Detection and identification of a Candidatus Liberibacter species from ash tree infesting psyllids

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Abstract

Candidatus Liberibacter species cause severe, economically important diseases. All known species of these pathogens are putatively insect-transmitted, specifically by psyllids. Detection of Liberibacter in plants is complicated by its uneven distribution in host plants and largely unculturable nature. The death of black (Fraxinus nigra) and mancana (Fraxinus mandshurica) ash trees in Saskatchewan, Canada has been associated with infestation with the cottony ash psyllid (Psyllopsis discrepans). We hypothesized that the symptoms and death could be due to a psyllid-transmitted Liberibacter. We used a combination of conventional PCR and Sanger sequencing of the 16S rDNA for detection of Liberibacter, and the genes CO1 and Cyt-b to determine species of psyllids. The 16S sequencing generated two sequences, NTHA 5 (GenBank accession number MK942379) and NTHA 6 (GenBank accession number MK937570) that were 1058 and 1085 bp long. A BLAST search for homology showed 99-100% sequence similarity to a Candidatus Liberibacter solanacearum sequence (GenBank accession number: KX197200) isolated from the Nearctic psyllid (Bactericera maculipennis) of US provenance. CO1 and Cyt-b gene sequencing of our psyllids yielded sequence information confirming that they were P. discrepans from comparisons with sequences in GenBank and BOLD. Confirmatory sequence comparison with a reference sample from the United Kingdom was concurrent. These results provide the first evidence on the likely cause of ash dieback in Saskatchewan. Further, they suggest a relatively rare example of a Liberibacter adapting a new host plant.

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Candidatus Liberibacter species cause severe, economically important diseases. All known species of these pathogens are putatively insect-transmitted, specifically by psyllids. Detection of Liberibacter in plants is complicated by its uneven distribution in host plants and largely unculturable nature. The death of black (*Fraxinus nigra*) and mancana (*Fraxinus mandshurica*) ash trees in Saskatchewan, Canada has been associated with infestation with the cottony ash psyllid (*Psyllopsis discrepans*). We hypothesized that the symptoms and death could be due to a psyllid-transmitted Liberibacter. We used a combination of conventional PCR and Sanger sequencing of the 16S rDNA for detection of Liberibacter, and the genes *CO1* and *Cyt-b*to determine species of psyllids. The 16S sequencing generated two sequences, NTHA 5 (GenBank accession number MK942379) and NTHA 6 (GenBank accession number MK937570) that were 1058 and 1085 bp long. A BLAST search for homology showed 99-100% sequence similarity to a *Candidatus* Liberibacter solanacearum sequence (GenBank accession number: KX197200) isolated from the Nearctic psyllid (*Bactericera maculipennis*) of US provenance.*CO1* and *Cyt-b* gene sequencing of our psyllids yielded sequence information confirming that they were *P. discrepans* from comparisons with sequences in GenBank and BOLD. Confirmatory sequence comparison with a reference sample from the United Kingdom was concurrent. These results provide the first evidence on the likely cause of ash dieback in Saskatchewan. Further, they suggest a relatively rare example of a Liberibacter adapting a new host plant.

Keywords : Psyllid, Ash disease, *Candidatus* Liberibacter species, DNA Barcoding, ecosystem jumping

Introduction

The *Candidatus* (*Ca* .) Liberibacter species are a group of mostly fastidious alpha proteo-bacteria. These species are associated with a series of devastating crop diseases including Zebra Chip (ZC) disease in potato (Crosslin *et al.* , 2011; Munyaneza, 2012), Vein Greening Disease in solanaceous crops (Crosslin *et al.* , 2010; Prager & Trumble, 2018), infections in carrots (Bertolini *et al.* , 2015) and celery (Teresani *et al.* , 2014) and most importantly Huanglongbing (HLB) a.k.a. 'citrus greening' in various citrus species (Batool *et al.* , 2007; da Graca, 1991). Since it was first detected in Florida in 2005, HLB has resulted in losses worth hundreds of millions of dollars to the citrus industry (Spreen *et al.* , 2014). Zebra Chip disease was first detected in Mexico in 1994 (Munyaneza *et al.* , 2007), where it has also resulted in millions of dollars of losses (Greenway, 2014). Zebra chip disease has now been found in almost all of the western United States, parts of Canada, and New Zealand (Crosslin *et al.* , 2010; Kalischuk & Johnson, 2019; Teulon *et al.* , 2009). In Israel and parts of Europe, *Ca.*Liberibacter solanacearum is associated with Carrot Yellows Disease (Alfaro-Fernández *et al.* , 2012; Haapalainen *et al.* , 2017; Holeva *et al.* , 2017; Mawassi *et al.* , 2018; Munyaneza, 2012; Munyaneza *et al.* , 2015; Tahzima *et al.* , 2014). Finally, *Ca.* Liberibacter-associated diseases have been found in crops in the Umbelliferae family in Spain, Greece, and on the African continent in Morocco (Alfaro-Fernández *et al.* , 2012, 2017; Holeva*et al.* , 2017; Munyaneza, 2010; Nelson *et al.* , 2013; Tahzima *et al.* , 2014; Teresani *et al.* , 2014).

There are at least five known species of *Ca.* Liberibacter, all of which have been identified through molecular techniques (Garnier*et al.* , 2000; Jagoueix *et al.* , 1996; Planet *et al.* , 1995). Three species, '*Ca.* Liberibacter asiaticus' (CLas), '*Ca* . L. americanus' (CLam), and '*Ca* . L. africanus (CLaf)' are associated with HLB (Munyaneza, 2010). Two species are associated with asymptomatic plants, '*Ca.* L. europaeus' (Leu) (Raddadi*et al.* , 2011) and '*Ca.* L. crescens' (Lcr) (Leonard*et al.* , 2012). Finally, *Candidatus* Liberibacter solanacearum (CLso): AKA pyllaurous is associated with ZC and various other diseases of vegetables (Munyaneza, 2012). There are at least seven haplotypes of CLso designated A-F (Grimm & Garczynski, 2019; Mauck*et al.* , 2019). All known *Ca.* Liberibacter species are transmitted by one or more species of psyllid (Haapalainen, 2014; Munyaneza, 2010).

Psyllids are small phytophagous hemipteran, phloem feeding insects in the suborder Sternorrhyncha and superfamily Psylloidea (Hodkinson, 1988). It is currently unclear how specific the relationships between a given Liberibacter species and psyllid vector are, but some species can have more than one psyllid vector (Haapalainen, 2014). In addition to being the primary and usually exclusive vectors of *Ca.*Liberibacter, psyllids are also common vectors of phytoplasmas, another group of mycoplasma-like bacterial plant pathogens (Ethmayer *et al.* , 2011; Jarausch *et al.* , 2019; Kaya *et al.* , 2016).

The cottony ash psyllid, *Psyllopsis discrepans* (Flor 1861) (Hemiptera: Psyllidae), is a specialist on several species of*Fraxinus* and is endemic to Europe. Its range, however, has included multiple reported introductions into North America (Hodkinson, 1988), including Nova Scotia, and multiple US States dating back to 1900s (USDA SEL Beltsville, MD, USA). Feeding by species of*Psyllopsis* is often associated with pseudogalls (Hodkinson, 1974) but not tree death. In 2000, this insect was detected in the cities of Edmonton and Calgary, Alberta followed by the death of many of the city-owned black ash trees in 2005 that was attributed to a combination of drought and psyllid damage. They were next found in Saskatoon, Saskatchewan and later found in North and South Dakota and Montana. In all instances, the insects were found infesting *Fraxinus nigra*(Black Ash), *Fraxinus manshurica* (Mancana Ash) and their hybrids. As with other *Psyllopsis*- infested trees, these species often demonstrate pseudogalls but also exhibit a decreasing leaf canopy (**Figure 1**) and eventually die. While the precise cause of this plant dieback and death is currently unknown, it has been attributed to an "unknown toxin" in the saliva. This explanation has previously been applied to other psyllid-tree syndromes which have since been at least partially linked to a *Ca.* Liberibacter species (Mauck *et al.* , 2019; Prager & Trumble, 2018). Here we report a new association between a plant host and an insect-vecored pathogen where the pathogen range is likely determined by the range of the vector.

Materials and Methods

Collection of Psyllids

Insects were trapped using standard or Asian Citrus Psyllid specific yellow colour sticky cards (Alpha Scents Inc., West Linn, OR) and by direct collection from trees via hand aspirators (Bioquip products, Rancho Dominguez, CA). Psyllid traps were deployed on Black (*Fraxinus nigra*), Mancana (*F. mandshurica*) and Hybrid ash trees owned and managed by the City of Saskatoon, the University of Saskatchewan Campus and Agriculture and Agri-Food Canada's Saskatoon Research and Development Centre. Traps were collected approximately every two weeks and stored at 4^{o}C. As soon as possible, the psyllids were recovered from traps and transferred to 5 ml glass vials containing 70% ethanol in the laboratory.

Molecular detection, sequence comparisons and phylogeny of the Liberibacter sequences

Molecular detection of Liberibacter was accomplished using 'general' Liberibacter primers (OA2 5'-GCGCTTATTTTTAATAGGAGCGGCA-3' and OI2c 5'- GCCTCGCGACTTCGCAACCCA-3') that amplified a 1160 bp region of the 16s ribosome gene as previously described (Jagoueix *et al.* , 1996; Liefting *et al.* , 2009). Amplicons generated were subjected to automated Sanger sequencing. Sequences generated were checked for homology in the GenBank via BLAST. Multiple sequence alignments were done using MUSCLE in Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7) (Kumar *et al.* , 2016) and phylogeny inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage differences between the nucleotide sequences of the isolates was obtained by a multiple sequence alignment with the MUSCLE algorithm in the Sequence Demarcation Tool software version 1.2 (Muhire *et al.* , 2014).

Psyllid species identification by molecular markers

DNA extraction was done using a DNeasy Blood and Tissue Kit (Qiagen GmbH, Mainz, Germany) and checked for quality using a Nanodrop® spectrophotometer (Thermo Scientific, USA). PCR was done using the extracted DNA as a template, 2X Biomix Red® (Bioline, UK) premix and universal primers for *Cytochrome oxidase subunit 1* (*CO1*) LCO1490/ HCO2198 (Folmer *et al.* , 1994). Confirmatory identification by PCR was conducted in the laboratory of Dr. Diane Percy using DNA from our insect samples and a reference *P. discrepans*sample from her lab collection (a voucher specimen collected in Essex, UK). Primers used targeting *CO1* were mtd6_F – 5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3' and mtd9_R – 5'-CCC GGT AAA ATT AAA ATA TAA ACT TC-3' or H7005P_R primer 5'-TGA GCT ACT ACR TAR TAT GTR TCA TG-3'. Those targeting *Cyt-b* were CytB_F – 5'-TGA GGN CAA ATA TCH TTY TGA-3' and CytB_R – 5' GCA AAT ARR AAR TAT CAT TCDG- 3'. All amplicons generated were subjected to automated Sanger sequencing using appropriate primers. The sequences were checked for homology in GenBank and Bar Code of Life databases (BOLD).

Results

16s gene sequences showed closest similarity to *Candidatus* Liberibacter solanacearum (psyllaurous) sequences

DNA from 20 psyllid samples from the trees were subjected to 16S gene sequencing, and of these, 11 showed the presence of *Ca.*Liberibacter (**Table 1**). Two samples, named NTHA 5 and NTHA 6, generated two high quality sequences which were that were 1058 and 1085 bp long, respectively **(Table 1).** These were deposited in GenBank (NTHA 5; GenBank accession number MK942379 and NTHA 6; GenBank accession number MK937570). A BLAST search for homology for these two sequences showed 99-100% sequence similarity to a *Ca.*Liberibacter solanacearum sequence (GenBank accession number: KX197200) isolated from a Nearctic psyllid (*Bactericera maculipennis*) from the US.

Phylogenetic reconstruction of evolutionary differences was conducted using sequences from GenBank representing different Liberibacter subspecies from different geographical locations and the NTHA 5 and NTHA

6 sequences (**Supplementary Table 1**). Though the other samples showed the presence of *Ca.* Liberibacter, the sequences generated were not of sufficient quality for use in phylogenetic analyses. Due to limited template, we were not able to repeat the PCR or sequencing. Phylogenetic analyses revealed four distinct clades based on subtypes and our samples clustered in the same clade with CLSo (**Figure 2**).

Pairwise comparisons of nucleotide sequences revealed sequence variation among the subspecies represented. The two samples from our study (NTHA5 and NTHA6) shared 99.9% nucleotide sequence similarity. The two sequences had closest similarity (approximately 94.4%), with *Ca.*Lib solanacearum sequences and were most dissimilar from those of*Ca.* Lib. americanus (approximately 54%) (**Figure 3, Supplementary Table 2**).

COI sequencing of psyllids suggests that psyllids on Ash trees are the cottony ash psyllid

Species identification by *COI* sequencing of psyllid samples collected from the same trees was done. Of these, 11 samples were successfully amplified and sequenced. A BOLD database search for similar sequences found ten samples (n=10) to be of the order Hemiptera and family Liviidae, with sequence similarities ranging from 98%-100%. Also, BLAST searches in the GenBank nucleotide database found all samples to be of the species *P. discrepans* , with homology ranges of 99.83%-100% (**Table 2**)

One of these samples, NTHA 5, which had tested positive for *Ca.*Liberibacter and for which there was sufficient DNA template, was sent*COI* and *Cyt-b* gene sequencing for independent confirmation of species identity against a reference species. The pairwise comparison revealed 100% sequence similarity between our sample and the reference*P. discrepans* sample. BLAST searches of *CO1* showed over 99.5% similarity to *P. discrepans* isolates of Swedish and Canadian origins (GenBank id: MT021807.1 and MF958492.1 respectively). The *Cyt-b* sequences showed 87.21% similarity to *P. repens*(GenBank ID: MG989141.1) and 86.42% to *P. flaxini* (GenBank id: MG989139.1). This discrepancy may be due to the absence of *Cyt-b*sequences for *P. discrepans* in GenBank. The percentage differences are indicative of clear species demarcation.

Discussion

Presented here is the first report of *Ca.* Liberibacter extracted from cottony ash psyllid (*Psyllopsis discrepans*) collected from ash trees. Following these results, a broader reaching survey of psyllids for *Ca.* Liberibacter has been initiated, and traps have now been deployed to collect psyllids in Saskatchewan and other provinces in Canada where ash death has been identified. A new design of 3D-printed traps is now in use to better preserve the trapped insects (Horton *et al.,* 2019). It was observed that the psyllids trapped on sticky cards degrade leading to low DNA recovery during extractions. This is also a known phenomenon with HLB and psyllid samples (Dr Sean Prager: Personal Communication).

From the data generated in this study, out best sequences grouped phylogenetically with CLso and had 99-100% sequence similarity in BLAST. Further, our sequences most closely matched a sequence of CLso extracted from the psyllid *Bactericera maculipennis* collected in Washington State USA. Previous detections of CLso have been from either vegetable crops or other wild solanaceous plants. In contrast, multiple other species of Liberibacter are associated with perennial trees in the*Rutaceae* (Halbert & Keremane, 2004) and one species, *L. crescens* , is associated with Babaco mountain papaya (*Carica stipulata* × *C. pubescens*) (Leonard *et al.* , 2012). The sequences presented here were extracted from psyllids, not plants, but as the insects were collected from ash trees, it is suggestive that this may be a new host for CLso.

The results presented here indicate strong genetic homology between psyllid specimens collected from infested trees in Saskatchewan and cottony ash psyllids. These genetic results are further supported by the samples sent to Dr. Diana Percy that were sequenced and compared to a known sample collected in Kent UK; the specimens were an exact genetic match based on *COI* and *Cyt-b* sequences. From the early 2000s, cities in western Canada and the United States began to suffer the loss of ash trees which were infested with a species of psyllid assumed to be the cottony ash psyllid. Collectively, this would indicate that those psyllids infesting ash trees in Western Canada are *P. discrepans.* This species is not endemic to North America despite having

been known in North America for over 100 years (Hodkinson, 1988). It is therefore likely that the infestation in western Canada and the United States is the result of introduction from either an established population in eastern North America or Europe.

The results presented here of *P. discrepans* collected from dying ash trees have important implications for both the biology of psyllid-Liberibacter pathosystems and in the context of ash loss in western Canada and the United States. These findings indicate that the loss of trees may be the result of infection with CLso rather than the effect of psyllid feeding alone. It is known that *P. discrepans*feeding causes leaf symptoms in ash, including rolled leaflets, reddening of leaf borders, and leaf swelling. *Psyllopsis discrepans* infestation also results in pseudogalls on infested trees (Burckhardt, 1994). Notably, while similar symptoms are observed in infested ash trees in western Canada, these trees also exhibit progressive defoliation over two to three seasons, eventually resulting in complete defoliation within four years (unpublished data). This progressing defoliation can be compared to HLB disease, which is also a disease in trees and often results in defoliation, dieback, and eventual death (Hu *et al.* , 2011). Notably, though it has been determined that citrus plants can become infectious with HLB 10-15 days post-inoculation by psyllids, the plant can remain asymptomatic for years while infected and not much is known about where the bacteria localizes *in planta* (Ann *et al.* , 2015). While the fastidious nature of all known *Ca.* Liberibacter species (other than *Ca.* Liberibacter crescens) complicates detection in plants; this can possibly be overcome through a combination of grafting experiments and PCR.

It is however highly likely that the extreme symptoms observed in psyllid infested ash trees in western Canada are also a result of*Ca.* Liberibacter infection transmitted by psyllids. In addition to describing a potential cause of ash loss, the findings presented here have direct implications for disease ecology and epidemiology. Transmission of Liberibacters across plant species by a single psyllid species is possible, though this is dependent on the transmission efficiency of the psyllid on and the ability of the bacteria to establish infection in the new host plant (Chaves de *et al.* , 2020). We hypothesize that an introduced population of *P. discrepans* may have acquired CLSo while sharing a common host plant with a population of *Bactericera maculipennis.* This shared host plant likely would have been field bindweed (*Convolvulus arvensis* , Solanales: Convolvulaceae) which is known to be co-inhabited by multiple psyllid species including *B. maculipennis* from which CLSo has been extracted previously (Borges *et al.* , 2017).*Bactericera maculipennis* has a known geographical range that includes the US states of Washington, Idaho, Oregon, and Montana (Horton*et al.* , 2017). Montana and Idaho both border Alberta, where these psyllids and the ash disease were both initially detected, while infected *B. maculipennis* was collected in Washington (Borges*et al.* , 2017). If this hypothesis is correct, this will be a somewhat rare example of a mycoplasma-like pathogen adopting a new vector and consequently a new host plant in what is a rather dramatic host shift. Furthermore, it also suggests that *Ca.* Liberibacter species may not be particularly host specific and that the actual restriction to their host range is the biology of their psyllid vectors. Collectivey, this is simiar to the "ecosystem jumping" pehenomenon descrived by da Graca et al. (2016); although, extraction and sequencing form infected plants will be required to further support this. Unfortunately, this is difficult process in trees as evidenced by multiple examples in the citrus and HLB pathosystem (Merfa *et al.* , 2019; Valdés *et al.* , 2016).

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Data Accessibility

Sequences for samples NTHA 5 (GenBank accession number MK942379) and NTHA 6 (GenBank accession number MK937570) are publicly available in GenBank.

Author contributions

SMP designed the research; FOW, NZ, RB and TW performed research. TW contributed reagents and data. FOW and SP analysed the Data. FOW and SP wrote the paper. All authors approved of the final submitted manuscript.

Table 1: Detection of *Ca.*Liberibacter-like sequences in psyllids

Sample	Host Plant	Sequencing Primer	Sequence length	Closest BLAST using FWD primer sequence	BLAST identity (%)	GenBank Number of closest identity
LB 3	Mancana ash	AO2	420	Ca. Lib. solanacearum	99.5	MG701017.1
NTHA 1	Northern Treasure Hybrid	AO2	245	Ca. Lib. solanacearum	99.11	KX185608.1
NTHA 2	Northern Treasure Hybrid	AO2	290	Ca. Lib. solanacearum	99.19	KX185608.1
NTHA 4	Northern Treasure Hybrid	AO2	478	Ca. Lib. solanacearum	97.03	KX185608.1
NTHA 5	Northern Treasure Hybrid	AO2	1058	Ca. Lib. solanacearum	100	KX197200
NTHA 6	Northern Treasure Hybrid	AO2	1085	Ca. Lib. solanacearum	99.91	KX197200
Y1	Mancana	AO2	1096	Ca. Liberibacter	98.18%	FJ914619.1
Y2	Mancana	AO2	450	Ca. Liberibacter	100%	FJ395219.1
Y4	Mancana	AO2	854	Ca. Liberibacter	99.76%	FJ914619.1
Y6	Mancana	AO2	373	Ca. Lib. solanacearum	99.72%	MN396643.1
Y10	Mancana	AO2	618	Ca. Liberibacter	99.19%	FJ914619.1

Table 2: BOLD and BLAST searches for *COI* sequences from psyllids

Sample ID		BOLD	Probability	BLAST	Probability
DowRd 1-1	Mancana ash	Hemiptera liviidae	100%	*Psyllopsis discrepans*	100%

Sample ID		BOLD	Probability	BLAST	Probability
DowRd 1-2	Mancana ash	Hemiptera liviidae	98%	*Psyllopsis discrepans*	100%
DowRd 1-3	Mancana ash	Hemiptera liviidae	98.55%	*Psyllopsis discrepans*	99.83%
DowRd 2-1	Mancana ash	Hemiptera liviidae	98.77%	*Psyllopsis discrepans*	99.85%
DowRd 2-2	Mancana ash	Hemiptera liviidae	100%	*Psyllopsis discrepans*	100%
DowRd 2-3	Mancana ash	Hemiptera liviidae	99.83%	*Psyllopsis discrepans*	99.85%
DowRd 3-1	Mancana ash	Hemiptera liviidae	100%	*Psyllopsis discrepans*	100%
DowRd 3-2	Mancana ash	Hemiptera liviidae	98.77%	*Psyllopsis discrepans*	100%
DowRd 3-3	Mancana ash	Hemiptera liviidae	98.77%	*Psyllopsis discrepans*	100%
NTHA 5	Northern Treasure Hybrid	Hemiptera liviidae	99.78	*Psyllopsis discrepans*	99.7%
UK Ref.	unknown	Hemiptera liviidae	99.78	*Psyllopsis discrepans*	99.5%

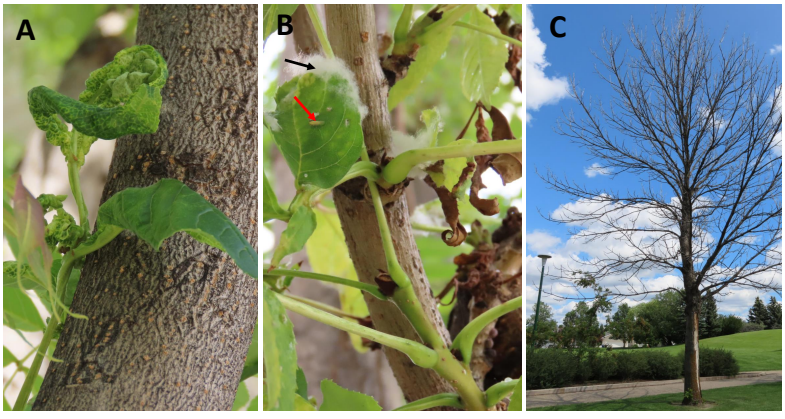
FIGURE CAPTIONS

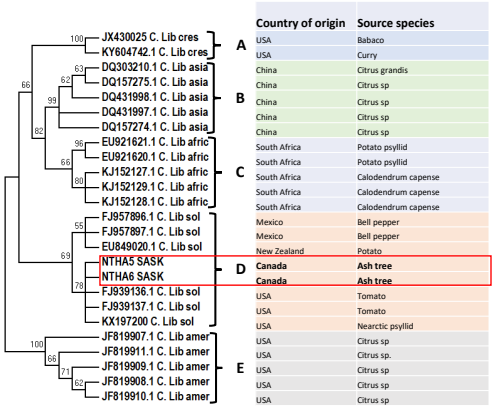
Figure 1: Typical disease symptoms and psyllids on ash tree.

Panel 'A': Leaf deformation caused by the disease on ash trees. Panel 'B': Psyllids were present on the leaves (red arrow), and there was the characteristic 'cotton' like depositions on the leaves (black arrow). Panel 'C': Completely defoliated ash tree associated with psyllid infestation.

Figure 2: Evolutionary relationships of different Liberibacter subspecies. Sequences used in this analysis were generated from the 16s ribosomal gene. Phylogenetically, they clustered in five clades (A-E) according to subtype. Sequences from our study (highlighted in the red box) clustered with *Ca.* Liberibacter solanacearum sequences in clade 'D'. The evolutionary history was inferred using the Neighbor-Joining method with bootstrapping (1000 replicates). Evolutionary distances were computed using the Kimura 2-parameter conducted in MEGA7.

Figure 3: Percentage pairwise sequence comparison of Liberibacter subspecies. Sequences from our study (red box) showed closest similarity to those of*Candidatus* Liberibacter solanacearum (grey box). They were most dissimilar to those of *Candidatus* Liberibacter americanus (light green colour). The computed percentages are shown in the additional information (Supplementary Table 2)





	Country of origin	Source species
A	USA	Babaco
	USA	Curry
B	China	Citrus grandis
	China	Citrus sp
	China	Citrus sp
	China	Citrus sp
	China	Citrus sp
C	South Africa	Potato psyllid
	South Africa	Potato psyllid
	South Africa	Calodendrum capense
	South Africa	Calodendrum capense
	South Africa	Calodendrum capense
	Mexico	Bell pepper
	Mexico	Bell pepper
	New Zealand	Potato
D	**Canada**	**Ash tree**
	Canada	**Ash tree**
	USA	Tomato
	USA	Tomato
	USA	Nearctic psyllid
E	USA	Citrus sp
	USA	Citrus sp.
	USA	Citrus sp
	USA	Citrus sp
	USA	Citrus sp