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MOLECULAR SURVEY OF ENDOSYMBIONTS IN FLORIDA POPULATIONS OF *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE) AND ITS PARASITOIDS *TAMARIXIA RADIATA* (HYMENOPTERA: EULOPHIDAE) AND *DIAPHORENCYRTUS ALIGARHENSIS* (HYMENOPTERA: ENCYRTIDAE)

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ABSTRACT

A molecular survey of endosymbionts was conducted in Florida populations of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), and its parasitoids *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agarwal) (Hymenoptera: Encyrtidae). Using a high-fidelity polymerase chain reaction (PCR), we detected 3 eubacterial species each in *D. citri* (the primary symbiont *Candidatus* Carsonella rudii, a secondary symbiont related to *Oxalobacter* and *Herbaspirillum* species, and *Wolbachia*), and *T. radiata* (*Caulobacter* sp., *Methylobacterium* sp. and a bacterium in the family Alcaligenaceae), whereas only 1 species was identified in *D. aligarhensis* (*Wolbachia*). Each eubacterial symbiont of *D. citri* was detected in the eggs, which is indicative of transovarial transmission. However, none of the eubacterial symbionts of *T. radiata* were detected in the eggs, suggesting that they are only transient associates. Stable horizontal transfer of these eubacterial species likely has not occurred between the host and its parasitoids because each insect hosts a different complement of Eubacteria. For example, different strains of *Wolbachia* were detected in *D. citri* and *D. aligarhensis*, based on evidence from the 16S rRNA and *wsp* gene sequences. Also, the *orf7* gene of the bacteriophage WO associated with *Wolbachia* was detected in *D. citri* but not in *D. aligarhensis*. No endosymbiotic Archaea, Helicosporidia, Microsporidia, Fungi, or Yeast-like symbionts were detected with PCR in these populations of *D. citri* or its parasitoids.

Key Words: *Diaphorencyrtus aligarhensis*, *Diaphorina citri*, endosymbiont, microbial diversity, symbiosis, *Tamarixia radiata*

RESUMEN

Se realizo un sondeo molecular de los endosimbiontes en poblaciones del sílido asiático de los cítricos, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) en la Florida y de sus parasitoides *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) y *Diaphorencyrtus aligarhensis* (Shafee, Alam y Agarwal) (Hymenoptera: Encyrtidae). Usando una reacción en cadena por la polimerasa (RCP) de alta fidelidad, nosotros detectamos 3 especies de eubacteria en cada uno de los *D. citri, T. radiata* y 1 especie en *D. aligarhensis*. Cada simbionte eubacterial de *D. citri* fue detectado en los huevos, lo cual indica la transmisión transovarial. Sin embargo, ningún de los simbiontes eubacteriales de *T. radiata* fue detectado en los huevos, lo cual sugiere que ellos solamente son socios transitorios. La transferencia horizontal estable de estas especies eubacteriales probablemente no ha ocurrido entre el hospedero y sus parasitoides por lo que cada insecto es un hospedero para un complemento diferente de Eubacteria. Por ejemplo, diferentes cepas de *Wolbachia* fueron detectadas en *D. citri* y *D. aligarhensis*, basado en la evidencia de las secuencias de los genes 16S rRNA ay *wsp*. También, el gene *ofr7* del bacteriófago WO asociado con *Wolbachia* fue detectado en *D. citri* pero no en *D. aligarhensis*. Ningún endosimbiotico de Archaea, Helicosporidia, Microsporidia, hongo, o un simbionte como levadura fue detectado en estas poblaciones de *D. citri* o en sus parasitoides.

Symbioses, ranging from mutualism to parasitism, exist between arthropods and a diverse biota of microorganisms (Dale & Moran 2006) including Eubacteria, Fungi, Microsporidia, Yeasts, Helicosporidia and viruses.Endosymbionts can be facultative or obligate and influence fundamental biological processes in their hosts such as metabolism (Douglas 2003), reproduction (O'Neill et al. 1997) and other fitness attributes (Grenier et al. 2002; Tagami et al. 2002; Huigens et al. 2004). Perhaps most importantly, numerous human, animal, and plant diseases result from the transmis-

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sion of pathogenic microbes harbored by arthropod vectors (Daly et al. 1998).

The number of surveys for arthropod endosymbionts has dramatically increased since the development of molecular tools used to identify uncultivated microbes (Darby & Welburn 2006). In general, comparative analyses of microbial diversity among economically-important arthropod pests and their parasitoids are rare. To address this, we conducted a molecular survey of endosymbionts in Florida populations of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), and its parasitoids *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agarwal) (Hymenoptera: Encyrtidae). Importantly, *D. citri* transmits citrus greening disease (Huanglongbing: HLB) which was reported in Florida in 2005 (Halbert 2006; Bouffard 2006). HLB is caused by the phloem-limited *Candidatus* Liberibacter asiaticus, and this disease poses a serious threat to the citrus industry because long-term infections result in a reduction of fruit quality and ultimately can kill citrus trees (Garnier 2000; Halbert 2006; Bouffard 2006). Knowledge of the microbial fauna in *D. citri* could provide information relevant to future studies aimed at controlling *D. citri* via manipulation of its endosymbionts (Douglas 2007) and examining HLB dynamics because the transmission of some pathogens is influenced by the presence of gut symbionts (Haines et al. 2002).

Colonies of *T. radiata* and *D. aligarhensis* were imported from Taiwan and Vietnam, respectively, reared in quarantine, and released in a classical biological control program against *D. citri* in Florida (Hoy & Nguyen 1998; Hoy et al. 1999; Skelley & Hoy 2004). Since their release, *T. radiata* populations, along with a complex of endemic natural enemies, have suppressed *D. citri* populations (Michaud 2002). Unfortunately, *D. aligarhensis* populations are rare for unknown reasons. *Tamarixia radiata* is an arrhenotokous ectoparasitoid while *D. aligarhensis* is a thelytokous endoparasitoid, and these biological differences may be tied to a different complex of endosymbionts. We hypothesized that the interaction and overlapping environments of *D. citri* with its parasitoids may facilitate stable horizontal transmission of microorganisms in this host-parasitoid system.

Several eubacterial endosymbionts associated with *D. citri* and its parasitoids have been reported. In an Asian population of *D. citri*, 5 bacterial symbionts were identified in a molecular survey including the primary (mycetocyte) symbiont *Candidatus* Carsonella rudii, a secondary (syncytium) symbiont related to species in the genera *Oxalobacter* and *Herbaspirillum,* an *Arsenophonus* species, *Ca.* L. asiaticus (the pathogen vectored by *D. citri* that causes HLB) (Garnier et al. 2000) and *Wolbachia* (Subandiyah et al. 2000). The primary and secondary symbionts were located in mycetocytes, microbe-containing structures commonly found in hemipteran insects (Baumann 2006), of *D. citri* based on *in situ* hybridization studies, but the other symbionts were not localized with this technique (Subandiyah et al. 2000). The primary and secondary gut symbionts of other psyllids and related insects are obligatory for their host's survival (Baumann 2006). Genome sequencing of *Ca*. C. ruddii revealed that its small genome (160 kb) has retained genes involved with synthesis of essential amino acids, presumably to compensate and contribute to the amino acid-deficient diet of the insect host (Nakabachi et al. 2006). In diagnostic assays, the primary and secondary symbionts were found in 100% of the Asian population of *D. citri*, but *Arsenophonus*, *Ca.* L. asiaticus, and *Wolbachia* were detected in 83, 45, and 76% of the psyllids tested, respectively (Subandiyah et al. 2000). *Wolbachia* was detected in the invasive Florida population of *D. citri*, which is of unknown origin, based on molecular methods (Jeyaprakash & Hoy 2000). Prior to the release of *T. radiata* and *D. aligarhensis* in Florida, subsets of these populations were screened for the HLB-causing bacterium, and all samples tested negative (Hoy et al. 1999; 2001). *Wolbachia* was detected in *D. aligarhensis* but not in *T. radiata* (Jeyaprakash & Hoy 2000). Antibiotic "curing" experiments indicated that thelytoky was associated with *Wolbachia* infection in *D. aligarhensis* (Meyer & Hoy 2007).

The previous research investigating microbial interactions in *D. citri* and its parasitoids was limited to surveys of Eubacteria and did not examine the possibility that other microorganisms could play important roles in the biology of this host-parasitoid system. This molecular survey complements prior studies and provides new survey data for endosymbiotic Archaea, Eubacteria, Fungi, Microsporidia, Helicosporidia and the bacteriophage WO of *Wolbachia* (Masui et al. 2000) based on a high-fidelity polymerase chain reaction (PCR) protocol. Finally, we used microbe-specific PCRs to determine if endosymbionts detected in the laboratory were present in field populations of *D. citri* and *T. radiata* and to investigate the transmission mechanisms of the Eubacteria associated with these insects because essential endosymbionts often are transmitted via the eggs (Douglas 2003).

Materials and Methods

Insect Colonies

Small citrus trees (20-50 cm tall) were grown in 15.2-cm diameter pots to maintain colonies of *D. citri* and its 2 parasitoids in wooden-framed, mesh-covered cages (0.76 m × 0.91 m × 1.11 m)

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held in a greenhouse at 20-32°C with a 16L:8D photoperiod (Skelley & Hoy 2004). Ten trees were pruned each week, fertilized with Peter's 20-20-20 (N-P-K) water-soluble fertilizer (United Industries, St. Louis, MO), and watered as necessary. Mated adult females of *D. citri* oviposited on the new growth (flush) that appeared approximately 2 weeks after the trees were pruned. Adult parasitoids were aspirated and separately released into the cages according to species when immature *D. citri* reached the first or second instar. Psyllid nymphs that were not attacked by the parasitoids and developed into adults were used to initiate the next generation.

DNA Extraction

Insects were treated with bleach prior to DNA extraction to remove DNA from external microbes that may contaminate the PCR (Meyer 2007). Treatment of insects was conducted in a plexiglass hood that was first washed with 70% EtOH, then with bleach (6% sodium hypochlorite), and DNA Away (Molecular BioProducts, San Diego, CA), according to the following protocol: (i) individual live insects were placed in sterile 15-mL centrifuge tubes containing 5 mL of the decontamination solution and vortexed vigorously for 1 min; (ii) the solution was discarded, leaving the insects in the tube; (iii) insects were rinsed 3× with 10 mL of sterile water, which was discarded after each rinse; (iv) insects were transferred to a sterile 1.5-mL centrifuge tube using a sterile pipette tip wetted with DNA extraction buffer prior to DNA extraction (Jeyaprakash & Hoy 2000). For the microbial surveys, 2 replicates of 10 newly emerged adult insects were pooled according to species before DNA extraction**.** DNA was extracted from insects by homogenization with a disposable blunt-ended sterile pipette tip with PUREGENE DNA isolation reagents (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol**.** DNA pellets were air dried, resuspended in 10-50 µL sterile water, and stored at -70°C. Ten individual *D. citri*, *T. radiata*, and *D. aligarhensis* from the laboratory colonies and 10 individual *D. citri* and *T. radiata* collected from the field in Polk county, Florida (28°03.656' N, 81°34.937' W) were used to isolate DNA for microbe-specific PCR. These insects were processed as described above, and their DNA was resuspended in 10 µL of sterile water. No field-collected *D. aligarhensis* were included because populations of this parasitoid are scarce in Florida. Twenty eggs of *D. citri* and *T. radiata* collected from the laboratory colonies were pooled, surface decontaminated, and the DNA was isolated as described above and resuspended in 10 µL of sterile water. No DNA was isolated from eggs of *D. aligarhensis* because the endoparasitoid deposits eggs inside of *D. citri* nymphs, and attempts to dissect and recover these eggs were unsuccessful.

High Fidelity Polymerase Chain Reaction

A high-fidelity PCR assay was used that can detect as few as 100 copies of the target template 100% of the time and as few as 10 copies 50% of the time (Hoy et al. 2001). For the molecular survey of endosymbionts and microbe-specific PCRs, the 50-µL reaction contained 34 µL of sterile water, 5 µL of PCR buffer consisting of 50 mM Tris, pH 9.2, 16 mM ammonium sulfate and 1.75 mM $MgCl_2$, 5 µL of 350 mM dNTPs, 2 µL each of the forward and reverse primers at a concentration of 800 pmol, and 1 µL of polymerase mix containing 1 unit of *Pwo* DNA polymerase and 5 units of *Taq* DNA polymerase (Roche Applied Science, Indianapolis, IN) Biochemicals) (Barnes 1994) to amplify 1 µL of template DNA. The primers and annealing temperatures used in the PCR for the survey of endosymbionts and microbe-specific analyses are listed in Table 1 (Hoy & Jeyaprakash 2005). Three linked profiles were used for the high-fidelity PCR that included (i) 1 cycle of denaturation at 94°C for 2 min; (ii) 10 cycles of denaturation at 94°C for 10 s, annealing (Table 1) for 30 s, and elongation at 68°C for 1 min; and (iii) 25 cycles of denaturation at 94°C for 10 s, annealing (Table 1) for 30 s, and extension at 68°C for 1 min plus an additional 20 s for each consecutive cycle (Hoy et al. 2001). Agarose gel electrophoresis (1% TAE gels) was used to separate PCR-amplified DNA, which was stained with ethidium bromide and visualized with UV light.

Cloning and Restriction Fragment Length Polymorphism Analysis

PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), ligated into the pCR2.1 TOPO plasmid (Invitrogen, Carlsbad, CA), and cloned into competent *E. coli* cells (Bioline USA, Inc., Randolph, MA). Plasmid DNA was extracted from *E. coli* colonies with QIAGEN Plasmid Mini columns (Valencia, CA). The presence and size of the inserted DNA was confirmed by gel electrophoresis of plasmids digested with *Eco*RI for 2 h at 37°C. Plasmids digested with *Rsa*I were used for the restriction fragment length polymorphism (RFLP) analyses (Jeyaprakash et al. 2003). Clones of interest were bidirectionally sequenced at the University of Florida Interdisciplinary Core Facility, Gainesville, FL.

Phylogenetic Analysis

Eubacterial DNA sequences obtained in the surveys were used in a BLAST search to identify related DNA sequences in GenBank with default settings. Retrieved sequences were aligned with CLUSTAL X v. 1.83 (Thompson et al. 1997), and manual adjustments of the alignment were conducted using MacClade 4.0 (Madison & Madison

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TABLE 1. PRIMER SEQUENCES USED IN THE HIGH-FIDELITY PCR SURVEY OF MICROORGANISMS ASSOCIATED WITH *D. CITRI*, *T. RADIATA*, AND *D. ALIGARHENSIS* AND FOR DIAGNOSTIC MICROBE-SPECIFIC PCR.

Microorganism: gene (primer name) Reference	Forward Primer Reverse Primer	Annealing temperature °C	Expected PCR product size (bp)
Archaea: 16S rRNA (Arch 21F, Arch 958R)	5'-TTCCGGTTGATCCYGCCGGA-3'	51	1000
DeLong (1992)	5'-YCCGGCGTTGAMTCCAATT-3'		
Eubacteria: 16S rRNA (27f and 1495r)	5'-GAGAGTTTGATCCTGGCTCAG-3'	55	1400
Weisburg et al. (1991)	5'-CTACGGCTACCTTGTTACGA-3'		
Fungi: LSU rRNA (LS1, LR5)	5'-AGTACCCGCTGAACTTAAG-3'	55	1000
Hausner et al. (1993); Rehner & Samuels (1995); Zhang et al. (2003)	5'-CCTGAGGGAAACTTCG-3'		
Helicosporidia: 16S rRNA (ms-5, ms-3)	5'-GCGGCATGCTTAACACATGCAAGTCG-3'	65	1300
Nedelcu (2001)	5'-GCTGACTGGCGATTACTATCGATTCC-3'		
Microsporidia: SSU rRNA (Mic-F, Mic-R)	5'-CACCAGGTTGATTCTGCCTGACGTAGACGC-3'	65	1200
Becnel et al. (2002)	5'-GATCCTGCTAATGGTTCTCCAACAGCAACC-3'		
WO phage: *orf7* (phgWOF, phgWOR)	5'-CCCACATGAGCCAATGACGTCTG-3'	65	400
Masui et al. (2000)	5'-CGTTCGCTCTGCAAGTAACTCCATTAAAAC-3'		
Yeast-like symbiont/ Fungi: SSU (NS1, FS2)	5'-GTAGTCATATGCTTGTCTC-3'	55	1500
Nikoh & Fukatsu (2000)	5'-TAGGNATTCCTCGTTGAAGA-3'		
Secondary (syncytium) symbiont: 16S rRNA	5'-GTGCCAGCAGCCGCGGTAATAC-3'	60	547
Subandiyah et al. (2000)	5'-CACCTGTGTTTAGATTCTTT-3'		
Primary symbiont: *Ca.* C. ruddii: 16S rRNA	5'-GTGCCAGCAGCCGCGGTAATAC-3'	60	560
Subandiyah et al. (2000)	5'-CACCTGTCTCAAAGCTAAAG-3'		
Ca. L. asiaticus: 16S rRNA	5'-GTGCCAGCAGCCGCGGTAATAC-3'	60	534
Subandiyah et al. (2000)	5'-CACCTGTGTAAAGGTCTCCG-3'		
Arsenophonus: 16S rRNA	5'-GTGCCAGCAGCCGCGGTAATAC-3'	60	533
Subandiyah et al. (2000)	5'-CACCTGTCTCAGCGCTCCCG-3'		
*Wolbachia wsp*A	5'-TGGTCCAATAAGTGATGAAGAAAC-3'	55	600
Braig et al. (1998)	5'-AAAAATTAAACGCTACTCCA-3'		
Caulobacter: 16S rRNA	5'-CTGGACCGCCACAGAGAT-3'	60	437
This work	5'-CCTTCGGGTAAAGCCAACTC-3'		
Methylobacter: 16S rRNA	5'-GAGATCCAGGGTCCTCTTCG-3'	60	423
This work	5'-CCGTCGGGTAAGACCAACT-3'		
Unknown β-proteobacterium (Alcaligenaceae): 16S rRNA	5'-ATGTCTGGAATGCCGAAGAG-3'	60	489
This work	5'-CCCAGTCATGAATCCTACCG-3'		

2000). The outgroup taxon was the Gram-positive bacterium *Bacillus subtilis*. Maximum likelihood (ML) and maximum parsimony (MP) analyses were conducted using heuristic searches implemented in PAUP* 4.0b4a (Swofford 2001). For ML, the data matrix was subjected to the MODELTEST 3.7 program (Posada & Crandall 1998) to select the best-fit nucleotide substitution model for the alignment. The substitution rate-matrix parameters and shape parameter (alpha) were estimated via ML. The bootstrap method (100 replicates) was used to support each branch in the ML tree in PAUP*.

Results and Discussion

High-Fidelity PCR Amplification of Endosymbionts in *D. citri* and its Parasitoids

Results from the high-fidelity PCR assays for the endosymbionts of surface-decontaminated *D. citri*, *T. radiata* and *D. aligarhensis* indicate that relatively few endosymbionts were present (Table 2). Amplification of the eubacterial 16S rRNA gene was detected with template DNA from each insect, but no amplification products were detected in any of the insects based on the primers for Archaea, Fungi, Helicosporida, or Microsporidia. The *orf7* gene from the bacteriophage WO of *Wolbachia* was amplified with DNA from *D. citri*, but not from *D. aligarhensis* or *T. radiata* as the template.

Endosymbionts of *D. citri*

The eubacterial 16S rRNA PCR products were cloned from 2 replicate high-fidelity PCR reactions, and a total of 15 clones were analyzed from each replicate by the RFLP technique. Analysis with *Eco*RI digests indicated that all clones from each replicate had an insert approximately 1.4-kb in length. Digests with *Rsa*I resulted in 2 and 3 unique banding patterns in the RFLP analysis according to replicate, respectively. A clone representing each unique banding pattern in both replicates was sequenced (5 total sequences). Two pairs of these sequences were 100% identical between each replicate, respectively. These comparisons indicated that there were at least 3 types of Eubacteria associated with our laboratory colony of *D. citri*. The 3 16S rRNA sequences obtained from *D. citri* were used in a BLAST search to identify related sequences in GenBank.

The first sequence (1466 bp, GenBank accession EF433792) was 100% identical over a 1463-bp homologous region of the 16S rRNA gene of the secondary (syncytium) symbiont of *D. citri* from an Asian population (GenBank accession AB038368) (Subandiyah et al. 2000), which is related to β-proteobacteria species in the genera *Oxalobacter* and *Herbaspirillum* (Fig. 1).

The second sequence was 1429 bp (GenBank accession EF433793) and produced significant alignments to the 16S rRNA gene of species in the α-proteobacterial family Rickettsiaceae. This sequence was 99% identical (1177/1185 bp) to a 1185-bp homologous region of a partial 16S rRNA gene sequence from the endosymbiont *Wolbachia* of *D. citri* from an Asian population (GenBank accession ABO38370) (Subandiyah et al. 2000). Five of the 8 nucleotide differences between the 2 *Wolbachia* sequences were attributed to errors (bases designated "N") in the sequences deposited by Subandiyah et al. (2000) which could have been due to their use of a standard PCR protocol because it does not include a proofreading polymerase.

The third sequence (1459 bp, GenBank accession EF450250) produced significant alignments to the 16S rRNA gene of a γ-proteobacterial species and was 99% identical to a homologous region of the 16S rRNA gene sequence from the primary (mycetocyte) symbiont, *Ca.* C. rudii, of *D. citri* from an Asian population (GenBank accession AB038367) (Subandiyah et al. 2000).

Subandiyah et al. (2000) reported 2 additional eubacterial symbionts, *Ca.* L. asiaticus and *Arsenophonus*, associated with an Asian population of *D. citri*, but no clones containing sequences related

Table 2. Summary of the high-fidelity PCR results for the survey of microbial endosymbionts in laboratory populations of *D. citri* and its 2 parasitoids.

	Species		
Endosymbiont	*D. citri*	*D. aligarhensis*	*T. radiata*
Archaea	—	—	—
Eubacteria	+	+	+
Fungi	—	—	—
Helicosporidia	—	—	—
Microsporidia	—	—	—
Yeast-like symbiont	—	—	—
Wolbachia (*wsp*)	+	+	—
Bacteriophage WO	+	—	—

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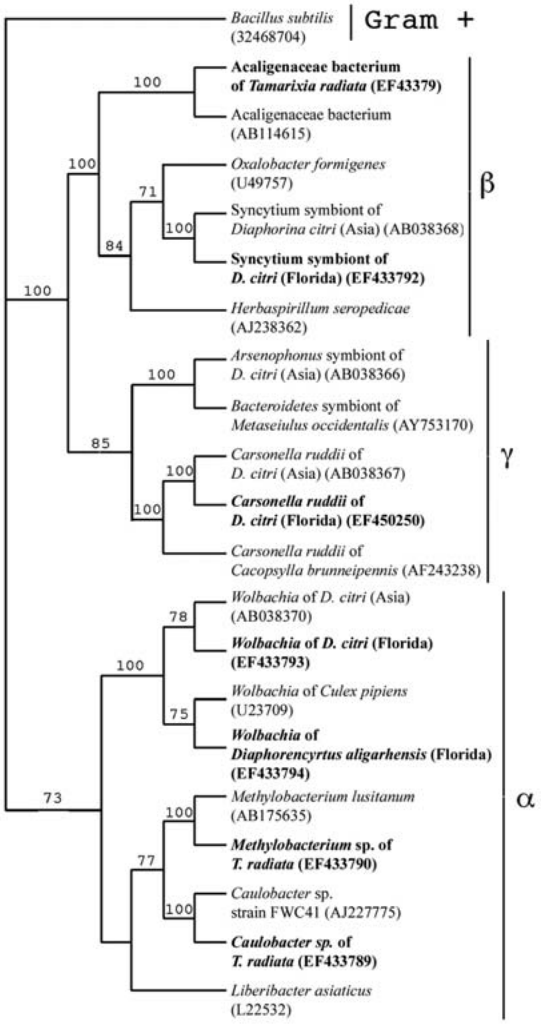


Fig. 1. Phylogenetic tree generated by maximum likelihood analysis based on 16S rRNA sequences from Eubacteria identified from *D. citri*, *T. radiata*, and *D. aligarhensis* compared to related sequences deposited in GenBank. The outgroup taxon is *Bacillus subtilis* (Gram-positive), and the numbers at branch points of the tree designate bootstrap values. The ingroup taxa are clustered in the α, β, and γ divisions of the proteobacteria.

to these symbionts were detected in this survey. To confirm that clones from these symbionts were not missed in our survey, the microbe-specific primers designed by Subandiyah et al. (2000) to amplify the 16S rRNA gene from *Ca.* L. asiaticus and *Arsenophonus* were tested on the 2 replicates of DNA samples isolated from the Florida population of *D. citri* and used in the molecular survey of endosymbionts. No positive PCR reactions were detected for *Ca*. L. asiaticus indicating that our laboratory colony of *D. citri* is free of the citrus greening pathogen. This confirmed prior results of Hoy et al. (2001) and was consistent with the findings of Meyer et al. (2007), in which none of 1793 and 179 field-collected *D. citri* adults and nymphs, respectively, tested positive for *Ca.* L. asiaticus. Three replicate PCR reactions with the *Arsenophonus*-specific primers did not produce amplification products of the expected size, and they produced non-specific products in the no-DNA control. Future research is needed to develop improved *Arsenophonus*-specific primers that do not produce artifacts in the PCR in order to confirm whether this symbiont is lacking in *D. citri* populations in Florida. Only 45% of the Asian population of *D. citri* that was analyzed had *Arsenophonus*, so this is not likely an essential endosymbiont and may be associated with the Asian environment.

To determine if there are multiple *Wolbachia* strains in the Florida population of *D. citri*, the *Wolbachia wsp* gene was amplified and 10 clones were sequenced. For each clone, an identical 539-bp sequence was obtained that was 100% identical to the *wsp* sequence previously reported from the Florida population of *D. citri* (GenBank accession AF217721) (Jeyaprakash & Hoy 2000). These results suggest that only a single *Wolbachia* strain is present in the laboratory population of *D. citri*.

A putative capsid protein gene (*orf7*) of the bacteriophage WO was PCR-amplified from DNA extracted from adult *D. citri*. In a BLAST search, the 409-bp *orf7* sequence (GenBank accession EF444818) produced significant alignments to *orf7* sequences of the bacteriophage WO of *Wolbachia* from multiple sources, and it was 99% identical (405/499 bp) to 2 deposited *orf7* sequences associated with *Wolbachia*-infected *Culex pipiens* (GenBank accession AY505105, AY505101**).** The *orf7* gene has been found in most, but not all *Wolbachia*-infected arthropods (Masui et al. 2000; Fujii et al. 2004; Gavotte et al. 2004; Braquart-Varnier et al. 2005; Hoy & Jeyaprakash 2005). Masui et al. (2000) and Braquart-Varnier et al. (2005) found that 100% of the *Wolbachia* strains tested had bacteriophage WO, and Gavotte et al. (2007) reported that 70% of the *Wolbachia* strains that were tested were positive for bacteriophage WO. Phylogenetic comparisons of the bacteriophage WO and host *Wolbachia* have suggested that the bacteriophage WO is horizontally transferred among different lineages of *Wolbachia* by unknown mechanisms (Masui et al. 2000; Gavotte et al. 2004, 2007).

Diagnostic, microbe-specific PCR was conducted to determine the proportion of laboratory and field-collected *D. citri* that were positive for each eubacterial species and for the bacteriophage WO. Amplification products of a portion of the 16S rRNA gene from the primary symbiont, *Ca.* C. ruddii, the secondary symbiont, *Wolbachia* and the bacteriophage WO of *Wolbachia* were detected in 100% of DNA samples extracted from adult *D.*

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citri from the laboratory colony (n = 10: 5 females, 5 males) and from 100% of the DNA isolated from psyllids collected from the field (n = 10: 5 females, 5 males). Both the primary and secondary symbionts were detected in 100% of *D. citri* in an Asian population by Subandiyah et al. (2000). The percentage of *Wolbachia* (100%) in the Florida population (Meyer et al. 2007) was higher than in the Asian population of *D. citri* (76%) (Subandiyah et al. 2000). This could reflect true differences in the endosymbiotic complex of *D. citri*, or could be related to the increased sensitivity of the high-fidelity PCR assay used in this study compared to the standard PCR protocol used by Subandiyah et al. (2000). Hoy et al. (2001) reported that the high-fidelity PCR assay was up to sevenfold more sensitive than standard PCR.

To determine the transmission mechanism of each symbiont, DNA isolated from *D. citri* eggs was used in a high-fidelity, microbe-specific PCR reaction. The primary symbiont, secondary symbiont, *Wolbachia* and the bacteriophage WO were detected both in samples that were not surface decontaminated with bleach and in samples that were bleach treated. This indicated that the symbiont DNA was present inside the eggs (transovarial transmission). Transovarially or maternally-transmitted symbionts often are obligatory to the host, such as in the case of *Buchnera* species that provide nutrients to their aphid hosts (Moran & Degnan 2006), or can manipulate their hosts' reproductive system to their advantage, as does *Wolbachia* in a variety of arthropods (O'Neill et al. 1997).

Endoymbionts of *T. radiata*

Amplification products from the eubacterial 16S rRNA gene were cloned from each of 2 replicate high-fidelity PCR reactions with DNA from surface-decontaminated adult *T. radiata*. A total of 15 clones were analyzed from each replicate by RFLP technique. Analysis with *Eco*RI digests indicated that all clones from each replicate had an insert approximately 1.4 kb in length. Digests with *Rsa*I resulted in a total of 2 and 3 unique banding patterns in the RFLP analysis according to replicate, respectively. A clone representing each unique banding pattern in both replicates was sequenced (5 total sequences). Two pairs of these sequences were 100% identical between each replicate, respectively. These comparisons indicated that there are at least 3 types of Eubacteria associated with the laboratory colony of *T. radiata*.

The three 16S rRNA sequences obtained from *T. radiata* were used in a BLAST search to identify related sequences in GenBank. The first sequence (1409 bp, GenBank accession EF433789) produced significant alignments to *Caulobacter* species of the α-proteobacterial family Caulobacteraceae. The sequence was 100% identical to the 16S rRNA gene sequence from *Caulobacter* sp. strain FWC41 (GenBank accession AJ227775) identified from activated sludge in a secondary treatment facility in Calgary, Alberta, Canada (Abraham et al. 1999). The sequence was also 100% identical to an uncultured eubacterial species (1283 bp, GenBank accession AY038618) from an "environmental sample". Other reports have associated *Caulobacter* species with the gut of arthropods, including a millipede (Abraham et al. 1999), *Acromyrmex* leafcutter ants (Van Borm et al. 2002), and the mite *Tetranychus urticae* (Koch) (Hoy & Jeyaprakash 2005). Thus, it is unclear whether this microorganism is associated with the gut of *T. radiata* as a permanent or transient component.

The second sequence (1410 bp, GenBank accession EF433790) produced significant alignments to *Methylobacterium* species of the α-proteobacterial family Methylobacteriaceae. This sequence was 100% identical to homologous portions of the 16S rRNA gene sequence from *Methylobacterium lusitanum* strain NCIMB 13779 (1435 bp, GenBank accession AB175635) (Kato et al. 2005) and from a *Methylobacterium* sp. strain TNAU12 (1340 bp, GenBank EF116590) reported from the phyllosphere of soybean. Species belonging to the genus *Methylobacterium* have been reported in a wide variety of environments (Kato et al. 2005), but are not known to be endosymbionts of parastioids.

The third sequence (1457 bp: GenBank EF43379) produced significant alignments to the 16S rRNA gene sequence from β-proteobacterial species in the family Alcaligenaceae. This sequence was 99% identical to the homologous portion of the 16S rRNA gene sequence from an unidentified bacterium in the family Alcaligenaceae (1452/1454 bp) found on the epiphytic surfaces of rice (Hiraoka et al. 2006). Again, we have no reason to believe that this microorganism is a permanent endosymbiont of *T. radiata*.

In the diagnostic PCR assay, using microbe-specific primers (Table 1), the *Caulobacter*, *Methylobacter* and Alcaligenaceae species were detected in 70, 30 and 20% of the individual *T. radiata* collected from the laboratory colony (N=10), respectively, and 20, 0 and 0% of the field collected specimens (N=10), respectively. None of the species were detected in the bleach-treated or untreated eggs of *T. radiata*. It appears that the eubacterial species detected in *T. radiata* are likely acquired from the environment and are not transovarially transmitted, with the possible exception of the *Caulobacter* species. Despite the efforts to surface-sterilize the insects and to use newly emerged adult *T. radiata* prior to DNA extraction, it is possible that these Eubacteria were acquired when adult *T. radiata* chewed a hole in the dorsum of the mummified and potentially contaminated *D. citri* host. If so, these Eubacteria would be protected from the bleach treatment because they were located in the gut of *T. radiata*, and likely are facultative or transient associates.

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Endosymbionts of *D. aligarhensis*

PCR amplification products for the eubacterial 16S rRNA gene were detected with template DNA from 2 separate DNA extractions of *D. aligarhensis*. Ten clones from each replicate were analyzed by RFLP technique. All clones had an approximate 1.4-kb insert as indicated by digestion with *Eco*RI. *Rsa*I digests resulted in a single banding pattern for all 10 clones in both replicates, and a single clone from each replicate was sequenced. Both sequences were 1429 bp and 100% identical, suggesting that only one endosymbiotic eubacterial species is associated with the laboratory colony of *D. aligarhensis*.

A BLAST search was conducted with this sequence (GenBank accession EF433794) that produced significant alignments to multiple *Wolbachia* species in the α-proteobacterial family Rickettsiaceae. This sequence was different than the 16S rRNA sequence from *Wolbachia* obtained from the Florida population of *D. citri* (GenBank accession EF433793), and a sequence alignment indicated the sequences were 99% identical (1416/1429 bp). The different *Wolbachia* infections in *D. citri* and *D. aligarhensis* indicated that horizontal transfer of *Wolbachia* did not occur between these species, at least not recently.

To determine if *D. aligarhensis* had multiple strains of *Wolbachia*, the *wsp* gene was PCR-amplified and the amplification products were cloned. Ten clones were sequenced and the sequences were 100% identical in size (542 bp) and nucleotide composition to the *wsp* gene previously reported in our laboratory population of *D. aligarhensis* (Jeyaprakash & Hoy 2000). This suggested that only a single strain of *Wolbachia* was present in this population of *D. aligarhensis*. The *wsp* gene sequences of *Wolbachia* from *D. citri* and *D. aligarhensis* were different, further supporting the conclusion based on differences in the 16S rRNA gene sequences that these 2 *Wolbachia* strains are different. Although the sequences are different, the 2 *Wolbachia* strains are classified in the same "Super-Group B" of *Wolbachia* (Jeyaprakash & Hoy 2000).

Amplification products for the *wsp* gene of *Wolbachia* were detected in 100% of DNA samples extracted from adult female *D. aligarhensis* from our laboratory colony (n = 10). No field-collected specimens were available for analysis because populations of this parasitoid are scarce in Florida. No eggs were sampled to confirm the transmission of *Wolbachia* in the eggs of *D. aligarhensis*, due to difficulties obtaining eggs from the endoparasitoid, which deposits them inside *D. citri* nymphs. However, we speculate that *Wolbachia* is transmitted inside the egg of *D. aligarhensis*, based on the results from this study regarding *Wolbachia* transmission in *D. citri* and on other reports showing such a transmission mechanism (Kose & Karr 1995). *Wolbachia* infection in *D. aligarhensis* is correlated with thelytokous reproduction, as demonstrated by male production following tetracycline treatment of parental females (Meyer & Hoy 2007).

Despite conducting replicate high-fidelity PCR reactions, no amplification products were obtained for the *orf*7 gene of the bacteriophage WO of *Wolbachia* with *D. aligarhensis* DNA as the template. This was in contrast to the presence of the bacteriophage WO in *D. citri* and indicated that the *Wolbachia* species in *D. aligarhensis* either does not have bacteriophage WO or that the sequence representing the *orf*7 gene is not amplifiable with the primers used in this survey. This finding is not uncommon; *Wolbachia* strains lacking the bacteriophage WO have been reported from parasitoids in the genus *Trichogramma* and nematodes (Gavotte et al. 2007).

Phylogenetic Analysis

The eubacterial 16S rRNA gene sequences obtained from *D. citri*, *T. radiata* and *D. aligarhensis* were used for phylogenetic analyses. The GTR + G nucleotide substitution model (Yang et al. 1994) was selected by MODELTEST for the data matrix and used for maximum likelihood (ML) analysis. The topologies of the ML and maximum parsimony (MP) trees were similar, and each method grouped the taxa according to their expected classification within the gram-negative Eubacteria and separated them from the outgroup taxon, *Bacillus subtilis*, a gram-positive eubacterium. Fig. 1 depicts the tree constructed by ML analysis. Three major clades were produced that included taxa belonging to the α–, β–, and γ-proteobacteria, respectively, and the separation of each major eubacterial division was strongly supported by the bootstrap method. The primary (mycetocyte) symbiont, *Ca.* C. ruddii of *D. citri* (Florida) was grouped in the γ-proteobacteria in a clade including the primary symbiont from *D. citri* (Asia) and the primary symbiont of *Cacopsylla brunneipennis* (Edwards) (Hemiptera: Psyllidae). Thao et al. (2000) provided a detailed phylogenetic analysis of additional psyllid species and their associated primary endosymbionts. The secondary symbiont of *D. citri* (Florida) was grouped in the β-proteobacteria with the secondary symbiont of *D. citri* (Asia) and related Eubacteria in the genera *Oxalobacter* and *Herbaspillium*. These findings were consistent with the phylogenetic analysis conducted by Subandiyah et al. (2000). *Wolbachia* of *D. citri* (Florida) was grouped in the α-proteobacteria in a clade consisting of 3 *Wolbachia* species from *D. citri* (Asia), *D. aligarhensis* and *Culex pipiens* Linnaeus (Diptera: Culicidae), respectively. The *Caulobacter* and *Methylobacter* microbes found in *T. radiata* were clustered with other species in the α-proteobacteria, and the bacterium in the family Alcaligenaceae was grouped with the β-proteobacteria. Separation in the clades includ-

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ing the eubacterial symbionts of *D. citri, T. radiata*, and *D. aligarhensis* was strongly supported by the bootstrap values.

We concluded that the endosymbiotic communities in our laboratory population of *D. citri* and its parasitoids are relatively simple. A total of 3 eubacterial species each were detected in *D. citri* and *T. radiata* and only 1 species was detected in *D. aligarhensis*, but none of these insects harbored any detectable endosymbionts belonging to the other taxa surveyed. The eubacterial 16S rRNA sequences obtained from these insects were not identical when compared between species, indicating that stable horizontal transfer of these microorganisms likely has not occurred in this host-parasitoid system.

Potential Caveats of the PCR-based Survey

The microbes identified in this study were detected with the sensitive high-fidelity PCR protocol but were not isolated in pure culture or located in the insects by *in situ* hybridization (or an equivalent technique). It is possible that we underestimated the diversity of endosymbionts if the "universal" PCR primers failed to amplify all microbial sequences in a particular taxonomic group. The authors recognize that additional endosymbionts may be associated with these insects for which no PCR primers have been developed. In addition, there may be endosymbionts associated with wild populations of *D. citri* and its parasitoids in Florida that were not identified in this survey, which was based on laboratory insect colonies and only limited numbers of field-collected insects. The titer of the microbial DNA may influence the sensitivity of the PCR. For example, microbial DNA sequences that are very rare may not compete with abundant microbial DNA sequences or could be below the sensitivity of the high-fidelity PCR assay (Hoy et al. 2001). The random selection of clones, number of clones analyzed, choice of restriction enzymes, and analysis of clones potentially containing different sequences but yielding identical RFLP profiles also could contribute to an underestimation of the total diversity present.

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