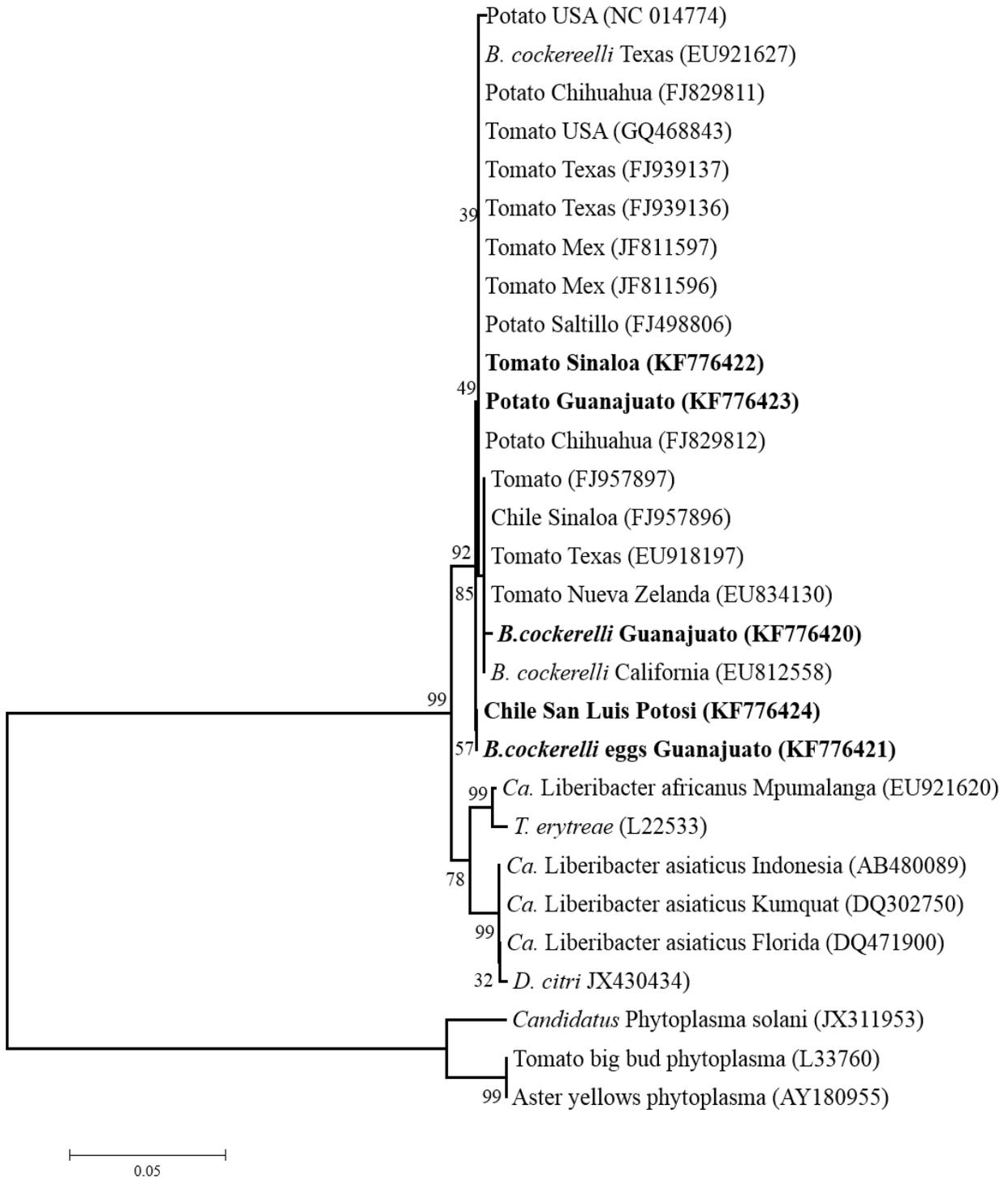
To date, five haplotypes of CLs have been reported, haplotypes A and B associated with diseases transmitted by *Bactericera cockerelli* in Solanaceous species, and haplotypes C, D and E, associated with diseases in apiaceas (carrots and celery), transmitted by *Trioza apicalis* and *Bactericera trogonica*, as well as the low probability that *Bactericera cockerelli* transmit CLs to carrots under field conditions (Munyaneza *et al.*, 2016).

**Table 2. Comparison of percentage identity between sequences of tomato (*S. lycopersicon* L.), potato (*S. tuberosum* L.), chili (*Capsicum annuum* L.) and *Bactericera cockerelli* Sulc, reported in this study with sequences described in GenBank of species of *Ca. Liberibacter* and *Ca. Phytoplasma***

GenBank	Host	% Identity	Pathogen	Host	GenBank (Others studies)
KF776422	<i>S. lycopersicum</i>	99.8	<i>Ca. Liberibacter</i>	<i>S. lycopersicum</i>	FJ829812, FJ829811,
KF776423	<i>S. tuberosum</i>		<i>solanacearum</i>	<i>S. tuberosum</i>	FJ498806, NC_014774,
KF776424	<i>C. annuum</i>			<i>C. annuum</i>	GQ468843, JF811596,
KF776421	<i>B. cockerelli</i> (Huevecillos)				JF811597, FJ939136, FJ939137, FJ957897,
KF776420	<i>B. cockerelli</i>				EU834130, EU918197, FJ957896
		98.5	<i>Ca. Liberibacter</i> <i>africanus</i>	<i>Trioza erytrae</i> <i>Citrus</i> sp	L22533 EU921620
		98.4	<i>Ca. Liberibacter</i> <i>asiaticus</i>	<i>Diaphorina citri</i> <i>Citrus</i> sp	GQ502291, JX430434 AB480089, DQ302750, DQ71900
		97.7	<i>Ca. Liberibacter</i> <i>americanus</i>	<i>Diaphorina citri</i>	AY742824
		97.2	<i>Ca. Liberibacter</i> <i>europaeus</i>	<i>Cacopsylla pyri</i>	FN678792, JX244258
		93.1	<i>Ca. Phytoplasma</i>	<i>S. lycopersicum</i>	JX311953, L33760, AY180955

The high identity between the sequences corresponding to eggs and adults of *B. cockerelli*, of diseased tissue of tomato, potato and chili in this study, allows us to confirm *B. cockerelli* as a transmitter of CLs, previously described as “non-culturable bacteria” (Garzón-Tiznado *et al.*, 2009), associated with diseases PT, ZC and BC, which demonstrates the epidemiological importance of the relationship between these organisms, and CLs placing this pathogen as responsible for large economic losses recorded in crops such as potatoes and tomato in Mexico (Garzón-Tiznado *et al.*, 1992; Garzón-Tiznado *et al.*, 2009; Munyaneza *et al.*, 2009). In addition, the identification of CLs in eggs of *B. cockerelli* supports the hypothesis of vertical or transovarial transmission of this pathogen.

Phylogenetic analysis. In Figure 3, the phylogeny corresponding to the sequences of tomato, potato, chili, adults and eggs of *B. cockerelli* is observed, the sequences analyzed in this study are part of the sequence group of *Ca. Liberibacter*, species *solanacearum* with 99% of reproducibility based on the Felsenstein index of 1000 replicas and separated from other species of *Ca. Liberibacter* and *Ca. Phytoplasma*.



**Figure 3. Phylogram based on the sequence comparison of the 16S gene of *Candidatus Liberibacter* and *Ca. Phytoplasma* species.** The phylogenetic tree was constructed with the Neighbor-Joining algorithm from distances calculated using the Tajima-Nei method, analyzing a total of 992 positions. In bold, the sequences of CLs obtained in this study are identified.

## Conclusions

The presence of *Candidatus Liberibacter solanacearum* was detected in the 14 sampled states of the Mexican Republic in samples of DNA extracted from tomato, chili and potato tissue, eggs, nymphs and adults of *Bactericera cockerelli*.

The presence of the bacteria was detected more frequently in tomato plants (62%), followed by potatoes (40.27%) and in a lower percentage in chili plants (33.33%).

Phylogenetic analyzes indicate the relationship between diseases of “tomato permanent” (PT), “zebra chip” (ZC) in potato, and “chlorotic outbreaks” (BC) in Chili with the non-culturable bacterium *Candidatus Liberibacter solanacearum*.

The presence of CLs in samples of eggs of *B. cockerelli* suggests that the transmission of the bacteria is transovarial.

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