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# Exploring the effect of the *Cardinium* endosymbiont on spiders

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## Abstract

Spiders have recently emerged as important diversity hot spots for endosymbiotic bacteria, but the consequences of these symbiotic interactions are largely unknown. In this article, we examined the evolutionary history and effect of the intracellular bacterium *Cardinium hertigii* in the marbled cellar spider *Holocnemus pluchei*. We showed that *Cardinium* infection is primarily transmitted in spider populations maternally via egg cytoplasm, with 100% of the progeny from infected mothers being also infected. Examination of a co-inherited marker, mitochondrial DNA (mtDNA), revealed that *Cardinium* infection is associated with a wide diversity of mtDNA haplotypes, showing that the interaction between *Cardinium* and *H. pluchei* has a long-term evolutionary dimension and that horizontal transmission among individuals could also occur. Although *Cardinium* is well known to exert sex ratio distortion or cytoplasmic incompatibility in various arthropod hosts, we show, however, that *Cardinium* does not interact with the reproductive biology of *H. pluchei*. A field survey shows a clear geographical structuring of *Cardinium* infection, with a marked gradual variation of infection frequencies from ca. 0.80 to 0. We discuss different mechanistic and evolutionary explanations for these results as well as their consequences for spider phenotypes. Notably, we suggest that *Cardinium* can either behave as a neutral cytoplasmic element within *H. pluchei* or exhibit a context-dependent effect, depending on the environmental conditions.

## Introduction

Although historically symbiosis has received less attention than other interactions such as predation or competition, it is increasingly recognized as an important selective force of evolution (Moran *et al.*, 2008; Werren *et al.*, 2008). Many terrestrial arthropods host various types of bacterial endosymbionts that are vertically inherited from mother to progeny through the egg cytoplasm (Duron *et al.*, 2008a; Hilgenboecker *et al.*, 2008). Molecular and experimental approaches have yielded an immensely rich understanding of the biological roles of these endosymbionts: obligate mutualists provide nutrients, facultative mutualists confer protection against natural enemies or abiotic stress, and reproductive parasites manipulate the host reproductive systems (Haine, 2008; Moran *et al.*, 2008; Werren *et al.*, 2008; Saridaki & Bourtzis, 2010). Endosymbionts have thus profound effects on arthropod phenotypes and are now regarded as major drivers of arthropod ecology and evolution (Engelstadter & Hurst, 2009; Fellous *et al.*, 2011; Jiggins & Hurst, 2011).

Perhaps, one of the most remarkable observations of these last few years has been that the spiders harbour one of the widest ranges of endosymbionts found in arthropods (Goodacre *et al.*, 2006; Duron *et al.*, 2008a,b). These symbionts belong to phylogenetically diverse lineages of bacteria, including *Cardinium hertigii* (Bacteroidetes), *Wolbachia pipientis* (alpha-proteobacteria), *Rickettsia* sp. (alpha-proteobacteria) and *Spiroplasma ixodetis* (Mollicutes). It is not yet well understood how these bacteria affect the spider's biology. Outside the spider order (Araneae), these bacteria are generally known as 'reproductive parasites' in the sense that they increase their own transmission by manipulating the reproductive phenotype of their hosts

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(Werren *et al.*, 2008; Engelstadter & Hurst, 2009). Because males represent a dead end for transmission, manipulations frequently involve biasing the sex ratio (SR) of infected females towards the production of daughters, via the induction of 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 cytoplasmic incompatibility (CI), a sperm-egg incompatibility between infected males and uninfected females. Each of these manipulations is of ecological and evolutionary importance to the particular host species that is infected, potentially inducing reproductive isolation or driving changes in sexuality (Werren *et al.*, 2008; Engelstadter & Hurst, 2009; Saridaki & Bourtzis, 2010; Jiggins & Hurst, 2011).

The importance of bacterial endosymbionts in shaping spider biology has rarely been investigated: *Wolbachia* has been shown to be involved in SR distortion in two spider species (Gunnarsson *et al.*, 2009; Vanthournout *et al.*, 2011), and the role of *Rickettsia* in the dispersal capacity has been established in one species of ballooning spider (Goodacre *et al.*, 2009). Current knowledge of other inherited bacteria, such as *Cardinium*, is weaker. This bacterium is exceptionally frequent in spiders, infecting ca. 20% of species (Duron *et al.*, 2008b; Martin & Goodacre, 2009; Perlman *et al.*, 2009), but was only discovered in the last decade (Zchori-Fein *et al.*, 2004). *Cardinium* was then isolated from mites (Weeks *et al.*, 2001), ticks (Kurtti *et al.*, 1996), parasitic wasps (Zchori-Fein *et al.*, 2001, 2004), biting midges (Nakamura *et al.*, 2009), planthoppers (Weeks *et al.*, 2003; Zchori-Fein & Perlman, 2004; Marzorati *et al.*, 2006) and plant-parasitic nematodes (Noel & Atibalentja, 2006). The list of manipulations induced by *Cardinium* is impressive: feminization in mites (Weeks *et al.*, 2001), thelytokous parthenogenesis in parasitic wasps (Zchori-Fein *et al.*, 2001, 2004) and scale insects (Provencher *et al.*, 2005), and CI in parasitic wasps (Hunter *et al.*, 2003) and mites (Gotoh *et al.*, 2007; Ros & Breeuwer, 2009). In addition, *Cardinium* spread is favoured by an adaptive manipulation of the oviposition behaviour of a parasitic wasp (Zchori-Fein *et al.*, 2001; Kenyon & Hunter, 2007) and by enhancing the fecundity of a predatory mite (Weeks & Stouthamer, 2004). This strongly suggests that *Cardinium* may also drive spider biology.

In this study, we explore how *Cardinium* interacts with the marbled cellar spider *Holocnemus pluchei* (Aranea: Pholcidae). First, we examined whether *Cardinium* alters reproductive traits of *H. pluchei*, by testing for SR distortion, CI and fecundity effects. Second, we report on the geographical variation of *Cardinium* prevalence in natural populations of *H. pluchei*. Third, because *Cardinium* depends on maternal transmission for spreading within *H. pluchei* populations, we also report on the diversity of mitochondrial DNA (mtDNA) haplotypes. We analyse how mtDNA variation is partitioned by infection status, as a means to investigate the evolutionary history of the *Cardinium* infection. This is an especially useful tool as some intracellular endosymbionts have lower rates of molecular evolution than their host's mtDNA (e.g. James & Ballard, 2000; Shoemaker *et al.*, 2003; Dyer & Jaenike, 2004). We discuss the importance of possible mechanisms leading to our results as well as the potential adaptive significance of the presence of *Cardinium* for spider evolution.

## Materials and methods

### Spider collection

*Holocnemus pluchei* specimens were field-collected from various sites in the native distribution range of the species, principally in the Mediterranean area (including France, Corsica, Spain, Crete, Israel and Jordan; 2009–2011). Specimens from California (2010) where *H. pluchei* has been recently introduced were also included in the analysis. The populations are presented in Table S1. Within each population, there were 2–159 individuals separately investigated for analysis. Spiders were fixed in 75% ethanol and stored at 4 °C until analysed.

### Effect of *Cardinium* infection

The effect of *Cardinium* infection on *H. pluchei* reproduction was examined using field-caught males and females from Montpellier (population I in Table S1). The infection status was determined using polymerase chain reaction (PCR) as described below. Females holding egg clutches in their chelicerae were captured alive and individually housed in plastic cups (0.7 $dm^3$). Egg clutches were checked daily until hatching; the eggs were then counted, and the hatching rate was recorded. The mother's body size was estimated by measuring the tibia-patella length of leg 1 with a micrometre NIKON Digital Counter CM-6S. Spiderlings were reared individually to adulthood, and their sex was recorded. Spiderlings were reared as follows: after their first moult, each spiderling was individually transferred with a paintbrush to its own plastic cup and fed *ad libitum* twice a week with living prey, that is, *Drosophila melanogaster* flies and *Nemobius* sp. crickets. All specimens were kept at ca. 25 ± 2 °C with 12-h/12-h light/dark cycle. PCRs of a random sample of 20 flies and crickets indicated that they were not infected by *Cardinium*.

### Screening and sequencing

Spider DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol (Rogers & Bendich, 1988). DNA extraction from adult specimens was performed on abdominal tissue to reduce the risk of missing infection with reproductive parasites when they are present (false negatives); DNA extraction from juvenile

specimens was performed on the entire body. The DNA quality was routinely tested by PCR amplification of a region of the mitochondrial *cytochrome oxidase I* (*COI*) gene. Two independent assays for *Cardinium* infection were performed by PCR amplification of two genes, the *16S rRNA* gene and the *DNA gyrase b* (*gyrb*) gene, using specific primers. Additional PCRs were conducted on a subsample of individuals to detect infections by four other endosymbionts found in other spider species: *Wolbachia*, *Rickettsia*, *Arsenophonus* and *Spiroplasma* (Goodacre *et al.*, 2006; Duron *et al.*, 2008a). Independent assays for detecting the infection by each of these endosymbionts were performed using specific PCR amplification as described by Duron *et al.* (2008a). Gene and primer features are listed in Table S2.

Polymerase chain reactions were performed under the following conditions: initial denaturation at 93 °C for 3 min, 35 cycles of denaturation (93 °C, 30 s), annealing (50–54 °C, depending on primers, cf. Table S2, 30 s), extension (72 °C, 1 min) and a final extension at 72 °C for 5 min. The PCR products were electrophoresed in a 1.5% agarose gel. Direct sequencing of PCR products was performed on an ABI Prism 3130 sequencer using the BigDye Terminator Kit (Applied Biosystems; Foster City, CA, USA) after purification with the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The chromatograms were manually inspected and cleaned with Chromas Lite (http://www.technelysium.com.au/chromas_lite.html), and sequence alignments were performed using MEGA (Kumar *et al.*, 2004). The sequences are deposited in GenBank (accession numbers, JN202549–JN202557).

### Phylogenetic analyses

Phylogenetic relationships were evaluated (i) for *Cardinium* sequences of the *16S rRNA* and *gyrb* genes and (ii) for *H. pluchei* mitochondrial *COI* sequences. For *Cardinium*, phylogenetic trees were constructed using our own data combined with other *Cardinium* sequences (groups A–C; Nakamura *et al.*, 2009) found in GenBank. Before analysing the sequences, the evolutionary model most closely fitting the data was determined using hierarchical likelihood ratio tests and Akaike information criterion with the program MODELTEST version 3.7 (Posada & Crandall, 1998). For the *16S rRNA*, *gyrb* genes and *COI* data sets, the best-fit approximation was the general time reversible model with invariant sites (GTR + I). To analyse phylogenetic relationships, maximum likelihood (ML) analyses were conducted using PAUP version 4.0 (Swofford, 2002). Model parameters were first estimated by ML on a neighbour-joining topology and then used in optimal tree searches, which consisted of heuristic searches with Tree-Bisection-Reconnection (TBR) branch swapping. Clade robustness was assessed by bootstrap analysis using 1000 replicates. The phylogenetic trees were visualized and edited in MEGA (Kumar *et al.*, 2004).

### Statistical analysis

All statistical analyses were carried out using the R statistical package (http://www.r-project.org/). To explain inter-population variation of *Cardinium* frequency, we built a statistical model by including distance of each population from a reference location as explanatory variable using a quasibinomial error distribution (*lmer*, LME4 package of R; http://lme4.r-forge.r-project.org) to correct for overdispersed errors. Differentiation among geographical mtDNA groups and heterogeneity between infected and uninfected hosts were tested using AMOVA (*ade4* package of R; http://pbil.univ-lyon1.fr/ADE-4).

## Results

### Effect of *Cardinium* infection

We first examined the mechanisms possibly used by *Cardinium* to spread through *H. pluchei* populations, that is, its mode of transmission, its effect on fecundity and on reproductive phenotype. That the *Cardinium* infection could be maternally transmitted was checked by examining the presence of infection in neonates from infected mothers. Thirteen infected mothers and five uninfected mothers, all holding clutches of eggs, were collected in Montpellier (population I), and 10 neonates per clutch were randomly sampled for PCR screening using *Cardinium*-specific primers. We found that all neonates ($n = 130$) from infected mothers were themselves infected, whereas no *Cardinium* infection was found in neonates ($n = 50$) from uninfected mothers. The mean transmission rate can be thus estimated at 1 (95% confidence interval, 0.972–1), demonstrating a very efficient maternal transmission of *Cardinium* in *H. pluchei*.

Next, we measured fecundity and body size of 67 females from Montpellier but found no significant effect of infection. The mean number of eggs produced by infected and uninfected females was almost identical (mean ± SE of infected, 25.2 ± 1.3 eggs, $n = 50$ females; uninfected, 23.9 ± 1.5 eggs, $n = 17$ females; Wilcoxon two-tailed test, $W = 419$, $P = 0.94$). There were no significant differences either between patella-tibia length of infected and uninfected females (infected, 11.7 ± 0.2 mm, $n = 50$ females; uninfected, 11.6 ± 0.2 mm, $n = 17$ females; Wilcoxon two-tailed test, $W = 483$, $P = 0.41$). Because patella-tibia length is correlated with body size in *H. pluchei* (Skow & Jakob, 2003), this suggests that *Cardinium* does not affect the host body size. The number of eggs was highly correlated with mother's patella-tibia length ($F_{1,65} = 23.60$, $R^2 = 0.25$, $P = 8.10^{-6}$), showing that larger female body size translates into increased reproductive success, but this correlation was not influenced by the infection status ($F_{1,65} = 0.25$, $P = 0.62$) (Fig. S1). Overall, these data show that infection by *Cardinium* does not alter body size and fecundity in *H. pluchei*.

We examined the Montpellier population for sex bias in infection prevalence, as an indication of potential SR distorting activity. However, *Cardinium* showed no evidence of sex-biased prevalence in adult hosts: 73 of 87 males (83.9%) and 53 of 72 females (74.6%) were infected showing that there was no evidence to reject the null hypothesis of equal prevalence in male and females (Fisher's exact test, $P = 0.63$). In addition, field females carrying egg clutches were assayed for two phenotypic indicators of the presence of SR distorter: low egg hatch rates (indicating male-killing) and female-biased progenic SR (indicating male-killing, feminization or thelytokous parthenogenesis). No hatch rate decrease was observed in the broods of 50 *Cardinium*-infected females compared with the broods of 17 uninfected females (infected, $0.99 \pm 0.01$, $n = 1258$ eggs; uninfected, $0.97 \pm 0.01$, $n = 407$ eggs; Wilcoxon two-tailed test, $W = 468$; $P = 0.41$). The progeny of six infected and of four uninfected mothers were also reared to adulthood to record their SR. The brood from infected females resulted in 64 : 60 male-to-female ratio, and the brood from uninfected females resulted in 40 : 45 male-to-female ratio, neither of which differ significantly from a 1 : 1 SR (binomial exact test, $P = 0.78$ and 0.66, respectively). Hence, *Cardinium*-infected females laid eggs with a high hatch rate, and the broods produced had a 1 : 1 SR, with resulting males infected. Overall, this shows that *Cardinium* does not exert a SR distorting activity in *H. pluchei*, excluding the possibility of parthenogenesis, feminization or male-killing.

It is more difficult to establish the presence or absence of CI. Unfortunately, our prior assays failed to obtain mating in laboratory population cages, preventing a direct test for CI through crossing experiments. However, the presence of CI is not corroborated by the field data given above. In its simplest form, CI results in increased embryonic mortality (up to 100%) in crosses between infected males and uninfected females. Hence, the mean hatching rate obtained from uninfected females should be lower than the mean hatching rate obtained from infected females, but as stated above, we did not observe such a pattern in *H. pluchei*. In addition, assuming random mating, one should expect that ca. 80% (i.e. the observed frequency of infected males in Montpellier) of the clutches from uninfected females suffer CI mortality. However, all clutches from uninfected females ($n = 17$) exhibit high hatch rate values (from 0.88 to 1). It is thus likely that crosses between infected males and uninfected females are fully fertile, a result not expected from a CI phenotype. Generally speaking, *Cardinium* does not act as a reproductive manipulator in *H. pluchei*.

### Distribution of *Cardinium* infection

To further investigate the population biology of *Cardinium*, we assayed for its presence in 510 *H. pluchei* individuals from 26 populations encompassing the native and introduced distribution area of this spider (Table S1 and Fig. 1). All spider DNA retained for analysis was positive for PCR amplification using the *COI* arthropod universal primers, indicating satisfactory DNA template quality. Of the 510 specimens examined, *16S rRNA* and *gyrb* PCR assays indicated the occurrence of *Cardinium* infection in 210 specimens (41%). Additional PCR screening did not reveal other endosymbionts (i.e. *Wolbachia*, *Rickettsia*, *Arsenophonus* and *S. ixodetis*) than *Cardinium* in *H. pluchei* (68 individuals were examined with 1–3 randomly sampled individuals per population).

Of the 26 populations examined, *Cardinium* was found in 16 populations from France (15 infected populations of 17 screened) and Israel (one of one) (Table S1 and Fig. 1). *Cardinium* was not found to infect populations from Spain (one population), Corsica (1), Crete (1), Jordan (1) and California (4). Where *Cardinium* infection was observed in a host population, a medium prevalence of infection was observed in all cases, and infection was never observed to be at fixation (*Cardinium* prevalence ranged from 7% to 86% of individuals; Table S1 and Fig. 1). Infection frequency is not homogeneous between the 16 infected populations as significant variation occurs between them (Fisher's exact test, $P > 10^{-6}$). It appears obvious that *Cardinium* infection is exceptionally frequent in the south of France, prompting us to examine how prevalence varies with geographical distance between populations.

We thus built a statistical model by including distance as an explanatory variable of *Cardinium* frequency. We arbitrarily used Montpellier as a reference location from where the distance of each population (populations A–R on the Fig. 1) was measured. The populations from Corsica (population S), Crete (T), Jordan (U), Israel (V) and California (W–Z) were not included in the analysis because of their geographical isolation. We found that the *Cardinium* frequency shows a gradual geographical variation: *Cardinium* is frequent around Montpellier (observed prevalence, 0.79; 95% confidence interval, 0.72–0.85; Table S1), but progressively declines from this area, and finally completely disappeared ($t_{16} = -2.822$; $P = 0.012$) (Fig. 2). The reliability of this observation was confirmed using some reference locations other than Montpellier (Fig. S2). The variation of *Cardinium* frequency is not yet strictly regular as exemplified by Saint Gilles (population J), which is close to Montpellier but exhibited a relatively low *Cardinium* frequency (observed prevalence, 0.07; 95% confidence interval, 0.01–0.24; Table S1). However, no *Cardinium*-infected specimen was found in populations more than 270 km from Montpellier (except one infected specimen from Israel).

### Evolutionary history of *Cardinium* infection

To ascertain *Cardinium* DNA variation, we sequenced the *16S rRNA* and *gyrb* gene fragments (954 and 483 bp, respectively) from 1 to 4 randomly sampled individuals

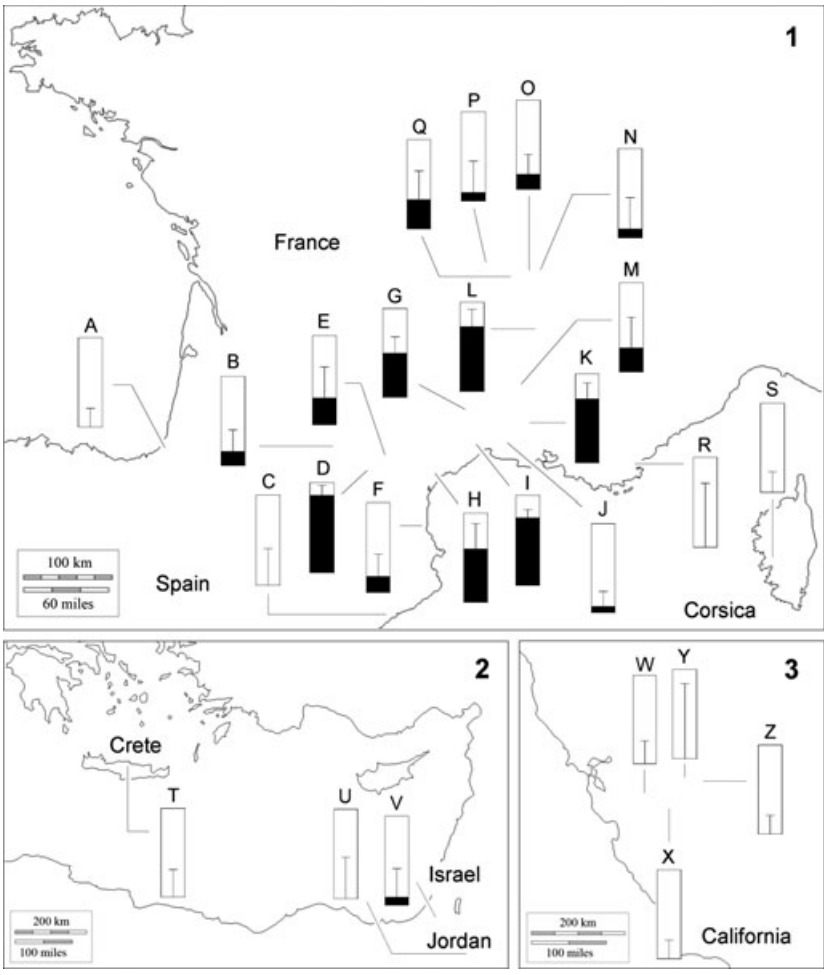


**Fig. 1** Prevalence of *Cardinium* across the populations of *Holocnemus pluchei*. 1, France, Spain and Corsica; 2, Eastern Mediterranean basin; 3, California. The bars show the prevalence of infection (white: uninfected, black: infected) and the 95% confidence interval calculated from the binomial distribution. Letters represent study sites listed in Table S1. Details on sample size and prevalence are given in Table S1.

per infected population. No *16S rRNA* and *gyrb* sequence variation was found between infected individuals ($n$ = 39), and the *16S rRNA* sequences we obtained were also strictly identical to the one deposited in GenBank (accession number, EU333930). Phylogenetically, this strain belongs to the A group within the *Cardinium* genus and clusters with the strains found in other spider species as well as with strains known to induce reproductive manipulations in mites and insects (Fig. S3a,b). Overall, this suggests that one *Cardinium* strain – or an assemblage of very closely related strains – occurs widely in *H. pluchei*, indicating that all of these *Cardinium* infections are derived from a single ancestral infection.

We also examined diversity in the co-inherited marker, *H. pluchei* mtDNA, and the partitioning of this between individuals of different infection status (Fig. S4). Partial *COI* sequences were obtained from 116 *H. pluchei* specimens, spanning 22 populations, and comprising 39 *Cardinium*-infected specimens and 77 uninfected specimens. Across these 116 *COI* sequences, 16 polymorphic sites were observed within the 488 bp sequenced that allowed us to distinguish eight different mtDNA haplotypes. The identity between pairs of *COI* sequences is moderate to high, ranging from 97.5% to 99.8%. The *Cardinium* infection was found in association with a wide diversity of haplotypes within *H. pluchei* (Figs 3 and S4). Indeed, five haplotypes were retrieved from the 39 *Cardinium*-infected specimens examined. No haplotype is confined to *Cardinium*-infected specimens (haplotypes found in association with *Cardinium* are also observed in uninfected individuals), except haplotype 5, which was observed in only one specimen. Most commonly, *Cardinium* is found associated with haplotype 6 (31 specimens) but is also found with haplotypes 3 (three specimens), 5 (1), 7 (1) and 8 (3).

We used AMOVA to formally assess and test for the association between *Cardinium* infection status and mitochondrial sequence variation. In our data set,

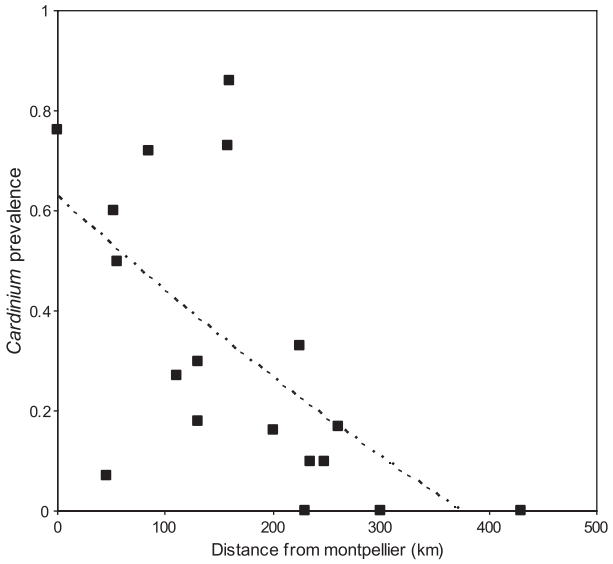


**Fig. 2** Variation of *Cardinium* frequencies in the south of France and Spain. The figure encompasses 18 populations (from A to R on Fig. 1); distances are measured from Montpellier (population I).

infection status explains only 11% of the mtDNA variance ($P = 0.02$ based on 1000 permutations), although a large fraction of the variation is found within populations (54%, $P = 0.001$) and between populations (35%, $P = 0.001$). No significant association was found between mtDNA haplotypes and *Cardinium* infection (Fisher's exact test, $P = 0.09$). This is not surprising given most mtDNA haplotypes found in association with *Cardinium* are also observed in uninfected individuals. There is thus no clear preferential infection of some mtDNA haplotypes, meaning that *Cardinium* infection is randomly distributed among mitochondrial lineages.

## Discussion

We used molecular variation and epidemiological variables to infer the evolutionary history and effect of *Cardinium* in *H. pluchei*. The incidence of infection is highly variable across the distribution range of *H. pluchei*: 16 populations of 26 harboured *Cardinium* and, when present, its prevalence ranged from 7% to 86%. *Cardinium* infection was, however, never observed at fixation as shown by the presence of a substantial number of uninfected *H. pluchei* specimens in each population examined. Although *Cardinium* infection was observed in geographically distant populations (France and Israel, i.e. ca. 2000 km apart), no *Cardinium* DNA variation was found, suggesting that only one *Cardinium* strain (or an assemblage of closely related strains) widely infects *H. pluchei* specimens. Three main conclusions can be drawn that we will discuss in more detail. First, the interaction between *Cardinium* and *H. pluchei* has a long-term evolutionary dimension, suggesting that both partners could have evolved complex interactions. Second, although *Cardinium* is primary described as a reproductive parasite of arthropods, it is not maintained in spider populations by manipulating reproductive phenotype or by enhancing fecundity. Third, the gradual variation of *Cardinium* frequencies across *H. pluchei* populations rather suggests either a neutral effect on or an adaptive response to geographical variation in natural selection.

*Cardinium* is transmitted through a very efficient maternal transmission in *H. pluchei* meaning that mtDNA variation can be used to infer the evolutionarily history of the infection. In a wide diversity of arthropods, maternally inherited symbionts are known to alter mtDNA genetic diversity through the linkage disequilibrium resulting from their common mode of inheritance

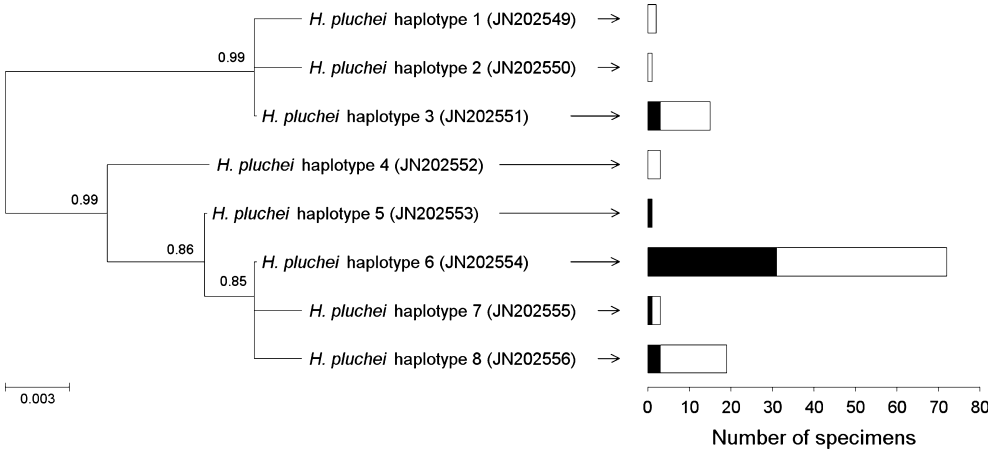


**Fig. 3** Phylogeny and distribution of *Cardinium* infections among *Holocnemus pluchei* mitochondrial DNA (mtDNA) haplotypes. The left part of the figure shows the phylogeny of the 8 *H. pluchei* mtDNA haplotypes via maximum likelihood. Numbers on branches indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values of 60% or more are shown). GenBank accession numbers are given in parenthesis. The scale bar is in units of substitutions/site. The right part of the figure gives the distribution of *Cardinium* infection observed with each mtDNA haplotype (black: infected specimens, $n = 77$; white: uninfected specimens, $n = 39$).

(see Hurst & Jiggins, 2005; for review). If the *Cardinium* invasion is recent in *H. pluchei* populations, only a single mtDNA haplotype should occur among infected individuals, and a variety of haplotypes should persist among uninfected individuals. Through cytoplasmic hitchhiking, this will increase the frequency of the *Cardinium*-associated mtDNA haplotype and thus decrease overall mtDNA diversity. Precisely, this pattern is found associated with some symbiont infections in arthropods (e.g. von der Schulenburg *et al.*, 2002; Jiggins, 2003; Gueguen *et al.*, 2010; Verne *et al.*, 2012), but the pattern found in *H. pluchei* stands in striking contrast to this scenario. This pattern revealed that this symbiotic association is evolutionarily ancient. First, there is no association between infection status and mtDNA haplotypes, indicating that incomplete maternal transmission can occur. Second, there are a substantial number of polymorphic sites in the mtDNA of *H. pluchei*, signifying that the infection is sufficiently old for many mutations to have occurred since the initial *Cardinium* invasion. This suggests that *Cardinium* is not expanding through *H. pluchei* populations but rather persists at intermediate frequencies, probably for a long evolutionary time. An alternative, but nonexclusive, hypothesis to account for the lack of disequilibrium between *Cardinium* and mtDNA is based on horizontal transmission among *H. pluchei* individuals. Horizontal transmission of *Cardinium* infection, which is thought to be rare (Weeks *et al.*, 2003; Zchori-Fein & Perlman, 2004), can explain the lack of disequilibrium: although horizontal transmission is probably not a primary factor driving symbiont spread, it could also explain the presence of *Cardinium* in diverse mtDNA lineages over a sufficient number of host generations.

The perfect maternal transmission of *Cardinium* implies that its transmission success should broadly depend on its effect on spider fitness. Surprisingly, *Cardinium* does not persist in *H. pluchei* populations using one of its known phenotypes. In insects and mites, *Cardinium* is well known to spread either through reproductive manipulation (Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001, 2004; Hunter *et al.*, 2003; Provencher *et al.*, 2005; Gotoh *et al.*, 2007; Ros & Breeuwer, 2009) or enhancing fecundity (Weeks & Stouthamer, 2004). In our case, we can be definite that *Cardinium* infection in *H. pluchei* does not fall into this evolutionary scheme. Although this *Cardinium* strain is genetically close to known reproductive manipulator strains, it does not either distort the SR or cause CI: males and females were found equally infected, and no variation in hatching rate was observed according to the infection status. *Cardinium* has also no detectable effect on fecundity and body size and is not required to support host development and reproduction. Although a more subtle effect can, however, exist, we thus found here no direct evidence for a *Cardinium* effect in *H. pluchei*. To our knowledge, very few similar cases have been reported in the literature (Gotoh *et al.*, 2007; White *et al.*, 2009). In the parasitic wasp *Encarsia inaron*, *Cardinium* was not able to induce CI, progeny SR distortion or clear fitness benefit (White *et al.*, 2009, 2010). It was therefore suggested that the maintenance of *Cardinium* within *E. inaron* could be directly attributable to co-infection with the CI-inducing *Wolbachia* also present in this host (White *et al.*, 2009). Perfect co-transmission of the two symbionts should confer the same CI transmission advantage to *Cardinium* as its *Wolbachia* partner. However, we rule out this possibility in the case of *H. pluchei*: we did not find infection by *Wolbachia* – or any other bacterial symbionts – which might induce a high frequency of *Cardinium*.

What then are the evolutionary forces governing *Cardinium* frequencies? A first possibility is that *Cardinium* has no effect, behaving as a neutral cytoplasmic element whose frequency is determined by stochastic processes. Although the relatively small sample sizes do not allow any definitive conclusion, it is thus clear that spatially close populations differ in their infection frequencies. The gradual variation of infection frequencies across the south of France is thus compatible with the isolation by distance (IBD) effect: the tendency for infected individuals to migrate between neighbouring populations, which results in decreasing *Cardinium* frequencies with increasing geographical distance. Under the hypothesis that the infection is not recent in *H. pluchei*, it is also likely that present-day *Cardinium* does not have the same effect as the *Cardinium* that initially invaded the spider populations. One possible process is the evolution of *Cardinium* towards weaker levels of manipulation: reproductive parasites may initially spread to reach high prevalence but then evolve towards an asymptomatic status, leading to the loss of infection (Hurst & McVean, 1996). This system is then expected to proceed to clearance of infection, at which point the population will have gone full circle, a process called 'reversible evolution'. Loss of manipulation implies a decrease in drive, which will be reflected in reduced prevalence, preceding infection loss. The progress towards the loss of infection is not expected to be rapid after the loss of reproductive manipulation: when the host population is dominated by a high frequency of a parasite without the capacity to modify host reproduction, parasite frequency can decline to extinction only if the infection is costly and/or imperfectly vertically transmitted. If not, a long-term coexistence of uninfected and infected individuals may then occur, and their frequencies would be driven by stochastic processes. This process thus fits well with the infection pattern observed within *H. pluchei*.

Alternatively, a second possibility is that *Cardinium* exhibits a context-dependent effect in *H. pluchei*. Facultative endosymbionts are generally believed to carry an intrinsic cost and, therefore, evolve compensatory adaptations to maintain their frequency (Oliver *et al.*, 2008; Jaenike *et al.*, 2010). Depending on environmental conditions, *Cardinium* could be a conditional mutualist, and its distribution would thus reflect an adaptive response to geographical variation in natural selection. Recent

literature provides striking examples with bacterial symbionts allowing environment-dependent effects, such as thermal tolerance (Russell & Moran, 2006) or resistance against natural enemies (Oliver *et al.*, 2003; Scarborough *et al.*, 2005; Hedges *et al.* 2008; Teixeira *et al.*, 2008; Jaenike *et al.*, 2010). In flies and aphids, experiments using population cages showed that, in host populations under an environmental stress, the symbiont frequency can rapidly increase, but in populations without this stress, the symbiont is not favoured and declines (Oliver *et al.*, 2008; Jaenike & Brekke, 2010). In this context, environmental stresses could directly influence *Cardinium* frequencies in *H. pluchei* populations. If true, the exact nature of this selective pressure remains to be characterized. Laboratory assay may, however, miss the critical selective agent (e.g. a combination of climatic variables), and it may be arduous to capture the factor maintaining infection. Another way to indirectly test this hypothesis would be to compare the *Cardinium* distribution with genetic differentiation at neutral *H. pluchei* markers: infection prevalence should reflect, at least partly, the effect of local selection, whereas the differentiation at *H. pluchei* loci will only be the result of neutral evolutionary processes, such as founder events or migrational patterns. The comparison of genetic differentiation levels at neutral *H. pluchei* loci with infection distribution could thus indicate if selection acts on *Cardinium*.

In conclusion, we would emphasize that symbiosis represents an important but insufficiently studied component of spider evolution. An exceptionally wide diversity of endosymbionts is present in spiders; what is unclear is what role do they play, calling into question the ecological processes that govern their distribution and abundance. Although reproductive manipulations have been reported for spider *Wolbachia* (Gunnarsson *et al.*, 2009; Vanthournout *et al.*, 2011), we showed here that the consequences of spider symbiosis are actually more diverse. The nature of the *Cardinium* effect remains now to be formally characterized to explain its wide distribution in spiders. Furthermore, the presence in the *Cardinium* genus of lineages with and without effects on host reproduction would be of great interest to study the evolutionary transitions that shape symbiotic associations.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Description of the *Holocnemus pluchei* samples: location, sample size and prevalence of *Cardinium*.

**Table S2** Genes and primer features.

**Figure S1** Numbers of eggs regressed against mother's tibia-patella length.

**Figure S2** Variation of *Cardinium* frequencies in the south of France and Spain.

**Figure S3** *Cardinium* phylogeny constructed via maximum-likelihood using (A) *16S rRNA* and (B) *gyrb* sequences.

**Figure S4** Distribution of mtDNA haplotypes among populations, partitioned by *Cardinium* infection status.

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