Journal of Invertebrate Pathology 105 (2010) 220–227

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

A divergent *Cardinium* found in daddy long-legs (Arachnida: Opiliones)

Jin Chang [1], Amber Masters [2], Amanda Avery, John H. Werren *

Biology Department, University of Rochester, Rochester, NY 14627, USA

ARTICLE INFO

Article history:
Received 11 September 2009
Accepted 26 May 2010
Available online 31 May 2010

Keywords:
16S rRNA
Cardinium
Endosymbiont
Opiliones

ABSTRACT

Recent studies indicate that a newly described bacterial endosymbiont, *Cardinium*, is widespread in arthropods and induces different reproductive manipulations in hosts. In this study, we used a portion of the 16S rRNA gene of the *Cardinium* to screen 16 Opilionid species from the suborder Palptores. We found the incidence of *Cardinium* in these Opiliones was significantly higher than in other pooled arthropods (31.2% versus 7.2%, $P = 0.007$). Phylogenetic analyses using maximum parsimony (MP) and Bayesian analysis revealed two distinct clades in Opiliones. One is a divergent monophyletic clade with strong support that has so far not been found in other arthropods, and a second one contains *Cardinium* both from Opiliones and other arthropods. There is not complete concordance of the *Cardinium* strains with host phylogeny, suggesting some horizontal movement of the bacteria among Opiliones. Although the divergence in the sequenced 16S rRNA region between the *Cardinium* infecting Opiliones and *Cardinium* from other arthropods is greater than among *Cardinium* found in other arthropods, all are monophyletic with respect to the outgroup bacteria (endosymbionts of *Acanthamoeba*). Based on high pairwise genetic distances, deep branch, and a distinct phylogenetic grouping, we conclude that some Opiliones harbor a newly discovered *Cardinium* clade.

1. Introduction

Endosymbionts such as *Wolbachia*, *Rickettsia*, and *Spiroplasma* are very widespread in arthropods and have been regarded as an important force for host ecology and evolution (Clark, 1982; O'Neill et al., 1992; Perlman et al., 2006; Werren et al., 2008). These symbionts can increase their frequency by directly increasing the fitness of their host (Moran and Telang, 1998), or by affecting host reproductive behavior in ways that increase bacterial transmission success (Charlat et al., 2003; Werren and O'Neill, 1997). Studies on incidence and phylogeny of endosymbionts in arthropods are revealing a rich assemblage of bacteria. Recently, a newly described endosymbiont, *Cardinium* has been shown to *be* widespread among arthropods (Duron et al., 2008; Weeks et al., 2003; Zchori-Fein and Perlman, 2004), and has been found to be involved in host reproduction manipulations. These manipulations include cytoplasmic incompatibility (CI) in *Encarsia* parasitoid wasp (Hunter et al., 2003) and spider mites (Gotoh et al., 2007; Ros and Breeuwer, 2009), parthenogenesis (Zchori-Fein et al., 2001), and feminization (Weeks et al., 2001). The studies on infection with *Cardinium* have mainly focused on insects (Perlman et al., 2008), spiders (Duron et al., 2008), and mites (Liu et al., 2006). However, there has been little attention given to other arthropods.

The arachnid order Opiliones (harvestmen, shepherd spiders, daddy longlegs, etc.) may be among the earliest diverging arachnids (Giribet et al., 2002; Shultz, 1990; Wheeler and Hayashi, 1998), and they are abundant and often widely distribute in ecosystems. Although the basic biology of most Opiliones is unexplored, many are known to have extraordinary differences in appearance between the sexes and show intraspecific polymorphic males. Recent work on Opiliones has mainly focused on the structure and evolution of mating systems (Martens, 1993; Ramires and Giaretta, 1994) and the evolution of genitalia (Shultz, 1998). It is still unknown whether these sexual variations are graded and what causes these polymorphisms. It is possible that endosymbiotic infection may play a role in Opiliones reproductive biology, although this has not yet been explored.

In this study we investigated whether *Cardinium* occur within Opiliones. In a preliminary survey of arachnids for endosymbionts, we detected a divergent *Cardinium-like bacterium* within Opiliones by 16S rDNA polymerase chain reaction (PCR) amplification using general 16S rDNA primers (Weisburg et al., 1991). Subsequently, we screened 16 species of Opiliones for *Cardinium* by PCR amplification using primers designed for *Cardinium* 16S rDNA (Gotoh et al., 2007), in order to estimate the incidence and distribution pattern of this endosymbiont in Opiliones. Secondly, a phylogenetic analysis for all known *Cardinium* 16S rRNA sequences from

* Corresponding author. Fax: +1 585 275 2070.
E-mail address: werr@mail.rochester.edu (J.H. Werren).
[1] Present address: Life Sciences Institute, University of Michigan, Ann Arbor, MI 48109, USA.
[2] Present address: Lake Champlain Fish and Wildlife Resources Office, Essex Junction, VT 05452, USA.

0022-2011/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2010.05.017

arthropods was performed, in order to reveal the genetic relationship among these bacteria strains from Opiliones compared to those found in other hosts.

2. Materials and methods

2.1. Specimen collection

Specimens from major families of Opiliones (Arachnida) were field collected from various sites (see Table 1). Species and sex were identified based on morphology of specimens. The species screened and their origins are presented in Table 1. All specimens were fixed in 95% ethanol and stored at −20 °C until dissection.

2.2. Screening and sequencing

DNA from abdominal tissue of specimens was extracted using the Qiagen DNeasy tissue extraction kit following the instructions of the manufacturer. One or two specimens per species were analyzed. The DNA quality was tested by polymerase chain reaction (PCR) amplification of a conserved region of the eukaryotic 28S rDNA using the universal primers 28Sf (5′-CCC TGT TGA GGT TGA CTC TAG TCT GGC-3′) and 28Sr (5′-AAG AGC CGA CAT CGA AGG ATC-3′) (Werren et al., 1995). A 466 bp fragment of the large subunit ribosomal RNA gene (16S rRNA) of *Cardinium* was amplified using the specific primers CLO-f1 (5′-GGAACCTTACCTGGGCTA GAATGTATT-3′) and CLO-r1 (5′-GCCACTGTCTTCAAGCTCTACCAA C-3′) (Gotoh et al., 2007). Amplification was performed in a total volume of 20 μL, containing 50 ng DNA template, 2 μL 10 × PCR buffer, 1 U of Taq polymerase, 0.5 mM $MgCl_2$, 0.2 mM dNTPs (10 mM each), and 1 mM of each primer. All PCRs were run with an initial denaturizing at 94 °C for 2 min, 36 cycles (94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min) and a final cycle of 72 °C for 7 min with a 4 °C hold. A *Cardinium*-positive individual from the first PCR screen was used as a positive control in subsequent PCR screens.

2.3. Sequences and strains analysis

Sequence comparisons and alignments were done with Clustal X 1.8 (Thompson et al., 1997) using default parameters (gap opening cost = 15; gap extension cost = 6.66; delay divergent sequences = 30%; DNA transition = 0.50), and then these output files from multiple sequences were converted into the Nexus file by the software DNAsp3.5 (Rozas and Rozas, 1999) to perform phylogenetic analyses. Several basic statistics (e.g., nucleotide composition, and pairwise distances between taxa) were calculated in MEGA 3.0 (Kumar et al., 1994). Uncorrected percent sequence divergences and base frequencies across taxa were calculated with PAUP*. In all the analyses, three endosymbionts of *Acanthamoeba* (GenBank accession number AF215634, AF366581, AY549546), which are the closest known relatives of *Cardinium*, were used as outgroups. Additional endosymbiont sequences from other arthropods (e.g. Acari, Diptera, Hymenoptera, Hemiptera, and Homoptera) were collected from GenBank, and unpublished spider *Cardinium* 16S rRNA sequences were obtained from our work (See Table 2). Analysis of frequency of *Cardinium* infection was performed using Fisher's exact test on data from Opiliones, spiders, mites and other arthropods (Duron et al., 2008; Nakamura et al., 2009; Weeks et al., 2003; Zchori-Fein and Perlman, 2004). Furthermore, each 28S rRNA sequence was compared with archived sequences in NCBI to confirm they were from Opiliones. DNA and arthropod remains are maintained as vouchers in the laboratory of John Werren (Department of Biology, University of Rochester).

2.4. Phylogenetic analysis

Both Maximum parsimony (MP) and Bayesian analysis were used for phylogenetic reconstruction with the 16S rRNA data sets of *Cardinium*. MODELTEST version 3.06 (Posada et al., 2003) was used to choose the most likely of 56 models of nucleotide evolution for 16S rRNA. The estimated best-fit models were used to estimate a tree using Bayesian inference. The phylogenetic estimation

Table 1
Results of screening of spider and Opiliones species for the presence of *Cardinium*.

Taxon	Specimen no.	Collection site	Sex	CLO infected
Araneae				
Araneidae	CLO153817	Fort Sherman, Colon Province, Panama	♀	+
Theridiidae	CLO155115	Cape Three Points, Ghana	♂	+
Tetragmathidae	5C	Olaa Forest, Hawaii, United States	unknown	+
Opiliones				
Caddidae				
Caddo agilis	CLO155489	Ashizu Tunnel, Tottori Prefecture, Japan	♀	–
Caddo agilis	CLO155490	Pisgah State Park, Cheshire Co, New Hampshire, United States	♀	–
Phalangiidae				
Phalangium opilio	CLO155713	Genessee Valley Park, Monroe County, New York, United States	♂	+
Phalangium opilio	CLO155716	Genessee Valley Park, Monroe County, New York, United States	♂	–
Rilaena triangularis	CLO152990	Genessee Valley Park, Monroe County, New York, United States	♂	+
Rilaena sp1.	CLO100934	Old Gate Site, Sonora, Rancho San Bernardino, Mexico	♀	–
Rilaena sp2.	CLO155712	Genessee Valley Park, Monroe County, New York, United States	♂	–
Rilaena sp3.	CLO155488	Rochester-general area, Monroe County, New York, United States	♂	–
Rilaena sp4.	CLO155714	Genessee Valley Park, Monroe County, New York, United States	♀	–
Rilaena sp5.	CLO155483	Old Gate Site, Sonora, Rancho San Bernardino, Mexico	♀	+
Sclerosomatidae				
Leiobunum bracchiolum	CLO101386	Mendon Ponds Park, Monroe County, New York, United States	♂	–
Leiobunum politum	CLO155711	Genessee Valley Park, Monroe County, New York, United States	♂	–
	CLO155484	Old Gate Site, Sonora, Rancho San Bernardino, Mexico	♀	–
Leiobunum nigropalpi	CLO155485	Old Gate Site, Sonora, Rancho San Bernardino, Mexico	♀	–
Leiobunum serratipalpe	CLO155748	Rochester-general area, Monroe County, New York, United States	♀	–
Leiobunum vittatum	CLO155410	Genessee Valley Park, Monroe County, New York, United States	♀	+
	CLO155487	Adirondacks, New York, United States	♂	–
Leiobunum sp1.	CLO155697	Genessee Valley Park, Monroe County, New York, United States	♀	–
Leiobunum sp2.	CLO101385	Ellison Park, Monroe County, New York, United States	♀	+
Leiobunum sp3.	CLO155486	Old Gate Site, Sonora, Rancho San Bernardino, Mexico	♀	–

Table 2
Distribution of *Cardinium* among different arthropod groups and GenBank no. of all sequences.

	Host Taxon		Symbiont Taxon	GenBank accession no. Host 28S rRNA	Cardinium 16S
Ingroup					
Arthropoda					
Arachinida					
Araneae					
Araneidae	(Araneidae)	CLO153817	*Candidatus Cardinium*	FJ875215[a]	FJ875201[a]
Theridiidae	(Theridiidae)	CLO155115	*Candidatus Cardinium*	FJ875217[a]	FJ875204[a]
Tetragmathidae	(Tetragmathidae)	5C	*Candidatus Cardinium*		FJ875202[a]
Opiliones					
Phalangiidae					
	Phalangium opilio	CLO155713	*Candidatus Cardinium*	FJ875211[a]	FJ875209[a]
	Rilaena triangularis	CLO152990	*Candidatus Cardinium*	FJ875214[a]	FJ875207[a]
	Rilaena sp5.	CLO155483	*Candidatus Cardinium*	FJ875210[a]	FJ875208[a]
Sclerosomatidae					
	Leiobunum vittatum	CLO155410	*Candidatus Cardinium*	FJ875212[a]	FJ875200[a]
	Leiobunum sp2.	CLO101385	*Candidatus Cardinium*	FJ875213[a]	FJ875206[a]
Acari					
Ixodidae	*Ixodes scapularis*		*Candidatus Cardinium*	AF200190	AB001518
Oppoidae	*Oppiella nova*		*Candidatus Cardinium*	DQ090822	AY279414
Erythraeidae	*Balaustium sp.*		*Candidatus Cardinium*		AY279411
Phytoseiidae	*Metaseiulus occidentalis*		*Candidatus Cardinium*		AY279413
Tetranychidae	*Tetranychus cinnabarinus 3*		*Candidatus Cardinium*		DQ369963
	Tetranychus puerariicola		*Candidatus Cardinium*	AB076372	AB241135
	Eotetranychus suginamensis		*Candidatus Cardinium*		AB241129
	Oligonychus ilicis		*Candidatus Cardinium*		AB241130
	Amphitetranychus quercivorus		*Candidatus Cardinium*		AB241131
	Tetranychus urticae red form A		*Candidatus Cardinium*	AB369894	AB241132
	Tetranychus urticae red form B		*Candidatus Cardinium*		AB241133
	Petrobia harti		*Candidatus Cardinium*	EU487054	AY279410
Tenuipaplidae	*Brevipalpus obovatus*		*Candidatus Cardinium*		AY279401
	Brevipalpus phoenicis		*Candidatus Cardinium*		AF350221
	Brevipalpus californicus		*Candidatus Cardinium*		AB116514
	Brevipalpus lewisi		*Candidatus Cardinium*		AB116515
Insecta					
Hemiptera/Homoptera					
Aleyrodidae	*Bemisia tabaci* (biotype A)		*Candidatus Cardinium*		AY279409
Cicadellidae	*Scaphoideus titanus*		*Candidatus Cardinium*		AM042540
Diaspididae	*Aspidiotus nerii* 1		*Candidatus Cardinium*	DQ145297	AY279402
	Aspidiotus nerii 2		*Candidatus Cardinium hertigii*		AY599768
	Aspidiotus paranerii		*Candidatus Cardinium*		AY327469
	Leucaspis pusilla		*Candidatus Cardinium hertigii*	DQ145357	AY599766
Delphacidae	*Dicranotropis hamata*		*Candidatus Cardinium*		AY279415
	Harmalia sirokata		*Candidatus Cardinium*		AB506773
	Sogatella furcifera		*Candidatus Cardinium*		AB506774
Hymenoptera					
Aphelinidae	*Aphytis sp.*		*Candidatus Cardinium*		AY327473
	Aphytis sp. 4		*Candidatus Cardinium*		AY279403
	Aphytis lingnanensis		*Candidatus Cardinium*	AY640361	AY279404
	Encarsia hispida 1		*Candidatus Cardinium*	AY599384	AY026334
	Encarsia inaron 1		*Candidatus Cardinium*	AY599399	DQ317667
	Encarsia lutea		*Candidatus Cardinium*	AY599389	AY279407
	Encarsia sp. 2		*Candidatus Cardinium*		AY279406
	Encarsia pergandiella asexual line		*Candidatus Cardinium*	AY599402	AF319783
	Encarsiella noyesi		*Candidatus Cardinium*	AY599381	AY279408
	Marietta sp.		*Candidatus Cardinium*		AY327470
Encyrtidae	*Plagiomerus diaspidis*		*Candidatus Cardinium*	AY599316	AY327472
Diptera					
Ceratopogonidae	*Culicoides arakawae*		*Candidatus Cardinium*		AB506776
	Culicoides lungchiens		*Candidatus Cardinium*		AB506777
	Culicoides ohmorii		*Candidatus Cardinium*		AB506778
	Culicoides peregrinus		*Candidatus Cardinium*		AB506779
Nematode					
Heteroderidae	*Heterodera glycines*		*Candidatus Paenicardiniu endonii*		DQ314214
Outgroup					
Acanthamoebidae	*Acanthamoeba sp.*		*Candidatus Amoebophilus*		AF215634
	Acanthamoeba sp.		*Candidatus Amoebophilus*		AF366581
	Acanthamoeba sp.		Endosymbiont of *Acanthamoeba*		AY549546

[a] Sequences obtained only in this study.

procedure of MP analyses was carried out using the program PAUP*, version 4.0 beta 10 (Swofford et al., 2001). A heuristic search (10 replicates) was used to estimate the most likely topology for MP methodology. Heuristic searches started with stepwise addition trees and were replicated 100 times, with each replicate beginning with a random order of sequences and saving no more than 1000 MP trees per search. Branch swapping was performed by the tree-bisection-reconnection (TBR) method using default

parameters. Relative clade support was assessed using nonparametric bootstrap analyses (Felsenstein, 1985). Bootstrap values for the maximum parsimony trees were calculated from 1000 replicates. For MP analyses, Gap states were treated as the fifth character state. In addition, a maximum-parsimony analysis for 28S rRNA sequences was also used for hosts' phylogeny reconstruction.

A Bayesian analysis was performed using MrBayes ver. 2.01 (Huelsenbeck and Ronquist, 2001), which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC3) sampling approach. The parameters rate matrix and gamma shape parameter from the estimated best-fit models were employed for the MrBayes analyses. In Bayesian analysis four MCMC chains were run for 2 million generations sampled every 100 generations. Burn-in period ranged between 25,000 and 30,000 generations. We applied the Bayesian estimation method to closely related species and assumed rate constancy among lineages. To determine whether there was topological differences between host and endosymbiont phylogeny, we used the Shimodaira–Hasegawa test (SH-test) (Shimodaira and Hasegawa, 1999) as implemented in PAUP*4.0b10 to compare the topologies of hosts and Cardinium MP trees based on 1000 bootstrap replicates using the re-estimated log likelihood (RELL) method.

3. Results

3.1. Incidence of the Cardinium

We screened for the presence of *Cardinium* from 20 specimens from 16 Opiliones species which belong to three major families of the suborder Palptores. Host DNA template quality was successfully tested by using the 28S rDNA universal primers. Of the 16 species examined, PCR assay showed *Cardinium* infection in five species (31.2%). Compared with the previous studies of *Cardinium* in insect and other arachnids (Duron et al., 2008; Nakamura et al., 2009; Weeks et al., 2003; Zchori-Fein and Perlman, 2004), the incidence of *Cardinium* in Opiliones was significantly higher in Opiliones than in other pooled arthropods ($P = 0.007$, Fisher's exact test), but did not differ from the incidence in planthoppers ($P = 0.59$, Fisher's exact test), spiders ($P = 0.72$, Fisher's exact test) or mites ($P = 0.12$, Fisher's exact test).

3.2. Relationships among Cardinium strains from Opiliones and other arthropods

Nucleotide sequences of the 16S rRNA genes amplified from all analyzed taxa showed 83 polymorphic sites and 45 parsimony sites. Using the test of homogeneity of base composition implemented in PAUP*, χ^2 analysis indicated that all observed base compositions in 16S rRNA were statistically homogeneous across taxa when all positions were analyzed together ($\chi^2 = 5.661$, $df = 14$, $P > 0.1$). Examination of sequences indicated the presences of two groups of bacteria amplified from Opiliones using the *Cardinium* specific primers, with one group containing sequences relatively divergent from *Cardinium* sequences found in other arthropods. The average pairwise genetic distance based on the Kimura 2-parameter model showed high genetic distance between each of these *Cardinium* strains to found among *Cardinium* in other arthropods (distances between Opiliones and other arthropods ranged from 0.055 to 0.087, but average distance among other arthropods was only 0.025). All pairwise sequence divergences among the divergent *Cardinium* strains from Opiliones and those from other hosts were over 5.3%, whereas none of those among the *Cardinium* strains from other hosts exceeded 4%. The only exception was the 16S rRNA sequence from a single Opiliones (CLO101385), which was more similar to that found in other arthropods (divergences < 4%).

To evaluate phylogenetic relationships among these bacteria, both Maximum Parsimony (MP) and Bayesian analyses were performed. The MP analysis for 16S rRNA of *Cardinium* resulted in 500 parsimonious trees of 172 steps (CI = 0.7326; RI = 0.8697). For the Bayesian analysis, the single tree selected using the K80 + G model as the best-fit model had a–ln likelihood score of 1516.09 (Ti/Tv = 2.4375, $\alpha = 0.4398$). Similar results were obtained from both the parsimony and Bayesian analyses (Figs. 1 and 2). The two trees showed that all but one strain from Opiliones cluster together in a monophyletic group with very strong bootstrap values and posterior probability (BP = 100%, PP = 0.99). The consensus trees obtained through maximum-parsimony analysis and Bayesian analyses are shown in Figs. 1 and 2, respectively, with support value for nodes.

3.3. Comparisons of host and bacteria phylogenies

The tree from parsimony analyses of the host 28S rDNA indicated that hosts from the same order clustered together (Fig. 3). However, there was lack of resolution for complete concordance of the *Cardinium* strains phylogeny with host phylogeny, and the SH-test indicated the *Cardinium* topology based on parsimony analysis is significant different with the hosts tree ($P < 0.05$). Second, the phylogenetic analyses showed that some *Cardinium* strains from closely related hosts often cluster together. Although there was not strong support (BP < 50%, PP < 0.50), most of strains either from Acari or from Hymenoptera were found in the same branches, intermingled with a few strains from other arthropods. Finally, the *Cardinium* strains infecting species of the suborder Palptores (Arachnida: Opiliones) formed a monophyletic group with strong support (BP = 100%, PP = 0.99), except for one unique sequence from Opiliones specimen CLO101385. This divergent clade contains *Cardinium* sequences from four different Opiliones species encompassing two different families and three genera. Members of this bacterial clade show an average pairwise divergence to nearest neighbor outside the clade (Acari, see Figs.1 1 and 2) of 6%, whereas the average divergence among all other *Cardinium* members is 2.6%. Nevertheless, the Cardinium-like bacteria of Opiliones and other *Cardinium* bacteria are monophyletic with respect to the nearest outgroup bacterium (endosymbiont of *Acanthamoeba sp.* AF215634) and have an average divergence to it of 14.3%. Therefore, we conclude that this is a divergent clade of *Cardinium*, so far only found in Opiliones.

4. Discussion

Although the effects of endosymbionts in arthropods have been widely researched (Moran et al., 2008; Werren et al., 2008), there has been little study of endosymbiotic bacteria in Opiliones. This study has revealed an incidence of *Cardinium* infection in Opiliones to be 31.2%, which is higher than in most other arthropods (Weeks et al., 2003; Zchori-Fein and Perlman, 2004), with the exception of planthoppers (Nakamura et al., 2009), mites (Gotoh et al., 2007) and spiders (Duron et al., 2008), in which the infection rates are similar. Our data support the viewpoint of Duron et al. (2008), who suggested that Arachnids represent a *Cardinium* infection hotspot. Our results suggest that *Cardinium* infect a larger proportion of Opiliones species than other arachnids, but this endosymbiont seems to occur in Opiliones without being associated without a significant sex-bias in hosts (Table 1). This implies that these *Cardinium* may not distort sex ratio (e.g., by parthenogenesis, feminization, and male killing), although further genetics studies on *Cardinium* and Opiliones are required to determine the phenotypic effects of these bacteria. The diversity found between the strains infecting Opiliones and other arthropods was up to 6.8% for the

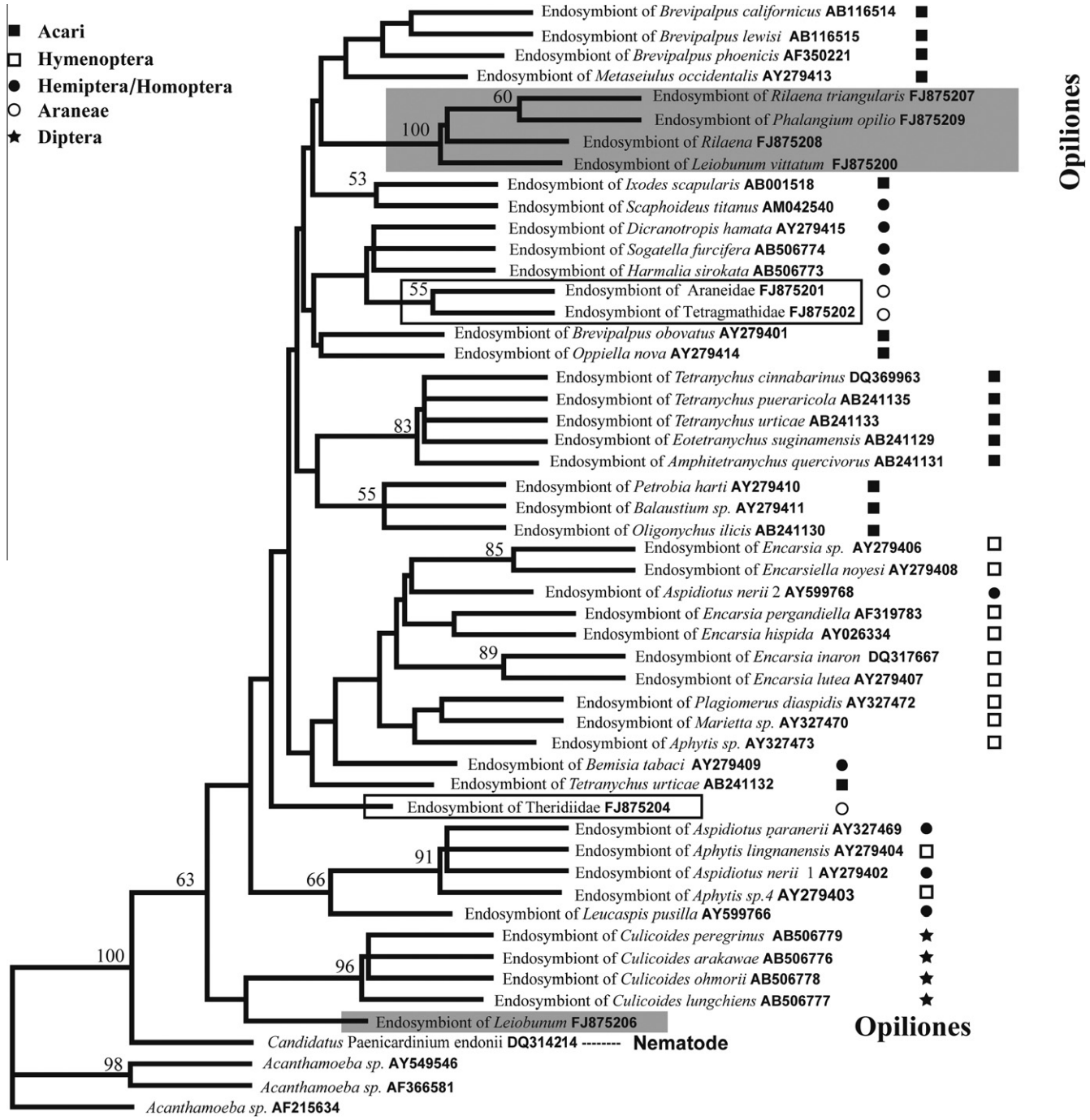

Fig. 1. Strict consensus tree from parsimony analysis of *Cardinium* strains based on 387 bp of 16S rRNA sequence. Nodal support is assessed by bootstrap values (above branches; values over 50% shown). *Cardinium* hosts are coded by shape symbols, Acari (black square), Hymenoptera (white square), Hemiptera/Homoptera (black circle), Araneae (white circle), and Diptera (black star). Gray shade areas and solid rectangular boxes designate *Cardinium* sequences obtained from Opiliones and from spiders respectively in this study.

16S rRNA region, which was higher than found in previous studies ($P = 0.007$). But these divergences did not exceed the differences between *Cardinium* and the outgroup endosymbiont of *Acanthamoeba sp.* (average divergent values = 10.9%). Therefore, we conclude that the strains infecting Opiliones still belong to the *Cardinium* clade. Also, the monophyletic clade with strong support value of *Cardinium* found in Opiliones is distinct from others previous found.

From the phylogenetic cladograms, there is little evidence for complete concordance of the *Cardinium* strains with host phylogeny (Fig. 3), which is strongly supported by an SH-test ($P < 0.05$). In addition, this evolutionary discordance between host and endosymbiont has been noted in previous studies of endosymbionts (O'Neill et al., 1992; Stouthamer et al., 1999; Werren and Windsor, 2000). This lack of concordance implies that there is the potential for horizontal transmission of the bacteria among hosts. In addition, *Cardinium* strains from closely related hosts (e.g., Acari, Hymenoptera, Homoptera and Opiliones, Figs. 1 and 2) often cluster together. This suggests that there may be a degree of physiological host specialization in some *Cardinium* types, whereas others

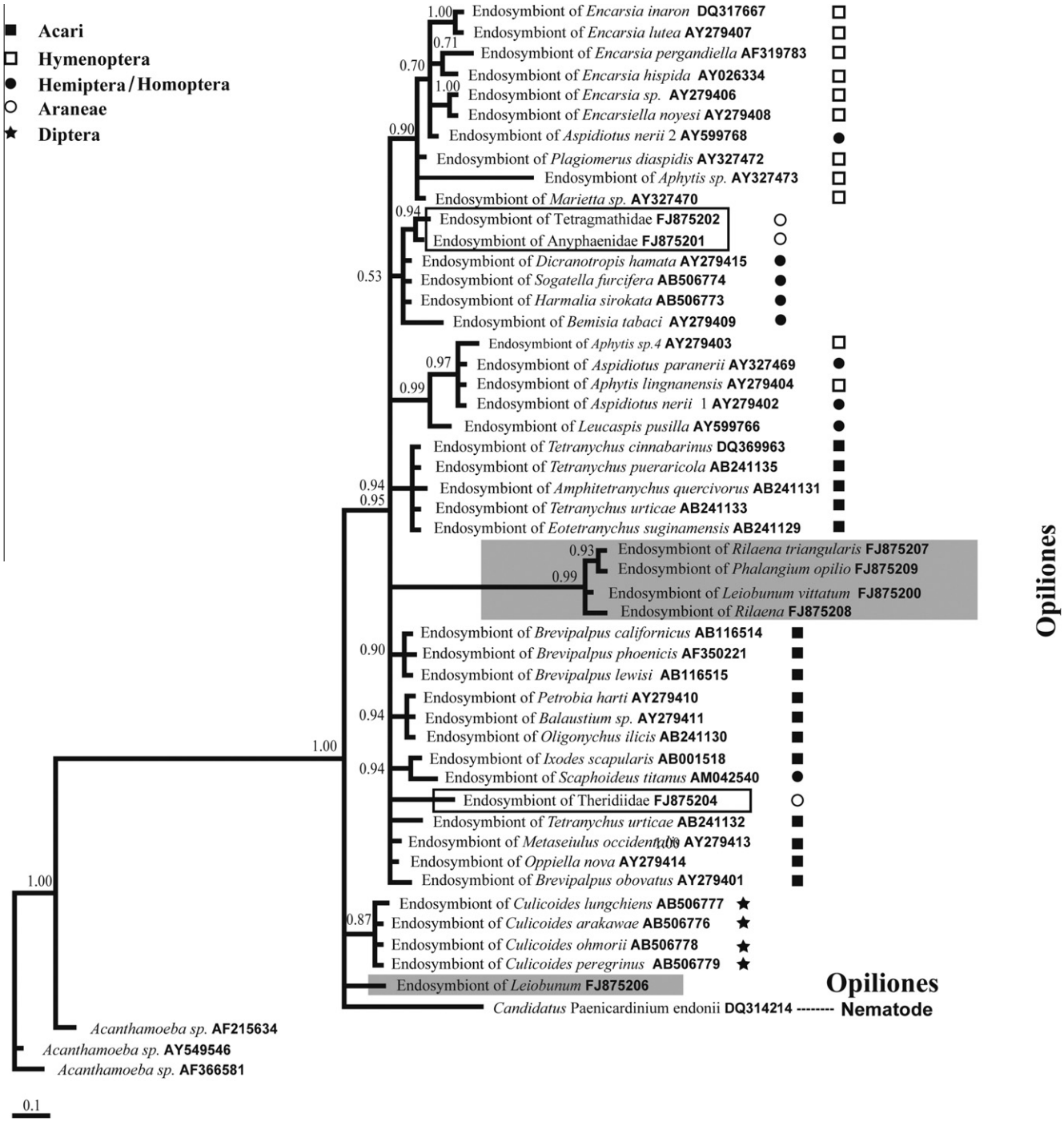

Fig. 2. Strict consensus tree of the Bayesian analysis for the *Cardinium* strains based on 16S rRNA sequences. Nodal support is assessed by posterior probability (above branches; Shown only > 0.50). The same shape symbols and shade marks are used as in Fig. 1. Bar is 0.1 substitutions per site.

are capable of broad host infection (Zchori-Fein and Perlman, 2004). The presence of very similar *Cardinium* strains from distantly related insect species (e.g., *Cardinium* strains from Opiliones, Araneae, Diptera, Hymenoptera, and Hemiptera clustered together in Fig. 2) as also shown in a previous study by Zchori-Fein and Perlman (2004), supports the idea that bacteria can be horizontal transmitted among arthropods rather than the theory of ancient vertical transmitted infection and secondary loss of bacteria during speciation. In our study, possibility of contamination with *Cardinium* from other specimens can be excluded because each specimen was preserved independently in 95% ethanol and genomic DNA was extracted only from the abdominal muscle tissues, and each strain found shows a distinct sequence. Horizontal transmission is also one of the best explanations for the phylogenetic position of CLO101385, since its closest relatives are all found in other arthropod taxa. Nakamura et al. (2009) suggested that current *Cardinium* should be divided into three groups (A, B and C), with group A being found in diverse arthropods, group B so far only in a single nematode *Heterodera glycines* (*Candidatus* Paenicardinium endonii), and group C in some midges. The position of our divergent Opiliones clade is uncertain with respect to this scheme, as it could represent a divergent member of group A or constitute yet another group (Figs. 1 and 2). In addition, the divergent *Cardinium* of Opiliones specimen CLO101385 (*Leiobunum*

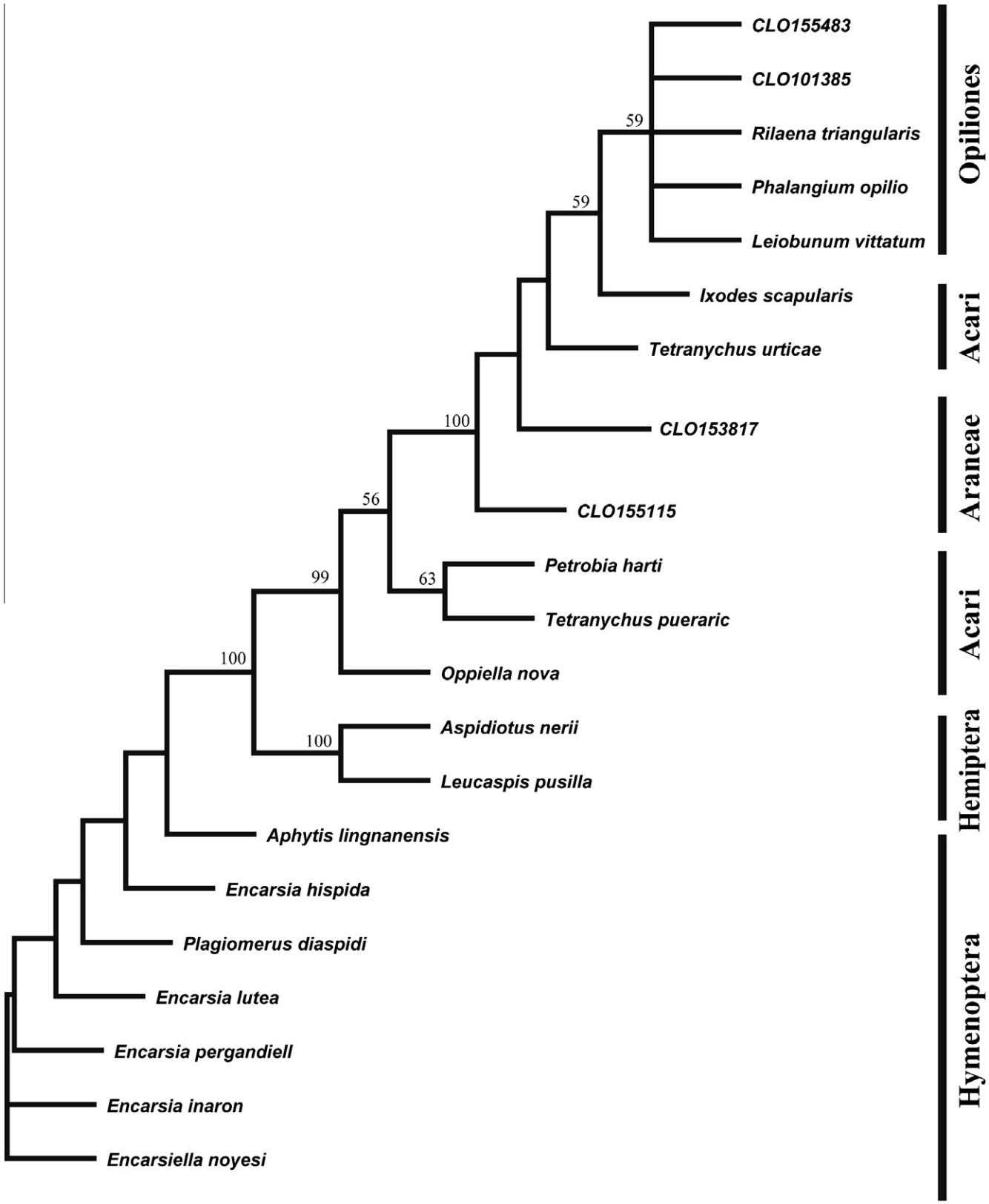

Fig. 3. Hosts 28S rRNA phylogeny constructed via parsimony analysis. Nodal support is assessed by bootstrap values (above branches; values over 50% shown).

sp2) has an ambiguous position. At this time, it is unclear whether the diversity of Cardinium strains falls within the current proposed typing of Nakamura et al. (2009), and it is likely that more groups are present and will be found.

Although our study shows high incidence of *Cardinium* in Opiliones, there is lack of fine scale resolution of the evolutionary relationships between hosts and symbionts. That may relate with a slow evolutional rate of the 16S rRNA gene in *Cardinium*.

Sequencing more rapidly evolving and phylogenetically informative *Cardinium* and Opiliones genes not only will help to infer actual evolutionary patterns between hosts and symbionts, but also can provide more information to reveal mechanisms by which these endosymbionts transfer between hosts.

Acknowledgments

We thank Cheryl Hayashi, Laura Baldo, Michael Clark, Rachel Edwards, Rhitoban Ray Choudhury, Jeffrey Shultz, and David Loehlin for sample collection or comments on this manuscript. We are grateful to one anonymous reviewer for valuable suggestions on a version of the manuscript. This work was supported by NSF EF-0328363 and NSF ID 0821936 to JHW.

References

Charlat, S. et al., 2003. Characterization of non-cytoplasmic incompatibility inducing Wolbachia in two continental African populations of *Drosophila simulans*. Heredity 90, 49–55.

Clark, T.B., 1982. Spiroplasmas: diversity of arthropod reservoirs and host-parasite relationships. Science 217, 57–59.

Duron, O. et al., 2008. High incidence of the maternally inherited bacterium *Cardinium* in spiders. Mol. Ecol. 17, 1427–1437.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Giribet, G. et al., 2002. Phylogeny and systematic position of Opiliones: a combined analysis of chelicerate relationships using morphological and molecular data. Cladistics 18, 5–70.

Gotoh, T. et al., 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98, 13–20.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Hunter, M.S. et al., 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc. Biol. Sci. 270, 2185–2190.

Kumar, S. et al., 1994. MEGA: molecular evolutionary genetics analysis software for microcomputers. Comput. Appl. Biosci. 10, 189–191.

Liu, Y. et al., 2006. Distribution of the endosymbiotic bacterium *Cardinium* in Chinese populations of the carmine spider mite *Tetranychus cinnabarinus* (Acari: Tetranychidae). J. Appl. Entomol. 130, 523–529.

Martens, J., 1993. Further cases of paternal care in Opiliones (Arachnida). Trop. Zool. 6, 97–107.

Moran, N.A. et al., 2008. Genomics and evolution of heritable bacterial symbionts. Annu. Rev. Genet. 42, 165–190.

Moran, N.A., Telang, A., 1998. Bacteriocyte-associated symbionts of insects. Bioscience 48, 295–304.

Nakamura, Y. et al., 2009. Prevalence of *Cardinium* bacteria in planthoppers and spider mites and taxonomic revision of "*Candidatus Cardinium* hertigii" based on detection of a new *Cardinium* group from biting midges. Appl. Environ. Microbiol. 75, 6757–6763.

O'Neill, S.L. et al., 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA 89, 2699–2702.

Perlman, S.J. et al., 2006. The emerging diversity of Rickettsia. Proc. Biol. Sci. 273, 2097–2106.

Perlman, S.J. et al., 2008. Population biology of cytoplasmic incompatibility: maintenance and spread of *Cardinium* symbionts in a parasitic wasp. Genetics 178, 1003–1011.

Posada, D., 2003. Using MODELTEST and PAUP* to select a model of nucleotide substitution. Curr. Protoc. Bioinformatics (Chapter 6, Unit 6 5).

Ramires, E.N., Giaretta, A.A., 1994. Maternal care in a neotropical harvestman, *Acutisoma proximum* (Opiliones, Gonyleptidae). J. Arachnol. 22, 179–180.

Ros, V.I., Breeuwer, J.A., 2009. The effects of, and interactions between, *Cardinium* and Wolbachia in the doubly infected spider mite *Bryobia sarothamni*. Heredity 102, 413–422.

Rozas, J., Rozas, R., 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15, 174–175.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Shultz, J.W., 1990. Evolutionary morphology and phylogeny of Arachnida. Cladistics 6, 1–38.

Shultz, J.W., 1998. Phylogeny of Opiliones (Arachnida): an assessment of the "Cyphopalpatores" concept. J. Arachnol. 26, 257–272.

Stouthamer, R. et al., 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53, 71–102.

Swofford, D.L., PAUP*. In: Phylogenetic Analysis Using Parsimony (*and Other Methods). S. Sinauer Associates, MA, 2001(Version 4).

Thompson, J.D. et al., 1997. The CLUSTAL_X windows interface. flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Weeks, A.R. et al., 2001. A mite species that consists entirely of haploid females. Science 292, 2479–2482.

Weeks, A.R. et al., 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc. Biol. Sci. 270, 1857–1865.

Weisburg, W.G. et al., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

Werren, J.H. et al., 2008. Wolbachia: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751.

Werren, J.H., Windsor, D., Guo, L.R., 1995. Distribution of Wolbachia among neotropical arthropods. Proc. R. Soc. Lond. B. 262, 197–204.

Werren, J.H., O'Neill, S., 1997. The evolution of heritable symbionts. In: O'Neill, S., Hoffman, A., Werren, J.H. (Eds.), Influential Passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford University Press.

Werren, J.H., Windsor, D.M., 2000. Wolbachia infection frequencies in insects: evidence of a global equilibrium? Proc. Biol. Sci. 267, 1277–1285.

Wheeler, W.C., Hayashi, C.Y., 1998. The phylogeny of extant chelicerate orders. Cladistics 14, 173–192.

Zchori-Fein, E. et al., 2001. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. Proc. Natl. Acad. Sci. USA 98, 12555–12560.

Zchori-Fein, E., Perlman, S.J., 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol. Ecol. 13, 2009–2016.