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Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods

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Many intracellular micro-organisms are now known to cause reproductive abnormalities and other phenomena in their hosts. The endosymbiont *Wolbachia* is the best known of these reproductive manipulators owing to its extremely high incidence among arthropods and the diverse host effects it has been implicated as causing. However, recent evidence suggests that another intracellular bacterium, a Cytophaga-like organism (CLO), may also induce several reproductive effects in its hosts. Here, we present the first survey of arthropod hosts for infection by the CLO. We use a sensitive hemi-nested polymerase chain reaction method to screen 223 species from 20 arthropod orders for infection by the CLO and *Wolbachia*. The results indicate that, although not as prevalent as *Wolbachia*, the CLO infects a significant number of arthropod hosts (*ca.* 7.2%). In addition, double infections of the CLO and *Wolbachia* were found in individuals of seven arthropod species. Sequencing analysis of the 16S rDNA region of the CLO indicates evidence for horizontal transmission of the CLO strains. We discuss these results with reference to future studies on host effects induced by intracellular micro-organisms.

Keywords: Cytophaga-like organism; *Wolbachia*; infection frequency; arthropods; phylogeny; 16S rDNA

1. INTRODUCTION

Intracellular micro-organisms are ubiquitous in nature and can play vital roles in host survival and evolution (Buchner 1965; Moran & Telang 1998; Stouthamer *et al.* 1999). Yet, owing to their fastidious nature, understanding these roles has often been difficult. Polymerase chain reaction (PCR) technologies have opened new avenues for research on these unusual but highly important organisms and have facilitated significant insights into relationships between host and endosymbiont. For example, over the past decade, the α-proteobacterium *Wolbachia* has become one of the most well known of all intracellular micro-organisms. This bacterium was known for several decades to be involved in reproductive abnormalities in its host (Yen & Barr 1971; Barr 1982; Wade & Stevens 1985; Hoffmann & Turelli 1988). It gained widespread notoriety only after a routine PCR-based screening study estimated that 16% of all insect species are infected with *Wolbachia* (Werren *et al.* 1995). A recent screening study has indicated that this may even be an underestimate and that *Wolbachia* infection levels are as high as 76% of all insect species (Jeyaprakash & Hoy 2000).

Wolbachia are known to induce four main reproductive abnormalities in their arthropod hosts: cytoplasmic incompatibility, parthenogenesis induction, feminization and male killing (see Stouthamer *et al.* (1999) for review). They have also been implicated in causing many other phenomena in their hosts, although the evidence for some of these is contentious (Weeks *et al.* 2002). Other micro-organisms are also known to induce reproductive abnormalities in their hosts. Microsporidia can cause feminization in some isopods (see Bandi *et al.* 2001) and there are three different intracellular bacteria and two spiroplasma species that can induce male killing (see Hurst & Jiggins 2000). However, owing to its high incidence among arthropods and its ability to induce multiple reproductive effects among its various hosts, *Wolbachia* is thought to be unique.

Recently, Weeks *et al.* (2001) showed that a bacterium from the Cytophaga–Flavobacterium–Bacteroides (CFB) phylum causes feminization in the false spider mite, *Brevipalpus phoenicis*. For clarity, we will refer to this bacterium throughout as a Cytophaga-like-organism (CLO). A similar strain of the CLO may also be associated with parthenogenesis in several species of parasitoids from the genus *Encarsia* (Zchori-Fein *et al.* 2001). Sequence analysis of the bacterial 16S rDNA gene shows these strains to be very similar (less than 3% sequence divergence) and related to an intracellular bacterium found in the tick *Ixodes scapularis* (Kurtti *et al.* 1996) where its effects are unknown. Based on transmission electron microscope (TEM) pictures of the cellular ultrastructure from the CLO strains infecting *Encarsia pergandiella* and *I. scapularis*, Zchori-Fein *et al.* (2001) and Weeks & Breeuwer (2003) suggested that a unique rod-like structure found within the bacterium's cells is characteristic of the CLO. Further, based on previous TEM pictures in the literature, Weeks & Breeuwer (2003) postulated that the CLO has previously been found infecting other invertebrate hosts, including several hemipterans, nematodes and mites. These recent results suggest that the CLO infects numerous arthropods and may induce multiple reproductive effects in its hosts, similar to *Wolbachia*.

In this study, we use a sensitive hemi-nested PCR screening method to determine the incidence of the CLO and *Wolbachia* among 223 species sampled from 20 arthropod orders. We then amplify and sequence a portion of the 16S rDNA gene of the CLO from all hosts infected to determine associations between host and symbiont. The results indicate that, although not as abundant as *Wolbachia*, the CLO infect a significant number of species

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from diverse arthropod orders. More interestingly, double infections of the CLO and *Wolbachia* are found within single individuals of several host species, highlighting problems with current research on the effects endosymbionts have on their hosts.

2. MATERIAL AND METHODS

(a) *Sample collection and DNA extraction*

One hundred and eighty-one arthropod species were sampled either directly from the field in southern California ($n = 119$), or obtained from cultures at the University of California, Riverside, CA ($n = 62$). An additional 42 species were obtained from several sources from around the world (a list of collection sites and collection dates can be obtained from the authors upon request). Between three and 10 individuals were collected for each species with a voucher specimen for each species stored at the Department of Entomology, University of California, Riverside, CA, USA.

DNA was extracted from either single, whole individuals (for arthropods 0.5 mm or less in size) or single, whole abdomens (arthropods greater than 0.5 mm in size). Predatory arthropods were starved for 24 h before DNA extraction to limit gut content contamination. Before DNA extraction, all specimens were washed three times in 95% ethanol and air-dried for 15 min. DNA was extracted using a phenol–chloroform extraction method with an RNase and proteinase-K treatment (see Sambrook & Russell (2001) for protocol). To check for contamination during extraction, a tube with no arthropod sample was included in each extraction procedure. We extracted DNA from between two and five individuals for each species.

(b) *PCR screening procedure for CLO and* Wolbachia *infection*

To screen DNA extractions for the presence of the CLO or *Wolbachia*, we used a hemi-nested PCR method. First, each DNA extraction was screened for the presence of bacteria by amplifying the 16S rDNA using the primers 27F and 1513R (Weisburg *et al.* 1991). These primers will amplify a region of *ca*. 1500 bp of the 16S rDNA of all eubacteria. PCR products were then diluted in water (1 µl of PCR product: 500 µl of sterile water) and screened, in two separate PCR reactions, for the CLO and *Wolbachia* using 16S rDNA primers specific for each bacterium. This procedure is extremely sensitive and allows detection of the CLO and *Wolbachia* in minute arthropods such as mites and parasitoids (A. R. Weeks and R. Stouthamer, unpublished data).

All PCRs were performed in a total volume of 25 µl containing: 1× buffer (Promega), 0.5 mM of each dNTP, 2 units of Taq polymerase (Promega), 1.5 mM of $MgCl_2$ and 5 µM of each primer. The primers for the initial PCR amplification of eubacterial 16S rDNA were 5′-AGAGTTTGATCMTGGCTCAG (27F) and 5′-ACGGYTACCTTGTTACGACTT (1513R). The CLO-specific 16S rDNA primers were 5′-GCGGTG TAAAATGAGCGTG (CLOf) and 5′-ACCTMTTCTTAAC TCAAGCCT (CLOr1). These primers were designed based on the currently known 16S rDNA sequences of the CLO (isolated from *Brevipalpus phoenicis*, *I. scapularis* and *E. pergandiella*) and amplify a product of *ca*. 450 bp. These primers never amplified bacteria other than the CLO in our study. The *Wolbachia*-specific 16S rDNA primers were taken from O'Neill *et al.* (1992) and amplify a product of *ca*. 900 bp. PCR conditions for the initial PCR were one cycle (4 min at 94 °C), 35 cycles (1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C) and one cycle (5 min at 72 °C). The CLO-specific PCR conditions were (4 min at 94 °C), 35 cycles (40 s at 94 °C, 40 s at 57 °C, 45 s at 72 °C) and one cycle (5 min at 72 °C) whereas the *Wolbachia*-specific PCR conditions were (4 min at 94 °C), 35 cycles (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) and one cycle (5 min at 72 °C). All amplifications were run on an Eppendorf Mastercycler with appropriate negative and positive controls for each reaction. After each PCR, 5 µl of amplified reaction product was run on a 1% agarose gel stained with ethidium bromide to determine the presence of an amplified DNA fragment. If a sample was negative for both the CLO and *Wolbachia*, then the 28S rDNA region of the host was amplified using primers, PCR mixtures and cycling conditions from Werren *et al.* (1995). If this did not yield a product of between 500 and 600 bp then failure of DNA extraction was assumed and the sample was not included in any further analysis. The overall proportion of DNA extraction failures was small (*ca*. 4.0%).

(c) *Sequencing and phylogenetic analysis of CLO strains*

For all species that were positive for the CLO infection, we sequenced *ca*. 1100 bp of the 16S rDNA of each CLO strain. Using Expand High Fidelity polymerase (Roche), a PCR was performed using genomic DNA as template, from the initial DNA extractions. PCRs were performed as above using the same cycling conditions as those used for the CLO-specific PCR screen. Primers used for PCR were CLOf and CLOr2 (5′-TGTGTACAAGGTCCGAGAACG). PCR products were purified using the Wizard PCR Preps DNA purification system (Promega) and sequenced using standard cycle sequencing PCR (ABI prism Big Dye terminator kit, Applied Biosystems, San Jose, CA, USA) with the CLOf and CLOr2 primers.

Sequences were aligned using the CLUSTAL W algorithm (Thompson *et al.* 1994) and adjustments made manually as needed. We included the CLO sequences isolated previously from *I. scapularis* and *B. phoenicis* (Brazil) (GenBank accession numbers AB001518 and AF350221, respectively). The sequence data were analysed using PAUP 4.0b10 (Swofford 1999). Both parsimony and maximum-likelihood methods were used to infer phylogenies. In all analyses, the endosymbiont of *Acanthomoeba* sp. (Hungarian isolate, GenBank accession number AF215634) was used as an outgroup, as it is the closest known relative of the CLO (Horn *et al.* 2001; Weeks & Breeuwer 2003). For parsimony analyses, we used heuristic search with tree bisection–reconnection branch swapping, random addition sequence for taxa, and 100 replicates per search. Bootstrap support for nodes was estimated using 1000 replicates with 100 random sequence additions per replicate. For maximum likelihood, we used likelihood-ratio tests to suggest a model with the best likelihood score (MODELTEST 3.0; Posada & Crandall 1998). The suggested model was the general time reversible (GTR) model with rates that vary over sites according to the invariable sites plus gamma (I+G) distribution. Likelihood parameters for heuristic searches were estimated using MODELTEST 3.0 (Posada & Crandall 1998). Bootstrap support for nodes was determined by using 500 replicates. 16S rDNA sequences representing each CLO strain are deposited in GenBank (accession numbers AY279401–AY279415 and AY285777).

3. RESULTS

(a) *Incidence of the CLO and* Wolbachia *among arthropods*

We screened 223 arthropod species from 20 orders for infection with either the CLO or *Wolbachia* (table 1). The CLO was found to infect 16 different species (7.2%) whereas *Wolbachia* was found to infect 49 species (22%). Therefore, over all species screened, *Wolbachia* was found to infect significantly more species than the CLO (*G*-test, $G = 20.4$, d.f. = 1, $p < 0.001$). The CLO was found in only three groups of arthropods (Acari, Hemiptera and Hymenoptera), whereas *Wolbachia* was found in all major groups surveyed (table 2). Infection frequencies were greatest in the Acari, with six and nine out of 19 species (31.6% and 47.4%) surveyed infected with the CLO and *Wolbachia*, respectively. The highest numbers of species were surveyed from the Hymenoptera, where we screened 67 species from 18 families. Interestingly, the CLO infections were found in only one family (Aphelinidae, seven species), whereas *Wolbachia* infections were found in four families (Aphelinidae, Encyrtidae, Halictidae and Trichogrammatidae). Three Hemipteran species were also infected with the CLO (*Bemisia tabaci*, *Aspidiotus nerii* and *Dicranotropis hamata*), whereas twelve hemipteran species were infected with *Wolbachia*.

Species infected with the CLO include four known asexual species (*Oppiella nova*, *Brevipalpus obovatus*, *Brevipalpus phoenicis* and *Aspidiotus nerii*), seven known sexual species (*Petrobia harti*, *Metaseiulus occidentalis*, *Bemisia tabaci*, *Dicranotropis hamata*, *Aphytis lingnanensis*, *Encarsia lutea* and *Encarsiella noyessi*) and five species for which their reproductive mode is unknown (*Balaustium* sp., *Aphytis* sp. 3, *Aphytis* sp. 4, *Encarsia* sp. 2 and *Encarsia* sp. 3). Therefore the CLO is not strictly associated with asexual hosts.

Interestingly, double infections of the CLO and *Wolbachia* were found in several species within single individuals. In the Acari, three double infections were found (*Balaustium* sp., *P. harti* and *M. occidentalis*), occurring at a frequency of 15.8%. The CLO and *Wolbachia* also infected the oribatid mite, *Oppiella nova*; however, owing to the small size of these mites, in each extraction we lumped five mites together. Therefore, it was not determined if individuals of this species were infected with both the CLO and *Wolbachia* or if the infections occurred in different individuals. In the Hymenoptera, individuals from three species were infected with both the CLO and *Wolbachia* (*Aphytis lingnanensis*, *Aphytis* sp. 3 and *Aphytis* sp. 4), and therefore double infections occurred at a frequency of 4.5%. Double infections of the CLO and *Wolbachia* occurred at a frequency of 3.1% (including *O. nova*) out of all arthropods screened. The random expectation for the frequency of double infections is 1.6%, which is significantly different from the observed frequency (Pearson $\chi^2 = 4.768$, d.f. = 1, $p < 0.05$). However, if only those groups where the CLO was found are considered (Acari, Hymenoptera and Hemiptera), then the observed frequency (5.2%) and the expected frequency (3.3%) are not significantly different (Pearson $\chi^2 = 2.185$, d.f. = 1, $p > 0.05$).

Precautions were taken to prevent false positives by: (i) washing samples with ethanol before DNA extraction; and (ii) including negative controls in all DNA extractions and PCR reactions. In addition, predatory arthropods were starved (when possible) for 24 h before DNA extraction. For all 16 species positive for the CLO, we confirmed infection with multiple extractions (at least three separate extractions on single individuals, except *Oppiella nova*), and contamination from other sources (such as host feeding) was ruled out by also testing hosts for infection (for predators/parasitoids). We also controlled for false negatives by amplifying the 28S rDNA of the host and included only those samples in our analysis.

(b) *Phylogenetic analyses of CLO strains*

Out of the 1109 sites included in the parsimony analysis, 941 (85%) were constant, 115 (10%) were autapomorphic and 53 (5%) were parsimony informative. Forty-seven most-parsimonious trees were found with a length of 229. For maximum-likelihood analysis, the single tree using the GTR+G+I model had a –ln likelihood score of 2742.67 (I = 53%, G = 0.71, A = 0.2799, C = 0.2026, G = 0.2875, T = 0.2300). Trees built by using both parsimony and maximum-likelihood methods revealed very similar topologies that were congruent where nodes were strongly supported. The tree obtained through maximum-likelihood analysis is shown in figure 1, with bootstrap support for nodes indicated for both parsimony and maximum-likelihood methods.

The diversity found between the 18 CLO strains infecting arthropods did not exceed 3.0% for the 16S rDNA region sequenced, although all strains were unique. Therefore, the resolution of the CLO strain relationships in the tree shown in figure 1 is quite low. However, there are several features evident from the tree. First, there is no evidence for complete concordance of the CLO strains with host phylogeny, suggesting horizontal transmission has occurred. In fact, three instances of possible horizontal transmission of the CLO strains between predators/parasites and their prey/hosts are evident from figure 1 (see § 4). The strain of the CLO infecting *B. phoenicis* collected from Riverside, CA, USA, was very similar (but not identical) to the strain of the CLO previously found to infect *B. phoenicis* collected from Brazil. However, the strain of the CLO infecting the sister species, *B. obovatus*, was not closely related to the CLO strains infecting *B. phoenicis* (1.4–1.7% difference) and could not be resolved from the CLO strains infecting other Acari and the hemipteran *Dicranotropis hamata*. Lastly, the CLO strains infecting species within the family Aphelinidae (Insecta: Hymenoptera) are monophyletic.

4. DISCUSSION

Weeks & Breeuwer (2003) recently suggested that the CLO would be found to infect numerous invertebrates based on an association they found between a unique cellular ultrastructure found in the CLO and previously documented TEM pictures of unidentified endosymbionts from various invertebrate species. Using a sensitive heminested PCR screening method we have confirmed this prediction, showing CLO infections in 16 species from five different arthropod orders (Oribatida, Prostigmata, Mesostigmata, Hymenoptera and Hemiptera), out of 223 species from 20 arthropod orders tested. Although CLO

Table 1. Arthropods tested for infection by the CLO and/or *Wolbachia* with PCR.
(Unidentified species/families are listed under their appropriate family/order.)

taxon	CLO	*Wolbachia*
Arthropoda		
Arachnida		
Araneae		
Dictynidae		
Dictyna calcarata	−	−
Ixodida		
Ixodidae		
Ixodes pacificus	−	−
Oribatida		
Oppoidea		
Oppiella nova	+	+
Prostigmata		
Erythraeidae		
Balaustium sp.	+	+
Penthaleidae		
Halotydeus destructor	−	−
Penthaleus major	−	−
Tenuipalpidae		
Brevipalpus obovatus	+	−
Brevipalpus phoenicis	+	−
Tetranychidae		
Bryobia sp.	−	+
Panonychus citri	−	−
Petrobia harti	+	+
Tetranychus cinnabarinus	−	−
Tetranychus urticae	−	+
Mesostigmata		
Phytoseiidae		
Galendromus annectens	−	+
Galendromus helveolus	−	−
Mesoseiulus longipes	−	+
Metaseiulus occidentalis	+	+
Neoseilus fallacis	−	−
Phytoseiulus macropilis	−	−
Phytoseiulus persmilis	−	+
Insecta		
Coleoptera		
Anthicidae		
Notoxus spatulifer	−	−
Bolboceratidae		
Bolbocerastes regalis	−	−
Cerambycidae		
Grammoptera militaris	−	+
Ceratocanthidae		
Germarostes globosus	−	−
Cleridae		
unidentified species	−	−
Coccinellidae		
Cycloneda sanguinea	−	−
Hippodemia convergens	−	−
Dermestidae		
Anthrenus sp. 1	−	+
Anthrenus sp. 2	−	−
Geotrupidae		
Geotrupes splendidus	−	−
Glaresidae		
Glaresis ecostata	−	−
Hybosoridae		
Hybosorus illigeri	−	−
Lucanidae		
Lucanus elephas	−	−
Melyridae		
Allonyx sp.	−	−
Listrus sp. 1	−	−
Listrus sp. 2	−	−
Pleocomidae		
Pleocoma dubitalis	−	−
Scarabaeidae		
Acoma glabrata	−	−
Anomala marginata	−	−
Diplotaxis sp.	−	−
Ligyrus ruginasus	−	−
Parathyce palpalis	−	−
Popillia japonica	−	−
Scarabaeus sp.	−	−
Serica sp.	−	−
Silphidae		
Nicrophorus sp.	−	−
Staphylinidae		
unidentified species 1	−	−
unidentified species 2	−	−
Tenebrionidae		
Cryptoglossa sp.	−	−
Trogidae		
Omorgus suberosus	−	−
Collembola		
Sminthuridae		
unidentified species	−	−
unidentified family		
unidentified species	−	−
Diplopoda		
unidentified family		
unidentified species	−	−
Diptera		
Blephariceridae		
Blepharicera sp.	−	−
Bombyliidae		
Bombylius sp.	−	+
Chironomidae		
unidentified species	−	−
Culicidae		
Culex pipiens	−	+
Culex quiquefasciatus	−	+
Culex tarsalis	−	−
Drosophilidae		
Drosophila hydei	−	−
Drosophila melanogaster	−	+
Drosophila wheeleri	−	−
Empididae		
unidentified species	−	−
Muscidae		
Musca domestica	−	−
unidentified species	−	−
Mycetophylidae		
unidentified species 1	−	−
unidentified species 2	−	−
Syrphidae		
unidentified species 1	−	−
unidentified species 2	−	−
Tachinidae		
unidentified species	−	−
Tephritidae		

(*Continued.*)

Table 1. (*Continued.*)

taxon	CLO	*Wolbachia*
Neotephritis sp.	−	−
Tipulidae		
unidentified species 1	−	−
unidentified species 2	−	−
unidentified family		
unidentified species	−	−
Hemiptera		
Aleyrodidae		
Aleurodicus dugessi	−	+
Bemisia argentifolia	−	−
Bemisia tabaci	+	−
Parabemisia myricae	−	−
Trialeurodes vaporariorum	−	−
unidentified species	−	−
Aphididae		
Sitobion miscanthi	−	−
Sitobion nr *fragariae*	−	−
unidentified species 1	−	−
unidentified species 2	−	−
unidentified species 3	−	−
Cercopidae		
Prosapia plagiata	−	−
Chermidae		
unidentified species	−	−
Cicadellidae		
Amblysellus grex	−	−
Euscelidius variegatus	−	−
Homalodisca coagulata	−	+
Listridea nuda	−	−
unidentified species 1	−	+
unidentified species 2	−	+
unidentified species 3	−	−
Cicadidae		
Okanagana utahensis	−	−
Cixiidae		
Oliarus hesperinus	−	+
Coccidae		
Coccus hesperidum	−	−
Coccus pseudomagnoliarum	−	−
Coreidae		
Anasa tristus	−	−
Delphacidae		
Dicranotropis hamata	+	−
Diaspididae		
Aonidiella aurantii	−	−
Aspidiotus nerii	+	−
Dictyopharidae		
Scolops fumida	−	−
Flatidae		
Siphanta acuta	−	−
Lygaeidae		
Nysius sp. 1	−	+
Nysius sp. 2	−	+
Membracidae		
Platycotis sp. 1	−	−
Platycotis sp. 2	−	−
Spissistilus festinus	−	+
Miridae		
Irbisia sp.	−	−
unidentified species	−	−
Peloridiidae		
Hemiowood wardia	−	−
Pentatomidae		
Nezara viridula	−	−
Thyanta pallidoventris	−	−
Phylloxeridae		
Daktulosphaira vitifoliae	−	−
Pseudococcidae		
Planococcus citri	−	−
Psyllidae		
Eucalyptolyma maidenii	−	+
Glycaspis brimblecombei	−	−
Trioza brevianten nata	−	+
Thyreocoridae		
Corimelaena lateralis	−	+
Tingidae		
unidentified species	−	+
unidentified family		
unidentified species	−	−
unidentified family		
unidentified species	−	−
Hymenoptera		
Andrenidae		
Andrena sp.	−	−
Aphelinidae		
Aphelinus sp. 1	−	−
Aphelinus sp. 2	−	−
Aphytis coheni	−	+
Aphytis lingnanensis	+	+
Aphytis melinus	−	+
Aphytis paramaculicornis	−	+
Aphytis vandenboschi	−	+
Aphytis yanonensis	−	+
Aphytis sp. 1	−	−
Aphytis sp. 2	−	−
Aphytis sp. 3	+	+
Aphytis sp. 4	+	+
Aphytis sp. 5	−	+
Aphytis sp. 6	−	−
Aphytis sp. 7	−	−
Coccophagous sp. 1	−	−
Encarsia lutea	+	−
Encarsia sp. 1	−	−
Encarsia sp. 2	+	−
Encarsia sp. 3	+	−
Encarsiella noyessi	+	−
Marietta sp. 1	−	+
Marietta sp. 2	−	+
Braconidae		
unidentified species 1	−	−
unidentified species 2	−	−
Chalcididae		
Acanthochalcis nigricans	−	−
Brachymeria sp.	−	−
Colletidae		
Hylaeus sp. 1	−	−
Hylaeus sp. 2	−	−
Encyrtidae		
Comperiella bifasciata	−	−
Metaphycus luteolus	−	−
Metaphycus nr *flavus*	−	−
Metaphycus stanleyi	−	−
unidentified species	−	+
Eurytomidae		

(*Continued.*)

Table 1. (*Continued.*)

taxon	CLO	*Wolbachia*	taxon	CLO	*Wolbachia*
Sycophila isis	−	−	*Synedoida* sp.	−	−
Formicidae			unidentified species	−	+
Linepithema humile	−	−	Nympalidae		
Gasteruptiidae			*Agraulis vanillae*	−	−
Gasteruption sp.	−	−	Papilionidae		
Halictidae			*Papilio rutulus*	−	−
Dialictus sp.	−	+	Pyralidae		
Sphecodes sp.	−	+	*Ephestia kuehniella*	−	+
Ichneumonidae			Riodinidae		
Therion longipes	−	−	*Apodemia mormo*	−	−
Mutillidae			Sphingidae		
Dasymutilla nocteus	−	−	*Manduca sexta*	−	−
Pteromalidae			**Neuroptera**		
Muscidifurax sp.	−	−	Chrysopidae		
Signiphoridae			*Chrysoperla* sp.	−	−
unidentified species	−	−	Coniopterygidae		
Tenthredinidae			*Conwentzia* sp.	−	+
unidentified species	−	−	Raphidiidae		
Tiphiidae			*Agulla* sp.	−	−
Brachycistis sp.	−	−	**Orthoptera**		
Trichogrammatidae			Acrididae		
Trichogramma alpha	−	−	*Melanoplus* sp.	−	−
Trichogramma aurosum	−	+	Gryllacrididae		
Trichogramma australicum	−	−	*Macrobaenetes* sp.	−	−
Trichogramma brevicapilum	−	−	Polyphagidae		
Trichogramma deion	−	+	*Arenivaga* sp.	−	−
Trichogramma flandersi	−	−	Tettigoniidae		
Trichogramma fuentesi	−	−	unidentified species	−	−
Trichogramma funestum	−	−	**Phasmida**		
Trichogramma kaykai	−	−	Phasmatidae		
Trichogramma minutum	−	−	*Bacullum* sp.	−	−
Trichogramma nr *deion*	−	−	*Extatosoma tiaratum*	−	−
Trichogramma nr *exiguum*	−	−	**Phthiraptera**		
Trichogramma nr *raoi*	−	−	Linognathidae		
Trichogramma ostriniae	−	−	*Solenopotes capillatus*	−	−
Trichogramma parkeri	−	−	Menoponidae		
Trichogramma pretiosum	−	+	*Menacanthus stramineus*	−	−
Trichogramma principium	−	−	Pediculidae		
Trichogramma retorridum	−	−	*Pediculus capitis*	−	−
Trichogramma sibericum	−	−	*Pediculus humanus*	−	−
Trichogramma thalense	−	−	Trichodectidae		
unidentified family			*Bovicola bovis*	−	−
unidentified species	−	−	**Psocoptera**		
Isoptera			unidentified family		
Rhinotermitidae			unidentified species	−	−
Reticulitermes hesperus	−	−	**Strepsiptera**		
Lepidoptera			Myrmecolacidae		
Bombycidae			*Caenocholax* sp.	−	−
Bombyx mori	−	−	*Stichotrema* sp.	−	−
Gracillariidae			**Thysanoptera**		
Marmara gulosa	−	−	Aeolothripidae		
Hesperiidae			*Franklinothrips orizabensis*	−	−
Hylephila phlaeas	−	+	Phlaeothripidae		
Lycaenidae			unidentified species	−	−
Strymon melinus	−	−	Thripidae		
Noctuidae			*Scirtothrips perseae*	−	+
Spodoptera exigua	−	−			

infections were found in a significant number of arthropods, infection frequencies and incidence among the different arthropod groups screened were less than those found for *Wolbachia* (49 infected species spanning 10 arthropod orders).

The results suggest that *Wolbachia* infects a greater number of arthropod species. However, the primers used to amplify the CLO were based on only three known 16S rDNA sequences of the CLO (from the hosts *B. phoenicis*, *E. pergandiella* and *I. scapularis*) and therefore may not

Table 2. Distribution of the CLO and *Wolbachia* among different arthropod groups.

group	number of species screened	CLO infection frequency (number of positives)	*Wolbachia* infection frequency (number of positives)	double infection frequency (number of positives)
Acari	19	31.6 (6)	47.4 (9)	21.1 (4)
Coleoptera	30	0	6.7 (2)	0
Diptera	21	0	19.0 (4)	0
Hemiptera	49	6.1 (3)	24.5 (12)	0
Hymenoptera	67	10.4 (7)	25.4 (17)	4.5 (3)
Lepidoptera	12	0	25.0 (3)	0
others	25	0	8.0 (2)	0
total	223	7.2 (16)	22.0 (49)	3.1 (7)

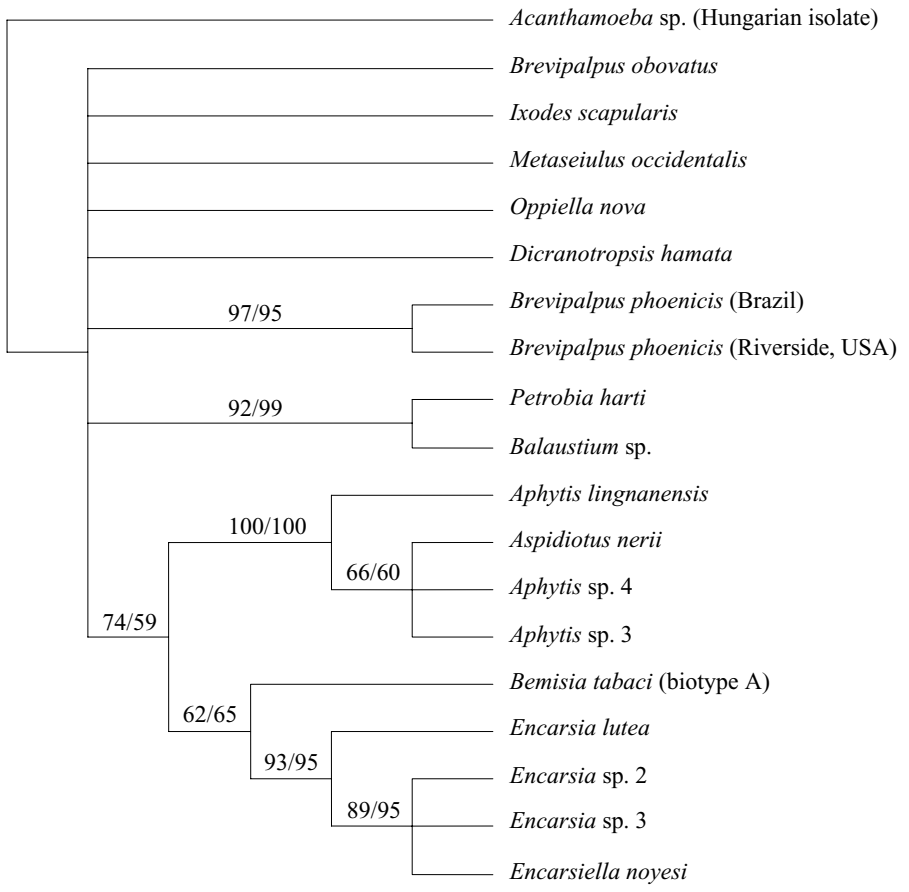

Figure 1. Consensus maximum-likelihood tree (using the GTR+I+G model) of the CLO strains based on 16S rDNA sequences (1109 bp). The CLO strains are identified by host species from which they were isolated. Bootstrap support for maximum likelihood (right) and maximum parsimony (left) are indicated above branches for nodes with greater than 50% support.

amplify all CLO strains. There can also be other reasons for false negatives such as template DNA concentration and the presence of PCR inhibitors (Jeyaprakash & Hoy 2000; Werren & Windsor 2000). In addition, our screening study will miss infections that are found at low prevalence within species (Jiggins *et al.* 2001). Screening studies can produce conflicting results for the above reasons and others (Jeyaprakash & Hoy 2000; Werren & Windsor 2000) and therefore should be used only as a guide. Interestingly, however, our *Wolbachia* infection frequency estimates are similar to those found by Werren *et al.* (1995), West *et al.* (1998) and Werren & Windsor (2000) and significantly less than that found by Jeyaprakash & Hoy (2000).

Many cytoplasmically inherited micro-organisms show strict mutualistic associations with their hosts which result in phylogenetic patterns such as co-cladogenesis (Moran & Baumann 1994; Clark *et al.* 2001). Although resolution of the CLO strains using the 16S rDNA marker was low in this study, there was no evidence of complete co-cladogenesis between host and the CLO strain, indicating horizontal transmission has occurred. In fact, there are three possible instances of horizontal transmission of the CLO strains between predators and prey shown in the tree in figure 1. The oleander scale insect, *A. nerii*, is infected with a strain of the CLO that forms a monophyletic group with the CLO strains infecting three species of the parasitoid genus *Aphytis*. *Aspidiotus nerii* is a common host for some *Aphytis* species both in the field and in the laboratory

(Rosen & De Bach 1979; Heimpel & Rosenheim 1995). Similarly, the whitefly *Bemisia tabaci* biotype A harbours a CLO strain that forms a monophyletic group with the CLO strains infecting three species of *Encarsia* and *Encarsiella noyessi*. Whiteflies are generally the primary host for most species of the heteronomous genera *Encarsia* and *Encarsiella* (Hunter & Woolley 2001). Lastly, the CLO strain infecting the phytophagous mite *Petrobia harti* forms a well-supported monophyletic group with the predatory mite *Balaustium* sp. Both species were collected from the same site and *Balaustium* species are known to prey on spider mites such as *P. harti* (Halliday 2001). Interestingly, both were also infected with *Wolbachia*.

Although the data indicate transmission is not strictly vertical, and horizontal transmission is another route that the CLO strains use to reach host species, the high incidence of infection (Zchori-Fein *et al*. 2001 and this study) and the monophyly of the *Encarsia* and *Encarsiella* CLO strains suggest strong associations between host and symbiont in these parasitoids. This could indicate strict vertical transmission within this group and co-speciation events occurring between host and symbiont. A similar situation was found with the parasitoid genus *Trichogramma* where numerous species are infected with *Wolbachia*, and 16S rDNA sequence analysis showed these strains to be monophyletic (Stouthamer *et al.* 1993). However, using a more phylogenetically informative molecular marker, Schilthuizen & Stouthamer (1997) found evidence for frequent horizontal transmission, and recently Huigens *et al.* (2000) showed experimentally that *Wolbachia* could be passed horizontally by superparasitism in *Trichogramma kaykai*. We are currently characterizing the bacterial gyrase subunit B gene from the CLO strains infecting 10 different *Encarsia* and *Encarsiella* species and using a recently published host phylogeny (Babcock *et al.* 2001) to help resolve strain/host relationships within this group.

Little is known about the effects the CLO has on its host. Weeks *et al.* (2001) have shown that the CLO causes feminization of genetic males in the mite *B. phoenicis*. We have shown here that the CLO also infects the asexual sister species *B. obovatus*, and based on results from Pijnacker *et al.* (1980) and Weeks *et al.* (2001), it is probable that the CLO also induces feminization in *B. obovatus*. The CLO may also be associated with causing parthenogenesis in *Encarsia hispida* and a host selection behavioural change in *E. pergandiella* (Zchori-Fein *et al.* 2001), although this needs clarification (Weeks & Breeuwer 2003). These are the only studies that have attempted to determine the effects of the CLO infection in their hosts.

Several species found infected by the CLO are known to have reproductive abnormalities that the CLO may induce. The whitefly *Bemisia tabaci* biotype A (infected) and biotype B (uninfected by the CLO, A. R. Weeks and R. Stouthamer, unpublished data) are known to be reproductively incompatible and it was postulated previously that this could be because of endosymbiont differences (Costa *et al.* 1995). It is also known that several *B. tabaci* biotypes harbour *Wolbachia*, which further complicates incompatibilities found in this species (Zchori-Fein & Brown 2002). The oleander scale, *A. nerii*, has sexual and asexual populations in the field (Normark 2003) and we have recently found a strict association of a CLO infection with asexual lineages (A. R. Weeks, L. Provencher and B. Normark, unpublished data).

Crosses between populations of the predatory mite *Metaseiulus occidentalis* are known to display reproductive incompatibilities resulting in reduced fecundity and F_1 survival (Croft 1970). Johanowicz & Hoy (1998) showed an association between *Wolbachia* and these incompatibilities and postulated that *Wolbachia* was responsible for these effects. However, Hess & Hoy (1982) had previously identified another endosymbiont in *M. occidentalis*, which has since been identified as the CLO (Weeks & Breeuwer 2003). Johanowicz & Hoy (1998) did not test for the CLO and so its effects on the reproductive incompatibilities cannot be excluded. Here, we found *M. occidentalis* individuals infected with both the CLO and *Wolbachia*.

The double infections of the CLO and *Wolbachia* found in individuals from six species (possibly seven including *Oppiella nova*) raises important concerns about current research on endosymbionts causing reproductive abnormalities and other phenomena in their hosts. With the advent of PCR technology, researchers typically screen hosts for *Wolbachia* using specific primers, cure infected individuals using antibiotics or heat treatment and then document the effects associated with *Wolbachia* infection (e.g. Johanowicz & Hoy 1998). This process ignores the presence of other intracellular micro-organisms that could be involved in any effects found. Many endosymbionts are known to cause reproductive abnormalities and other effects in their hosts (Weeks *et al.* 2002). The double infections found here of two micro-organisms known to induce reproductive abnormalities highlight the complexity in some host–endosymbiont(s) systems.

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