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INVERTEBRATE MICROBIOLOGY

# 16S rRNA Sequencing Detected *Profftella*, *Liberibacter*, *Wolbachia*, and *Diplorickettsia* from Relatives of the Asian Citrus Psyllid

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## Abstract

The Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psylloidea) is a serious pest of citrus species worldwide because it transmits *Candidatus* Liberibacter spp. (Alphaproteobacteria: Rhizobiales), the causative agents of the incurable citrus disease, huanglongbing or greening disease. *Diaphorina citri* possesses a specialized organ called a bacteriome, which harbors vertically transmitted intracellular mutualists, *Ca.* Carsonella ruddii (Gammaproteobacteria: Oceanospirillales) and *Ca.* Profftella armatura (Gammaproteobacteria: Betaproteobacteriales). Whereas *Carsonella* is a typical nutritional symbiont, *Profftella* is an unprecedented type of toxin-producing defensive symbiont, unusually sharing organelle-like features with nutritional symbionts. Additionally, many *D. citri* strains are infected with *Wolbachia*, which manipulate reproduction in various arthropod hosts. In the present study, in an effort to obtain insights into the evolution of symbioses between *Diaphorina* and bacteria, microbiomes of psyllids closely related to *D. citri* were investigated. Bacterial populations of *Diaphorina* cf. *continua* and *Diaphorina lycii* were analyzed using Illumina sequencing of 16S rRNA gene amplicons and compared with data obtained from *D. citri*. The analysis revealed that all three *Diaphorina* spp. harbor *Profftella* as well as *Carsonella* lineages, implying that *Profftella* is widespread within the genus *Diaphorina*. Moreover, the analysis identified *Ca.* Liberibacter europaeus and *Diplorickettsia* sp. (Gammaproteobacteria: Diplorickettsiales) in *D.* cf. *continua*, and a total of four *Wolbachia* (Alphaproteobacteria: Rickettsiales) lineages in the three psyllid species. These results provide deeper insights into the interactions among insects, bacteria, and plants, which would eventually help to better manage horticulture.

**Keywords** *Diaphorina* · Sternorrhyncha · Insect · Bacterial endosymbiont · Microbiome · Molecular phylogeny

## Introduction

Psyllids or jumping plant lice (Hemiptera: Sternorrhyncha: Psylloidea) are plant sap-sucking insects encompassing about 4000 described species worldwide [1]. They exclusively feed on phloem sap [2], a diet that is deficient in essential amino acids [3] and some vitamins [4, 5]. This nutritional deficiency is compensated for by vertically transmitted intracellular symbionts. Psyllids possess a specialized organ called a bacteriome [6], which typically harbors two distinct bacterial symbionts [7–22]. One is *Candidatus* Carsonella ruddii (Gammaproteobacteria: Oceanospirillales) [17], which provides the host with essential amino acids that are scarce in the phloem sap [9, 23]. *Carsonella* is assumed to be present in all psyllid species and is thus categorized as the "primary symbiont" [7–17, 19–23]. Molecular phylogenetic analyses demonstrated congruence between the host and *Carsonella* trees [12, 16, 17, 20, 21], indicating a single acquisition of an ancestor of *Carsonella* by a common ancestor of psyllids, followed by strict vertical transmission of symbionts resulting

The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers DRR190968–DRR190970 (MiSeq output) and TAAA01000001–TAAA01000013 (dereplicated sequence variants).

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in cospeciation between the host and symbiont lineages. In addition, most psyllid species harbor another bacterial lineage in the bacteriome, which is categorized as a "secondary symbiont" [8, 9, 11, 12, 14, 16, 18, 20]. The secondary symbionts in the psyllid bacteriome are varied depending on psyllid species or genera, suggesting multiple infections and replacements of the symbionts during the evolution of Psylloidea. Whereas the secondary symbionts of various insect lineages have diverse range of associations, from parasitic to mutualistic, with the host [24–30], those residing in the psyllid bacteriome appear to consistently have obligate mutualistic, organelle-like features like the primary symbionts [9, 11, 12, 14]. Such features are characteristic of nutritional symbionts [24, 31–39]. Indeed, whole genome analyses showed that the secondary symbionts of two psyllid species *Ctenarytaina eucalypti* (Aphalaridae: Spondyliaspidinae) and *Heteropsylla cubana* (Psyllidae: Ciriacreminae) are nutritional symbionts that complement incomplete amino acid biosynthetic pathways of *Carsonella* [9]. In contrast, an unprecedented type of secondary symbiont that falls out of this category was found in the Asian citrus psyllid, *Diaphorina citri* (Liviidae: Euphyllurinae).

*Diaphorina citri* is an important agricultural pest that transmits *Candidatus* Liberibacter spp. (Alphaproteobacteria: Rhizobiales), primarily *Ca.* Liberibacter asiaticus (*C*Las), the causative agent of a devastating citrus disease known as huanglongbing (HLB) or greening disease [40–42]. *Diaphorina citri* and *C*Las were originally distributed in tropical and subtropical South to East Asia but were relatively recently introduced into the Arabian Peninsula, Mascarenes, Oceania, and Caribbean, South, Central, and North America [40–42]. Because HLB is currently incurable, controlling *D. citri* as the vector is presently the most crucial part of HLB management [42]. Whereas the association with *Liberibacter* is transient, *D. citri*, like other psyllid species, has more intimate and evolutionarily long-lasting relationships with bacteriome-associated bacteria. Along with the primary symbiont *Carsonella*, *D. citri* possesses *Ca.* Profftella armatura (Gammaproteobacteria: Betaproteobacteriales) as a bacteriome-residing secondary symbiont [43, 44]. *Profftella* is an intracellular resident found in all *D. citri* individuals across global populations and has a drastically reduced genome of much less than 1 Mb, which is characteristic of bacteriome-associated nutritional symbionts [24, 31, 32, 45]. However, the genome encodes only a few genes required to supplement the host's diet [43]. Instead, a large part of the genome is devoted to a gene set for synthesizing a secondary metabolite, diaphorin, a polyketide exhibiting significant cytotoxicity to various organisms [43, 46–48]. Thus, *Profftella* is considered to be an unprecedented type of defensive symbiont with organelle-like features. Furthermore, genomic and phylogenetic analyses demonstrated that the *Liberibacter* lineage has horizontally acquired a gene from the *Profftella* lineage, showing ecological and evolutionary interactions between the HLB pathogen and the bacteriome symbiont [49]. In addition to these symbionts, many *D. citri* strains are infected with *Wolbachia* (Alphaproteobacteria: Rickettsiales) [19, 50–57]. *Wolbachia* have various effects on arthropod hosts behaving as reproductive manipulators, defensive symbionts, or nutritional symbionts [58–60]. Although the role of *Wolbachia* in *D. citri* is not known, interactions between *Wolbachia* and other symbionts, including *Carsonella*, *Profftella*, and *Liberibacter*, are suggested [53, 61–64].

*Diaphorina citri* is a member of a species-rich psyllid genus, which currently includes 78 described and many undescribed species restricted to the Old World and mainly distributed in its warm and dry regions, e.g., the Mediterranean Basin, the Sahel region, South and South West Africa, the Middle East, and the arid parts of the Indian subcontinent and Central Asia; these species are associated with many different plant families [65–70]. *Diaphorina* has been formally classified as a member of Liviidae: Euphyllurinae [71], but recent molecular phylogenomic analyses place the genus as sister to Psyllidae or Triozidae, i.e., outside of Liviidae [1]. Except for *D. citri*, the microbial symbionts of *Diaphorina* spp. are unknown. Revealing bacterial flora in different *Diaphorina* spp. would provide deeper insights into the evolution of symbioses between psyllids and bacteria. This would enhance our understanding of *D. citri* biology, eventually aiding to improve the efficiency of HLB control.

For this purpose, in the present study, we analyzed bacterial populations in two European species, *Diaphorina* cf. *continua* and *Diaphorina lycii*, using the 16S rRNA gene sequencing technique. For comparison, the bacterial flora of *D. citri* was also analyzed.

## Materials and Methods

### Insects

*Diaphorina* cf. *continua* was collected from *Thymelaea tartonraira* subsp. *thomasii* (Thymelaeaceae) in a pine forest 1.8 km west of Moltifao village (42°29′12″N, 9°8′22″E, 300 m.a.s.l.), Haute-Corse department, Corsica island, France, on April 9, 2017. Morphologically, these specimens are similar to *D. continua*, originally described from Morocco [72]. *Diaphorina continua* was also recorded from Algeria and Canary Islands (without host plant data) [66] and Sardinia. In Sardinia, which is geographically close to Corsica, *D. continua* was reported from *Thymelaea tartonraira* [73, 74], i.e., the same host plant with the material analyzed in this study. However, the adults from Corsica differ

from the published descriptions of *D. continua* [66, 72] in some details, such as the male terminalia. Thus, the species identity of *Diaphorina* specimens collected on *T. tartonraira* in Corsica and Sardinia needs to be confirmed by a detailed taxonomic revision.

*Diaphorina lycii* was collected from *Lycium barbarum* (Solanaceae) in the floodplain of the Stara river 2.3 km northeast of Byaga village (42°4′45″N, 24°24′11″E, 250 m.a.s.l.), Pazardzhik Region, Bulgaria, on June 18, 2017. *Diaphorina lycii* is narrowly oligophagous on *Lycium* spp. and widely distributed in Southern Europe, North Africa, Middle East, Central Asia, and Mongolia [65, 66, 70].

The material of *D. citri* was used from a laboratory stock free of *Ca.* Liberibacter spp. The established colony of *D. citri*, originally collected from Amami Oshima Island, Kagoshima, Japan (28°23′46″N, 129°31′46″E, 5 m.a.s.l.), was maintained on *Murraya paniculata* (Rutaceae) kept in incubators set at 28 °C with a 16-h light:8-h dark photoperiod.

## DNA Extraction

DNA was extracted from whole bodies of adult *D. citri* (5 males and 5 females), *D.* cf. *continua* (3 males and 8 females), and *D. lycii* (5 males and 5 females) using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of extracted DNA was assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the quantity was assessed using a Qubit 2.0 Fluorometer with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

## Construction and Sequencing of Amplicon Libraries

Bacterial populations in *D. citri*, *D.* cf. *continua*, and *D. lycii* were analyzed using the MiSeq System (Illumina, San Diego, California, USA). The sequencing libraries targeting V3 and V4 regions of the 16S rRNA gene were constructed according to the instructions by Illumina [75] but with some modifications. Briefly, amplicon PCR was performed using the genomic DNA extracted from *Diaphorina* spp., KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, Massachusetts, USA), and the primer set 16S_341Fmod (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGYYTAMGGRNGGCWGCAG-3') and 16S_805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Running parameters were 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension step of 72 °C for 5 min. PCR product sizes were analyzed by agarose gel electrophoresis. Amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA). Dual indices and Illumina sequencing adapters were attached to the amplicons with index PCR using Nextera XT Index Kit v2 (Illumina). Running parameters were 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension step of 72 °C for 5 min. Amplicons were purified again using Agencourt AMPure XP beads, which were then quantified using a Qubit 2.0 Fluorometer with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The libraries were combined with PhiX Control v3 (Illumina), and both ends of 250 bp were sequenced on the MiSeq platform (Illumina) with a MiSeq Reagent Kit v2 (500 cycles; Illumina).

## Computational Analysis of Bacterial Populations

Amplicon sequence reads were demultiplexed using MiSeq Reporter (Illumina). The output sequences in FASTQ files per sample were imported into the QIIME2 platform (version 2019.7) [76] and processed using a set of plugins. Primer sequences were removed using the cutadapt plugin [77] with the following options: –p-front-f ^YYTAMGGRNGGCWGCAG –p-front-r ^GACTACHVGGGTATCTAATCC –p-discard-untrimmed. Paired-end sequences were trimmed, denoised, joined, and dereplicated using the dada2 plugin [78] with the following options: –p-max-ee 2 –p-trunc-len-f 230 –p-trunc-len-r 230. During this step, chimeric sequences were detected in samples individually, and sequences found to be chimeric in a sufficient fraction of samples were removed. The q2-feature-classifier plugin [79], a Naive Bayes classifier based on a probabilistic machine learning algorithm, was trained using V3 and V4 regions of 16S rRNA gene sequences in SILVA database ver. 132 (SILVA_132_QIIME_release/taxonomy/16S_only/99/taxonomy_7_levels.txt) that were clustered at 99% sequence similarity. Subsequently, denoised and dereplicated amplicon reads were classified, and taxonomic information was assigned using the trained q2-feature-classifier. Obtained sequence variants (SVs) were manually checked by performing BLASTN searches against the National Center for Biotechnology Information (NCBI) non-redundant (nr) database [80].

## Phylogenetic Analysis of Detected Bacteria

SVs after dereplication were aligned with related sequences using SINA (v1.2.11) according to the global SILVA alignment for rRNA genes [81]. Nucleotide sites corresponding to alignment gap(s) were omitted from the data set. Phylogenetic trees were inferred by the maximum likelihood (ML) method using RAxML (version 8.2.12) [82]. The GTR + Γ model was used with no partitioning of the data matrix, with 1000 bootstrap iterations (options -f a -m GTRGAMMA -# 1000).

## Results and Discussion

### All Three *Diaphorina* Spp. Have *Profftella* and *Carsonella*

The MiSeq sequencing of the amplicon libraries yielded 90,796 pairs of forward and reverse reads for *D. citri*, 71,195 paired reads for *D.* cf. *continua*, and 32,231 paired reads for *D. lycii*. Denoising and joining of the paired-end reads along with removal of low-quality or chimeric reads resulted in 69,657 reads for *D. citri*, 55,673 reads for *D.* cf. *continua*, and 30,004 reads for *D. lycii* (Table S1). Dereplication of these reads resulted in 49 independent SVs, among which only 12 SVs accounted for > 1% of total reads (Table S1). Extremely simple bacterial communities of this type have been reported for sternorrhynchan insects with the bacteriome, including aphids, whiteflies, and other psyllid species [11, 14, 50, 83–86]. Taxonomic classification by QIIME2 (Fig. 1) followed by independent BLAST searches and phylogenetic analyses (Fig. 2) revealed that all three *Diaphorina* spp. possess distinct lineages of *Profftella*. The q2_feature_classifier plugin assigned SV1, SV2, and SV3 to *Ca.* Profftella armatura. SV1, which was derived from 55.6% of denoised *D. citri* reads (Table S1), was 100% identical to the corresponding sequence of *Profftella* previously reported from *D. citri* populations from Japan (CP003468), China (CP012591), and the USA (EF433792). SV2, which was derived from 40.6% of denoised *D.* cf. *continua* reads, was 98.4% identical to SV1. SV3, which was derived from 71.8% of denoised *D. lycii* reads, was 98.4% and 99.5% identical to SV1 and SV2, respectively. Molecular phylogenetic analysis showed that SV2 and SV3 form a clade with SV1 corresponding to *Profftella* of *D. citri* with good bootstrap support (Fig. 2), verifying that both *D.* cf. *continua* and *D. lycii* possess bacterial lineages that are sister to *Profftella* of *D. citri*. The finding implies that *Profftella* lineages are widespread within the genus *Diaphorina*. Further studies including more psyllid taxa are required to confirm this and to reveal if *Profftella* is unique to *Diaphorina* or it also occurs in other psyllid genera.

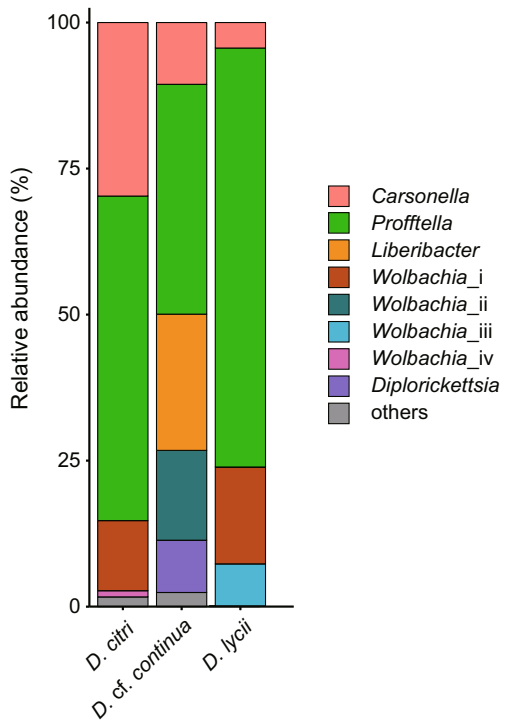


**Fig. 1** Composition of bacterial populations in *Diaphorina* spp. Relative abundances of Illumina reads belonging to assigned bacterial taxa are shown

As expected, the analyses showed that all three *Diaphorina* spp. possess distinct lineages of *Carsonella* (Table S1, Figs. 1, S1) that is assumed to be universal in Psylloidea [7–17, 19–23]. The q2_feature_classifier assigned SV4, SV8, and SV11 to *Ca.* Carsonella ruddii. SV4, which was derived from 30.4% of denoised *D. citri* reads (Table S1), was 100% identical to the corresponding sequence of *Carsonella* previously reported from *D. citri* populations from Japan (CP003467) and China (CP012411) and 99.8% identical to that from the USA (AF211136). SV8, which was derived from 10.8% of denoised *D.* cf. *continua* reads, was 99.5% identical to the corresponding sequence of *Carsonella* previously reported from *D. lycii* (AF280097) [21] and 98.6% identical to SV4. SV11, which was derived from 4.3% of denoised *D. lycii* reads, was 99.8% identical to SV8 and the *Carsonella* sequence previously reported from *D. lycii* (AF280097) and 98.4% identical to SV4. Phylogenetic analysis showed that SV4, SV8, and SV11 form a strongly supported clade with the *Carsonella* sequences previously reported from *D. citri* and *D. lycii* (Fig. S1), verifying that these SVs correspond to *Carsonella* lineages. As previously reported [12, 16, 17, 20, 21], the phylogeny of *Carsonella* showed general congruence with the relationships of their psyllid hosts [1, 87]. Whereas some previous studies that analyzed psyllid microbiomes using "universal primers" detected only a trace amount of *Carsonella* reads [14, 50, 84], the present study succeeded in detecting a large percentage of *Carsonella* reads using primers appropriately modified for highly AT-biased symbiont genes [9, 23, 43]. The ratio of *Carsonella* reads to *Profftella* reads in *D. citri* was 0.55, which was consistent with previous reports of quantitative PCR using target-specific primers [51–53, 88].

### *Diaphorina* cf. *continua* has *Liberibacter* and *Diplorickettsia*

Taxonomic classification by QIIME2 (Fig. 1) followed by independent BLAST searches and phylogenetic analyses (Fig. 3) identified *Ca.* Liberibacter europaeus in *D.* cf. *continua*. The q2_feature_classifier assigned SV6, which was derived from

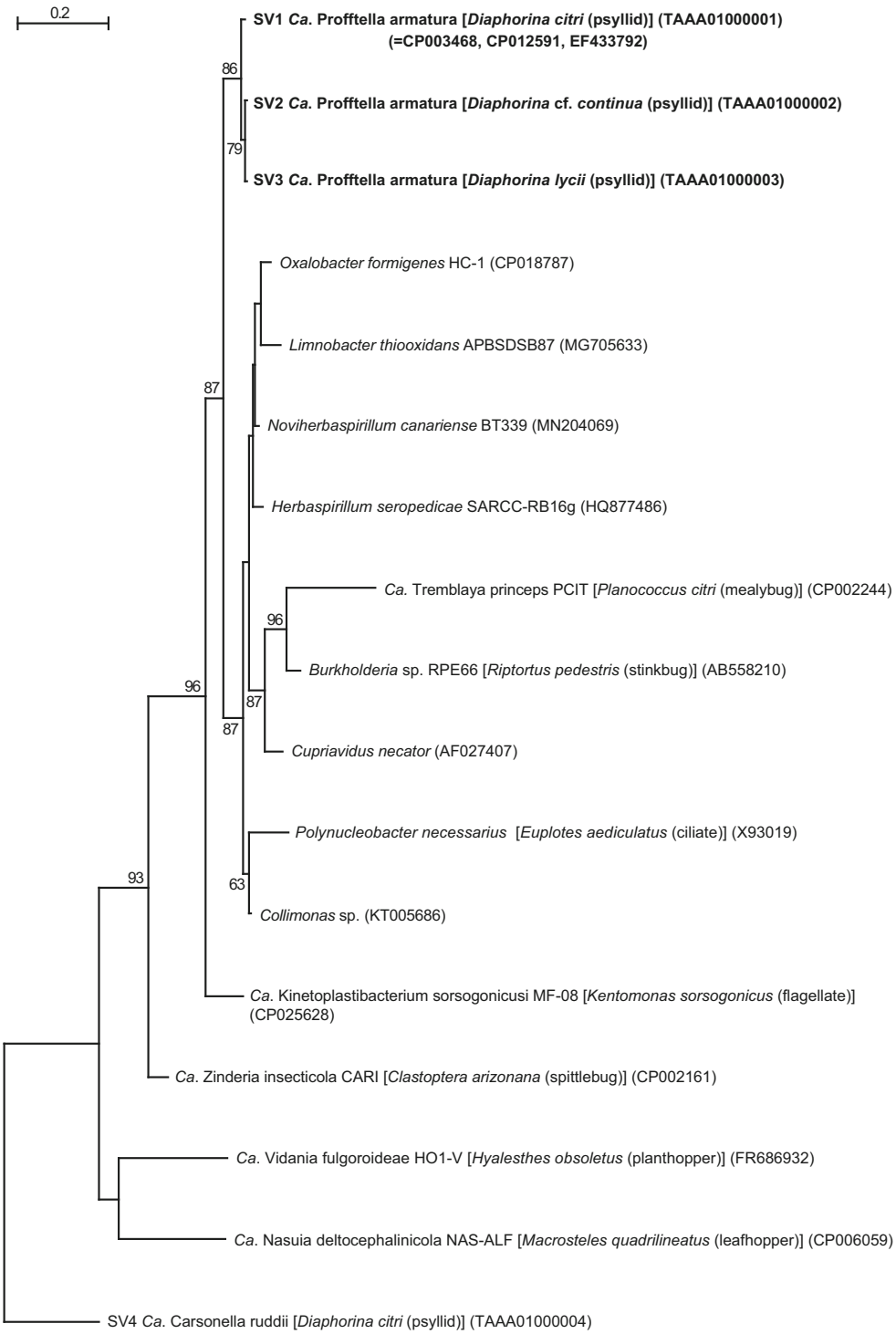


**Fig. 2** Phylogenetic position of *Profftella* lineages within Betaproteobacteriales inferred by the maximum likelihood method. A total of 427 unambiguously aligned nucleotide sites of 16S rRNA genes were subjected to the analysis. On each branch, bootstrap support values over 50% are shown. The scale bar indicates substitutions per site. For symbiotic bacteria, host organisms are shown in brackets. *Profftella* sequences from this study are shown in bold. DDBJ/EMBL/GenBank accession numbers are provided in parenthesis. *Carsonella* was used as an outgroup

23.8% of denoised *D.* cf. *continua* reads, to *Ca.* Liberibacter europaeus (Alphaproteobacteria: Rhizobiales) (Table S1, Fig. 1). SV6 was 100% identical to the corresponding sequence of *Ca.* Liberibacter europaeus NR-01 (FN678792) and 99.3% identical to *Ca.* Liberibacter europaeus, isolate Psy6 (JX244258), and isolate BrS (JX244259). Molecular phylogenetic analysis showed that these sequences form a robustly supported clade within *Ca.* Liberibacter spp. (Fig. 3).

The genus *Liberibacter* currently includes eight species: *Ca.* L. asiaticus (*C*Las), *Ca.* L. americanus (*C*Lam), and *Ca.* L. africanus (*C*Laf), which cause HLB in citrus plants (Rutaceae) in Asia, the Americas, and Africa [41, 42]; *Ca.* L. caribbeanus (*C*Lca) that was identified in citrus in Columbia but the pathogenicity of which is uncertain [89]; *Ca.* L. solanacearum (*C*Lso), which causes diseases in solanaceous plants in North and Central Americas and New

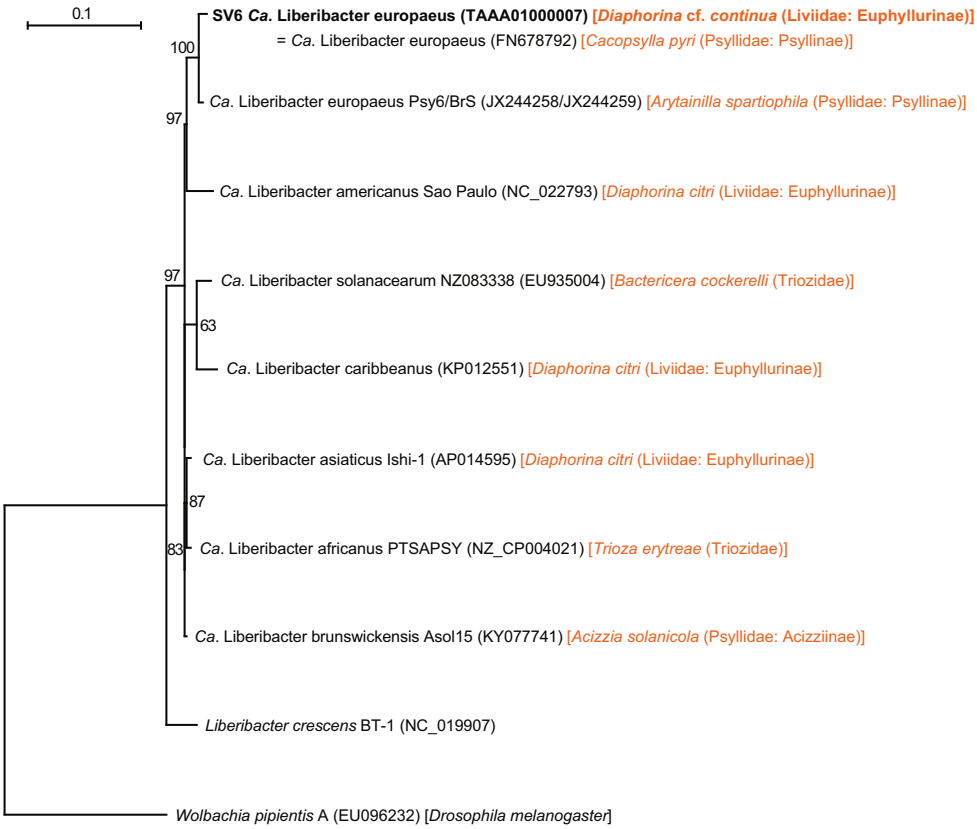


**Fig. 3** A maximum likelihood phylogram of *Liberibacter* spp. A total of 402 unambiguously aligned nucleotide sites of 16S rRNA genes were subjected to the analysis. On each branch, bootstrap support values over 50% are shown. For insect-associated bacteria, host insects are shown in brackets. Psyllids are shown in orange. Families and subfamilies of the host psyllids are shown in parenthesis. The sequence from this study is shown in bold. DDBJ/EMBL/GenBank accession numbers for sequences are provided in parenthesis. The scale bar represents nucleotide substitutions per position. *Wolbachia* was used as an outgroup

Zealand and in carrot and celery (Apiaceae) in Europe and North Africa [90–93]; *Ca.* L. brunswickensis (*C*Lbr), a probable endophyte of solanaceous plants in Australia [94]; *L. crescens* (*Lcr*) that is nonpathogenic and the only culturable species in the genus, which was isolated from Babaco papaya (Caricaceae) in Puerto Rico [95]; and *Ca.* L. europaeus (*C*Leu) [96–98].

*C*Leu NR-01 (FN678792), which was detected in *Cacopsylla* spp. (Psyllidae: Psyllinae) and their host rosaceous plants in Italy and Hungary, was described as an endophyte, as it caused no apparent symptoms to the plants [96, 97]. Subsequently, another *C*Leu lineage (JX244258/JX244259) was found from the broom psyllid *Arytainilla spartiophila* (Psyllidae: Psyllinae) and its host, the Scotch broom *Cytisus scoparius* (Fabaceae) with disease symptoms in New Zealand [98]. *Arytainilla spartiophila* was introduced to New Zealand from the UK for a biological control of the Scotch broom [99]. The *C*Leu lineage with the sequence (MN176610) identical to that from New Zealand was later confirmed in *A. spartiophila* and *C. scoparius* in the UK [100]. The present study adds another example of *C*Leu from another psyllid species, *D.* cf. *continua*, in Corsica island. As field observations suggested that *Thymelaea tartonraira* (Thymelaeaceae) is the only host plant species for *D.* cf. *continua* in Corsica, it would be interesting to assess if this plant species, which is distantly related to *C. scoparius* (Fabaceae) and rosaceous plants, is also infected with *C*Leu and if it shows disease symptoms. In the Scotch broom, the presence of *C*Leu is associated with stunted growth of shoots, shortened internodes, leaf dwarfing, and leaf tip chlorosis [98]. At the moment, data on its possible pathogenicity in *T. tartonraira* are lacking.

It appears that *Ca.* Liberibacter lineages have evolved in close associations with Psylloidea, and all known vectors for all *Ca.* Liberibacter spp. are psyllids. The vectors reported thus far are *D. citri* for *C*Las, *C*Lam [41, 42], and *C*Lca [89]; *Trioza erytreae* (Triozidae) for *C*Laf [41, 42]; *Bactericera cockerelli*, *B. trigonica*, and *Trioza apicalis* (all Triozidae) for *C*Lso [90, 93]; *Acizzia solanicola* (Psyllidae: Acizziinae) for *C*Lbr [94]; and *Cacopsylla* spp. [96, 97], *Arytainilla spartiophila* [98], and *D.* cf. *continua* (this study) for *C*Leu. Interactions between *Ca.* Liberibacter spp. and psyllids are assumed to have evolved multiple times independently because of a lack of congruence between the phylogenies of both groups (Fig. 3). This is also the case for associations between *Liberibacter* and plants. Pelz-Stelinski and Killiny reported that *D. citri* harboring *C*Las are more fecund than their uninfected counterparts and overall population fitness of infected psyllids is better [101]. This observed beneficial effect may account for the close associations between *Ca.* Liberibacter spp. and psyllids. Further studies are required to assess if this hypothesis is applicable to other *Ca.* Liberibacter -psyllid combinations in general.

The analysis also detected *Diplorickettsia* sp. (Gammaproteobacteria: Diplorickettsiales) from *D.* cf.

*continua*. The q2_feature_classifier assigned SV9, which was derived from 9.1% of denoised *D.* cf. *continua* reads (Table S1), to *Diplorickettsia*. SV9 was 98.8% identical to the corresponding sequence of *Diplorickettsia* sp. MSebKT1 (AB795342), 98.6% identical to the sequence of *Diplorickettsia massiliensis* 20B (NR_117407), and 97.7% identical to the sequence of *Diplorickettsia* sp. NS15 (JN606082). Molecular phylogenetic analysis showed that SV9 forms a well-supported clade with these *Diplorickettsia* spp. (Fig. 4). To our knowledge, this is the first report of *Diplorickettsia* detected in psyllids.

*Diplorickettsia massiliensis* was first isolated from the European sheep tick *Ixodes ricinus* (Arachnida: Acari: Ixodidae) collected in Slovakia and proposed to be the type species of a newly described genus *Diplorickettsia* [102]. The following study detected *D. massiliensis* from serum samples of human patients with suspected tick-borne disease, suggesting that the bacterium is a human pathogen, like most other bacteria and viruses found in *I. ricinus* [103]. *Diplorickettsia* sp. MSebKT1 was found in the leafhopper *Macrosteles sexnotatus* (Hemiptera: Auchenorrhyncha: Cicadellidae) in Japan, a plant sap-sucking insect that is closely related to psyllids [104]. The clade of *Diplorickettsia* clustered with *Rickettsiella* spp. (Gammaproteobacteria: Diplorickettsiales) with a high level of bootstrap support (Fig. 4), corroborating that the genus *Diplorickettsia* is closely related to the genus *Rickettsiella* comprising intracellular bacteria that are associated with various arthropods (insects, arachnids, and isopods), including also psyllids [14, 20]. Whereas many *Rickettsiella* spp. are pathogenic to arthropods, *Ca.* Rickettsiella viridis [105] found in the aphid *Acyrthosiphon pisum* (Hemiptera: Sternorrhyncha: Aphidoidea: Aphididae), which is also a close relative of psyllids, alters the aphid body color, potentially affecting the attractiveness of aphids to natural enemies including parasitoids and ladybirds [106]. As little is known about the functions of *Diplorickettsia* on host arthropods, it would be worth assessing ecological effects of *Diplorickettsia* to *Diaphorina* spp., including *D. citri*.

## Four *Wolbachia* Strains Reside in *Diaphorina* spp.

Taxonomic classification by QIIME2 (Fig. 1) followed by independent BLAST searches and phylogenetic analyses (Fig. 5) identified four SVs corresponding to distinct lineages of *Wolbachia* (Alphaproteobacteria: Rickettsiales). Namely, both *D. citri* and *D. lycii* were shown to have two strains of *Wolbachia*, one of which was shared by the two psyllid species, whereas *D.* cf. *continua* possessed a single *Wolbachia*

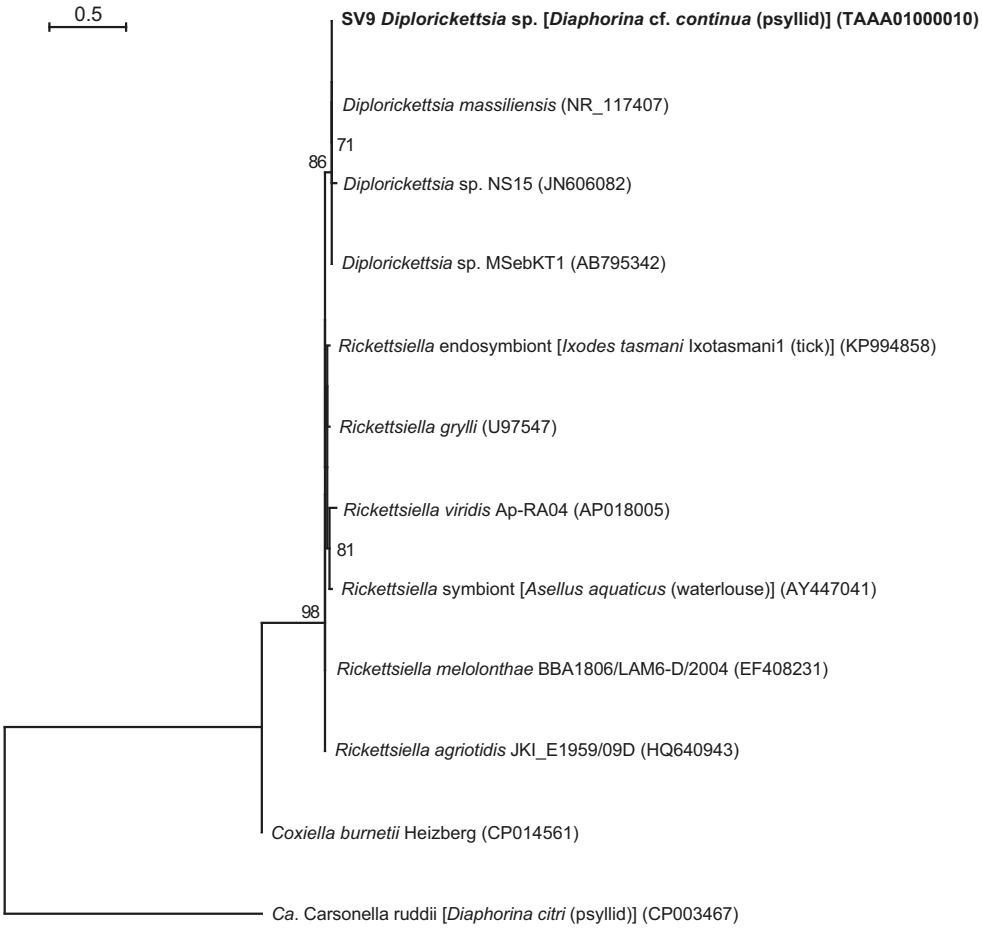


**Fig. 4** Phylogenetic position of *Diplorickettsia* lineages inferred by the maximum likelihood method. A total of 427 unambiguously aligned nucleotide sites were subjected to the analysis. On each branch, bootstrap support values over 50% are shown. The scale bar indicates substitutions per site. For symbiotic bacteria, host organisms are shown in brackets. The sequence from this study is shown in bold, and DDBJ/EMBL/GenBank accession numbers are provided in parenthesis. *Carsonella* was used as an outgroup

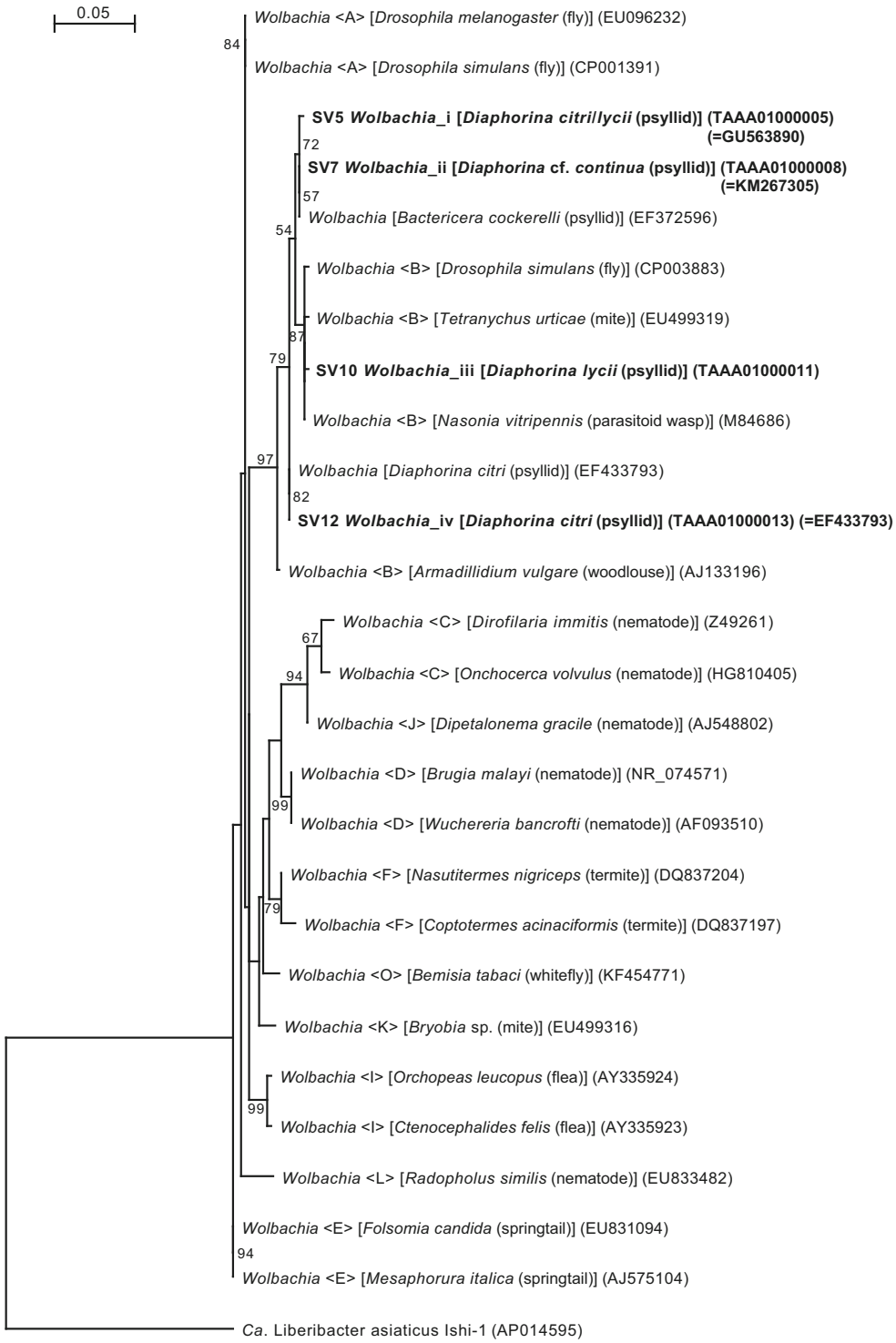


**Fig. 5** A maximum likelihood phylogram of *Wolbachia*. A total of 402 unambiguously aligned nucleotide sites of 16S rRNA genes were subjected to the analysis. On each branch, bootstrap support values over 50% are shown. Host organisms are shown in brackets. The sequence from this study is shown in bold. DDBJ/EMBL/GenBank accession numbers for sequences are provided in parenthesis. Supergroups of *Wolbachia* are shown in angle brackets. The scale bar represents nucleotide substitutions per position. *Liberibacter* was used as an outgroup

strain that was previously reported from another psyllid genus. SV5 (*Wolbachia*_i), which was derived from 12.4% of denoised *D. citri* reads and 16.6% of denoised *D. lycii* reads (Table S1), was 100% identical to the sequence of *Wolbachia* previously reported from *D. citri* in China (GU563890), the planthopper (Hemiptera: Auchenorrhyncha: Delphacidae) *Nilaparvata lugens* in China (FJ774974) [107], and the aphids (Hemiptera: Sternorrhyncha: Aphidoidea: Aphididae) *Phloeomyzus passerinii* in China (HQ843849), *Cervaphis quercus* in China (JN635325), and *Cinara cedri* in Israel (JN384059) [108]. SV7 (*Wolbachia*_ii), which was derived from 15.7% of denoised *D.* cf. *continua* reads (Table S1), was 100% identical to the sequence of *Wolbachia* detected from the following insects: the psyllid *Bactericera cockerelli*

(Triozidae) in the USA (KM267305) [109], the whiteflies (Hemiptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae) *Bemisia tabaci* (MG977008) and *Bemisia tuberculata* (MG977007) in Brazil, the aphid *Cinara cedri* in Israel (JN384060) [108], the planthopper *Nilaparvata lugens* in China (KX280764), the leafhoppers (Hemiptera: Auchenorrhyncha: Cicadellidae) *Homalodisca coagulata* in the USA (AF501664) [110] and *Hishimonoides sellatiformis* in Japan (AB073729) [111], the spittlebugs (Hemiptera: Auchenorrhyncha: Aphrophoridae) *Philaenus maghresignus* in Spain (AB772263) and *Aphrophora quadrinotata* in the USA (AB772260) [112], the grasshoppers (Orthoptera: Acrididae) *Chorthippus parallelus* in the Pyrenees (FJ438533) [113] and *Stenobothrus lineatus* in the UK (EU727131) [114], the mosquito (Diptera: Culicidae) *Aedes fluviatilis* in Brazil (GQ981315) [115], and the weevil (Coleoptera: Curculionidae) *Naupactus cervinus* in Brazil (GQ402143) [116]. SV10 (*Wolbachia*_iii), which was derived from 7.2% of denoised *D. lycii* reads (Table S1), was 99.8% identical to the sequence of *Wolbachia* detected in various arthropod hosts including the psyllid *Mycopsylla fici* (Homotomidae) in Australia (KT273277). SV12 (*Wolbachia*_iv), which was derived from 1.1% of denoised *D. citri* reads (Table S1), was 100% identical to the sequence of *Wolbachia* in *D. citri* in the USA (EF433793) [117] and *Bemisia tabaci* in the Philippines (MK157177), Bangladesh (MH370786), Indonesia (KM404233-KM404238), India (KM404186, KM404191, KM404193), Japan (AB981359), China (AY850932, KF454756), and Australia (KF454754).

*Wolbachia* are rickettsial bacteria widely distributed among various clades of arthropods and nematodes [58–60], and the strains are currently classified into supergroups A–Q [118]. Supergroups A and B are monophyletic and are the most common supergroups that infect arthropods, while supergroups C and D infect nematodes. Supergroups E–Q infect a variety of hosts including nematodes, springtails, termites, fleas, aphids, and mites [60]. The molecular phylogenetic analysis in the present study placed SV5, SV7, SV10, and SV12 from *Diaphorina* spp. in the robustly supported clade of *Wolbachia* supergroup B (Fig. 5).

Many *Wolbachia* strains manipulate the reproduction of arthropod hosts through cytoplasmic incompatibility, feminization, male killing, and parthenogenesis, to increase the frequency of infected females in host populations [58–60]. With this ability of promoting dissemination, *Wolbachia* are proposed as promising agents to control insect pests by affecting host traits or microbiomes, including pathogens therein [119, 120]. Because infectious rates of *Wolbachia* are high in world populations of *D. citri* [19, 50–57], and interactions between *Wolbachia* and other symbionts, including *Carsonella*, *Profftella*, and *Liberibacter*, are suggested [53, 61–64], the application of *Wolbachia* to control *D. citri* and/or HLB is anticipated [52, 53, 55, 57, 62]. The present study suggests rampant horizontal transmissions of *Wolbachia* among various insect lineages including *Diaphorina* spp., implying that artificial infection and/or removal of *Wolbachia* are feasible in *D. citri*. Such techniques would facilitate exploitation of *Wolbachia* as a tool to control *D. citri* and/or HLB.

## Conclusion

The present study revealed that all three *Diaphorina* spp. examined harbor *Profftella* as well as *Carsonella* lineages, implying that *Profftella* is widespread within the genus *Diaphorina*. Moreover, the analysis identified *Ca.* Liberibacter europaeus and *Diplorickettsia* sp. in *D.* cf. *continua* and a total of four *Wolbachia* supergroup B lineages in the three psyllid species. These results provide deeper insights into the evolution of interactions among insects, bacteria, and plants, which could eventually help to better manage horticulture.

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