Environmental Entomology, 52(2), 2023, 243–253
https://doi.org/10.1093/ee/nvad015
Advance Access Publication Date: 4 March 2023
Research

ENTOMOLOGICAL SOCIETY OF AMERICA
SHARING INSECT SCIENCE GLOBALLY

OXFORD

Insect-Symbiont Interactions

Bacterial Endosymbionts Identified From Leafhopper (Hemiptera: Cicadellidae) Vectors of Phytoplasmas

William Rodney Cooper,[1,7] William B. Walker III,[1] Gina M. Angelella,[1] Kylie D. Swisher Grimm,[1] Jillian J. Foutz,[2] Scott J. Harper,[3] Louis B. Nottingham,[4] Tobin D. Northfield,[4] Carrie H. Wohleb,[5] and Carl A. Strausbaugh[6]

[1]USDA-ARS Temperate Tree Fruit and Vegetable Research Unit, 5230 Konnowac Pass Road, Wapato, WA 98951, USA, [2]Department of Entomology, Washington State University, 166 FSHN Building, Pullman, WA 99164, USA, [3]Department of Plant Pathology, Washington State University, Irrigated Agriculture Research and Education Center, Prosser, WA 99350, USA, [4]Department of Entomology, Washington State University, Tree Fruit Research and Extension Center, 1100 N. Western Avenue, Wenatchee, WA 98801, USA, [5]Washington State University Extension, 1525 E. Wheeler Road, Moses Lake, WA 98837, USA, [6]USDA-ARS Northwest Irrigation and Soils Research Laboratory, Kimberly, ID 83341, USA, and [7]Corresponding author, e-mail: Rodney.Cooper@usda.gov

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture. USDA is an equal opportunity provider and employer.

Subject Editor: Jiri Hulcr

Received 19 September 2022; Editorial decision 19 January 2023.

Abstract

Insects often harbor bacterial endosymbionts that provide them with nutritional benefit or with protection against natural enemies, plant defenses, insecticides, and abiotic stresses. Certain endosymbionts may also alter acquisition and transmission of plant pathogens by insect vectors. We identified bacterial endosymbionts from four leafhopper vectors (Hemiptera: Cicadellidae) of *'Candidatus* Phytoplasma' species by direct sequencing 16S rDNA and confirmed endosymbiont presence and identity by species-specific conventional PCR. We examined three vectors of *Ca.* Phytoplasma pruni, causal agent of cherry X-disease [*Colladonus geminatus* (Van Duzee), *Colladonus montanus reductus* (Van Duzee), *Euscelidius variegatus* (Kirschbaum)] – and a vector of *Ca.* Phytoplasma trifolii, the causal agent of potato purple top disease [*Circulifer tenellus* (Baker)]. Direct sequencing of 16S identified the two obligate endosymbionts of leafhoppers, *'Ca.* Sulcia' and '*Ca.* Nasuia', which are known to produce essential amino acids lacking in the leafhoppers' phloem sap diet. About 57% of *C. geminatus* also harbored endosymbiotic *Rickettsia*. We identified *'Ca.* Yamatotoia cicadellidicola' in *Euscelidius variegatus,* providing just the second host record for this endosymbiont. *Circulifer tenellus* harbored the facultative endosymbiont *Wolbachia,* although the average infection rate was only 13% and all males were *Wolbachia*-uninfected. A significantly greater percentage of *Wolbachia*-infected *Ci. tenellus* adults than uninfected adults carried *Ca.* P. trifolii, suggesting that *Wolbachia* may increase this insect's ability to tolerate or acquire this pathogen. Results of our study provide a foundation for continued work on interactions between leafhoppers, bacterial endosymbionts, and phytoplasma.

Key words: *Colladonus montanus*, western X, beet leafhopper, BLTVA, purple top disease

Species of Cicadellidae (Hemiptera) are important pests of crops as vectors of bacterial or viral plant pathogens (Nault and Ammer 1989, Weintraub and Beanland 2006). Many leafhoppers are known to harbor bacterial endosymbionts that can alter the insects' biology and behavior. All leafhoppers harbor the obligate endosymbiont, '*Candidatus* Sulcia muelleri' (Bacteroidota), that resides within a specialized organ called the bacteriome and produces at least eight essential amino acids that are lacking in the insects' phloem sap diet

(Moran et al. 2005, McCutcheon et al. 2009). Most leafhoppers harbor at least one other obligate bacteriome-inhabiting endosymbiont that synthesizes the remaining essential amino acids (Takiya et al. 2006, McCutcheon et al. 2009, Noda et al. 2012). Many leafhoppers also carry nonobligate facultative endosymbionts such as *Wolbachia* (Rickettsiales: Ehrlichiaceae) or *Rickettsia* (Rickettsiales: Rickettsiaceae) that may provide them with protection against pathogens, insecticides, and plant defenses, or alter their ability to transmit plant pathogens (Davis et al. 1998, Mitsuhashi et al. 2002, Bove et al. 2003, Kambris et al. 2009, Ferrater et al. 2013, Jain et al. 2017, Gonella et al. 2018, Li et al. 2018, Shchuler et al. 2022).

At least two leafhopper-transmitted strains of '*Candidatus* Phytoplasma sp.' currently threaten the tree fruit and vegetable industries of the Pacific Northwest (USA States of Washington and Oregon): *Candidatus* Phytoplasma pruni and *Candidatus* Phytoplasma trifolii. *Ca.* P. pruni (16SrIII-A) causes X-disease of cherry and other stone fruits (Wright et al. 2021). This bacterium is transmitted by several leafhopper vectors, including the native species, *Colladonus montanus reductus* (Van Duzee) (hereafter referred to as *C. reductus*) and *Colladonus geminatus* (Van Duzee) (Wolfe et al. 1950, Jensen 1969). Previous studies from California suggest that the nonnative leafhopper *Euscelidius variegatus* (Kirschbaum) is also a vector of *Ca.* P. pruni (Davis et al. 2013). Phytoplasma-infected trees develop undersized, unripe, and unmarketable fruit (Wright et al. 2021). Visible symptoms are not apparent for several years after infection, allowing the bacterium to initially spread unnoticed throughout an orchard. *Ca.* P. trifolii (16SrVI) is transmitted by the beet leafhopper, *Circulifer tenellus* (Baker) and causes disease symptoms in potato and other vegetables (Crosslin et al. 2005, Munyaneza et al. 2006). *Circulifer tenellus* is native to Europe and is also a vector of beet curly top virus (BCTV) and the bacterium, *Spiroplasma citri* Saglio (Mycoplasmatales: Mycoplasmataceae) (Liu et al. 1983, Chen and Gilbertson 2016). All three *Ci. tenellus*-transmitted pathogens cause economic losses in vegetable crops. There are no direct means to control these leafhopper-vectored pathogens or to cure plants of the diseases, so management practices rely largely upon the use of insecticides to suppress vector populations.

Most research on the biology and ecology of *C. geminatus* and *C. reductus* was completed more than 40 yr ago before the identification of *Ca.* P. pruni as the causal agent of cherry X-disease. There have been no prior investigations on the microbial communities of these two important vectors of X-disease Phytoplasma. Work on *E. variegatus* and *Ci. tenellus* has primarily focused on their natural or experimental roles as vectors of phytoplasmas and viruses (Liu et al. 1983, Gellato et al. 2009, Munyaneza et al. 2010, Chen and Gilbertson 2016). Less is known of other bacteria harbored by these two introduced insect vectors. The goal of our study was to identify and compare bacterial endosymbionts from *C. geminatus, C. reductus, E. variegatus*, and *Ci. tenellus*. We used high-throughput sequencing of the 16S ribosomal RNA gene (16S rDNA) of bacteria to identify bacterial endosymbionts, and sequence analyses to compare bacterial communities among the leafhopper vectors. We had two primary objectives: 1) identify bacterial endosymbionts of these leafhopper vectors of phytoplasma, and 2) investigate patterns among the presence of the most abundant endosymbionts and infections by plant pathogens, especially phytoplasma. Documentation of bacterial endosymbionts of these important vectors of phytoplasma provides a foundation for further work on how endosymbionts potentially alter leafhopper biology or vector competence.

Materials and Methods

Direct Sequencing of 16S Ribosomal DNA

Colladonus geminatus, *C. reductus*, and *Ci. tenellus* were collected from various broadleaf plants from locations in central Washington and Idaho using an insect vacuum collection device constructed from an inverted leaf blower. Table 1 lists locations of leafhopper collections used for direct sequencing of 16S rDNA. *Ci. tenellus* were also obtained from a colony originally collected

Table 1. Sources of leafhopper vectors screened for presence of endosymbionts using directed sequencing of 16S rDNA gene

Leafhopper vector	Location	Date	Habitat	DNA extraction method	N[a]
Colladonus geminatus					
Rep 1	Yakima County	May-2020	Kochia/Tumble mustard	CTAB	3
Rep 2	Yakima County	June-2020	Flixweed/Tumble mustard	CTAB	3
Rep 3	Grant County	Oct.-2020	Kochia/Russian thistle	CTAB	3
Colladonus reductus					
Rep 1	Yakima/Adams Counties, WA	May-2020	Kochia/Russian thistle/dandelion	CTAB/DNeasy kit	3
Rep 2	Yakima/Grant Counties, WA	May/June-2020	Kochia/dandelion	CTAB/DNeasy kit	2
Rep 3	Yakima/Grant Counties, WA	Aug./Oct.-2020	Pigweed	CTAB	3
Euscelidius variegatus					
Rep 1	Chelan County, WA	Aug.-2020	Groundcover within an organic apple orchard; mallow, clover, grasses, dandelion	CTAB	14
Circulifer tenellus					
Rep 1	Franklin County, WA	Mar.-2021	Tumble mustard	DNeasy kit	4
Rep 2	Yakima County, WA	July-2021	Kochia/Russian thistle	DNeasy kit	3
Rep 3	Elmore County, ID	July-2021	Kochia	DNeasy kit	6
Rep 4	Colony	Nov.-2021	Radish	DNeasy kit	6

[a]Indicates the number of insects pooled in the replication.

Table 2. PCR primer sets used to screen leafhoppers for bacterial endosymbionts

Target gene	Primer sequence, 5'-3'	PCR conditions	Amplicon size (bp)	Reference
CO1 gene of psyllids	LCO1490: GGT CAA CAA ATC ATA AAG ATA TTG G HCO2198: TAA ACT TCA GGG TGA CCA AAA AAT CA	35 × 94°C for 30 s, 41°C for 30 s, and 72°C for 45 s	710	
16S gene of eubacteria	27F: GAG AGT TTG ATC CTG GCT CAG 1495R: CTA CGG CTA CCT TGT TAC GA	35 × 94°C for 30s, 55°C for 30 s, and 72°C for 90 s	~1500	
wsp gene of *Wolbachia*	wsp81F: TGG TCC AAT AAG TGA TGA AGA AAC wsp691R: AAA AAT TAA ACG CTA CTC CA	40 × 95°C for 20 s, 55°C for 10 s, and 72°C for 35 s	~600	
ftsZ gene of *Wolbachia*	ftsZF1: ATY ATG GAR CAT ATA AAR GAT AG ftsZR1: TCR AGY AAT GGA TTR GAT AT	35 × 95°C for 30 s, 46°C for 30 s, and 72°C 60 s	481	
16S of '*Ca*. Sulcia'	SulF: AAA CGC TAG CGG AGG GCT TAA C SulR: CTT ACC GCG ACT GCT GGC AC	35 × 95°C for 30 s, 58°C for 30 s, and 72°C 60 s	500	Wangkeeree et al. (2012)
16S gene Rickettsia	RbF: GCT CAG AAC GAA CGC TAT C RbR: GAA GGA AAG CAT CTC TGC	34 × 95C for 30 s, 49°C for 30 s, 72°C for 30 s	800–1,000	Gottlieb et al. (2006)
Phytoplasma trifolii	z-R16R2-wfB-F: AAA TAT TTC TCG GGG TTT GTA CAC ACC GCC CGT CA BLTVA-int-wfB-R: AAT TAT CTC TGA TGA TTT TAG TAT ATA TAG TCC	20 × 95°C for 10 s, 65°C for 10 s, (touchdown, Δ-0.5°C), 72°C for 10 s, then 20 × 95°C for 10 s, 55°C for 10 s, 72°C for 10 s	213	Swisher Grimm unpublished
Phytoplasma pruni	sXd-F: GGA ATC TCC TCG CTC GCT AAC sXd-R: AAT ACC GTT TCC TAY CCC TTT AGA AG sXd-Probe: AGT GGT CGG AGC CTT CAT TAG CAT TTG G	45 × 95°C for 15 s, 60°C for 60 s		Kogej et al. (2020)
Spiroplasma citri	Scitri1-GGT CTG CTG CTT TAA TTT CTA C Scitri2-TGC AGC ACC TGC AAC TGT AG	20 × 95°C for 15 s, 65°C for 30 s (touchdown, Δ-0.5°C), 72°C for 20 s, then 20 × 95°C for 15 s, 55°C for 30 s, 72°C for 20 s	347	Crosslin unpublished
Beet curly top virus	BCTV2F-GTG GAT CAA TTT CCA GAC AAT TAT C BCTV2R-CCC ATA AGA GCC ATA TCA AAC TTC	20 × 95°C for 15 s, 65°C for 30 s (touchdown, Δ-0.5°C), 72°C for 20 s, then 20 × 95°C for 15 s, 55°C for 30 s, 72°C for 20 s	519	Stausbaugh et al. (2008)

from radish near Moxee, WA (Yakima County) and reared under laboratory conditions (~25°C with a 16:8 [L:D] hr photoperiod) on sugar beet. *Euscelidius variegatus* were collected from ground cover within an organic apple orchard near Wenatchee, WA (Chelan County) using a sweep net (Table 1). DNA was extracted using either a cetryltrimethylammonium bromide (CTAB) precipitation method (Zhang et al. 1998) or a DNeasy Blood and Tissue Kit (Qiagen) (Table 1). All leafhoppers were screened for the presence of parasitoids by Sanger sequencing the cytochrome oxidase I (COI) gene (MC Laboratories, San Francisco, CA) amplified using universal primers, LCO1490/HCO2198 (Table 2) (Folmer et al. 1994). We considered leafhoppers to be likely nonparasitized if Sanger sequences were of high quality. In contrast, leafhoppers with low quality sequences were considered to be potentially parasitized and were not included in direct sequences of 16S.

Universal 16S PCR primers 27F/1495R (Table 2) (Weisburg et al. 1991) with barcode adapter sequences were used to generate PCR amplicons for sequencing with the Oxford Nanopore MinION Mk1C sequencer [Oxford Nanopore Technologies (ONT), Oxford, United Kingdom]. For initial PCR amplification, all DNA input samples were normalized to a concentration of 50 ng/µl, and 100 ng were used as template for amplification in 50 µl with the Advantage 2 Taq polymerase system (Takara Bio Inc., Kusatsu, Japan). PCR amplicons were purified with the Qiaquick PCR Amplification Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol and barcoded with ONT barcodes from the PCR barcode kit I (EXP-PBC001). Sequencing libraries were then generated with the Ligation Sequencing Kit (SQK-LSK110) according to manufacturer's protocols. A mixture containing normalized aliquots of all samples was then loaded in a MinION Flow Cell v. R9.4.1 and directly sequenced. Each MinION sequencing replicate consisted of a pool of 2–6 individual insects depending upon availability, except for *E. variegatus* which consisted of only a single MinION replicate consisting of 14 insects (Table 1). *E. variegatus* included only a single replication because our initial focus was on *Colladonus* spp. and *Ci. tenellus*, which are known to be important Phytoplasma vectors in the Pacific Northwest. The sequencing run was terminated after 196 minutes. The 'high-accuracy basecalling' model was used, and

low-quality reads that did not pass the minimum q-score threshold of nine were excluded. Sequence reads were grouped by barcode, further filtered and trimmed using Geneious Prime, and assembled into operational taxonomic units (OTUs) with 98% sequence identity using the de novo assembler of Geneious Prime. Each OTU was analyzed using BLAST application of the NCBI sequence database (Altschul et al. 1990).

Phylogenetic relationships among endosymbionts were assessed using a Bayesian phylogenetic analysis using the MrBayes 3.2.6 application of Geneious Prime (Huelsenbeck and Ronquist 2021) using the Jukes and Cantor (JC69) substitution model carried out in four chains each for 1,000,000 generations, with subsampling every 1,000 generations and a burn-in length of 100,000. The best substitution model was selected by comparing summary statistics of various models. The purpose of these trees was to confirm identification of bacterial taxa from BLAST analysis. 16S rDNA sequences of specific endosymbionts were obtained from the NCBI database, and 16S of *Arsenophonus* endosymbiont of *Macrosteles laevis* was used as an outgroup.

Confirmation of Endosymbiont-Presence Using Conventional PCR

Conventional PCR targeting genes of *Ca.* S. muelleri (Wangkeeree et al. 2012), *Wolbachia* (Braig et al. 1998), and *Rickettsia* (Gottlieb et al. 2006) were used to confirm results of direct sequencing of 16S products (Table 2). *Colladonus geminatus* and *C. reductus* were additionally screened pruni (Kogej et al. 2020) while *Ci. tenellus* was screened for *Ca.* P. trifolii (Swisher Grimm unpublished), BCTV (Strausbaugh et al. 2008), and *Spiroplasma citri* (Crosslin unpublished) (Table 2).

Confirmation of endosymbionts using conventional PCR was conducted on all individual insects that were examined by direct sequencing of 16S rDNA (Table 1) and also additional leafhopper specimens that were captured in orchards, crop fields, and noncrop habitats throughout central WA. The number of additional specimens examined in this study depended upon availability for a given species. Additional *C. reductus* and *C. geminatus* (30 specimens per species) were collected from cherry and apple orchards near Wapato, WA and Wenatchee, WA in 2021. Additional *Ci. tenellus* specimens were captured directly from noncrop hosts using a vacuum collection device in 2020 (n = 75) or captured on sticky card traps placed in vegetable fields in 2021 (n = 367) near Eltopia, WA (45), Moses Lake, WA (N = 43), Ephrata, WA (N = 36), Paterson, WA (N = 71), Burbank, WA (N = 41), or Mattawa, WA (N = 131). These additional specimens were not screened for possible presence of parasitoids before conventional PCRs. Only a subset 67 of arbitrarily selected *Ci. tenellus* specimens were tested for '*Ca.* Sulcia' or *Rickettsia* since these were expected to expected to by 100% infected with Sulcia or 0% infected with *Rickettsia*. A subset of amplicons produced by each primer set was Sanger-sequenced to confirm target specificity (MC Laboratories). The GLIMMIX procedure of SAS 9.4 was used to compare phytoplasma-infection or BCTV infection rates among *Wolbachia*-infected and uninfected *Ci. tenellus* adults. The analyses assumed a binomial error distribution and a logit-link function, with the chi-square approximation for likelihood ratio tests.

Results

Direct Sequencing of 16S Ribosomal DNA

Direct sequencing using the minION Nanopore sequencer produced a total of 846,540 raw sequences that were initially filtered down to 675,045 sequences after quality control assessment (Table 3). These filtered sequences were assembled into an average of 124 OTUs per sample, and the OTUs were identified as at least 15 unique bacterial taxa, excluding unidentified bacteria (Table 3). Known endosymbionts of insects that were identified from 16S sequencing included *Ca.* S. muelleri, '*Candidatus* Nasuia deltocephalinicola', *Burkholderia, Rickettsia, Symbiopectobacterium purcellii,* '*Candidatus* Yamatotia cicadellidicola', and *Wolbachia* (Table 3). Several other bacteria were identified as likely contaminates, including *Cutibacterium, Lactococcus, Massilia, Ralstonia* (also present in blank controls), and *Sphingomonas* (Table 3).

The obligate endosymbiont of leafhoppers, *Ca.* Sulcia, was detected in all pooled replications of all four leafhopper species (Table 3). Sequences putatively identified as Sulcia by BLAST analysis fell within a well-defined clade that included Sulcia of Deltocephalinae leafhoppers (Fig. 1).

Ca. Nasuia deltocephalinicola, an obligate endosymbiont associated with Deltocephalinae, was detected in just one replication of *C. geminatus* and in *E. variegatus,* but not in other pooled samples (Table 3). These sequences identified as Nasuia of *C. geminatus* and *E. variegatus* fell within clades that included Nasuia of other leafhoppers, confirming initial identification (Fig. 2). Phylogenetic analyses of 16S sequences of Nasuia obtained from NCBI revealed substantial sequence variation among Nasuia from different leafhopper hosts, but also revealed that phylogeny of Nasuia 16S gene largely tracks broad-scale phylogeny (based on tribes of Deltocephalinae) of the leafhopper hosts (Fig. 2).

Rickettsia occurs as insect endosymbionts or as free-living environmental bacteria. Several variants of 16S of *Rickettsia* were identified from *C. geminatus, C. reductus*, and *Ci. tenellus* (Table 3). One 16S variant was detected in all three pooled replications of *C. geminatus,* while other variants were not consistently detected in other leafhopper hosts. Phylogenetic analyses aligned the 16S sequences of *Rickettsia* of *C. geminatus* with endosymbiotic *Rickettsia* strains from other insects (Fig. 3). Different 16S variants of *Rickettsia* were identified from *C. reductus* and *Ci. tenellus,* but both sequences appeared to align more closely with *Rickettsia* isolated from environmental samples (Fig. 3).

Other known endosymbionts were inconsistently detected in pooled replications of various leafhopper species. *Burkholderia* sequences were detected in very low numbers in just two replications of *C. geminatus* (Table 3). *Ca.* Yamatotia cicadellidicola and *Symbiopectobacterium purcellii* was detected in *E. variegatus* only. *Wolbachia* sequences were abundant in *Ci. tenellus but* were detected in just two of the four replications of this leafhopper host (Table 3).

Confirmation of Endosymbiont-Presence Using Conventional PCR

Sulcia was confirmed to be present in 100% of leafhoppers using Sulcia-specific conventional PCR (Table 4). *Rickettsia* was detected in >56% of *C. geminatus* using species-specific conventional PCR (Table 4). Detection of *Rickettsia* in *C. geminatus* confirms 16S sequencing results suggesting that this insect harbors endosymbiotic *Rickettsia.*

Rickettsia and *Wolbachia* were both detected using species-specific conventional PCR from 2 specimens of *C. reductus* (Table 4). One of these specimens was co-infected with both bacteria. CO1 of insects, along with 16S of *Rickettsia* or the *wsp* gene of *Wolbachia* were PCR-amplified and sequenced from these *C. reductus* specimens. *Rickettsia* sequences obtained from the two *C. reductus* specimens varied from each other by 4% (34 base pair differences) but were confirmed to align with other *Rickettsia* sequences obtained from

Table 3. Bacteria identified from assembled reads produced by direct sequencing of 16S rDNA amplified by universal PCR primers from leafhopper vectors of Phytoplasma listed in Table 1. Values refer to the total number of sequence reads identified as each bacterial taxa

	Colladonus geminatus			*Colladonus reductus*			*Euscelidius variegatus*	*Circulifer tenellus*			
Bacteria Taxa	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3		Rep 1	Rep 2	Rep 3	Rep 4
Known Endosymbionts											
Burkholderia	2	–	2	–	–	–	–	–	–	–	–
Ca. Sulcia muelleri	1	11	711	7,774	1,371	5,751	8,283	4,031	10,419	7,380	10,088
Ca. Nasuia deltocephalinicola[a]	–	–	4	–	–	–	3,007	–	–	–	–
Ca. Yamatotia cicadellidicola	–	–	–	–	–	–	46	–	–	–	–
Rickettsia 16S var. 1	10,182	10,085	7,393	–	–	–	–	–	–	–	–
Rickettsia 16S var. 2	–	–	–	–	5,886	–	–	–	–	–	–
Rickettsia 16S var. 3	–	–	–	–	–	–	–	–	–	4	595
Spiroplasma citri								–	12	–	–
Symbiopectobacterium purcellii	0	0	0	0	0	0	2				
Wolbachia	–	–	–	–	–	–	–	4,908	–	2,554	–
Other Bacteria											
Cutibacterium sp.	–	–	–	4	–	9	–	–	25	–	–
Lactococcus sp.	–	–	–	–	–	4	–	–	–	–	–
Massilia sp.	2	–	–	–	–	–	–	2	–	–	–
Ralstonia insidiosa	771	1,586	844	2,889	221	1,641	13	578	170	50	41
Sphingomonas sp.	–	–	6	–	–	–	–	–	–	–	–
Unidentified	21	14	38	2	–	2	–	2	–	4	–
Total No. Sequences	5,9102	64,790	54,407	68,547	59,547	53,074	72,925	54,759	63,968	60,238	63,688
Filtered/trimmed reads	28,761	30,104	26,421	32,694	26,215	23,307	37,400	26,480	33,755	29,863	31,400
Assembled Reads	10,977	11,696	8,998	10,669	7,478	7,407	11,351	9,521	10,626	9,992	10,724

[a]Nausia deltocephalinicola was not well amplified using universal primers for 16S rDNA, likely due to primer mismatches.

the NCBI database. In fact, 16S of *Rickettsia* obtained from one *C. reductus* specimen was 100% identical to those obtained from *C. geminatus*. Sequences of *wsp* genes also confirmed presence of *Wolbachia* in both samples, but these two sequences shared only 76% identity with each other, and <90% identity to *wsp* from *Ci. tenellus*. CO1 sequences suggested that all three *C. reductus* specimens were parasitized. The specimen harboring both *Rickettsia* and *Wolbachia* was likely parasitized by *Tomosvaryella* sp. (Diptera; Pipunculidae) based on the CO1 sequence. The specimen harboring *Rickettisia* was likely parasitized by an unidentified Hymenoptera. CO1 sequences from the remaining *C. reductus* specimen that harbored *Wolbachia* were too low quality to analyze, suggesting that it too was parasitized. It is likely that *Rickettsia* and *Wolbachia* detected in these three *C. reductus* were endosymbionts of the parasitoids.

Wolbachia was detected by species-specific conventional PCR (*wsp* gene) in just 13% of *Ci. tenellus* adults (Table 4). The sex of 74 *Ci. tenellus* collected directly from noncrop hosts was determined before DNA extraction, allowing us to investigate sex ratio between *Wolbachia*-infected and uninfected insects. The overall population was female biased, with 50 females and 24 males. The sex ratio of *Wolbachia*-uninfected *Ci. tenellus* was nearly 1:1, but 100% of *Wolbachia*-infected adults were female (Fig. 4A).

Overall, about 25% of *Ci. tenellus* captured in vegetable fields harbored either *Ca*. P. trifolii or BCTV, while <2% harbored *S. citri* (Table 4). We did not observe significant differences in the proportion of *Wolbachia*-infected or -uninfected adults carrying BCTV (X^2 = 0.01; df = 1; P = 0.962; Fig. 4B), but significantly more *Wolbachia*-infected than uninfected *Ci. tenellus* harbored *Ca*. P. trifolii (X^2 = 5.4; df = 1; P = 0.021; Fig. 4C)

Discussion

We used high-throughput sequencing of the 16S rDNA gene of eubacteria to identify endosymbionts in leafhopper vectors of *Ca*. P. pruni (*C. geminatus, C. reductus*, and *E. variegatus*) or *Ca*. P. trifolii (*Ci. tenellus*), then used species-specific conventional PCR to examine infection rates of several known endosymbionts. All leafhoppers harbored *Ca*. Sulcia, as expected. *Colladonus geminatus* also harbored *Burkholderia, Rickettsia* and *Ca*. Nasuia. We did not detect presence of facultative endosymbionts of *C. reductus* using our methods. The endosymbionts *Ca*. Nasuia *and Ca*. Yamatotia were both detected in *E. variegatus*, and *Wolbachia* was detected in many specimens of *Ci. tenellus*. Other bacteria identified from 16S sequencing were considered contaminates, and were excluded from further analysis and discussion.

Obligate Endosymbionts '*Ca*. S. muelleri' and '*Ca*. N. deltocephalinicola'

Most leafhoppers harbor at least two obligate endosymbionts. *Ca*. S. muelleri is found in all Auchenorrhyncha and produces at least 8 of the 10 essential amino acids that are lacking in the leafhoppers' diet (Moran et al. 2005, McCutcheon et al. 2009). *Ca*. N. deltocephalinicola is a second obligate endosymbiont associated with leafhoppers in the subfamily Deltocephalinae that synthesizes the essential amino acids that are not produced by *Ca*. S. muelleri (Bennett and Moran 2013). Thus, Sulcia and Nasuia are capable of synthesizing all 10 essential amino acids, as observed for other pairs of obligate endosymbionts occurring in other groups of Auchenorrhyncha (McCutcheon et al. 2009, Bennett and Moran 2013).

Ca. N. deltocephalinicola was detected from just a single pooled replication of *C. geminatus* and from *E. variegatus*. This bacterium was not detected in the other leafhopper species by direct sequencing of 16S. *Ca*. N. deltocephalinicola has previously been detected in several other species of Deltocephalinae including *Nephotettix cincticeps, Matsumuratettix hiroglyphicus*, and *Macrosteles striifons* (Noda et al. 2012, Wangkeeree et al. 2012, Ishii et al. 2013). Ishii

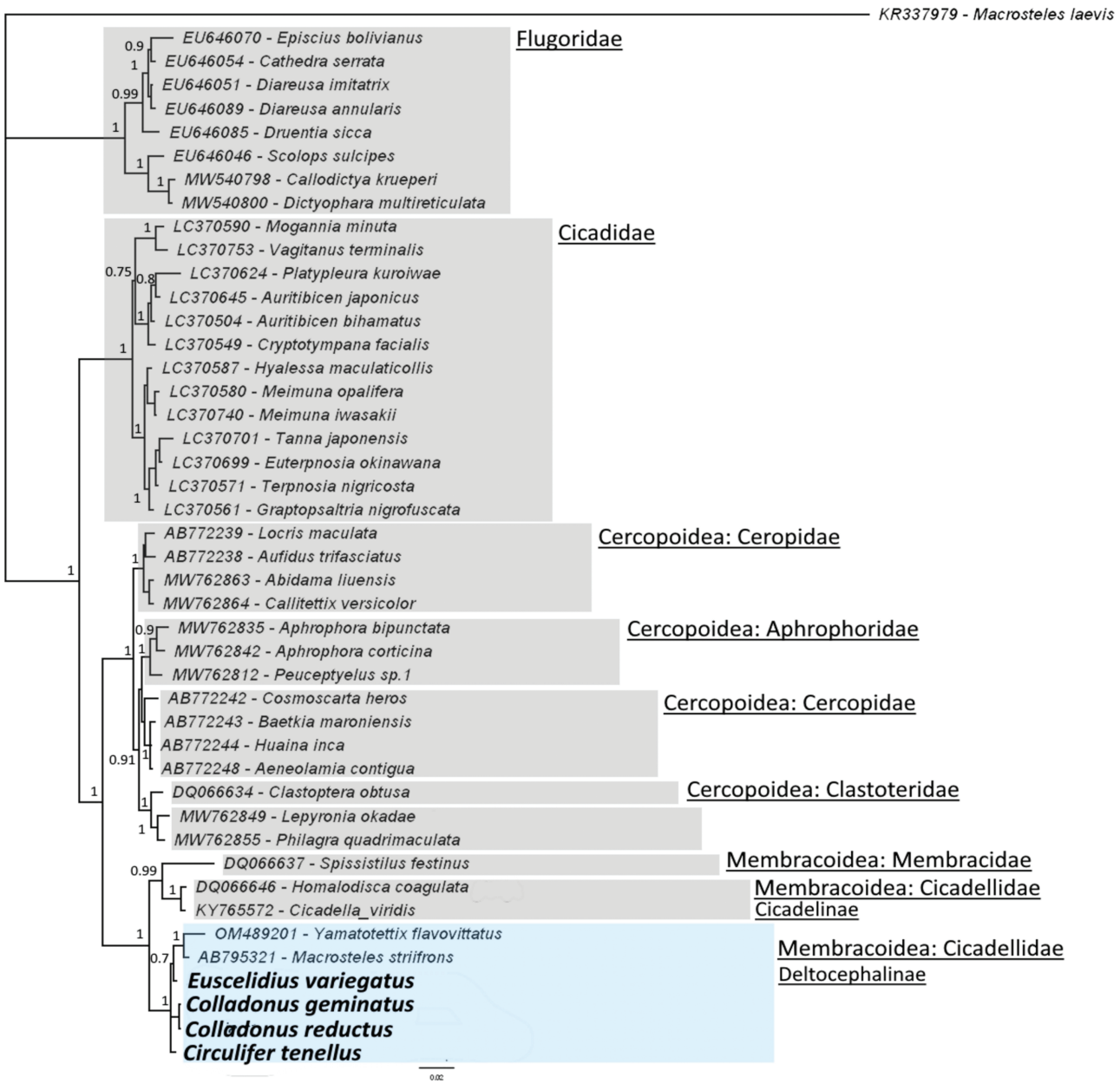

Fig. 1. Phylogenetic relationships among 16S sequences identified as Sulcia from *Colladonus geminatus*, *C. reductus*, *Euscelidius variegatus*, and *Circulifer tenellus* (large bold print) and from other Hemiptera obtained from the NCBI database. Values denote posterior probabilities. Arsenophonus of *Macrosteles laevis* was included as an outgroup.

et al. (2013) found extensive primer mismatches and low PCR efficiency in 16S of Nasuia from *Macrosteles*, perhaps due to high AT-rich genes found in this bacterium. *Ca.* N. deltocephalinicola was likely present yet undetected in the remaining two replications of *C. geminatus*, and perhaps also present in *C. reductus* and *Ci. tenellus*.

Rickettsia spp.

Rickettsia includes many transovarially-transmitted symbionts of various insects, and can also occur as free-living bacteria (Perlman et al. 2006). Endosymbiotic *Rickettsia* can alter the biology and behavior of their insect hosts, including their susceptibility to certain insecticides (Ghanim and Kontsedalov 2009), production of male offspring (Weinert et al. 2009), and adaptation to temperature extremes (Maes et al. 2012). Results of our study suggest that over 50% of *C. geminatus* harbored endosymbiotic *Rickettsia*. We were unable to examine for patterns between infection with *Rickettsia* and *Ca.* Phytoplasma pruni due to low sample size and low Phytoplasma infection rates. Further research is required to examine the potential biological effects of *Rickettsia* infection of *C. geminatus*.

Interspecific plant-mediated transmission of *Rickettsia* appears to occur frequently when insects feed on host plants in proximity (Weinert et al. 2009, Caspi-Fluger et al. 2012). Both *C. geminatus* and *C. reductus* co-occur on various broadleaf hosts including dandelion (*Taraxacum* spp.; Asterales: Asteraceae) and mallow (*Malva neglecta* Wallr.; Malvales: Malvaceae) (Clarke 2021, Cooper et al. 2022), providing the opportunity for plant-mediated transmission of *Rickettsia* between these two species. *Rickettsia* sequences from *C. geminatus* and one specimen of *C. reductus* were 100% identical, but it remains unknown whether these data suggest plant-mediated transmission occured.

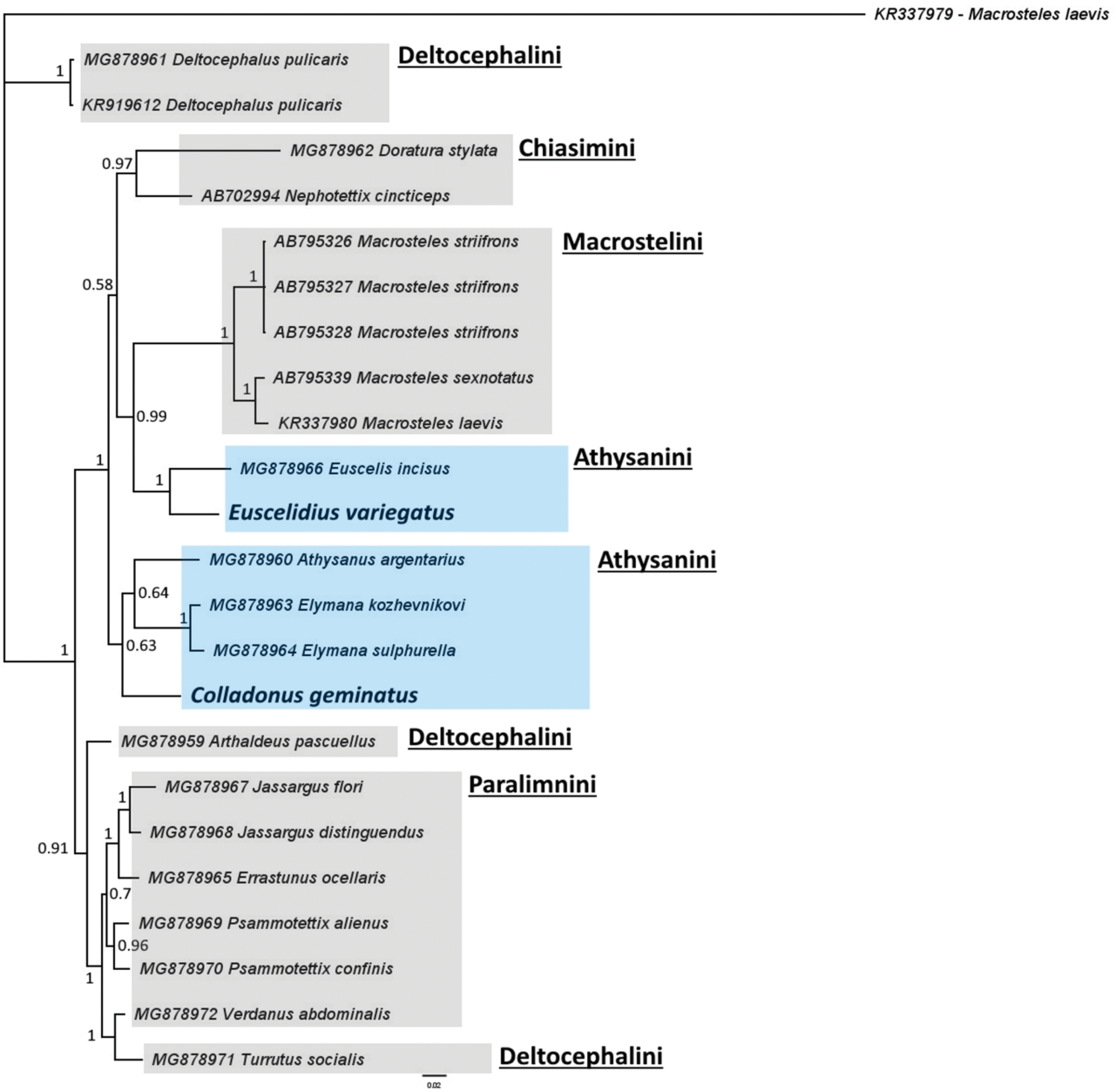

Fig. 2. Phylogenetic relationships among 16S sequences identified as Nasuia from *Colladonus geminatus* and *Euscelidius variegatus,* (large bold print) and from other Hemiptera obtained from the NCBI database. Tribes are labels in the right column. Values denote posterior probabilities. Arsenophonus of *Macrosteles laevis* was included as an outgroup.

Burkholderia Endosymbiont of *C. geminatus*

Sequences identified as *Burkholderia* were detected in very low abundance from two replications of *C. geminatus*. *Burkholderia* is a highly diverse group of bacteria that is widespread in highly variable environments and habitats. Some strains of *Burkholderia* are symbiotic, and provide benefits to insect hosts, including nutrition and resistance against insecticides and pathogenic fungi (Kaltenpoth and Florez 2020). *Burkholderia* is unique among insect symbionts because they are acquired and transmitted by multiple modes, including acquisition from environmental sources (Kaltenpoth and Florez 2020). Stink bugs (Hemiptera: Pentatomidae) acquire *Burkholderia* symbionts *de novo* from environmental sources every host generation (Kikuchi et al. 2007, Olivier-Espejel et al. 2011, Xu et al. 2016). *Burkholderia* has been found in several populations of the leafhopper species, *Macrosteles striifrons* (Ishii et al. 2013), but the impact of *Burkholderia* on leafhopper fitness remains unknown. Further work is required to validate whether *Burkholderia* detected from *C. geminatus* is indeed symbiotic in origin or is environmental contamination.

Symbiopectobacterium purcellii Endosymbiont of *E. variegatus*

The endosymbiont *Symbiopectobacterium purcellii* (Enterobacteriaceae) was detected from *E. variegatus*, albeit from just two sequence reads. *Symbiopectobacterium* is closely related to *Pectobacterium*, a bacterial group that includes many insect-vectored plant pathogens (Martinson et al. 2020). This group is diverse and widespread, and includes at least four independently evolved symbioses in nematodes and in Hemiptera (Kuechler et al. 2011, 2012; Husnik and McCutcheon 2016; Martinson et al.

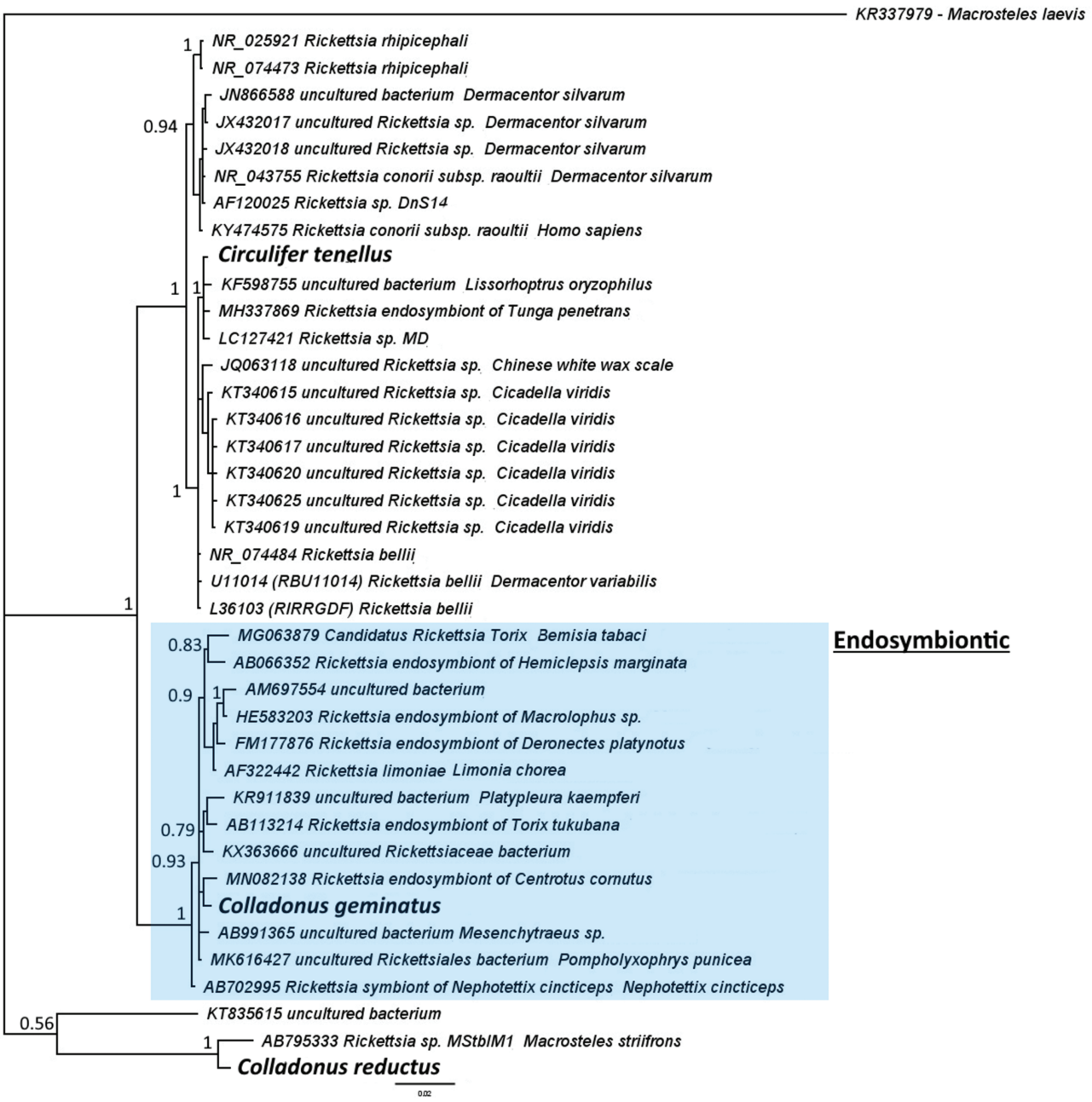

Fig. 3. Phylogenetic relationships among 16S sequences of ancestral and endosymbiotic Rickettsia obtained from NCBI and sequences identified as Rickettsia from *Colladonus geminatus, C. reductus,* and *Circulifer tenellus* (large bold print). Values denote posterior probabilities.

Table 4. Proportion of insects harboring bacterial endosymbionts detected using species-specific conventional PCR

Leafhopper vector[a]	Sulcia	*Rickettsia*	*Wolbachia*	Phytoplasma	BCTV	*Spiroplasma*
Colladonus geminatus	39/39	22/39	0/39	7/39	–	–
Colladonus reductus	38/38	2/38[c]	2/38[c]	5/38	–	–
Euscelidius variegatus	18/18	0/18	0/18	0/18	–	–
Circulifer tenellus	67/67[b]	0/67[b]	61/460	104/460	124/460	8/460

[a]Specimens examined by specific-specific conventional PCR included those detailed in Table 1 and additional leafhoppers captured from crops, orchards, and noncrop hosts in central Washington.

[b]Only a subset of *Ci. tenellus* specimens were examined for presence of Sulcia or Rickettsia.

[c]One *C. reductus* specimen was co-infected with both *Rickettsia* and *Wolbachia*, the other two were infected with either *Rickettsia* or *Wolbachia*. CO1 sequences obtained from these specimens suggested that all three were parasitized.

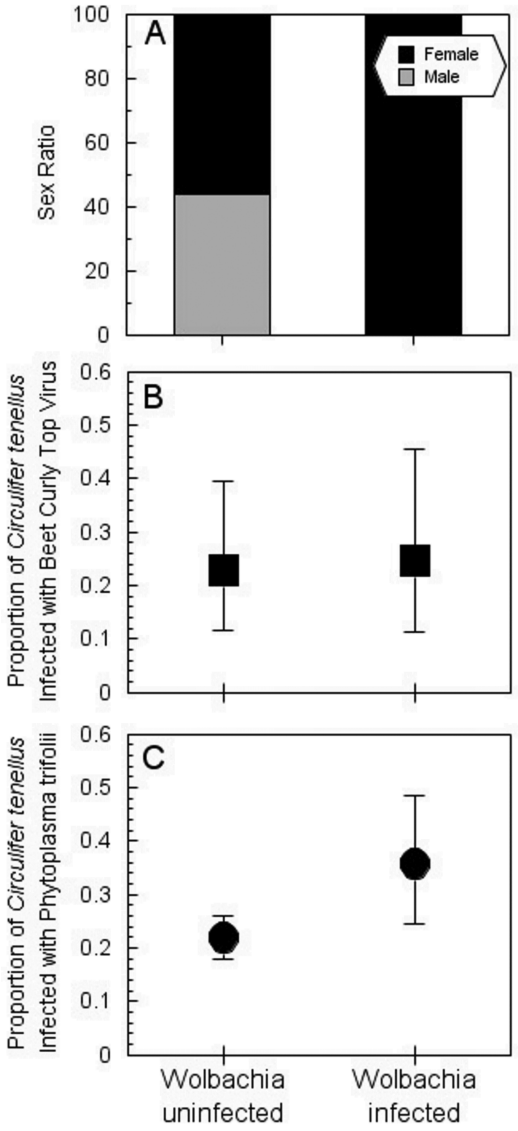

Fig. 4. Sex ratio of Wolbachia-infected and -uninfected *Ci. tenellus* (A), proportion of Wolbachia-infected and -uninfected *Ci. tenellus* adults harboring either Beet curly top virus (B) or '*Candidatus* Phytoplasma trifolii' (C).

2020; Nadal-Jimenez et al. 2022) including *E. variegatus* (Purcell et al. 1986). In fact, *Symbiopectobacterium* (referred to originally as 'Bacterium of *Euscelidius variegatus*'), was an early model for evolution of insect symbiosis from free-living bacteria (Purcell et al. 1986, Degnan et al. 2011). *Symbiopectobacterium* reduces fitness of other leafhoppers when transferred from *E. variegatus* (Purcell et al. 1986, Purcell and Suslow 1987, Cheung and Purcell 1999). The bacterium was also initially harmful to *E. variegatus*, but those effects have diminished over time under laboratory conditions (Purcell and Suslow 1987, Degnan et al. 2011). This bacterium is often transovarially transmitted, but can spread by plant-mediated transmission (Purcell and Suslow 1987, Purcell et al. 1994)

Ca. Yamatotia cicadellidicola Endosymbiont of *E. variegatus*

Sequences that aligned with *Ca.* Y. cicadellidicola were also identified from *E. variegatus* but not from other leafhopper species. This recently discovered bacterium has thus far only been found in the Old World leafhopper *Yamatotettix flavovittatus* Matsumura (Cicadellidae: Deltocephalinae), which is a vector of the phytoplasma that causes white leaf disease in sugarcane (Wangkeeree et al. 2019). This bacterium inhabits the bacteriome of *Y. flavovittatus*, is present throughout the entire life cycle of the host, and occurred in all populations of *Y. flavovittatus* that were surveyed (Wangkeeree et al. 2019). *Euscelidius variegatus* is the second leafhopper host shown to harbor this bacteriome-inhabiting endosymbiont.

Wolbachia Endosymbiont of *Ci. tenellus*

Wolbachia is well known for causing reproductive manipulations in insect populations, including cytoplasmic incompatibility, male-killing, and feminization (Stouthamer et al. 1999). The most documented manipulation in insects is cytoplasmic incompatibility resulting in production of inviable eggs by uninfected females mated by *Wolbachia*-infected males. Because infected females gain a reproductive advantage from cytoplasmic-incompatibility-inducing *Wolbachia*, this endosymbiont spreads quickly to infect nearly all individuals within a population. At just 13%, *Wolbachia* infection rates in *Ci. tenellus* were lower than expected if this strain were causing cytoplasmic incompatibility. The total absence of *Wolbachia* in males suggests that *Ci. tenellus* harbors a sex-ratio-distorting *Wolbachia* strain, which would be consistent with the low overall rate of *Wolbachia* infection in *Ci. tenellus* (Bouchon et al. 1998, Hurst and Jiggins 2000). Work is currently underway to investigate whether *Wolbachia* causes reproductive manipulations in *Ci. tenellus*.

Wolbachia has previously been shown to alter the acquisition and transmission of plant and animal pathogens by various insect vectors. A greater proportion *of Wolbachia*-infected versus uninfected *Ci. tenellus* harbored Phytoplamsa trifolii, suggesting that *Wolbachia* may increase the vector's ability to tolerate or acquire this pathogen. While previous studies have suggested that *Wolbachia* alters vector competency, results of those studies have been inconsistent; some studies suggest that *Wolbachia* reduces vector competence (Hedges et al. 2008, Moreira et al. 2009, Blagrove et al. 2012, Krstic et al. 2018, Gong et al. 2020, Schuler et al. 2022) while others suggest that *Wolbachia* aids pathogen acquisition or transmission (Fagen et al. 2012, Cooper et al. 2023). A *Wolbachia* strain harbored by *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) produces a protein that suppresses phage lytic cycle genes of the plant pathogen, *Candidatus* Liberibacter citri, thus suppressing systemic innate immune response from psyllid vector (Jain et al. 2017). This suppression of immune responses in the psyllid host increases the survival of both bacterial species (Jain et al. 2017). It is possible that a similar interaction reported by Jain et al. (2017) occurs between *Wolbachia* and *Ca.* P. trifolii in *Ci. tenellus*. More targeted research is needed to explore this association.

Conclusions

The research findings presented here provide a foundation for additional research on interactions among leafhoppers and bacterial endosymbionts, including phytoplasmas. Further research is needed to explore the relationships between *Rickettsia*, *C. geminatus*, and other community associates. *Colladonus* are attacked by the parasitoid *Tomosvaryella lepidipes* (Diptera: Pipunculidae) and *Ufens niger* (Ashm.) (Hymenoptera: Trichogrammatidae) (Kaloostian 1953, 1955), but we did not investigate for correlations between *Rickettsia* and parasitism rates. Another area in need of addition research is the potential relationship between *Wolbachia* and phytoplasma infection of *Ci. tenellus*. We observed a positive correlation between *Wolbachia* and *Ca.* P. trifolii infections, but further

work is needed to confirm this result and to explore the molecular underpinnings for this association. Our report demonstrates that *E. variegatus* harbors *Ca*. Y. cicadellidicola, becoming only the second species known to harbor this symbiont (Wangkeeree et al. 2019). How widespread this endosymbiont occurs among cicadellids, and its impact on leafhopper biology remain unknown.

Acknowledgments

Pauline Anderson, Rachel Cook, Chris Gorman, Millie Heidt, Jennifer Stout, and Heather Headrick provided technical assistance.

Authors Contributions

William Cooper (Conceptualization-Equal, Formal analysis-Lead, Investigation-Equal, Methodology-Equal, Project administration-Equal, Resources-Equal, Supervision-Equal, Writing – original draft-Lead), Willam Walker (Conceptualization-Equal, Formal analysis-Supporting, Methodology-Equal, Supervision-Equal, Writing – original draft-Supporting), Gina Angelella (Conceptualization-Equal, Formal analysis-Equal, Methodology-Supporting, Writing – original draft-Supporting), Kylie Swisher Grimm (Methodology-Equal, Resources-Equal, Writing – review & editing-Supporting), Jillian Foutz (Investigation-Supporting, Resources-Equal), Scott Harper (Methodology-Supporting, Resources-Supporting, Writing – review and editing-Supporting), Louis Nottingham (Resources-Supporting, Writing – review and editing-Supporting), Tobin D. Northfield (Resources-Supporting, Writing – review and editing-Supporting), Carrie Wohleb (Resources-Supporting, Writing – review and editing-Supporting), Carl Strausbaugh (Resources-Supporting, Writing – review and editing-Supporting)

References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol*. 215: 403–410.

Bennett, G. M., and N. A. Moran. 2013. Small, smaller, smallest: The origins and evolution of ancient dual symbiosis in a phloem-feeding insect. *Gen. Biol. Evol*. 5: 1675–1688.

Blagrove, M. S. C., C. Arias-Goeta, A. B. Failloux, and S. P. Sinkins. 2012. Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. *Proc. Natl Acad. Sci. USA*. 109: 255–260.

Bouchon, D., T. Rigaud, and P. Juchault. 1998. Evidence for wide-spread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. B-Biol. Sci*. 265: 1081–1090.

Bove, J. M., I. Renaudin, C. Saillard, X. Foissac, and M. Garnier. 2003. *Sprioplasma citri*, a plant pathogenic mollicute; relationships with its two hosts, the plant and the leafhopper vector. *Annu. Rev. Phytopathol*. 41: 483–500.

Braig, H. R., W. G. Zhou, S. L. Dobson, and S. L. O'Neill. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *J. Bacteriol*. 180: 2373–2378.

Caspi-Fluger, A., M. Inbar, N. Mozes-Daube, N. Katzir, V. Portnoy, E. Belausov, M. S. Hunter, and E. Zchori-Fein. 2012. Horizontal transmission of the insect symbiont Rickettsia is plant-mediated. *Proc. R. Soc. B Biol. Sci*. 279: 1791–1796.

Chen, L. F., and R. L. Gilbertson. 2016. Transmission of Cutoviruses *(beet curly top virus)* by the beet leafhopper *(Circulifer tenellus)*, pp. 463. *In* J. K. Brown (ed.), *Vector-mediated transmission of plant pathogens*. The American Phytopathological Society, St. Paul, MN.

Cheung, W. W. K., and A. H. Purcell. 1999. Invasion of bacteroids and BEV bacterium into oocytes of the leafhopper *Euscelidius variegatus* Kirschbaum (Homoptera: Cicadellidae): An electron microscopic study. *Zool. Stud*. 38: 69–75.

Clarke, A. E. 2021. *Host use and trapping of X-disease vector Colladonus montanus (Van Duzee) (Hemiptera; Cicadellidae)*. Master of Science, Washington State University Pullman, WA.

Cooper, W. R., K. D. Swisher Grimm, G. M. Angelella, and T. Mustafa. 2023. Acquisition and transmission of '*Candidatus* Liberibacter solanacearum' differs among *Wolbachia*-infected and -uninfected haplotypes of *Bactericera cockerelli*. *Plant Dis*. in press. doi:10.1094/PDIS-11-22-2701-RE

Cooper, W. R., A. Marshall, J. Foutz, M. R. Wildung, T. Northfield, D. W. Crowder, H. Leach, T. Leskey, S. E. Halbert, and J. Snyder. 2022. Directed sequencing of plant specific DNA identifies the dietary history of four species of Auchenorrhyncha (Hemiptera). *Ann. Entomol. Soc. Am*. 115: 275–284.

Crosslin, J. M., J. E. Munyaneza, A. Jensen, and P. B. Hamm. 2005. Association of beet leafhopper (Hemiptera: Cicadellidae) with a clover proliferation group phytoplasma in Columbia basin of Washington and Oregon. *J. Econ. Entomol*. 98: 279–283.

Davis, M. J., Z. Ying, B. R. Brunner, A. Pantoja, and F. H. Ferwerda. 1998. Rickettsial relative associated with papaya bunchy top disease. *Curr. Microbiol*. 36: 80–84.

Davis, R. E., Y. Zhao, E. L. Dally, I. M. Lee, R. Jomantiene, and S. M. Douglas. 2013. '*Candidatus* Phytoplasma pruni', a novel taxon associated with X-disease of stone fruits, Prunus spp.: multilocus characterization based on 16S rRNA, secY, and ribosomal protein genes. *Int. J. Syst. Evol. Microbiol*. 63: 766–776.

Degnan, P. H., L. S. Bittleston, A. K. Hansen, Z. L. Sabree, N. A. Moran, and R. P. P. Almeida. 2011. Origin and examination of a leafhopper facultative endosymbiont. *Curr. Microbiol*. 62: 1565–1572.

Fagen, J. R., A. Giongo, C. T. Brown, A. G. Davis-Richardson, K. A. Gano, and E. W. Triplett. 2012. Characterization of the relative abundance of the citrus pathogen *Ca*. Liberibacter asiaticus in the microbiome of its insect vector, *Diaphornia citri*, using high throughput 16S rRNA sequencing. *Open Microbiol. J*. 6: 29–33.

Ferrater, J. B., P. W. de Jong, M. Dicke, Y. H. Chen, and F. G. Horgan. 2013. Symbiont-mediated adaptation by planthoppers and leafhoppers to resistant rice varieties. *Arthropod-Plant Interact*. 7: 591–605.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*. 3: 294–299.

Gellato, L., M. Nardi, P. Saracco, A. Bressan, C. Marzachi, and D. Bosco. 2009. Varation in vector competency depends on chrysanthemum yellows phytoplasma distribution within *Euscelidius variegatus*. *Entomol. Exp. Appl*. 131: 200–207.

Ghanim, M., and S. Kontsedalov. 2009. Susceptibility to insecticides in the Q biotype of *Bemisia tabaci* is correlated with bacterial symbiont densities. *Pest Manag. Sci*. 65: 939–942.

Gonella, E., E. Crotti, L. Mandelco, D. Daffonchio, and A. Alma. 2018. *Asaia* symbionts interfere with infection by Flavescence doree phytoplasma in leafhoppers. *J. Pest Sci*. 91: 1033–1046.

Gong, J. T., Y. J. Li, T. P. Li, Y. K. Liang, L. C. Hu, D. J. Zhang, C. Y. Zhou, C. Yang, X. Zhang, S. S. Zha, *et al*. 2020. Stable introduction of plant-virus-inhibiting Wolbachia into planthoppers for rice protection. *Curr. Biol*. 30: 4837–+.

Gottlieb, Y., M. Ghanim, E. Chiel, D. Gerling, V. Portnoy, S. Steinberg, G. Tzuri, A. R. Horowitz, E. Belausov, N. Mozes-Daube, *et al*. 2006. Identification and localization of a *Rickettsia* sp in *Bemisia tabaci* (Homoptera: Aleyrodidae). *Appl. Environ. Microb*. 72: 3646–3652.

Hedges, L. M., J. C. Brownlie, S. L. O'Neill, and K. N. Johnson. 2008. Wolbachia and virus protection in insects. *Science*. 322: 702–702.

Huelsenbeck, J. P., and F. Ronquist. 2021. MRBAYES: Bayesian inference of phylgenetic trees. *Bioinformatics*. 17: 754–755.

Hurst, G. D. D., and F. M. Jiggins. 2000. Male-killing bacteria in insects: Mechanisms, incidence, and implications. *Emerg. Infect. Dis*. 6: 329–336.

Husnik, F., and J. P. McCutcheon. 2016. Repeated replacement of an intrabacterial symbiont in the tripartite nested melay bug symbiosis. *Proc. Natl. Acad. Sci. U. S. A*. 113: E5416–E5424.

Ishii, Y., Y. Matsuura, S. Kakizawa, N. Nikoh, and T. Fukatsu. 2013. Diversity of bacterial endosymbionts associated with *Macrosteles* leafhoppers

vectoring phytopathogenic phytoplasmas. *Appl. Environ. Microbiol*. 79: 5013–5022.

Jain, M., L. A. Fleites, and D. W. Gabriel. 2017. A small *Wolbachia* protein directly represses phage lytic cycle genes in '*Candidatus* Liberibacter asiaticus' within psyllids. *Msphere*. 2: e00171–e00117.

Jensen, D. D. 1969. Comparative transmission of western x-disease virus by *Colladonus montanus*, *C. geminatus* and a new leafhopper vector, *Euselidius variegatus*. *J. Econ. Entomol*. 62: 1147–1150.

Kaloostian, G. H. 1953. *Tomosvaryella lepidipes* – a parasite of geminate leafhopper. *J. Econ. Entomol*. 46: 1098–1099.

Kaloostian, G. H. 1955. *Ufens niger* (Ashm), an egg parasite of the geminate leafhopper. *J. Econ. Entomol*. 49: 140.

Kaltenpoth, M., and L. V. Florez. 2020. Versatile and dynamic symbioses between insects and *Burkholderia* bacteria. *Annu. Rev. Entomol*. 65: 7.1–7.26.

Kambris, Z., P. E. Cook, H. K. Phuc, and S. P. Sinkins. 2009. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science*. 326: 134–136.

Kikuchi, Y., T. Hosokawa, and T. Fukatsu. 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl. Environ. Microbiol*. 73: 4308–4316.

Kogej, Z., M. Dermastia, and N. Mehle. 2020. Development and validation of a new TaqMan real-time PCR for detection of '*Candidatus* Phytoplasma pruni'. *Pathogens*. 9: 642.

Krstic, O., T. Cvrkovic, M. Mitrovic, S. Radonjic, S. Hrncic, and I. Tosevski. 2018. Wolbachia infection in natural popualtiosn of *Dictyophara europaea*, an alternative vector of graevine Flavesence doree phytoplasma: effects and interactions. *Ann. Appl. Biol*. 172: 47–64.

Kuechler, S. M., K. Dettner, and S. Kehl. 2011. Characterization of an obligate intracellular bacterium in the midgut epithelium of the bulrush bug *Chilacis typhae* (Heteroptera, Lygaeidae, Artheneinae). *Appl. Environ. Microbiol*. 77: 2869–2876.

Kuechler, S. M., P. Renz, K. Dettner, and S. Kehl. 2012. Diversity of symbiotic organs and bacterial endosymbionts of lygaeoid bugs of the families blissidae and lygaeidae (Hemiptera: Heteroptera: Lygaeoidea). *Appl. Environ. Microbiol*. 78: 2648–2659.

Li, Y. T., X. D. Liu, and H. F. Guo. 2018. Variations in endosymbiont infection between buprofezin-resistant and susceptible strains of *Laodelphax striatellus* (Fallen). *Curr. Microbiol*. 75: 709–715.

Liu, H. Y., D. J. Gumpf, G. N. Oldfield, and E. C. Calavan. 1983. The relationship of *Spiroplasma citri* and *Circulifer tenellus*. *Phytopathology*. 73: 585–590.

Maes, S., T. Machtelinckx, M. Moens, J. C. Gregoire, and P. De Clercq. 2012. The influence of acclimation, endosymbionts and diet on the supercooling capacity of the predatory bug *Macrolophus pygmaeus*. *Biocontrol*. 57: 643–651.

Martinson, V. G., R. M. R. Gawryluk, B. E. Gowen, and S. J. Perlman. 2020. Multiple origins of obligate nematode and insect symbionts by a clade of bacteria closely related to plant pathogens. *Proc. Natl. Acad. Sci. U. S. A*. 117: 31979–31986.

McCutcheon, J. P., B. R. McDonald, and N. A. Moran. 2009. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci. U. S. A*. 106: 15394–15399.

Mitsuhashi, W., T. Saiki, W. Wei, H. Kawakita, and M. Sato. 2002. Two novel strains of *Woblachia* coexisting in both species of mulberry learhoppers. *Insect. Mol. Biol*. 11: 577–584.

Moran, N. A., P. Tran, and N. M. Gerardo. 2005. Symbiosis and insect diversification: An ancient symbiont of sap-feeding insects from the bacterial Phylum *Bacteroidetes*. *Appl. Environ. Microbiol*. 71: 99028810.

Moreira, L. A., I. Iturbe-Ormaetxe, J. A. Jeffery, G. J. Lu, A. T. Pyke, L. M. Hedges, B. C. Rocha, S. Hall-Mendelin, A. Day, M. Riegler, *et al*. 2009. A Wolbachia symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. *Cell*. 139: 1268–1278.

Munyaneza, J. E., J. M. Crosslin, and J. E. Upton. 2006. Beet leafhopper (Hemiptera: Cicadellidae) transmits the Columbia basin potato purple top phytoplasma to potatoes, beets, and weeds. *J. Econ. Entomol*. 99: 268–272.

Munyaneza, J. E., J. M. Crosslin, J. E. Upton, and J. L. Buchman. 2010. Incidence of the beet leafhopper-transmitted virescence agent phytoplasma in local populations of the beet leafhopper, *Circulifer tenellus*, in Washington State. *J. Insect Sci*. 10: 18.

Nadal-Jimenez, P., S. Siozios, N. Halliday, M. Camara, and G. D. D. Hurst. 2022. *Symbiopectobacterium purcellii*, gen. nov., sp. nov., isolated from the leafhopper *Empoasca decipiens*. *Int. J. Syst. Evol. Microbiol*. 72: 005440.

Nault, L. R., and E. D. Ammer. 1989. Leafhopper and planthopper transmission of plant viruses. *Annu. Rev. Entomol*. 34: 503–529.

Noda, H., K. Watanabe, S. Kawai, G. Yukuhiro, T. Miyoshi, M. Tomizawa, Y. Koizumi, N. Nikoh, and T. Fukatsu. 2012. Bacteriome-associated endosymbionts of the green rice leafhopper *Nephotettix cincticeps* (Hemiptera: Cicadellidae). *Appl. Environ. Zool*. 47: 217–225.

Olivier-Espejel, S., Z. L. Sabree, K. Noge, and J. X. Becerra. 2011. Gut microbiota in nymph and adults of the giant mesquite bug (*Thasus neocalifornicus*) (Heteroptera: Coreidae) is dominated by *Burkholderia* acquired de novo every generation. *Environ. Entomol*. 40: 1102–1110.

Perlman, S. J., M. S. Hunter, and E. Zchori-Fein. 2006. The emerging diversity of *Rickettsia*. *Proc. R. Soc. B-Biol. Sci*. 273: 2097–2106.

Purcell, A. H., and K. G. Suslow. 1987. Pathogenicity and effects on transmission of a mycoplasmalike organism of a transovarially infective bacterium on the leafhopper *Euscelidius variegatus* (Homoptera, Cicadellidae). *J. Invertebr. Pathol*. 50: 285–290.

Purcell, A. H., K. G. Suslow, and M. Klein. 1994. Transmission via plants of an insect pathogenic bacterium that does not multiply or move in plants. *Microb. Ecol*. 27: 19–26.

Purcell, A. H., T. Steiner, F. Megraud, and J. M. Bove. 1986. In vitro isolation of a transovarially transmitted bacterium from the leafhopper *Euscelidius variegatus* (Hemitpera: Cicadelliae). *J. Invertebr. Pathol*. 48: 66–73.

Schuler, H., J. Dittmer, L. Borruso, J. Galli, S. Fischnaller, G. Anfora, O. Rota-Stabelli, T. Weil, and K. Janik. 2022. Investigating the microbial community of *Cacopsylla* spp. as potential factor in vector competence of phytoplasma. *Environ. Microbiol*. 24: 4771–4786.

Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst. 1999. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol*. 53: 71–102.

Strausbaugh, C. A., W. M. Wintermantel, A. M. Gillen, and I. A. Eujayl. 2008. Curly top survey in the Western United States. *Phytopathology*. 98: 1212–1217.

Takiya, D. M., P. L. Tran, C. H. Dietrich, and N. A. Moran. 2006. Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol. Ecol*. 15: 4175–4191.

Wangkeeree, J., T. A. Miller, and Y. Hanboonsong. 2012. Candidates for symbiotic control of sugarcane white leaf disease. *Appl. Environ. Microbiol*. 78: 6804–6811.

Wangkeeree, J., P. Tewaruxsa, and Y. Hanboonsong. 2019. New bacterium symbiont in the bacteriome of the leafhopper *Yamatotettix flavovittatus* Matsumura. *J. Asia-Pac. Entomol*. 22: 889–896.

Weinert, L. A., J. H. Werren, A. Aebi, G. N. Stone, and F. M. Jiggins. 2009. Evolution and diversity of *Rickettsia* bacteria. *BMC Biol*. 7: 6. doi:10.1186/1741-7007-7-6.

Weintraub, P. G., and L. Beanland. 2006. Insect vectors of phytoplasmas. *Annu. Rev. Entomol*. 51: 91–111.

Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16s ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*. 173: 697–703.

Wolfe, H. R. E., E. W. Anthon, and L. S. Jones. 1950. Transmission of western x-disease of peaches by the leafhopper, *Colladonus geminaus* (Van Duzee). *Phytopathology*. 40: 971.

Wright, A. A., M. K. Shires, C. Beaver, G. Bishop, S. T. DuPont, R. Naranjo, and S. Harper. 2021. Effect of '*Candidatus* Phytoplasma pruni' infection on sweet cherry fruit. *Phytopathology*. 111: 2195–2202.

Xu, Y., E. A. Buss, and D. G. Boucias. 2016. Environmental transmission of the gut symbiont *Burkholderia* to phloem-feeding *Blussus insularis*. *PLoS One*. 11: e0161699.

Zhang, Y. P., J. K. Uyemoto, and B. C. Kirkpatrick. 1998. A small-scale procedure for extracting nucleic acid from woody plants infected with various phytoplathogens for PCR assay. *J. Virol. Methods*.: 7145–7150.