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**Permalink**

https://escholarship.org/uc/item/6d98p9qd

**Journal**

Phytopathology, 114(7)

**ISSN**

0031-949X

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**Publication Date**

2024-07-01

**DOI**

10.1094/phyto-01-24-0025-r

Peer reviewed

# Hiding in Plain Sight: A Widespread Native Perennial Harbors Diverse Haplotypes of '*Candidatus* Liberibacter solanacearum' and Its Potato Psyllid Vector

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Accepted for publication 10 April 2024.

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## Abstract

The unculturable bacterium '*Candidatus* Liberibacter solanacearum' (CLso) is responsible for a growing number of emerging crop diseases. However, we know little about the diversity and ecology of CLso and its psyllid vectors outside of agricultural systems, which limits our ability to manage crop disease and understand the impacts this pathogen may have on wild plants in natural ecosystems. In North America, CLso is transmitted to crops by the native potato psyllid (*Bactericera cockerelli*). However, the geographic and host plant range of the potato psyllid and CLso beyond the borders of agriculture are not well understood. A recent study of historic herbarium specimens revealed that a unique haplotype of CLso was present infecting populations of the native perennial *Solanum umbelliferum* in California decades before CLso was first detected in crops. We hypothesized that this haplotype and other potentially novel CLso variants are still present in *S. umbelliferum* populations. To test this, we surveyed populations of

*S. umbelliferum* in Southern California for CLso and potato psyllid vectors. We found multiple haplotypes of CLso and the potato psyllid associated with these populations, with none of these genetic variants having been previously reported in California crops. These results suggest that CLso and its psyllid vectors are much more widespread and diverse in North American natural plant communities than suggested by data collected solely from crops and weeds in agricultural fields. Further characterization of these apparently asymptomatic haplotypes will facilitate comparison with disease-causing variants and provide insights into the continued emergence and spread of CLso.

*Keywords*: disease control and pest management, diseases in natural plant populations, pathogen detection

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'*Candidatus* Liberibacter solanacearum' (CLso) is a species of unculturable alphaproteobacteria that is responsible for multiple emerging crop diseases around the world. CLso was first identified in 2008 from diseased potato and tomato plant tissue in the United States and New Zealand (Hansen et al. 2008; Liefting et al. 2008). This research, and subsequent studies, established that CLso is the causal agent of a condition called "zebra chip disease" in potato, which is characterized by foliar discoloration, necrosis, and dieback, as well as darkened medullary rays of tubers that lead to a "zebra stripe" appearance after tubers are fried (Crosslin et al. 2010). One of these studies also determined that CLso reproduces within the tissues of its psyllid vectors and may be transmitted vertically between psyllid generations (Hansen et al. 2008). Since the discovery of CLso, diverse haplotypes have been identified, associated with a growing list of host plant families and species of insect hosts and vectors (psyllids) in the superfamily Psylloidea (Hemiptera) (Fig. 1). However, we know very little about the diversity, distribution, and abundance of CLso and its psyllid vectors outside of agricultural systems. This limits efforts to forecast and manage the increasingly rapid emergence of novel CLso variants, and their vec-

tors, in crop settings. We also have little to no information on how CLso infections may affect the physiology and ecology of native plants in the natural habitats we are striving to preserve.

The discovery of CLso as a causal agent of disease in solanaceous crops sparked an uptick in psyllid and CLso surveillance in agricultural settings (Mauck et al. 2024; Wenninger and Rashed 2024). This surveillance revealed a total of three genetic variants or haplotypes of CLso infecting solanaceous crops in North America, CLso A, CLso B, and CLso F (Nelson et al. 2011; Swisher Grimm and Garczynski 2019), as well as four additional haplotypes associated with disease in carrots and other cultivated members of the *Apiaceae* throughout Europe and Northern Africa, CLso C, CLso D, CLso E, and CLso H (Alfaro-Fernández et al. 2012a, b, 2017a, b; Haapalainen et al. 2020) (Fig. 1; Munyaneza et al. 2010, 2012a, b, 2015; Nelson et al. 2013; Tahzima et al. 2014; Teresani et al. 2014). In several studies, common weeds and non-pest psyllid species sampled in and along the margins of CLso-affected agricultural fields were included in CLso surveys or controlled CLso transmission assays, confirming that CLso can also infect a wide variety of wild plant and psyllid species in both Europe and North America (Cooper et al. 2019; Haapalainen et al. 2018, 2020; Lethmayer and Gottsberger 2020; Murphy et al. 2014; Sumner-Kalkun et al. 2020; Swisher Grimm and Garczynski 2019; Wen et al. 2009). Furthermore, in many cases, the CLso haplotypes infecting these wild plant and psyllid species were unique from those found in the adjacent crops, such as CLso haplotype U found only in stinging nettle and nettle-associated psyllids (Haapalainen et al. 2018; Sumner-Kalkun et al. 2020) and CLso Cras1-2 and Aph1-3 found only in non-pest psyllid species collected within or along the margins of fields of carrot and potato, respectively (Grimm et al. 2022; Sumner-Kalkun et al. 2020). The discovery of so many distinct genetic variants of CLso in this limited sampling of wild plants and psyllids found

in agricultural areas suggests that CLso is likely also widespread and diverse in natural plant communities, with yet undocumented effects on the ecology of native plant and psyllid hosts.

In North America, CLso is transmitted among solanaceous crop plants by the potato psyllid (*Bactericera cockerelli*). Although the potato psyllid is considered native across central and western North America, published records of the temporal and spatial dynamics of wild host plant use beyond the borders of agriculture are incomplete (Horton et al. 2015; Mauck et al. 2019); significant progress in addressing this knowledge gap has recently been made in the major potato-growing regions of Texas and the Pacific Northwest (Cooper et al. 2023; Delgado-Luna et al. 2024; Murphy et al. 2014; Reyes Corral et al. 2021; Thinakaran et al. 2017; Wen et al. 2009; Wenninger et al. 2019), but for the rest of North America, data remain sparse. In contrast, the role of potato psyllids as crop pests has been studied intensively for at least a century, with dense populations on potato or tomato having long been associated with a pathology known as "psyllid yellows" (Compere 1916; Crawford 1914; Pletsch 1947; Wallis 1955). With the exception of the namesake darkened medullary rays of tubers affected by zebra chip disease, symptoms of psyllid yellows and zebra chip disease largely overlap (Eyer 1937; Richards 1928; Sengoda et al. 2010). This led to the hypothesis that CLso may have been involved in historical outbreaks of psyllid yellows (Hansen et al. 2008; Mauck et al. 2019). Unfortunately, these historical outbreaks predated PCR and sequencing-based diagnostics, and there are no preserved plant specimens from affected crop fields available to directly test this hypothesis.

To circumvent the lack of historic specimens from crops, Mauck et al. (2019) screened for CLso infections in herbarium specimens of native perennial *Solanum* species known to serve as hosts for potato psyllids. These specimens were collected from dates and locations aligning with historical outbreaks of psyllid yellows in California. Several specimens of one species, *Solanum umbelliferum* (blue-witch nightshade), collected as far back as 1970 and as recently as 2016, tested positive for CLso infection (Mauck et al. 2019). All specimens that tested positive for CLso were collected in western Riverside County, California. However, phylogenetic analyses revealed that these specimens were not infected with CLso genetic variants known to cause disease in crops (haplotypes A and B) as predicted. Instead, they were infected with a novel genetic variant of CLso, which the authors named haplotype G (Mauck et al. 2019). Furthermore, herbarium specimens testing positive for CLso G did not show symptoms of CLso-induced disease conditions typically found in crops (Mauck et al. 2019).

Based on evidence from herbarium specimens that *S. umbelliferum* hosts have harbored apparently asymptomatic CLso infections since at least 1970, we hypothesized that variants of CLso are still present in contemporary populations of this host. We also hypothesized that the potato psyllid vector feeds and reproduces on perennial *S. umbelliferum* when it is in leaf and bloom (fall through spring). To test these hypotheses, we performed extensive surveys to determine the prevalence of CLso infections in populations of *S. umbelliferum* growing in natural plant communities in southwestern California, the region from which CLso G was originally detected in herbarium specimens. We then characterized the genetic diversity of recovered CLso variants using multilocus sequence typing (MLST) (Haapalainen et al. 2018) and quantified year-to-year retention of infections in tagged CLso-positive plants. To explore associations with vectors, we used active and passive collections to quantify the temporal dynamics of potato psyllid visitation to *S. umbelliferum* hosts over the growing season (fall through spring). Using the specimens recovered from these efforts, we then studied relationships between potato psyllid genotype, psyllid CLso infection status, and CLso haplotype. Our study provides the first systematic survey of contemporary populations of a native perennial potato psyllid host plant, *S. umbelliferum*, for both CLso and potato psyllid vectors over multiple years and seasons.

## Materials and Methods

### Sampling locations

We collected plant and potato psyllid samples from five populations of *S. umbelliferum* (bluewitch nightshade) growing in natural reserves throughout western Riverside County (Fig. 2A to C; Table 1). This geographic area was targeted for sampling because it



**Fig. 1.** General geographic distribution, pathology, and associated plant and psyllid host species of each known 'Candidatus Liberibacter solanacearum' (CLso) haplotype. D = CLso has been detected in this species; V = confirmed CLso vector species; ZCD = zebra chip disease; PY = psyllid yellows; and CYD-like = symptoms resembling carrot yellows disease.

| Region | Lso Haplotype | Pathology | Host Plant Families | Aphalara curta | Aphalara loca | Aphalara persicaria | Craspedolepta nebulosa | Craspedolepta subpunctata | Bactericera cockerelli | Bactericera dorsalis | Bactericera maculipennis | Bactericera nigricornis | Bactericera tremblayi | Bactericera trigonica | Heterotrioza chenopodii | Trichochermes walkeri | Trioza apicalis | Trioza anthrisci | Trioza urticae | Psyllopsis discrepans | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| North America | A | ZCD/PY | Solanaceae | | | | | | V | | | | | | | | | | | | Hansen et al. 2008; Liefting et al. 2008 |
| North America | B | ZCD/PY | Solanaceae Convolvulaceae | | | | | | V | V | D | | | | | | | | | D | Borges et al. 2017; Cooper et al. 2023; Nelson et al. 2011; Wamonje et al. 2022 |
| North America | F | ZCD/PY | Solanaceae | | | | | | | | | | | | | | | | | | Swisher-Grimm and Garczynski 2019 |
| North America | G | *unknown* | Solanaceae | | | | | | | | | | | | | | | | | | Mauck et al. 2019 |
| North America | Aph1 | *unknown* | *unknown* | D | D | | | | | | | | | | | | | | | | Swisher-Grimm et al. 2022 |
| North America | Aph2 | *unknown* | *unknown* | D | | D | | | | | | D | | | | | | | | | Swisher-Grimm et al. 2022 |
| North America | Aph3 | *unknown* | *unknown* | D | | | | | | | | | | | | | | | | | Swisher-Grimm et al. 2022 |
| Europe | C | CYD-like symptoms | Apiaceae Solanaceae | | | | | | | | | | | | | | V | D | | | Haapalainen et al. 2018; Sumner-Kalkun et al. 2020 |
| Europe | D | CYD-like symptoms | Apiaceae | | | | | | | | | V | | | | | | | | | Antolinez et al. 2017 |
| Europe | E | CYD-like symptoms | Apiaceae Solanaceae | | | | | | | | | V | D | V | | | | | | | Antolinez et al. 2017; Moreno et al. 2021 |
| Europe | H | CYD-like symptoms | Apiaceae Polygonaceae | | | | | | | | | | | | | | | | | | Haapalainen et al. 2019 |
| Europe | U | *unknown* | Urticaceae | | | | | | | | | | | | | | | | D | | Haapalainen et al. 2018 |
| Europe | Cras1 | *unknown* | *unknown* | | | | D | D | | | | | | | | | | | | | Sumner-Kalkun et al. 2020 |
| Europe | Cras2 | *unknown* | *unknown* | | | | D | D | | | | | | | | | | | | | Sumner-Kalkun et al. 2020 |
| Europe | Tw | *unknown* | *unknown* | | | | | | | | | | | | | D | | | | | Kwak et al. 2021 |

encompasses the locations from which herbarium specimens testing positive for CLso haplotype G were collected (Mauck et al. 2019). It is located within a Mediterranean climate zone characterized by hot, dry summers and mild, wet winters, and it is situated between several important regions of commercial solanaceous crop production (Supplementary Fig. S1): the high desert of Los Angeles County, where winter potatoes are grown; the Coachella Valley in Riverside County, where winter potatoes and peppers are grown; the Imperial Valley in Imperial county, where winter potatoes are grown; the Oxnard Plain and Santa Clara River Valley in Ventura County, where peppers are produced spring through summer; and northern San Diego County, where fresh market tomatoes are produced almost year-round.

### Plant tissue sampling

**2020 shoots.** In the spring of 2020, we collected shoot tissue from approximately 10% of individual *S. umbelliferum* plants in each of the five populations (Table 1). Sampled individuals were spaced at least 5 m apart. We labeled the individual sampled plants by securing a metal plant tag around the base of the main stem, then recorded GPS coordinates. Using a clean glove for each plant, we collected one to two apical stems with 5 to 10 leaves each by hand and placed them in 15-ml Falcon tubes on ice for transport back to the lab. Tubes were then stored at –80°C until DNA extraction could be performed.

**2021 shoots.** In the spring of 2021, we used GPS coordinates and metal plant tags to relocate the same individual plants across all five populations originally sampled in the spring of 2020. Once plants were relocated, we recorded whether they were alive or dead, and, if alive, we collected new tissue samples following the same protocol. If plants were dead or metal tags were found broken off on the ground, the next closest plant was sampled, so that the same total number of plants were included each year to estimate CLso prevalence.

**2020 roots.** In October of 2020, while *S. umbelliferum* plants were still dormant and leafless from the summer drought period,
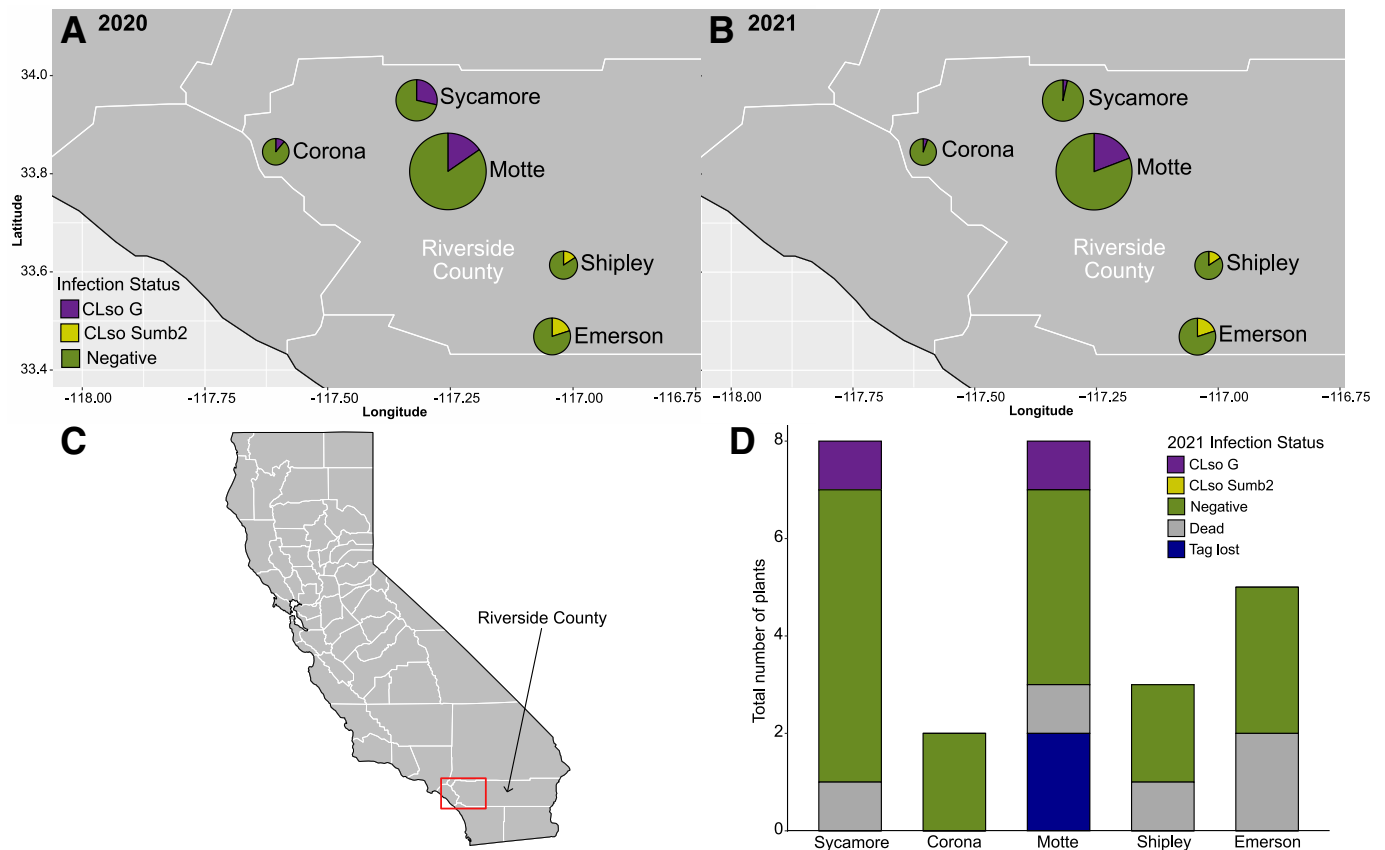


Fig. 2. **A**, Prevalence and diversity of '*Candidatus* Liberibacter solanacearum' (CLso) haplotypes in five *Solanum umbelliferum* populations during the spring of 2020. The total numbers of plants positive for CLso at each location were as follows: Sycamore, 8/28; Corona, 2/18; Motte, 8/52; Shipley, 3/19; and Emerson, 5/25. One of the CLso Sumb2-positive plants at Shipley was coinfected with CLso B. **B**, Prevalence and diversity of CLso haplotypes in five *S. umbelliferum* populations during the spring of 2021. The total numbers of plants positive for CLso at each location were as follows: Sycamore, 1/28 (CLso G); Corona, 1/18 (CLso G); Motte, 10/52 (CLso G); Shipley, 3/19 (CLso Sumb2); and Emerson, 5/25 (CLso Sumb2). CLso B was not detected. **C**, Map showing the location of the sampling area within California (Western Riverside County). **D**, The 2021 infection status of individual plants that had tested positive for CLso in 2020. Figures were created using the ggplot2 package in R version 4.3.0.

TABLE 1. Name and location of the sampling sites and number of *Solanum umbelliferum* plants sampled at each site per year

| Population name | Reserve name (DOI) | Location | Latitude | Longitude | Total number of plants sampled per year |
|---|---|---|---|---|---|
| Corona | Cleveland National Forest | Corona, CA | 33°50′43.6″N | 117°36′51.0″W | 18 |
| Emerson | Emerson Oaks Reserve (10.21973/N34H2G) | Temecula, CA | 33°27′59.4″N | 117°02′33.1″W | 25 |
| Motte | Motte Rimrock Reserve (10.21973/N31T0W) | Perris, CA | 33°48′25.6″N | 117°15′18.7″W | 52 |
| Shipley | Southwestern Riverside County Multi-Species Reserve | Winchester, CA | 33°36′54.6″N | 117°01′09.3″W | 19 |
| Sycamore | Sycamore Canyon Wilderness Park | Riverside, CA | 33°56′45.8″N | 117°19′11.4″W | 28 |

we collected root tissue to test whether CLso may be retained in this part of the plant from one growing season to the next. We sampled root tissue from the two populations of *S. umbelliferum* located in University of California natural reserves, Motte Rimrock Reserve and Emerson Oaks Reserve, as we were permitted to sample root tissue in these locations. We sampled all plants testing positive for CLso in shoot tissue in the spring of 2020. To collect root tissue, we used a sharp trowel to remove the soil covering the main taproots and excise approximately two inches of tissue. The trowel was cleaned with 100% ethanol between plants. Root samples were stored in 15-ml tubes on ice for transport back to the laboratory, where they were then held at –80°C until DNA extraction.

### Psyllid surveys

**Active sampling.** To conduct active sampling for potato psyllids, we shook *S. umbelliferum* shoots over a canvas beating sheet, then collected psyllids into 9-dram vials using an insect aspirator (BioQuip). We sampled plants for psyllids concurrent with collection of shoot tissue in the spring of 2020 and 2021. We transported psyllids back to the lab on ice, then photographed all psyllids under a dissecting scope, placed them in 95% ethanol, and stored them at –80°C to await DNA extraction.

**Passive sampling.** At one *S. umbelliferum* population, Motte Rimrock Reserve, we performed passive psyllid sampling to determine how potato psyllid activity and genetic diversity varied throughout and across years. We placed one yellow sticky card at foliage height in each of three stands of *S. umbelliferum* and collected them on a weekly basis. This yellow sticky card trapping protocol was originally performed at Motte Rimrock Reserve in March and April of 2018 and 2019 for another study monitoring hemipteran vectors of plant viruses (aphids and whiteflies). When these cards were reexamined for psyllids, it was determined that potato psyllids were present throughout this entire trapping period. Thus, in 2020, we started trapping at the beginning of March and continued trapping through June of 2020, when psyllids stopped appearing in our traps. Because this trapping period still did not appear to capture the arrival of potato psyllids at the beginning of the *S. umbelliferum* growing season in winter, we performed a final, more thorough trapping effort from October 2020 through September 2021. Once collected from the field, yellow sticky cards were stored in Ziplock bags at 4°C until psyllids could be removed for DNA extraction.

### Plant DNA extraction and PCR detection of CLso

We removed one 5 to 10 mm$^2$ leaf, including the midrib and petiole, from each plant tissue sample for DNA extraction using a previously optimized, column-based CTAB DNA extraction method (Mauck et al. 2019). All PCR experiments in this study were conducted using Phusion HF DNA polymerase, allowing us to use the same PCR recipe (20 μl total reaction volume: 1 μl of template DNA, 4 μl of 5× HF buffer [Thermo Fisher Scientific], 2 μl of 2 mM dNTP mix, 1 μl of each 10 μM primer, and 0.2 μl of Phusion HF DNA polymerase [Thermo Fisher Scientific]) and PCR program (initial denaturation at 98°C for 5 min followed by 40 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 1 min, and then 72°C for a 10-min final elongation) for all primer sets, as described in Mauck et al. (2019). We performed initial screening of plant and insect samples for 'Candidatus Liberibacter' using Las606/Lss-2 primers that amplify the 16S-23S intergenic spacer region (Mauck et al. 2019). For samples that produced amplicons consistent with CLso, we then used the same PCR recipe and program with an additional set of primers (rpo01F/R) to amplify a portion of the 50S ribosomal protein rplJ/rplL gene previously used for distinguishing between CLso haplotypes (Haapalainen et al. 2018). During previous work with *S. umbelliferum* DNA extracts, we observed that DNA amplification sometimes failed, presumably due to the presence of PCR inhibitors. Thus, for all plant samples, we also performed a round of PCR with primers targeting plant ITS2 (Chen et al. 2010) to confirm the success of DNA extraction and prevent false negatives during our CLso screening. DNA from plant samples from which we were initially unable to amplify ITS2 was serially diluted by a factor of 10, until ITS2 successfully amplified, and then rescreened for CLso. We visualized all PCR products on a 1% agarose gel. Bands of the expected size were excised from the gel, and the PCR products were purified using a ZymoClean Gel DNA Recovery Kit. We sent purified PCR products to Retrogen for Sanger sequencing. The resulting sequences were manually quality checked and trimmed, and forward and reverse sequences were merged using BioEdit version 7.0.5.3 (Hall 1999).

### Psyllid DNA extraction and determination of mitochondrial haplotypes

For psyllid specimens, DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's recommendations for use with insect specimens, with one modification: Due to the psyllids' small size, DNA was eluted in only 50 μl of AE buffer to increase the concentration of DNA in the final eluate. Potato psyllid haplotypes were determined using one of two methods, Sanger sequencing or high-resolution melt analysis. For Sanger sequencing, a 500 base pair portion of the mitochondrial cytochrome oxidase I (mtCOI) gene was amplified using the primers COI-F3 (5′-TACGCCATACTAGCAATCGG-3′) and COI-R3 (5′-GAGTAACGTCGTGGTATTCC-3′) (Crosslin et al. 2011) and the PCR reagents and conditions described above. The resulting sequences were matched with previously published representative sequences for each haplotype (Swisher et al. 2012, 2014a) via BLAST searches. For the high-resolution melt analysis, two smaller regions of the mtCOI gene were amplified, and previously established differences in melting behavior were used to determine the haplotype of each specimen, using the primers and protocol described in Swisher et al. (2014a). Each psyllid sample was also screened for CLso with the same PCR and Sanger sequencing protocols used for plant samples.

### CLso MLST

We selected samples with positive detections of each CLso haplotype from plants, and, when possible, also from one potato psyllid from each *S. umbelliferum* population for MLST. For these samples, we amplified seven additional loci from throughout the CLso genome (Haapalainen et al. 2018; see Supplementary Data Set S1 for the full list of primers) using the same PCR recipe and program described above in "Plant DNA extraction and PCR detection of CLso infection." After aligning the sequences from all CLso-positive samples for each MLST locus, we manually identified all existing single-nucleotide polymorphisms (Supplementary Data Sets S2 to S8). For every locus, each possible combination of single-nucleotide polymorphisms was assigned a unique allele identifier (1, 2, 3, etc.). These allele identifiers were entered into a table, and each different combination of allele identifiers was designated as a unique sequence or strain type.

### Phylogenetic analysis of CLso haplotypes

We built one phylogenetic tree using CLso 50S rpIJ/rpIL sequences and one using seven concatenated MLST loci. Using BioEdit, we aligned the sequences generated in this study with all available CLso 50S rpIJ/rpIL or MLST sequences available on GenBank. Model selection was performed using IQTree, and maximum likelihood trees were constructed using the IQTree web server (Nguyen et al. 2015) with 1,000 bootstrap replicates. All CLso sequences used for phylogenetic analyses (50S and MLST loci) were deposited in GenBank. GenBank accession numbers are provided in Supplementary Data Set S9.

# Results

## Prevalence and persistence of CLso in Riverside County

### *S. umbelliferum*

**2020 shoots.** In the spring of 2020, CLso was detected in the shoots of plants from all five populations of *S. umbelliferum* (Fig. 2A). A total of 26 of 142 *S. umbelliferum* plants tested positive for CLso, with prevalence ranging from 11% in the Cleveland National Forest at Corona to about 29% at Sycamore Canyon Park. In the three more northerly populations, we only detected CLso G, the haplotype originally identified from historic herbarium specimens collected in this same region. In the two southernmost populations, we never detected haplotype G, but we did detect an additional novel haplotype. This novel haplotype was named CLso Sumb2 in reference to the fact that it is the second CLso haplotype first detected in *S. umbelliferum* (the first being CLso G). Following the example of other CLso researchers, we opted not to continue using the alphabetical system of naming CLso haplotypes (Grimm et al. 2022; Sumner-Kalkun et al. 2020), as this previously resulted in the same letter inadvertently being used to describe multiple different haplotypes of CLso (Contreras-Rendón et al. 2020; Haapalainen et al. 2020). We did not detect the crop-associated CLso haplotypes A and B as single infections in any of the plants tested. However, we did detect haplotype B in coinfection with haplotype Sumb2 in one individual plant from the Shipley-Skinner Reserve population.

**2021 shoots.** In the spring of 2021, a total of 20 of 142 *S. umbelliferum* plants tested positive for CLso, with prevalence in each population ranging from about 4% at Sycamore Canyon Park to 20% at both Motte Rimrock Reserve and Emerson Oaks Reserve (Fig. 2B). The same pattern of CLso haplotype distribution was recapitulated: We only detected CLso G in the three more northern populations and only detected CLso Sumb2 in the two southernmost populations. No plants tested positive for CLso A or B. Of the 26 individual plants that tested positive for CLso in the spring of 2020, two could not be relocated, as their metal tags had been broken off and were found on the ground. Five of the remaining plants were apparently dead. Thus, we were only able to resample shoot tissue from 19 plants that tested positive for CLso in 2020. Of these, only two tested positive for CLso (both haplotype G; one at Sycamore Canyon Park and one at Motte Rimrock Reserve), whereas the remaining 17 plants tested negative (Fig. 2D).

**2020 roots.** We were able to relocate and sample roots from all the 13 plants at Emerson Oaks Reserve and Motte Rimrock Reserve that tested positive for CLso by shoot screening in 2020. At Emerson Oaks Reserve, we detected CLso in the roots of three of five resampled plants. At Motte Rimrock Reserve, we detected CLso in five of eight resampled plants. However, when shoot tissue was sampled from these plants in the spring of 2021, none of the plants at Emerson Oaks Reserve and only one of the eight resampled plants at Motte Rimrock Reserve tested positive for CLso (Table 2).

## Genetic diversity and CLso infection status of *S. umbelliferum*-associated psyllids

**Active sampling.** Through active psyllid sampling in the spring of 2020, we confirmed that potato psyllids colonize *S. umbelliferum* in all five bluewitch nightshade populations. The majority of collected psyllids were of the Northwestern haplotype, with smaller numbers of the Southwestern haplotype detected at Sycamore Canyon Park, Motte Rimrock Reserve, and Shipley-Skinner Reserve (Fig. 3A). None of the psyllids captured in the Cleveland National Forest at Corona (five total) or Emerson Oaks Reserve (five total) tested positive for CLso. Five of 25 psyllids from Motte Rimrock Reserve and 5 of 18 psyllids from Sycamore Canyon Park tested positive for CLso G, and 1 of 18 psyllids from Sycamore Canyon Park and 1 of 12 from Shipley-Skinner Reserve tested positive for the crop-associated haplotype CLso B (Fig. 3C). All the psyllids that tested positive for CLso in 2020 were of the North-

western haplotype, with the exception of one individual of the Southwestern haplotype at Sycamore Canyon Park that was carrying CLso G.

We did not recover any potato psyllids from the Cleveland National Forest at Corona and Emerson Oaks Reserve *S. umbelliferum* populations during spring 2021 sampling. However, we collected the Northwestern haplotype at all three of the remaining reserves and also collected the Southwestern haplotype at Sycamore Canyon Park and Motte Rimrock Reserve (Fig. 3B). None of the psyllids collected at Shipley-Skinner Reserve tested positive for CLso (six total, all Northwestern haplotype). Five of 20 psyllids from Sycamore Canyon Park (all Northwestern haplotype) and 6 of 37 of the psyllids captured during the spring of 2021 at Motte Rimrock Reserve (all Southwestern haplotype) tested positive for CLso G (Fig. 3D). No other CLso haplotypes were detected in psyllids in 2021.

**Passive sampling.** Through long-term passive sampling at Motte Rimrock Reserve (2018 to 2021), we corroborated single-time-point sampling results while documenting the seasonal dynamics of potato psyllids associated with *S. umbelliferum* in southwestern California. We detected both Northwestern and Southwestern haplotype potato psyllids in all 3 years at this location by screening a subset of individuals captured on yellow sticky cards each year. During our year-long sampling effort, from fall 2020 to fall 2021, a total of 92 psyllids were captured on yellow sticky cards at Motte Rimrock Reserve. We determined the haplotypes of these psyllids and screened them for CLso. Both Northwestern and Southwestern haplotypes were detected (21 Northwestern, 71 Southwestern; Fig. 4). Approximately 10% of these psyllids tested positive for CLso. All CLso-infected psyllids were of the Southwestern haplotype, and all were carrying CLso G. Potato psyllids were occasionally captured from November through January and consistently detected from the beginning of February through June, with activity peaking in early March. No potato psyllids were detected from the end of June through October. This pattern matches the phenology of *S. umbelliferum* at this location (primarily dormant and leafless during the hot, dry summer and fall). The results of the more limited 2018 to 2020 spring sampling and haplotyping efforts were similar and are provided in Supplementary Table S1. An overview of the results of both active and passive potato psyllid sampling combined can be found in Supplementary Table S2.

## Evolutionary relationships between haplotypes of CLso associated with *S. umbelliferum*

We initially identified haplotypes of CLso based on a maximum likelihood tree generated using sequences from the 50S rpIJ/rpIL gene region (Fig. 5). We found a total of three CLso haplotypes

TABLE 2. Overview of results of retesting plants from Emerson Oaks (EO) and Motte Rimrock (MR) reserves whose shoots tested positive for '*Candidatus* Liberibacter solanacearum' in the spring of 2020 at subsequent time points[a]

| Plant | Spring 2020 infection status (shoots) | Fall 2020 infection status (roots) | Spring 2021 infection status (shoots) |
|---|---|---|---|
| EO 10 | Positive | Positive | Dead |
| EO 12 | Positive | Positive | Negative |
| EO 13 | Positive | Positive | Dead |
| EO 15 | Positive | Negative | Negative |
| EO 21 | Positive | Negative | Negative |
| MR 7 | Positive | Positive | Negative |
| MR 8 | Positive | Negative | Negative |
| MR 19 | Positive | Negative | Negative |
| MR 20 | Positive | Positive | Dead |
| MR 25 | Positive | Positive | Tag broken off |
| MR 27 | Positive | Negative | Positive |
| MR 34 | Positive | Positive | Tag broken off |
| MR 49 | Positive | Positive | Negative |

[a] In the fall of 2020, root tissue was tested (as shoots were not present), and in the spring of 2021, new shoots were tested.

infing *S. umbelliferum* plants and associated potato psyllids in western Riverside County, with haplotypes partitioned geographically. With the exception of one psyllid sample from Sycamore Canyon Park, all sequences from the three northernmost populations, Motte Rimrock Reserve, Sycamore Canyon Park, and Cleveland National Forest at Corona, clustered with the CLso G sequences from historical herbarium specimens of *S. umbelliferum*. Except for one plant and one psyllid sample, all the sequences from the two more southern reserves, Shipley-Skinner and Emerson Oaks, did not match any GenBank accessions and clustered together in the 50S tree, forming a unique clade. These sequences represent a novel haplotype, CLso Sumb2, which is most closely related to the crop-associated haplotype CLso B. The remaining sequences from one psyllid at Sycamore Canyon, as well as sequences from one psyllid and one plant at Shipley-Skinner Reserve, were identified as belonging to a third haplotype, CLso B.
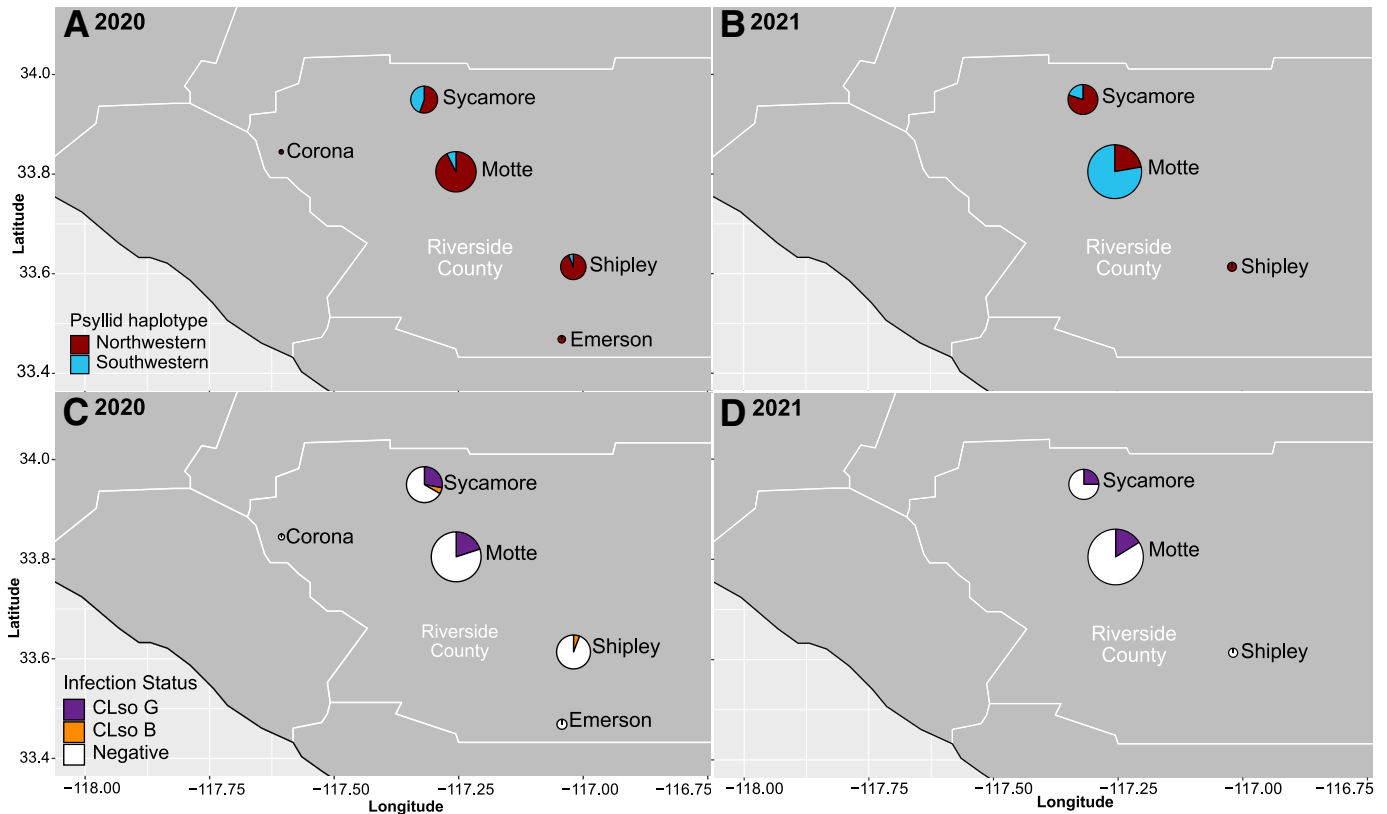


**Fig. 3.** Results of active psyllid sampling: **A,** 2020 potato psyllid diversity by reserve; **B,** 2021 potato psyllid diversity by reserve; **C,** 2020 potato psyllid '*Candidatus* Liberibacter solanacearum' (CLso) infection status by reserve; and **D,** 2021 potato psyllid CLso infection status by reserve. Motte 2021 samples were the result of weekly trapping over the course of a year, whereas all other reserves were sampled actively during only the spring of 2021. Thus, only April and May samples from 2021 at Motte Rimrock are included here, to correspond with the period during which the other reserves were sampled. More detailed results from year-long trapping at Motte are presented in Figure 5. Figures were created using the ggplot2 package in R version 4.3.0.

**Fig. 4.** Results of passive psyllid sampling. Potato psyllid abundance, diversity, and '*Candidatus* Liberibacter solanacearum' (CLso) infection status at Motte Rimrock Reserve during a period of weekly trapping running from October 2020 to September 2021 (no psyllids were caught before November 2020 or after June 2021, so these weeks have been omitted here). All nine CLso-positive psyllids were of the Southwestern haplotype and infected with CLso haplotype G.
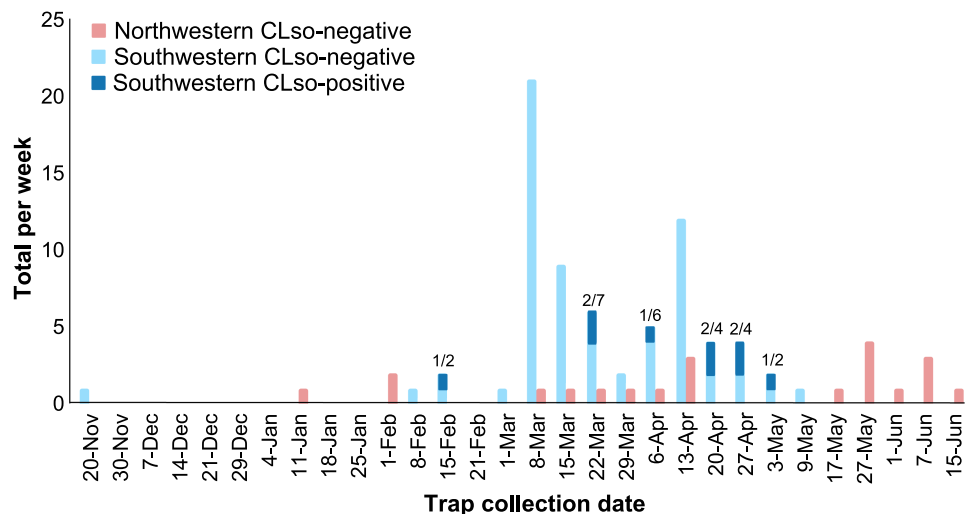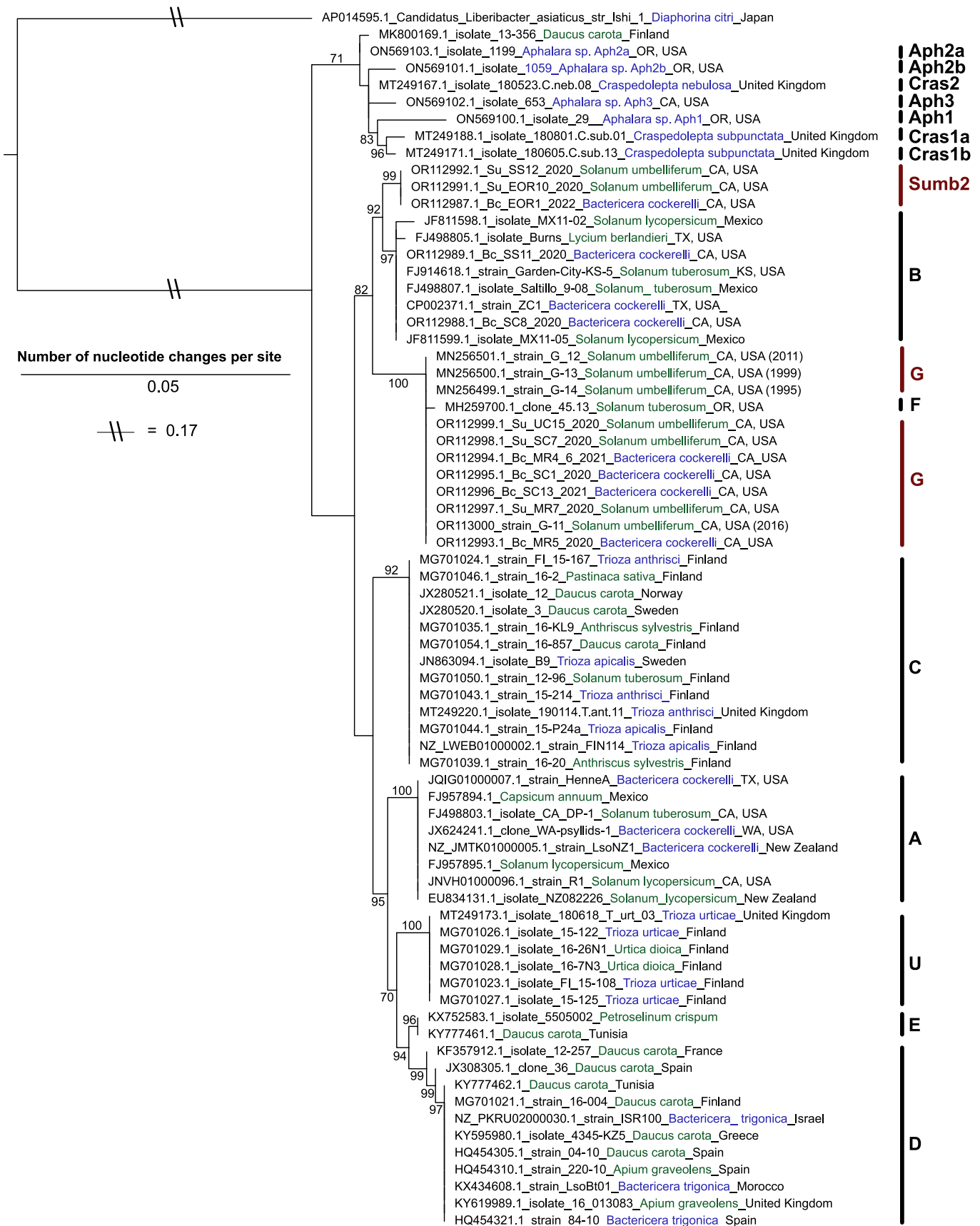
**Fig. 5.** Maximum likelihood tree based on alignment of the 633 nt '*Candidatus* Liberibacter solanacearum' 50S rpIl- rpIJ region; 1,000 bootstraps (only values ≥70% shown here). Some identical variants of haplotype C were omitted for ease of visualization. The host name is given in green if the sample originated from a plant and in blue if the sample originated from a psyllid.

Within these three haplotypes, MLST revealed seven unique CLso sequence or strain types (Table 3). The maximum likelihood tree constructed using the seven concatenated MLST loci closely matched the 50S tree (Fig. 6), with one primary caveat: Based on 50S sequences, CLso F, detected in one sample from a symptomatic potato tuber in Oregon, was initially placed among CLso G sequences, but in our MLST tree, all CLso G sequences clustered separately from CLso F, justifying its designation as a unique haplotype. It should be noted that the CLso sequences from the 2020 plant sample from Shipley-Skinner Reserve that appeared to be coinfected with both CLso Sumb2 and CLso B could not be included in any of the phylogenetic analyses. Su_EOR10 (from *S. umbelliferum* at Emerson Oaks Reserve) was also omitted from the final MLST table because it was from a plant infected with more than one sequence type of CLso Sumb2.

## Discussion

Prior research showed that both potato psyllids and CLso colonize native *Solanaceae* (Mauck et al. 2019; Reyes Corral et al. 2020; Thinakaran et al. 2015; Wallis 1955). However, the geographic distribution, prevalence, and genetic diversity of CLso and potato psyllids in native vegetation was still virtually unknown throughout most of western North America. The present study found that both CLso and potato psyllids are commonly associated with the native perennial *S. umbelliferum* in natural plant communities in southwestern California. Whereas CLso infections can persist across seasons in the roots of *S. umbelliferum*, the prevalence of CLso in *S. umbelliferum* shoot tissue and associated psyllid populations is relatively low. Furthermore, CLso and potato psyllids associated with native nightshade populations were both genetically distinct from, and more diverse than, those previously documented in California crops (Liu and Trumble 2007; Liu et al. 2006).

Previous studies found CLso infecting *S. umbelliferum* as far back as 1970 and documented the presence of potato psyllids on this host plant species in Southern California as far back as 1955 (Mauck et al. 2019; Wallis 1955). Our results revealed that CLso is ubiquitous in contemporary southwestern *S. umbelliferum* populations and confirmed that native plants may play an important role in shaping patterns of transmission, as well as evolution, of this pathogen in North America. We also reported distinct partitioning of CLso haplotypes by habitat; the crop disease-associated CLso haplotypes A and B were almost completely absent from *S. umbelliferum* populations, with a single plant testing positive for CLso B once over 2 years of screening. Instead, only CLso haplotypes unique to native nightshades, CLso G and CLso Sumb2, were detected. Similarly, our potato psyllid and CLso DNA sequencing and phylogenetic analyses indicates that crop-to-wild and wild-to-crop plant movement is also rare for the vector. Prior studies only ever detected Western haplotype potato psyllids in California crops growing throughout the same region in which we performed our study (Arp et al. 2014; Liu et al. 2006; Swisher et al. 2012). However, over multiple years of sampling and mtCOI haplotyping, we failed to detect a single Western haplotype potato psyllid on *S. umbelliferum*. Instead, two haplotypes that had never before been documented in the state of California—the Northwestern and Southwestern haplotypes—were repeatedly found in association with these native plants.

Taken together, these results suggest that potato psyllids—and, therefore, CLso—in Southern California may only rarely move back and forth between native and crop plants. Western haplotype psyllids in the Southwest may be dependent on certain crops, or other non-crop hosts not evaluated here, for reproduction. In contrast, Northwestern and Southwestern haplotype populations may retain adaptations that tie their reproduction to the availability of specific native host plants. The different distributions of populations of these haplotypes observed here may be due to these differing preferences for crop versus native plant hosts. There is certainly prior evidence that differences in the specific host plant preferences of distinct psyllid species or populations can act to maintain isolation of unique genetic variants of CLso in cultivated versus wild plants within the same geographic region. For example, in Finland, unique strains of CLso haplotype C were found infecting cultivated carrot and parsnip versus wild cow parsnip, even when growing right next to each other (Haapalainen et al. 2018). Screening of psyllids associated with these hosts revealed that these strains of CLso C were also cleanly partitioned between the two species; CLso strains in cultivated carrot and parsnip were detected in *Trioza apicalis*, a known vector of CLso in crops, and wild cow parsnip strains were detected in *T. anthrisci* (Haapalainen et al. 2018). Furthermore, in both Finland and Scotland, CLso U has been detected in wild *Urtica dioica* and the associated psyllid species *T. urticae* along the margins of carrot fields, but never from carrots or *T. apicalis,* which were, in contrast, often infected with CLso C (Haapalainen et al. 2018; Sumner-Kalkun et al. 2020). These studies suggest that preference-performance relationships with hosts may

TABLE 3. Unique allele identifiers and sequence types of all North American '*Candidatus* Liberibacter solanacearum' haplotypes for which complete multilocus sequence typing (MLST) data are available and for selected samples collected in this study

| Haplotype | Sample designation[a] | Allele identifiers for each MLST locus | | | | | | | Sequence type |
| | | adk | atpA | fbpA | ftsz | glyA | groEL | gyrB | |
|---|---|---|---|---|---|---|---|---|---|
| A | HenneA | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| A | Strain LsoNZ1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| A | Strain R1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| B | ZC1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 |
| B | Bc_SC8_2020 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 |
| B | Bc_SS11_2020 | 2 | 2 | 2 | 3 | 2 | 3 | 3 | 4 |
| Sumb2 | Su_SS12_2020 | 3 | 3 | 3 | 4 | 3 | 4 | 4 | 5 |
| Sumb2 | Bc_EOR1_2022 | 4 | 3 | 3 | 5 | 3 | 5 | 5 | 6 |
| G | Su_MR7_2020 | 5 | 4 | 4 | 6 | 4 | 6 | 6 | 7 |
| G | Bc_MR5_2020 | 5 | 5 | 4 | 6 | 4 | 6 | 6 | 8 |
| G | Bc_MR4_2020 | 5 | 5 | 4 | 6 | 4 | 6 | 6 | 8 |
| G | Bc_SC1_2020 | 5 | 5 | 4 | 6 | 4 | 6 | 6 | 8 |
| G | Su_SC7_2020 | 5 | 5 | 4 | 6 | 4 | 6 | 6 | 8 |
| G | Bc_SC13_2021 | 5 | 5 | 4 | 7 | 4 | 6 | 6 | 9 |
| G | Su_UC15_2020 | 5 | 5 | 4 | 7 | 4 | 6 | 6 | 9 |
| G | Herb-61 (G11) | 6 | 5 | 5 | 7 | 4 | 6 | 6 | 10 |
| F | Clone 45.13 | 6 | 6 | 6 | 8 | 5 | 6 | 7 | 11 |

[a] Samples designated as Bc were recovered from *Bactericera cockerelli*, and those designated as Su originated from *Solanum umbelliferum* plants.

**Fig. 6.** Maximum likelihood tree based on 3,917 nt alignment of seven concatenated multilocus sequence typing genes amplified from 'Candidatus Liberibacter solanacearum'; 1,000 bootstraps (only values ≥70% shown here). Haplotypes are listed on the right. The host name is given in green if the sample originated from a plant and in blue if the sample originated from a psyllid.

also play a major role in determining the respective importance of each potato psyllid haplotype as a driver of crop pathogen transmission in North America, which could be explored through behavioral work.

In addition to revealing sharp delineations of potato psyllid haplotypes by host plant, our study illuminated seasonal activity patterns in relation to *S. umbelliferum*. Weekly monitoring of potato psyllid activity in an *S. umbelliferum* population revealed that Northwestern and Southwestern potato psyllids visit *S. umbelliferum* from fall through spring, but not in the summer. This finding confirms that *S. umbelliferum* is not a suitable reproductive host for potato psyllids during summer dormancy, when shoots lose their leaves and may die back to the soil level. We had hypothesized that when *S. umbelliferum* senesces for the season in late spring, associated potato psyllids likely migrate to freshly planted California tomato, pepper, or potato fields to pass the summer, moving back onto native nightshades as crops are harvested and seasonal rains revive *S. umbelliferum* in the fall. However, our mtCOI potato psyllid haplotyping results refute this idea because only Western haplotype potato psyllids have been detected in California crops (Arp et al. 2014; Liu et al. 2006; Swisher et al. 2012), whereas only the Northwestern and Southwestern haplotypes were present in *S. umbelliferum* populations. However, it should be noted that before this study, only a handful of potato psyllid samples, all collected over a decade ago from just a few crop fields in Southern California, had been characterized for their haplotype via mtCOI sequencing (Arp et al. 2014; Liu et al. 2006; Swisher et al. 2012). Thus, expanded sampling and sequencing of crop-associated psyllids will be critical to draw any final conclusions about potato psyllid movement across the agro-ecological interface. Nonetheless, there is precedent for the idea that different potato psyllid haplotypes may exist in sympatry, but remain genetically separate, due to differing preferences for wild versus crop plant hosts. In the Pacific Northwest, mtCOI sequencing of potato psyllids collected from crop fields and introduced solanaceous weeds demonstrated that Western haplotype potato psyllids were dominant in potato fields but almost completely absent from nearby stands of wild *S. dulcamara*, where the Northwestern haplotype was found to dominate year-round (Swisher et al. 2013, 2014b). In this case, the source of Western haplotype potato psyllids entering agricultural fields in Oregon, Washington, and Idaho each spring remained unclear. Here, however, we are faced with the opposite mystery: If Northwestern and Southwestern haplotype potato psyllids are not utilizing crop plant hosts when native nightshades are unavailable in the summer, where are they going?

One possible explanation is that native nightshade-associated potato psyllids are not going anywhere at all. Perhaps, like many other psyllid species, they diapause as eggs or nymphs on the bare stems or even roots of their dormant host plants (Bird and Hodkinson 1999; Hodkinson 2009; Lal 1934; Lauterer 1993; Loginova 1970, 1976; Mustafa and Najjar 1985; Rapisarda 1990; Wheeler and Rawlins 1993). However, such behavior has never been observed in potato psyllids. On the contrary, adult potato psyllids have been observed during the summer months (June to September) on evergreen trees and shrubs in the mountains of California (>2,000 m), often in large numbers (Crawford 1914). Thus, we propose that Northwestern and Southwestern potato psyllids may undertake a migration to nonreproductive shelter plants at higher elevations to survive the summer and then return to native nightshades as temperatures drop in the fall. Other psyllid species in the Triozidae and beyond employ a similar strategy for surviving the winter, when their reproductive hosts are unavailable in more temperate climes (Jarausch and Jarausch 2016; Thébaud et al. 2009; Valterová et al. 1997). However, again, none of the potato psyllids observed during the summer in the mountains of California has been haplotyped, so we can only speculate about the genetic composition of these psyllids or the nature and duration of associations with the evergreen plants they are visiting.

During our initial screening of *S. umbelliferum* shoot tissue for CLso infection, we detected CLso in all of the native nightshade populations we sampled. We also detected CLso in *S. umbelliferum* roots the subsequent fall before these plants had broken their summer dormancy and had any opportunity to become reinfected. However, the following spring, the majority of new shoots sprouting from infected roots did not test positive for CLso. This is consistent with results of CLso retention studies performed on another native, perennial host of the potato psyllid in the American Southwest, *S. elaeagnifolium* (Thinakaran et al. 2015), and could partially explain the relatively low prevalence (∼15%) of CLso we observed in wild *S. umbelliferum* populations. Furthermore, it suggests that *S. umbelliferum*, and other North American nightshades, may encode some genetic resistance to or tolerance of CLso. As coevolution between host and pathogen is expected to result in increased host resistance and lower pathogen virulence over time, these results suggest that the relationship between CLso and native North American nightshades, including *S. umbelliferum*, is likely quite longstanding. It may also be the case that *S. umbelliferum*-associated CLso haplotypes (CLso G and CLso Sumb2) are mild or avirulent. Here, it is worth remembering that because CLso and most other Liberibacter species are unculturable, we currently rely on comparative genomics studies to identify potential genes dictating host range and conferring virulence within this taxon (Batarseh et al. 2023; Levy et al. 2020; Thapa et al. 2020; Wang et al. 2017). Thus, because CLso G and Sumb2 are closely related to the highly virulent CLso B, but potentially only mild or avirulent, further characterizing their biology and genomes could accelerate identification of genetic determinants of virulence within this taxon, facilitating the development of highly specific and effective CLso management tools.

Although the present study focused on a subset of populations of one host plant species, the results suggest that CLso and its psyllid vectors are much more widespread and diverse in North American natural plant communities than suggested by data collected solely from agricultural fields. Future studies should expand on our CLso sampling and sequencing efforts to include crops and native habitats throughout western North America. If our study is any indication, these efforts would vastly improve our understanding of CLso evolutionary history and ecology in this region of the world. The presence of a diversity of strains of CLso in *S. umbelliferum* and associated potato psyllid populations means that even occasional movement of potato psyllid vectors between native and crop plant hosts could eventually lead to the emergence of new pathogenic CLso haplotypes in both crops and natural plant communities. Thus, it would also be enlightening to perform a more thorough investigation of the potato psyllid population structure in California to determine the frequency with which potato psyllids move back and forth between native and crop plant hosts, potentially carrying CLso with them. Such a study could also include potato psyllid samples from nonreproductive evergreen shelter plants to determine whether native and crop-associated potato psyllid populations do, indeed, exhibit divergent summer migration tactics. Finally, future work could include controlled transmission assays to compare the virulence and host range of native nightshade-associated CLso G and Sumb2 with crop disease-associated CLso A and B. These could be coupled with comparative genomics studies to identify specific virulence factors within this taxon. Overall, this study demonstrates that expanding and continuing field surveillance for potato psyllids and CLso in both wild and agricultural settings is imperative if we aim to improve our ability to predict and manage future outbreaks of this pathogen and vector in crops, as well as in natural plant communities of conservation concern.

and Kylie Swisher-Grimm for providing sequences of CLso haplotype F for the MLST analysis and for her assistance with high-resolution melting methods.

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