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High incidence of the maternally inherited bacterium *Cardinium* in spiders

OLIVIER DURON,* GREGORY D. D. HURST,*† EMILY A. HORNETT,*† JAMES A. JOSLING* and JAN ENGELSTÄDTER*‡

**Department of Biology, University College London, 4 Stephenson Way, London NW1 2HE, UK, †School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK, ‡Institute of Integrative Biology (IBZ), ETH Zurich, Universitätsstrasse 16, ETH Zentrum, CHN K12.1, CH-8092 Zurich, Switzerland*

Abstract

Inherited bacteria are now recognized as important players in arthropod evolution and ecology. Here, we test spiders, a group recently identified as possessing inherited bacteria commonly, for the presence of two reproductive parasites, *Cardinium hertigii* (Bacteroidetes group) and *Wolbachia* (α-proteobacteria), estimating incidence, prevalence, any sex bias in infection, and infection diversity, for a panel of field-collected specimens. We identify spiders as a hotspot for *Cardinium*. Present in 22% of the sampled species, incidence was significantly higher than that previously recorded in insects. Where present, *Cardinium* infection occurred at medium prevalence without evidence of sex bias in prevalence that would indicate sex-ratio distortion activity. *Wolbachia* was present in 37% of species, but revealed a gradation from being rare to very common. In one case, *Wolbachia* was found significantly more commonly in females than males, indicating it may act as a sex-ratio distorter in some species. Breeding work conducted on two species confirmed that *Wolbachia* and *Cardinium* were transmitted maternally, which represents the first proof of inheritance of these symbionts in spiders. Overall, this study demonstrates that the majority of spider species are infected with inherited bacteria, and their role in host biology clearly requires determination.

Keywords: *Cardinium*, reproductive parasitism, spider, symbiosis, *Wolbachia*

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Introduction

Hereditary symbioses are extremely widespread and constitute an important motive force in evolution, because symbiont transmission success is tightly linked to host fitness. While many of these associations have evolved towards a mutualistic pathway, with both host and symbiont either requiring the presence of the other, or at least being mutually beneficial, some maternally inherited microorganisms have evolved into reproductive parasites of arthropods (Bandi *et al*. 2001). Such parasites spread within host populations by manipulating the host reproductive processes to enhance their own transmission.

The maternal inheritance of reproductive parasites has selected for a variety of phenotypes associated with promoting the production and fitness of infected daughters (i.e. the transmitting sex) via negative effects on the fitness of individuals not involved in the transmission (Werren 1997; Stouthamer *et al*. 1999; Bandi *et al*. 2001; Stevens *et al*. 2001). Because males represent a dead-end for reproductive parasites, manipulations frequently involve biasing the sex ratio (SR) of infected females towards the production of daughters, via the induction of either thelytokous parthenogenesis (production of all female progeny from unfertilized eggs), feminization of genetic males, or male killing. However, some reproductive parasites do not distort the host SR but induce sterility with hosts carrying different types of cytoplasmic infection, a phenotype called cytoplasmic incompatibility (CI). CI results in abortive embryonic development when infected males mate either with uninfected females or with females infected by another reproductive parasite. The death of progeny from uninfected or differently infected females means that females infected

Correspondence: Olivier Duron, Fax: +44 20 7679 5052;
E-mail: o.duron@ucl.ac.uk

by a CI-inducing parasite have a reproductive advantage, enhancing the spread of infection (Caspari & Watson 1959; Fine 1978; Rousset & Raymond 1991). Such manipulations have then profound effects on host phenotypes and reproductive parasites are now regarded as very important cryptic drivers of host ecology and evolution (Werren 1998; Hurst & Werren 2001).

Until recently, work in the field has been biased to a single bacterium, *Wolbachia*. *Wolbachia* has been observed to be common in arthropods, and to demonstrate a variety of interactions with hosts, including cytoplasmic incompatibility, parthenogenesis induction, feminization and male killing (Werren 1997; Stouthamer *et al*. 1999; Stevens *et al*. 2001). However, other bacteria are known to induce reproductive manipulations in arthropods, such as *Rickettsia* (male killing, cf. Von der Schulenburg *et al*. 2001; or parthenogenesis, cf. Hagimori *et al*. 2006), *Spiroplasma* (male killing, cf. Jiggins *et al*. 2000) and *Arsenophonus* (male killing, cf. Werren *et al*. 1986). More recently, the bacterium *Cardinium hertigii* was described, a bacterial reproductive parasite of the Bacteroidetes group (Zchori-Fein *et al*. 2004). Its array of host manipulations has been found to be second only to *Wolbachia* with three distinct reproductive manipulations recorded to date. These manipulations include feminization in the *Brevipalpus* mite (Weeks *et al*. 2001), thelytokous parthenogenesis in the *Encarsia* parasitic wasps (Zchori-Fein *et al*. 2001; Zchori-Fein *et al*. 2004) and in the hemipteran *Aspidiotus nerii* (Provencher *et al*. 2005), and CI in sexual populations of the wasp *Encarsia pergandiella* (Hunter *et al*. 2003) and in the spider mite *Eotetranychus suginamensis* (Gotoh *et al*. 2007). In addition to these reproductive manipulations, *Cardinium* spread is favoured by an adaptive manipulation of the oviposition behaviour of *E. pergandiella* (Zchori-Fein *et al*. 2001; Kenyon & Hunter 2007) and by enhancing the fecundity in the predatory mite *Metaseiulus occidentalis* (Weeks & Stouthamer 2003). Host manipulation was thus always observed in previous studies of *Cardinium*–host associations. However, recent surveys have shown *Cardinium* to be rarer than *Wolbachia*, and strictly restricted to Hymenoptera, Hemiptera and Acari (Weeks *et al*. 2003; Zchori-Fein & Perlman 2004).

Although *Wolbachia* demonstrate the potential importance of inherited parasites, the current knowledge of other reproductive parasites — like *Cardinium* — is much weaker. *Cardinium* infection has been observed in an extensive range of hymenopteran and hemipteran insects and mites (Weeks *et al*. 2003; Zchori-Fein & Perlman 2004; Enigl & Schausberger 2007). Spiders are well known to be a haven for some reproductive parasites, such as *Wolbachia*, *Rickettsia* and *Spiroplasma* bacteria (Rowley *et al*. 2004; Goodacre *et al*. 2006). The combination of the presence of these infections commonly, with the occurrence of strong SR bias and parthenogenesis in some spider families, produced the hypothesis that manipulation by reproductive parasites could be an important component of spider biology (Goodacre *et al*. 2006). In this study, we test a range of spiders for the presence of *Cardinium* and *Wolbachia*, estimating incidence, prevalence, and geographical variations between natural populations. By separately testing males and females, we also estimate any sex bias in prevalence that would indicate SR distortion activity associated with infection. Lastly, the relatedness of *Cardinium* and *Wolbachia* strains from spiders with strains of these bacteria found in other host species is estimated. Our results indicate that *Cardinium* is a very important associate of spiders, but there is little evidence from our results that it is involved in sex-ratio distortion. *Wolbachia*, in contrast, does show a pattern of sex-biased prevalence in one case that is compatible with sex-ratio distortion activity.

Materials and methods

Spider collection

Specimens from major families of spiders (Araneae) were field collected from various sites, principally in Northern Europe (2004–2006). A species of harvestmen (Opiliones) was also included in the analysis. Species and sex were identified based on morphology of specimens. The species screened and their origins are presented in Table 1. One population per species was generally analysed, except for four species for which distinct populations were collected. Within each species, there were 2 to 28 individuals separately investigated for analysis. Spiders were fixed in 95% ethanol and stored at 4 °C until analysed. Specimens were separated during storage so as to avoid any potential interindividual transmission.

Screening and sequencing

Spider DNA was extracted using the Promega Wizard SV 96 Genomic DNA Purification System following the instructions of the manufacturer. DNA extraction was performed on abdominal tissue to reduce the risk of missing infection with reproductive parasites when they are present (false-negatives). In addition, one leg from each individual was harvested and pooled in groups of five legs (i.e. five individuals) for DNA extraction to permit assessment of the possibility of any false-positives from reproductive parasites from spider prey present in the digestive system.

The DNA quality was systematically tested by polymerase chain reaction (PCR) amplification of a conserved region of the eukaryotic 18S rDNA using the universal primers NSF4/18 (5′-CTGGTTGATYCTGCCAGT) and NSF399/19 (5′-TCTCAGGCTCCYTCTCCGG) (Hendriks *et al*. 1989; Hendriks *et al*. 1991). A 473-bp fragment of the large subunit ribosomal RNA gene (16S rDNA) of spider gene was

Table 1 Results of screening of spider species for the presence of *Cardinium* and *Wolbachia*. Difference in prevalence between sexes was tested using Fisher's exact test (*, $P < 0.05$; **, $P < 0.01$). Only *Meta mengei* displayed a significant difference in prevalence after a Bonferroni correction for multiple comparisons. RP, Richmond Park; TP, Trent Park. For some species, specimens were kindly provided by †Susan Riechert (University of Tennessee), ‡Jan Bosselaers, §Jutta Schneider (University Hamburg), ¶Martin Schmidt (University Bern), ††Klaus Birkhofer (then University Darmstadt) and ‡‡Dries Bonte (Ghent University)

		Sample size		Prevalence of *Cardinium*			Prevalence of *Wolbachia*			Prevalence of superinfection		
Taxon	Population	♂	♀	overall	♂	♀	overall	♂	♀	overall	♂	♀
ARANEAE												
Amaurobiidae												
Amaurobius fenestralis	Montpellier, France, 2006	6	10	—	—	—	—	—	—	—	—	—
Araneidae												
Agelenopsis aperta	Tennessee, USA, 2005†	10	10	—	—	—	—	—	—	—	—	—
Araneus diadematus	Beerse, Belgium, 2005‡	10	10	—	—	—	—	—	—	—	—	—
	London, UK, 2005	3	5	—	—	—	—	—	—	—	—	—
Argiope bruennichi	Hamburg, Germany, 2005§	10	10	—	—	—	—	—	—	—	—	—
Argiope lobota	Israel, 2005§	5	4	—	—	—	—	—	—	—	—	—
	Spain, 2005§	3	4	—	—	—	—	—	—	—	—	—
Cyclosa conica	Berlin, Germany, 2004–05	10	10	0.40	0.60	0.20	—	—	—	—	—	—
Larinioides cornutus	London (RP), UK, 2005	10	10	—	—	—	—	—	—	—	—	—
Larinioides sclopetarius	Hamburg, Germany, 2005§	10	10	—	—	—	—	—	—	—	—	—
Dysderidae												
Dysdera crocata	Montpellier, France, 2006	1	1	—	—	—	—	—	—	—	—	—
Linyphiidae												
Linyphia triangularis	Berlin, Germany, 2004	1	8	0.44	1.00	0.38	—	—	—	—	—	—
	London (RP), UK, 2004–05	1	7	0.63	1.00	0.57	0.38	0.00	0.43	0.00	0.00	0.00
	London (TP), UK, 2004–05	2	7	0.25	0.50	0.14	0.78	0.00	0.88	0.00	0.00	0.00
Neriene clathrata	Beerse, Belgium, 2005‡	10	10	—	—	—	—	—	—	—	—	—
Lycosidae												
Alopecosa pulverulenta	Bern, Switzerland, 2005¶	10	10	0.75	0.70	0.80	0.25	0.10	0.40	0.25	0.10	0.40
Pardosa lugubris	Darmstadt, Germany, 2005††	10	10	—	—	—	—	—	—	—	—	—
Pardosa pullata	Bern, Switzerland, 2005¶	10	10	—	—	—	0.05	0.10	0.00	—	—	—
Pardosa purbeckensis	Salt march, Belgium, 2005‡‡	10	10	—	—	—	—	—	—	—	—	—
Pholcidae												
Holocnemus pluchei	Montpellier, France, 2006	10	10	0.4	0.60	0.20	—	—	—	—	—	—
Pholcus phalangioides	Berlin, Germany, 2004–05	10	10	—	—	—	0.90	0.90	0.90	—	—	—
	London, 2007	10	10	—	—	—	0.95	0.90	1.00	—	—	—
Pisauridae												
Pisaura mirabilis	London (TP), UK, 2004	2	10	—	—	—	—	—	—	—	—	—
Salticidae												
Evarcha falcata	Beerse, Belgium, 2005‡	10	10	0.45	0.40	0.50	—	—	—	—	—	—
Tetragnathidae												
Meta mengei	London (TP), UK, 2005	10	10	—	—	—	0.5	0.10	0.90**	—	—	—
Meta segmenta	Berlin, Germany, 2004	10	10	—	—	—	0.15	0.10	0.20	—	—	—
Pachygnatha degeeri	Bern, Switzerland, 2004¶	10	10	0.45	0.40	0.50	0.05	0.00	0.10	0.00	0.00	0.00
Pachygnatha listeri	Beerse, Belgium, 2005‡	10	10	—	—	—	0.25	0.20	0.30	—	—	—
Tetragnatha montana	London (TP), UK, 2005	10	10	—	—	—	0.25	0.00	0.50*	—	—	—
Theridiidae												
Enoplognatha ovata	London (TP), UK, 2004–05	10	10	—	—	—	0.05	0.00	0.10	—	—	—
Thomisidae												
Xysticus cristatus	Cambridge, UK, 2005	8	8	—	—	—	—	—	—	—	—	—
OPIOLONES												
Leiobunidae												
Leiobunum rotundum	Feurs, France, 2006	10	6	—	—	—	—	—	—	—	—	—

amplified in some cases using the universal primers 16SA (5′-CGCCTGTTTATCAAAAACAT) and 16SB (5′-CCGGTTGAACTCAGATCA) (Hwang *et al*. 2001). Two independent assays for *Cardinium* infection were performed by PCR amplification of two distinct regions of the 16S rDNA gene using the specific primers (i) CLO-f1 (5′-GGAACCTTACCTGGGCTAGAATGTATT) and CLO-r1 (5′-GCCACTGTCTTCAAGCTCTACCAAC), which amplified 466-bp fragment (Gotoh *et al*. 2007); and (ii) ChF (5′-TACTGTAAGAATAAGCACCGGC) and ChR (5′-GTGGATCACTTAACGCTTTCG) which amplified 394-bp fragment (Zchori-Fein & Perlman 2004). *Wolbachia* infection was examined using the 16S rDNA specific primers 16Swolb76-99f (5′-TTGTAGCCTGCTATGGTATAACT) and 16Swolb1012-994r (5′-GAATAGGTATGATTTTCATGA), which amplified 895–897-bp fragment (O'Neill *et al*. 1992). All the PCRs were run for 35 cycles (94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min). A *Cardinium*-positive individual from the first PCR screen was used as a positive control in subsequent PCR screens. *Wolbachia*-infected *Culex pipiens* served as template for positive controls in *Wolbachia* PCRs. The PCR products were electrophoresed in a 1.5% agarose gel. Amplicons from putative positives were sequenced directly to confirm infection. *Cardinium* sequences were obtained by sequencing the 953-bp fragment of 16S rDNA gene amplified with the ChF and CLO-r1 primers. *Wolbachia* sequences were obtained using the 16Swolb76-99f and 16Swolb1012-994r primers. PCR products of two randomly sampled individuals per infected species were sequenced and analysed.

Sequences were first aligned and modified visually using MEGA version 3.1 (Kumar *et al*. 2004). Neighbour-joining phylogenies were constructed using MEGA version 3.1 (Kumar *et al*. 2004) based upon unambiguously aligned sites using the Tajima–Nei model of nucleotide substitution (Tajima & Nei 1984) with support for the tree topology assessed by bootstrap resampling (1000 replicates).

We also, in one case for each bacterium, confirmed the maternal transmission of the bacterium through assaying neonate progeny derived from infected females for the presence of the infection. Eggs laid by five *Wolbachia*-infected female *Pholcus phalangioides* and three *Cardinium*-infected female *Holocnemus pluchei* were obtained in the laboratory, neonates collected, washed externally, DNA prepared, and then the template assayed for presence of *Wolbachia* and *Cardinium*, respectively.

Results

Distribution of infection

We assayed for the presence of *Cardinium* and *Wolbachia* in 523 individual spiders from 27 species (encompassing 22 genera and 13 families, including the harvestman *Leiobunum rotundum*) (Table 1). Host DNA was successfully amplified from all specimens using the 18S rDNA universal primers, indicating satisfactory DNA template quality. Of the 27 species examined, PCR assay indicated the presence of *Cardinium* in six species (22%) and *Wolbachia* in 10 (37%). Infections were detected in 12 spider families (54%) and there was no evidence of restriction to particular clades. Within the infected species, there were three cases of co-infection at the species level, but in only one case did this reflect co-infection at the individual level (Table 1). Results from assays of individuals via template derived from abdomen were reflected precisely by template derived from legs, indicating infections that were detected were internalized within the host, and not derived from gut contents. That these infections could be maternally transmitted was confirmed for the cases of *Wolbachia* in *Pholcus phalangioides* and *Cardinium* in *Holocnemus pluchei* by examining presence of infection in neonates derived from infected mothers. For *P. phalangioides*, five infected mothers collected from the field produced 106 eggs (mean clutch size: 21.2 ± 2.7 eggs) and 101 neonates (mean hatching rate: 0.96 ± 0.03). Ten neonates per clutch (i.e. a total of 50 neonates) were randomly sampled for PCR analysis. For *H. pluchei*, three infected mothers collected from the field produced 56 eggs (mean clutch size: 18.7 ± 2.3 eggs) and 56 neonates (mean hatching rate: 1.00 ± 0.00). Ten neonates per clutch (i.e. a total of 30 neonates) were randomly sampled for PCR analysis. In each case, all neonates from infected parents were themselves infected showing a very good maternal transmission in these associations.

We compared our results to previous surveys of *Cardinium* (incidence of 7.2% in Weeks *et al*. 2003 and 6% in Zchori-Fein & Perlman 2004) and found the incidence of *Cardinium* to be significantly higher in spiders than in all arthropods ($P = 0.001$, Fisher's exact test) but did not differ from the incidence in mites ($P = 0.99$, Fisher's exact test). In our sample of spiders, *Cardinium* incidence did not differ statistically from that of *Wolbachia* ($P = 0.37$, Fisher's exact test), although we lack the statistical power to conclude that they are equally common. The high incidence of *Wolbachia* observed in this study lies in the range of values previously reported in spider (incidence of 37% in the synthesis established by Goodacre *et al*. 2006; $P > 0.99$, Fisher's exact test).

Where *Cardinium* infection was observed in a host species, a medium prevalence of infection was observed in all cases, and infection was never observed to be at fixation (infection prevalence ranged from 22% to 75% of all individuals; Table 1). *Wolbachia* prevalence similarly ranged from rare (5%) to very common (95%) at the population level.

We then examined the data for sex bias in infection prevalence, as an indication of potential sex-ratio distorting activity. *Cardinium* infection showed no evidence of sex bias in prevalence in any case (Fisher's exact test, all

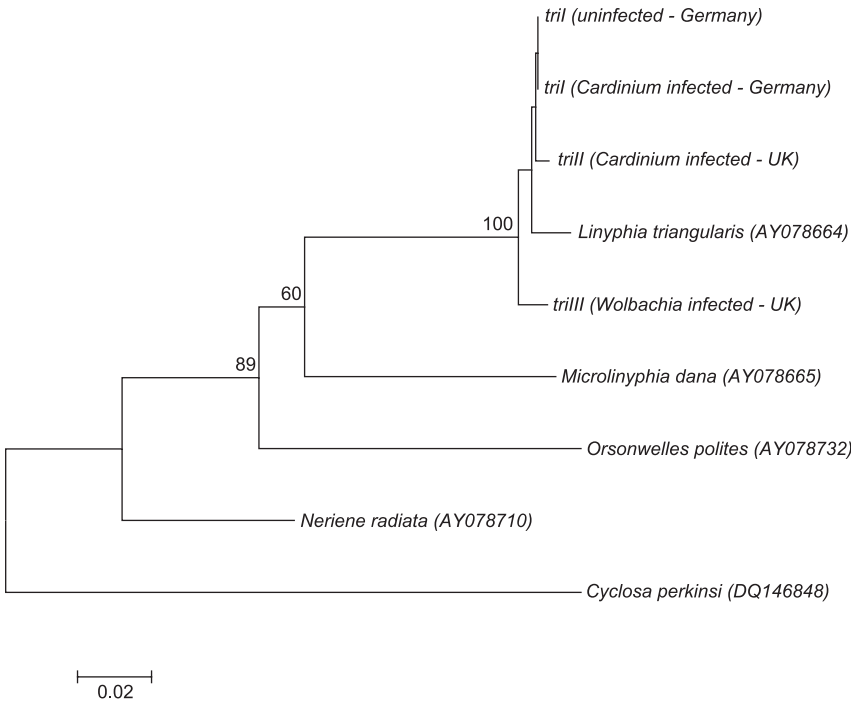

Fig. 1 Mitochondrial phylogeny of *Linyphia triangularis* based on 16S rDNA sequences, constructed via neighbour joining as implemented on MEGA version 3.1. Three mitotypes were identified in this study (*tri*I, *tri*II, *tri*III). The outgroup sequences belong to Linyphiidae (*Microlinyphia dana, Orsonwelles polistes and Neriene radiata*) and to Araneidae (*Cyclosa perkinsi*). Numbers on branches indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values ≥ 60% are shown).

$P > 0.08$; Table 1). By contrast, *Wolbachia* did show evidence for sex-biased prevalence. Two species, *Meta mengei* and *Tetragnatha montana* carried infection more commonly in females than males ($P = 0.001$ and 0.03, respectively, Fisher's exact test) but only *M. mengei* displayed a significant difference after sequential Bonferroni correction for multiple comparisons. While there was no evidence to reject the null hypothesis of equal prevalence in male and females in other cases, we would note that in all species aside from *P. phalangioides*, infection was generally rarer in males than females, suggesting more intensive sampling would be useful.

We examined specimens from different locations in four species. Three of the species showed no variation between samples. In the case of *Linyphia triangularis*, the infection pattern varied between the three populations sampled. While 10 of the 17 individuals from UK populations were infected by *Wolbachia*, this infection was not detected in any of the nine individuals collected in Germany (Fisher's exact test, $P = 0.004$; Table 1). *Cardinium* infection was observed to be common in both UK and German populations of this species (7 of 17 and 4 of 9 individuals, respectively), and the frequency of infection did not differ significantly between populations (Fisher's exact test, $P = 0.27$).

Surprisingly, no superinfected individual was observed in *L. triangularis*. We investigated this interesting pattern further to ascertain that the individuals sampled did truly belong to a single monophyletic group, and that the pattern was not due to errors in taxonomy. To this end, the 16S rDNA mitochondrial gene was sequenced for two *L. triangularis* individuals per population and of each infection type (i.e. uninfected, *Cardinium* infected and *Wolbachia* infected). Sequence analysis revealed the occurrence of three different mitotypes in *L. triangularis* (i.e. mitochondrial haplotype, called *tri*I, *tri*II and *tri*III; GenBank Accession nos EU333942–EU333944). *tri*I was found in *Cardinium* infected and uninfected individuals from the German population but was absent in UK populations. *tri*II was found in *Cardinium* infected individuals from the UK populations and *tri*III in *Wolbachia* infected individuals from the UK. Mitotypes displayed 0.4% to 1.3% divergence, and no insertion/deletion events, with *tri*I and *tri*II more close in sequence (99.6% identity) than either is with *tri*III (98.7% identity) (Fig. 1). An *L. triangularis* sequence available in GenBank clearly falls within the group of our samples (Fig. 1). While mtDNA sequence cannot be used to delimit species, this divergence level is of the order of that typically found within species, and indicates no reason to suspect erroneous taxonomic identification by the collectors as the cause of the variation in infection state observed between the two populations.

Phylogenetic analysis

Bacterial sequences were taken from two individuals from each species that was positive for infection by *Cardinium* or by *Wolbachia* (GenBank Accession nos EU333926–EU333941). The sequence of all the putative *Cardinium* or *Wolbachia* PCR products were those of *Cardinium* or *Wolbachia*, respectively, indicating the PCR assays were robust and not suffering

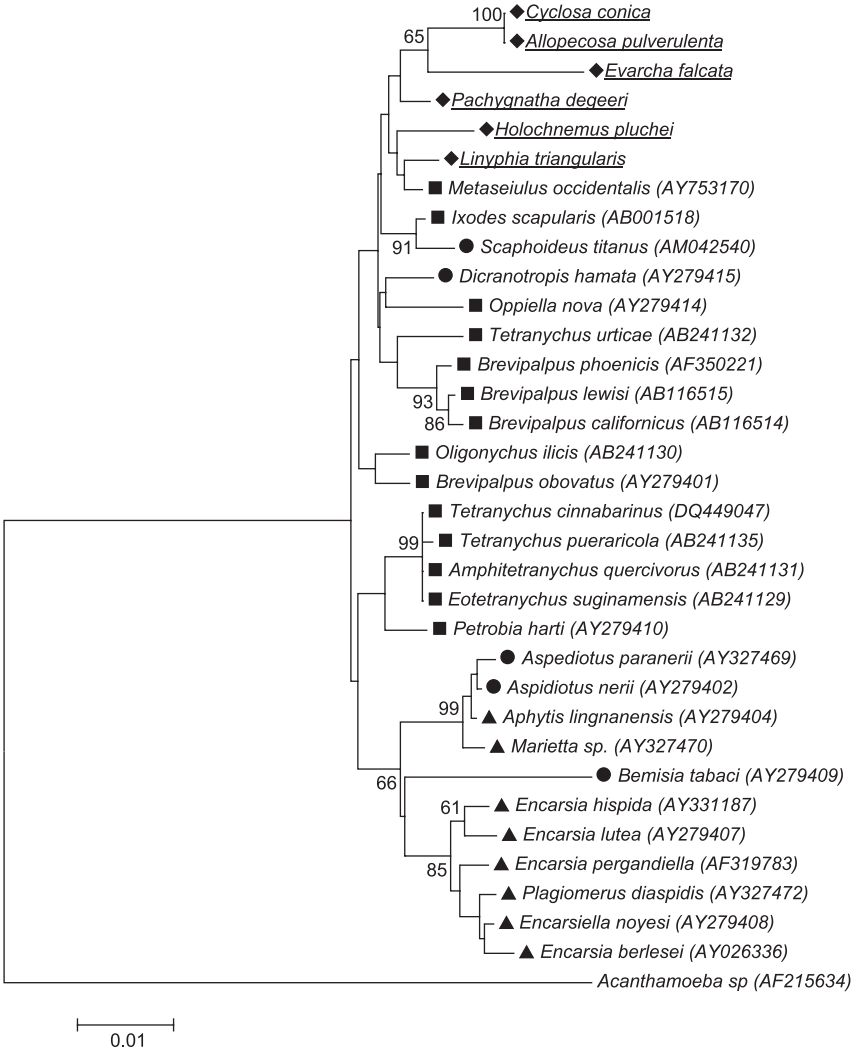

Fig. 2 *Cardinium* 16S rDNA phylogeny constructed via neighbour joining as implemented on MEGA version 3.1. Previously published *Cardinium* sequences are shown in plain type and sequences from this study are underlined. *Cardinium* hosts are coded with shape symbols, with Hymenopteran hosts designated by a triangle, Hemiptera by a circle, Acari by a square and Araneae by a diamond. Numbers on branches indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values ≥ 60% are shown).

from false-positives. The sequences were easily readable without messy peaks, indicating that there is no co-infection of different strains of the same bacterium in any of the individuals. In no case was any sequence variation of the symbiont observed within a host species, even between different strains from different populations of *L. triangularis* and *P. phalangiodes*, suggesting that only one strain of *Cardinium* and/or *Wolbachia* was present in a host species. Five *Cardinium* strains were identified from the six infected spider species, that is, each spider species was infected by a distinct *Cardinium* strain excepted for *Alopecosa pulverulenta* and *Cyclosa conica* which shared the same strain. Four *Wolbachia* strains were identified from the 10 infected species, that is, spider species shared frequently very closely related *Wolbachia* strains.

The *Cardinium* sequences of spiders have at least 96% identity to known *Cardinium* 16S rDNA sequences. A phylogeny of the group was constructed using the spider *Cardinium* 16S rDNA sequences obtained in this study, as well as *Cardinium* sequences (from Hymenoptera, the Hemiptera and the Acari) available in GenBank, using the closest known relative of *Cardinium*, the *Acanthamoeba* symbiont *Amoebophilus asiaticus*, as an outgroup (Fig. 2). The spider *Cardinium* strains clearly fall within the *Cardinium hertigii* species group and especially with *Cardinium* strains from Acari species. However, the power of the phylogeny to resolve relationships between the strains collected to date is poor, owing to a dearth of informative characters in the slow evolving 16S rDNA sequence.

Spiders from this study carried four *Wolbachia* strains belonging to three supergroups (A, B and G) of the eight currently recognized (A to H; cf. Lo *et al*. 2007) but the *L. triangularis Wolbachia* could not be attached to any supergroup currently described (Fig. 3). *Wolbachia* from

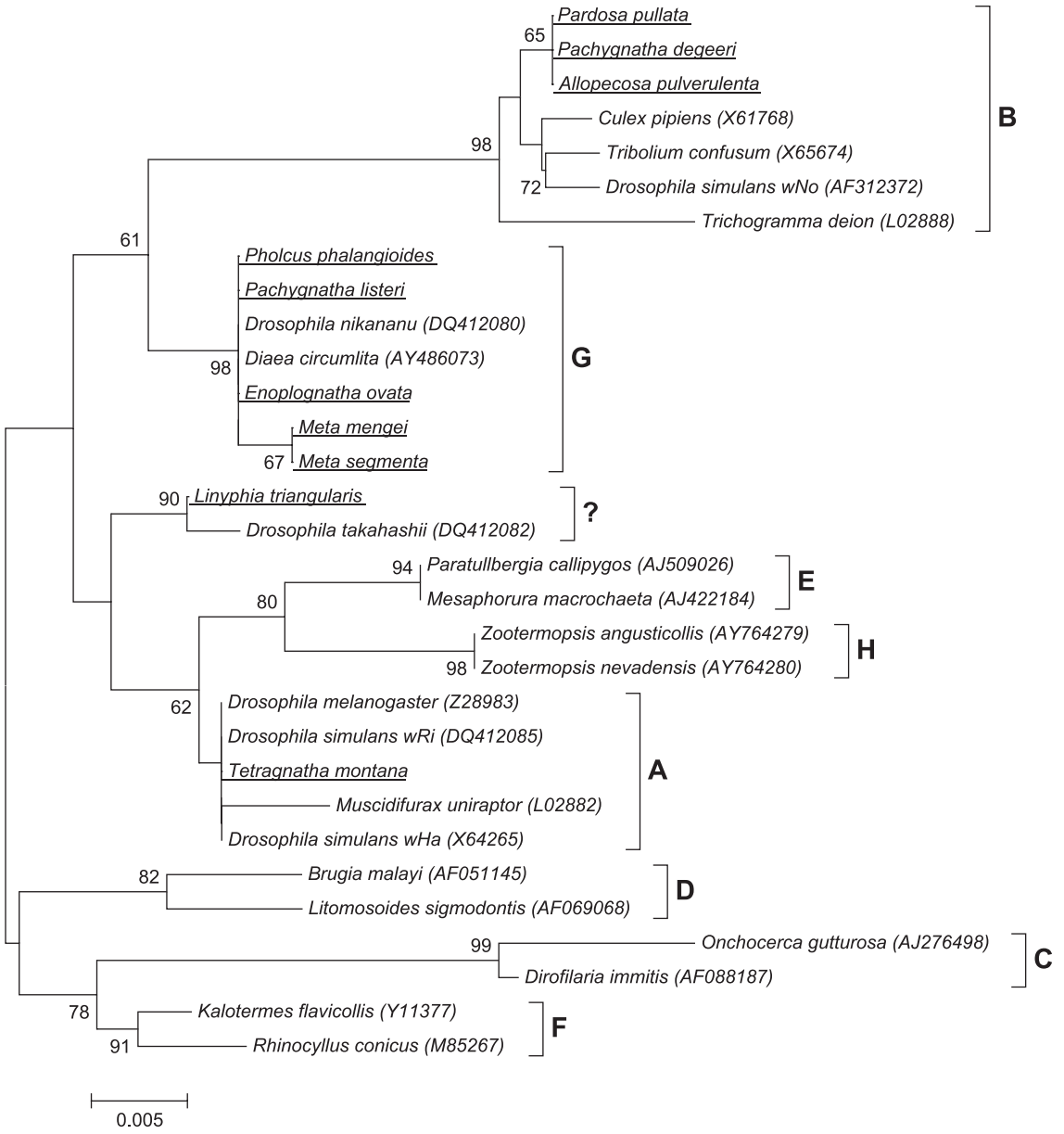

Fig. 3 *Wolbachia* 16S rDNA phylogeny constructed via neighbour joining as implemented on MEGA version 3.1. Previously published *Wolbachia* sequences are shown in plain type and sequences from this study are underlined. Major supergroup lineages are reported (A–H). Numbers on branches indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values ≥ 60% are shown).

Meta segmenta, *M. mengei*, *P. phalangoides*, *Enoplognatha ovata* and *Pachygnatha listeri*, were most closely related to previously described spider *Wolbachia* and belonged to the G supergroup of *Wolbachia* which principally comprises spider-hosted *Wolbachia* (Rowley *et al*. 2004). The other spider-hosted *Wolbachia* sequences were most closely related to *Wolbachia* from various other insects and belonged either to the A (i.e. *Wolbachia* from *Tetragnatha montana*) or to the B (i.e. *Wolbachia* from *A. pulverulenta*, *Pardosa pullata* and *Pachygnatha degeeri*) supergroups.

Discussion

Establishing the inventory of reproductive parasites and their hosts is a crucial first step in understanding the evolution of hereditary symbiosis and their contribution to the variation in reproductive biology observed across arthropods. In this study, we sampled a medium number of male and female individuals of 27 species of spider for *Cardinium*, a bacterium whose incidence was unknown in spiders, and *Wolbachia*, a bacterium known to be common

in spiders, but whose prevalence and phenotype in infected species is unknown.

The estimated incidence of infection for spiders was 22% and 37% for *Cardinium* and *Wolbachia*, respectively. Both bacteria are present in a wide range of spider species and seem to infect the major taxa of spiders without discernable pattern. In comparison with previous surveys (Weeks *et al*. 2003; Zchori-Fein & Perlman 2004), *Cardinium* incidence was significantly higher in spiders than in all arthropods, but similar to that found in mites. Thus, Arachnida and especially spiders represent a *Cardinium* hotspot. However, the differences between surveys could in some part be also associated with differences in screening intensity and methods. While we standardly screened 20 individuals per species, Weeks *et al*. (2003) used between 3 and 10 individuals from natural populations, and Zchori-Fein & Perlman (2004) do not indicate sample size but used individuals from laboratory colonies. This stated, the 3–10 individuals of Weeks *et al*. (2003) should make for a roughly comparable survey, given prevalence within infected species in our survey was around 50%. This means that *Cardinium* is certainly common in spiders, although a definitive comparison with other arthropod taxa is currently not possible.

In our survey, as in previous ones, *Wolbachia* was also common in spiders (Oh *et al*. 2000; Cordaux *et al*. 2001; Rowley *et al*. 2004; Goodacre *et al*. 2006). Furthermore, infection of other reproductive parasites, that is, of the *Spiroplasma ixodetis* group and the *Rickettsia* genus, has been reported (Goodacre *et al*. 2006). This argues that the incidence of microorganisms commonly associated with reproductive parasitism is exceptionally high in spiders. This could be explained by ecological factors facilitating acquisition via horizontal transmission. Indeed, spiders are exceptional because all species of this group depend completely on predation, while some other arthropods exploit various strategies of feeding (Coddington & Levi 1991). Predation could be a potential mechanistic hypothesis for bacterial transfer between nonrelated hosts, but this hypothesis remains currently speculative.

The factors maintaining *Cardinium* within host species are not clear. There was no evidence of a significant sex bias in *Cardinium* infection that would imply SR distorting activity by the bacterium. Thus, this bacterium is unlikely to induce parthenogenesis, feminization or male killing in the spider species we sampled. It is more difficult to establish the presence/absence of cytoplasmic incompatibility. It is clear that the prevalence levels observed are different from those classically associated with strong CI: strong CI has been observed to produce near fixed infections in many species (Kitrayapong *et al.* 2002; Kondo *et al.* 2002; Duron *et al.* 2005). However, weak CI inducers can exist at medium prevalence (as in *Drosophila melanogaster*: Hoffmann *et al*. 1998), and the spider infections may all fall in this category.

Alternative mechanism for the invasion of *Cardinium* relies on direct advantage or horizontal transmission. The patchy distribution of *Cardinium* infections within spider families suggests that spiders are not dependent upon these bacteria for survival or reproduction, a result also supported by the lack of fixed infections within species. It is possible that *Cardinium* is a secondary (facultative) symbiont (S-endosymbiont) in this group: the infection may have no negative effects on their host, but may confer slight facultative benefit under particular environmental conditions. For example, S-endosymbionts are known to confer resistance to parasitoids in the pea aphid *Acyrthosiphon pisum* (Oliver *et al*. 2005). Finally, and intriguingly, the maintenance of *Cardinium* in spiders could be associated with horizontal transmission rates that are higher than usually found, allowing invasion without any manipulation/direct effect on the host. Only cage test experiments controlling for endosymbiont infection — including breeding experiments and antibiotic treatments — will definitely allow the identification of the infectious phenotypes of *Cardinium* in spiders.

The phenotype demonstrated by *Wolbachia* can also be conjectured. By contrast to *Cardinium*, there was evidence for sex bias in prevalence in one case, indicating potential sex ratio distorting activity. In *Meta mengei*, 9 of 10 females sampled were infected compared to 1 of 10 males. The occurrence of infected males would indicate that any sex-ratio distorting activity occurring had incomplete penetrance, a phenomenon reported previously in insects (e.g. Hurst *et al*. 2000). Because of a general pattern of sex-biased infection, sex-ratio distortion will be worth investigating further in other species, with a need to obtain larger sample sizes to allow greater statistical power to delineate any sex-biased patterns. Again, in contrast to *Cardinium*, strong CI is possible in one case, with infection near fixation in *Pholcus phalangioides.* Aside from these contrasts, the alternate explanations for infection invasion postulated for *Cardinium* (weak CI, secondary symbiont activity, horizontal transmission) all bear investigation.

That some spider species possess more than one type of reproductive parasite has been reported previously, with respect to *Rickettsia*, *Spiroplasma* and *Wolbachia* infection (Goodacre *et al*. 2006). In the present study, intraspecific co-infection with both *Cardinium* and *Wolbachia* was observed in three spider species (Table 1). Individuals were co-infected in one species, *Alopecosa pulverulenta* where individuals were either infected by *Wolbachia* and *Cardinium*, *Cardinium* alone, or were uninfected. In the other two co-infected species, *Linyphia triangularis* and *Pachygnatha degeeri*, there was no co-infection at the individual level. Multiple infections with reproductive parasites are not unexpected and have been documented in other arthropods (for example see Rousset & Solignac 1995; Majerus *et al*. 2000; Dale *et al*. 2006). However, theory predicts that multiple infections on

the species level should only persist if either multiple infections on the individual level occur (Rousset *et al*. 1991; Frank 1998; Engelstädter *et al*. 2004), or if the host population is spatially structured (Keeling *et al*. 2003; Telschow *et al*. 2005; Engelstädter *et al*. in press). Thus, the cases of *L. triangularis* and *P. degeeri*, where both *Cardinium* and *Wolbachia* co-occur within host populations as single infections represent a conundrum. At present, we cannot say whether doubly infected individuals are present but have not been detected, whether co-existence represents an unstable state (e.g. with one of the symbionts about to spread in the population and driving the other one extinct), or if as yet unknown mechanisms maintain a stable polymorphism.

Population structure may limit the spread of both *Cardinium* and *Wolbachia* infection and result in co-existing infected and uninfected populations within a same species (Telschow *et al*. 2005; Telschow *et al*. 2007; Engelstädter *et al*. in press). Geographical variation in infection frequency was thus investigated in four species for which individuals from distant populations have been collected. No variation was found for three species, individuals from different populations being all uninfected for *Araneus diadematus* and *Argiope lobota*, or almost all infected for *P. phalangioides*. However, the infection pattern in *L. triangularis* varied significantly between the three populations sampled. No individuals from Germany were infected by *Wolbachia*, while infection is commonly found in the two UK populations. By contrast, *Cardinium* infection was observed at the same prevalence in all populations. The existence of mtDNA sequence–infection association provides another case where mtDNA alone cannot be used to reliably infer population history of symbiont infected species, as there is a very high probability of an incorrect conclusion due to indirect selection arising from the presence of an inherited symbiont (Hurst & Jiggins 2005).

We could not resolve the phylogeny of *Cardinium* infection sufficiently to permit inference on patterns of horizontal transfer and specialization. For *Wolbachia*, a more robust picture is available. On the basis of 16S rDNA sequence, strains for spiders come from a variety of supergroups, especially A, B and G, and have probably arisen through horizontal transmission. Interestingly, the G supergroup represents mostly *Wolbachia* from spiders, suggesting specialization. The *Wolbachia* infection in *L. triangularis* appears to be phylogenetically distinct from the A–H clades. The consistency of the cluster is strengthened by the inclusion of *Wolbachia* strain infecting an unidentified *Drosophila* species of the *takahashii group* (Fig. 3). However, only the *Wolbachia* strains from these two species belong to this putative new clade. The independence of this clade should be re-examined by using the *Wolbachia* multilocus strain typing system (MLST) which uses sequence data from five loci (Baldo *et al*. 2006).

Although understudied, the order Araneae ranks seventh in global diversity after the five largest insect orders (Coleoptera, Hymenoptera, Lepidoptera, Diptera, Hemiptera) and Acari among the arachnids in terms of species described or anticipated (Coddington & Levi 1991). In this study, we have demonstrated that *Cardinium* is an important symbiont of spiders as well as mites, potentially as common as *Wolbachia* in pure incidence terms. What is unclear is what phenotype or role they play. While our study provided no evidence for sex-ratio distortion for *Cardinium*, the inference of cytoplasmic incompatibility is not strong either. For *Wolbachia*, there was evidence of sex biased in prevalence in some host species, making sex-ratio distortion a potential manipulation phenotype. Future work will need to focus on the manipulations and the role these bacteria play in the biology of spiders.

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References

Baldo L, Hotopp JCD, Jolley KA *et al*. (2006) Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology*, **72**, 7098–7110.

Bandi C, Dunn AM, Hurst GDD, Rigaud T (2001) Inherited microorganisms, sex-specific virulence and reproductive parasitism. *Trends in Parasitology*, **17**, 88–94.

Caspari E, Watson GS (1959) On the evolutionary importance of cytoplasmic sterility in mosquitoes. *Evolution*, **13**, 568–570.

Coddington JA, Levi HW (1991) Systematics and evolution of spiders (Araneae). *Annual Review of Ecology and Systematics*, **22**, 565–592.

Cordaux R, Michel-Salzat A, Bouchon D (2001) *Wolbachia* infection in crustaceans: novel hosts and potential routes for horizontal transmission. *Journal of Evolutionary Biology*, **14**, 237–243.

Dale C, Beeton M, Harbison C, Jones T, Pontes M (2006) Isolation, pure culture, and characterization of '*Candidatus Arsenophonus arthropodicus*', an intracellular secondary endosymbiont from the Hippoboscid louse fly *Pseudolynchia canariensis*. *Applied and Environmental Microbiology*, **72**, 2997–3004.

Duron O, Lagnel J, Raymond M, Bourtzis K, Fort P, Weill M (2005) Transposable element polymorphism of *Wolbachia* in the mosquito *Culex pipiens*: evidence of genetic diversity, super-infection and recombination. *Molecular Ecology*, **14**, 1561–1573.

Engelstädter J, Telschow A, Hammerstein P (2004) Infection dynamics of different *Wolbachia*-types within one host population. *Journal of Theoretical Biology*, **231**, 345–355.

Engelstädter J, Telschow A, Yamamura N (2008) Coexistence of cytoplasmic incompatibility and male-killing inducing endosymbionts, and their impact on host gene flow. *Theoretical Population Biology*, **73**, 125–133.

Enigl M, Schausberger P (2007) Incidence of the endosymbionts *Wolbachia*, *Cardinium* and *Spiroplasma* in phytoseiid mites and associated prey. *Experimental and Applied Acarology*, **42**, 75–85.

Fine PE (1978) On the dynamics of symbiote-dependent cytoplasmic incompatibility in culicine mosquitoes. *Journal of Invertebrate Pathology*, **31**, 10–18.

Frank SA (1998) Dynamics of cytoplasmic incompatibility with multiple *Wolbachia* infections. *Journal of Theoretical Biology*, **192**, 213–218.

Goodacre SL, Martin OY, Thomas CFG, Hewitt GM (2006) *Wolbachia and* other endosymbiont infections in spiders. *Molecular Ecology*, **15**, 517–527.

Gotoh T, Noda H, Ito S (2007) *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity*, **98**, 13–20.

Hagimori T, Abe Y, Date S, Miura K (2006) The first finding of a *Rickettsia* bacterium associated with parthenogenesis induction among insects. *Current Microbiology*, **52**, 97–101.

Hendriks L, Goris A, Neefs J, Van de Peer Y, Hennebert G, De Wachter R (1989) The nucleotide sequence of the small ribosomal subunit RNA of the yeast *Candida albicans* and the evolutionary position of the fungi among the eukaryotes. *Systematic and Applied Microbiology*, **12**, 223–229.

Hendriks L, De Baere R, Van de Peer Y, Neefs J, Goris A, De Wachter R (1991) The evolutionary position of the Rhodophyte *Porphyra umbilicalis and* the Basidiomycete *Leucosporidium scotii* among other Eukaryotes as deduced from complete sequences of small ribosomal subunit RNA. *Journal of Molecular Evolution*, **32**, 167–177.

Hoffmann AA, Hercus M, Dagher H (1998) Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics*, **148**, 221–231.

Hunter MS, Perlman SJ, Kelly SE (2003) A Bacteroidetes-group bacterial symbiont induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 2185–2190.

Hurst GDD, Jiggins JM (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 1525–1534.

Hurst GDD, Johnson AP, von der Schulenburg JHG, Fuyama Y (2000) Male-killing *Wolbachia* in *Drosophila*: A temperature-sensitive trait with a threshold bacterial density. *Genetics*, **156**, 699–709.

Hurst GDD, Werren JH (2001) The role of selfish genetic elements in eukaryotic evolution. *Nature Reviews Genetics*, **2**, 597–606.

Hwang UW, Friedrich M, Tautz D, Park CJ, Kim W (2001) Mitochondrial protein phylogeny joins myriapods with chelicerates. *Nature*, **413**, 154–157.

Jiggins FM, Hurst GDD, Jiggins CD, Von Der Schulenburg JH, Majerus MEN (2000) The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology*, **120**, 439–446.

Keeling MJ, Jiggins FM, Read JM (2003) The invasion and coexistence of competing Wolbachia strains. *Heredity*, **91**, 382–388.

Kenyon SG, Hunter MS (2007) Manipulation of oviposition choice of the parasitoid wasp, *Encarsia pergandiella*, by the endosymbiotic bacterium *Cardinium*. *Journal of Evolutionary Biology*, **20**, 707–716.

Kitrayapong P, Baimai V, O'Neill SL (2002) Field prevalence of *Wolbachia* in the mosquito vector *Aedes albopictus*. *American Journal of Tropical Medicine and Hygene*, **66**, 108–111.

Kondo N, Ijichi N, Shimada M, Fukatsu T (2002) Prevailing triple infection with *Wolbachia* in *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Molecular Ecology*, **11**, 167–180.

Kumar S, Tamura K, Nei M (2004) MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

Lo N, Paraskevopoulos C, Bourtzis K *et al*. (2007) Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. *International Journal of Systematics and Evolutionary Microbiology*, **57**, 654–657.

Majerus MEN, Hinrich J, Schulenburg GVD, Zakharov IA (2000) Multiple causes of male-killing in a single sample of the two-spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae) from Moscow. *Heredity*, **84**, 605–609.

O'Neill SL, Giordano R, Colbert AME, Karr TL, Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences, USA*, **89**, 2699–2702.

Oh HW, Kim MG, Shin SW, Bae KS, Ahn YJ, Park HY (2000) Ultrastructural and molecular identification of a *Wolbachia* endosymbiont in a spider, *Nephila clavata*. *Insect Molecular Biology*, **9**, 539–543.

Oliver KM, Russell JA, Moran NA, Hunter MS (2005) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences, USA*, **102**, 12795–12800.

Provencher LM, Morse GE, Weeks AR, Normark BB (2005) Parthenogenesis in the *Aspidiotus nerii* complex (Hemiptera: Diaspididae): a single origin of a worldwide, polyphagous lineage associated with *Cardinium* bacteria. *Annals of the Entomological Society of America*, **98**, 629–635.

Rousset F, Raymond M (1991) Cytoplasmic incompatibility in insects: why sterilize females? *Trends in Ecology & Evolution*, **6**, 54–57.

Rousset F, Solignac M (1995) Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex. *Proceedings of the National Academy of Sciences, USA*, **92**, 6389–6393.

Rousset F, Raymond M, Kjellberg F (1991) Cytoplasmic incompatibilities in the mosquito *Culex pipiens*: how to explain a cytotype polymorphism? *Journal of Evolutionary Biology*, **4**, 69–81.

Rowley SM, Raven RJ, McGraw EA (2004) *Wolbachia pipientis* in Australian spiders. *Current Microbiology*, **49**, 208–214.

Stevens L, Giordano R, Fialho RF (2001) Male-killing, nematode infections, bacteriophage infection, and virulence of cytoplasmic bacteria the genus *Wolbachia*. *Annual Review of Ecology and Systematics*, **32**, 519–545.

Stouthamer R, Breeuwer JAJ, Hurst GDD (1999) *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annual Review of Microbiology*, **53**, 71–102.

Tajima F, Nei M (1984) Estimation of evolutionary distance between nucleotide sequences. *Molecular Biology and Evolution*, **1**, 269–285.

Telschow A, Yamamura N, Werren JH (2005) Bidirectional cytoplasmic incompatibility and the stable coexistence of two *Wolbachia* strains in parapatric host populations. *Journal of Theoretical Biology*, **235**, 265–274.

Telschow A, Flor M, Kobayashi Y, Hammerstein P, Werren JH (2007) *Wolbachia*-induced unidirectional cytoplasmic incompatibility and speciation: mainland-island model. *PLoS One*, **2**, e701.

Von der Schulenburg JHG, Habig M, Sloggett JJ *et al*. (2001) Incidence of male-killing *Rickettsia spp*. (alpha-proteobacteria) in the ten-spot ladybird beetle *Adalia decempunctata* L. (Coleoptera: Coccinellidae). *Applied and Environmental Microbiology*, **67**, 270–277.

Weeks AR, Stouthamer R (2003) Increased fecundity associated with infection by a Cytophaga-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. *Proceedings of the Royal Society B: Biological Sciences*, **271**, S193–S195.

Weeks AR, Marec F, Breeuwer JA (2001) A mite species that consists entirely of haploid females. *Science*, **292**, 2479–2482.

Weeks AR, Velten R, Stouthamer R (2003) Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 1857–1865.

Werren JH (1997) Biology of *Wolbachia*. *Annual Review of Entomology*, **42**, 587–609.

Werren JH (1998) *Wolbachia* and speciation. In: *Endless Forms: Species and Speciation* (eds Howard D, Berlocher S), pp. 245–260. Oxford University Press, Oxford, UK.

Werren JH, Skinner SW, Huger AM (1986) Male-killing bacteria in a parasitic wasp. *Science*, **231**, 990–992.

Zchori-Fein E, Perlman SJ (2004) Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molecular Biology*, **13**, 2009–2016.

Zchori-Fein E, Gottlieb Y, Kelly SE *et al*. (2001) A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proceedings of the National Academy of Sciences, USA*, **98**, 12555–12560.

Zchori-Fein E, Perlman SJ, Kelly SE, Katzir N, Hunter MS (2004) Characterization of a *Bacteroidetes* symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus cardinium hertigii*'. *International Journal of Systematics and Evolutionary Microbiology*, **54**, 961–968.

The authors are interested in the evolutionary and ecological effects of parasites on host populations, with a particular focus on parasites that affect host reproduction.
