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ORIGINAL ARTICLE

Cardinium symbionts cause cytoplasmic incompatibility in spider mites

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Intracellular symbiotic bacteria belonging to the *Cytophaga–Flavobacterium–Bacteroides* lineage have recently been described and are widely distributed in arthropod species. The newly discovered bacteria, named *Cardinium* sp, cause the expression of various reproductive alterations in their arthropod hosts, including cytoplasmic incompatibility (CI), induction of parthenogenesis and feminization of genetic males. We detected 16S ribosomal DNA sequences similar to those of *Cardinium* from seven populations of five spider mite species, suggesting a broad distribution of infection of *Cardinium* in spider mites. To clarify the effect of *Cardinium* on the reproductive traits of the infected spider mites, infected mites were crossed with uninfected mites for each population. In one of the populations, *Eotetranychus suginamensis*, CI was induced when infected males were crossed with uninfected females. The other six populations of four species showed no reproductive abnormalities in the F_1 generation, but the possibility of CI effects in the F_2 generation remains to be tested. One species of spider mite, *Tetranychus puerariicola*, harbored both *Cardinium* and *Wolbachia*, but these symbionts seemed to have no effect on the reproduction of the host, even when the host was infected independently with each symbiont.
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Introduction

Bacterial symbionts of arthropods can cause reproductive abnormalities in their hosts. *Wolbachia*, which belong to α-Proteobacteria, are well-known sex ratio distorters. Their effects include cytoplasmic incompatibility (CI) in crosses between infected males and uninfected females; feminization, in which genetic males develop as females; parthenogenesis induction by diploidization of unfertilized eggs in haplodiploids; and male killing owing to the death of either male embryos or male larvae (O'Neill *et al*, 1997; Bourtzis and Miller, 2003). CI is the most common effect of *Wolbachia* infection and is considered to be related to a delay in chromosome condensation and alignment of the male pronucleus (Tram and Sullivan, 2002; Zabalou *et al*, 2004). An abnormality in paternal chromosome behavior results in embryonic death in diplodiploids (Hoffmann and Turelli, 1988), and in either increased male production or embryonic death in haplodiploids (Breeuwer and Werren, 1990; Gotoh *et al*, 2003). Conversely, some *Wolbachia* strains have no effect on host reproduction in both *Drosophila* spp (Hoffmann *et al*, 1994, 1996; Giordano *et al*, 1995) and spider mites (Gomi *et al*, 1997; Gotoh *et al*, 2003). In *Drosophila*, these *Wolbachia* strains are referred to as the *mod*$^-$ *resc*$^+$ strains (Bourtzis *et al*, 1998; Merçot and Poinsot, 1998; Zabalou *et al*, 2004), and they are considered to be prevalent in arthropods. The *mod*$^-$ *resc*$^+$ strains do not modify the sperm but instead rescue the detrimental modification caused by a *mod*$^+$ *resc*$^+$ strain.

Other symbiotic bacteria belonging to the *Cytophaga–Flavobacterium–Bacteroides* (CFB) lineage are found in many arthropods. Flavobacteria are found in termites and cockroaches (Bandi *et al*, 1994, 1995), and those infecting *Adonia variegata* and *Coleomegilla maculata* (Coleoptera) cause male killing in the host offspring (Hurst *et al*, 1997, 1999). CFB bacteria have been found in false spider mites – *Brevipalpus* spp (Weeks *et al*, 2001) – and parasitoid wasps – *Encarsia* spp (Zchori-Fein *et al*, 2001). These CFB bacteria are phylogenetically distinct from CFB bacteria known at present, and their closest relative is a bacterium isolated from the tick *Ixodes scapularis* (Kurtti *et al*, 1996). They are called *Cytophaga*-like organisms (CLOs) and a new genus name, *Cardinium*, has been proposed (Zchori-Fein *et al*, 2004). *Cardinium* spp cause parthenogenesis (Zchori-Fein *et al*, 2001) and CI (Hunter *et al*, 2003) in parasitoid wasps, *Encarsia* spp and feminization in false spider mites, *Brevipalpus* spp (Weeks *et al*, 2001).

Infections of both *Cardinium* and *Wolbachia* in a single host are known in four mite species and three *Aphitis* species (Hymenoptera; Weeks *et al*, 2003) and one hymenopteran species and one mite species (Zchori-Fein and Perlman, 2004). As both *Cardinium* and *Wolbachia* cause reproductive abnormalities in their host arthropods, it is of interest to know whether they work independently, cooperatively or interferingly. To understand the interaction between the endosymbionts and their host arthropods, we created mite populations that were infected with *Cardinium*, *Wolbachia* and both

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Cardinium and *Wolbachia* for crossing experiments between infected and uninfected individuals.

In some spider mites, *Wolbachia* have been reported to induce CI (Breeuwer, 1997; Gotoh *et al*, 2003) or parthenogenesis (Weeks and Breeuwer, 2001), whereas in other spider mites, no reproductive effects were reported (Gomi *et al*, 1997; Gotoh *et al*, 2003). The aim of this study was to clarify whether the *Cardinium* sp in the spider mites affects reproduction. We determined the phylogenetic relationships of the *Cardinium* strains found in seven populations of five spider mite species, tested the effect of *Cardinium* on the reproductive traits of infected spider mites and evaluated the effects of *Cardinium* infection, *Wolbachia* infection and infection with both.

Materials and methods

Spider mites

Seven populations representing five species of spider mite were used in this study (Table 1). The species were *Eotetranychus suginamensis*, *Oligonychus ilicis*, *Amphitetranychus quercivorus*, *Tetranychus urticae* (three populations) and *Tetranychus pueraricola*. A PCR survey of 94 populations comprising 27 spider mite species showed that some of these populations were infected with *Cardinium* (H Noda *et al*, unpublished data). Mites were reared on leaf discs of either of the original host plants or of kidney bean – for *T. urticae* and *T. pueraricola* – in a climate-controlled room (25°C, L:D = 16:8, RH 60%). The Taiwanese population of *E. suginamensis* was imported to Japan with the authorization of the Ministry of Agriculture, Forestry and Fisheries of Japan (no. 14-Y-583) on 25 October 2002.

Polymerase chain reaction

The DNA template was prepared by homogenizing a single female adult in a 25 µl mixture of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 2 µl proteinase K (10 mg/ml). The mixture was incubated at 37°C for 30 min, and proteinase K was inactivated at 95°C for 5 min. The samples were centrifuged briefly in a microfuge tube and used immediately for the PCR reaction, or stored at −30°C for later use. All PCR reactions were performed in 20 µl of buffer: 14 µl H_2O, 2 µl 10 × buffer, 1 µl dNTP (2.5 mM each), 5 U/µl *Taq* polymerase (TAKARA r*Taq*, Tokyo), 1 µl sample and 1 µl of primers (10 pmol each). The primers used for detection of *Cardinium* were CLO-f1 (5′-GGA ACC TTA CCT GGG CTA GAA TGT ATT) and CLO-r1 (5′-GCC ACT GTC TTC AAG CTC TAC CAA C), which amplified 468 bp of 16S ribosomal DNA (rDNA). The primers used for obtaining 16S rDNA for sequencing were fD1 (5′-AGA GTT TGA TCC TGG CTC AG) and rP2 (5′-ACG GCT ACC TTG TTA CGA CTT) (Weisburg *et al*, 1991) and CLO-specific primers (CLO-f2, 5′-GGT GCG TGG GCG GCT TAT T; CLO-r2, 5′-AAA GGG TTT CGC TCG TTA TAG). The 16S rDNA of *A. quercivorus* was amplified using fD1 and rP2. Two parts of 16S rDNA were separately amplified using primer combinations of fD1/CLO-r2 and CLO-f2/rP2 in other spider mite species. These *Cardinium*-specific primers were designed on the basis of the 16S rDNA sequences of *Cardinium* from *I. scapularis* (Kurtti *et al*, 1996), *Brevipalpus phenicis* (Weeks *et al*, 2001), *Encarsia* spp (Zchori-Fein *et al*, 2001) and *A. quercivorus* (this study). Reactions were cycled 35 times for 30 s at 95°C, 30 s at 54°C and 90 s at 72°C. The PCR products were electrophoresed in a 1.0% agarose gel in TAE.

Wolbachia infection was examined using *Wolbachia*-specific 16S rDNA (99F-992R; O'Neill *et al*, 1992), *fts*Z (*fts*Z f1-r1; Holden *et al*, 1993) and *wsp* (*wsp*f-*wsp*r; Zhou *et al*, 1998).

Sequencing and phylogenetic analysis

The phylogenetic relationship of *Cardinium* strains in spider mites was based on the nucleotide sequences of the 16S rDNA genes, which were amplified by primers fD1/rP2, fD1/CLO-r2 and CLO-f2/rP2. The PCR product was cloned into a pGEM-T Vector (Promega). The template DNA was amplified by PCR using M13-20 and reverse primers. The sequence was determined by the dye terminator sequencing method by using a DNA sequencer (models 377 and 3700, PE Applied Biosystems).

The 16S rDNA sequences of 32 *Cardinium* strains were aligned by using the CLUSTAL X program (Thompson *et al*, 1997). The aligned data set consisted of 1108 residues. A neighbor-joining analysis was performed with CLUSTAL X, and a bootstrap analysis was performed with 1000 replications.

Antibiotic treatment

Small discs (ca 1 cm^2) of the original host or kidney bean leaves were placed on a cotton bed soaked in either tetracycline hydrochloride (0.1%, w/v; TC, Wako)

Table 1 Mite populations collected for this study

Species	*Location*	*Col. date*	*Host plant*
Eotetranychus suginamensis	Mia-oli, Taiwan 24°23′N–120°43′E	October 2002	Mulberry *Morus bombycis*
Oligonychus ilicis	Chiran, Kagoshima 31°22′N–130°27′E	October 2000	Tea *Thea sinensis*
Amphitetranychus quercivorus	Sapporo, Hokkaido 43°09′N–141°18′E	May 2002	Deciduous oak *Quercus mongolica*
Tetranychus urticae (red form) A	Nanae, Hokkaido 41°53′N–140°41′E	September 2002	Carnation *Dianthus* sp
Tetranychus urticae (red form) B	Matsukawa, Nagano 36°25′N–137°51′E	August 2001	Carnation
Tetranychus urticae (red form) C	Iida, Nagano 35°29′N–137°49′E	May 2001	Carnation
Tetranychus pueraricola	Hitachiohta, Ibaraki 35°01′N–140°12′E	August 2001	Kudzu vine *Pueraria lobata*

(to eliminate both *Cardinium* and *Wolbachia*) or in penicillin G (0.1%, w/v; PCG, Sigma) (to eliminate *Cardinium* only; Morimoto *et al*, 2006). The procedure was carried out in plastic dishes (9 cm in diameter), 1 day before the start of rearing. Newly hatched larvae were placed on the leaf discs, and distilled water was added daily to keep the cotton bed wet. The cotton and the leaf discs were replaced every 4 days, and the mites were reared in the antibiotic environment for one generation (Gotoh *et al*, 1995). Mites were checked for infection with *Cardinium* and/or *Wolbachia* by PCR after three generations, by using the specific primers mentioned above. Offspring from adults that were found to be *Cardinium*-free and/or *Wolbachia*-free were used in the crossing experiments.

Heat treatment

Wolbachia was eliminated by heat treatment (van Opijnen and Breeuwer, 1999). To determine the most effective heat treatment, *T. pueraricola* eggs laid during a 24 h period were kept at two different temperatures (35 and 40°C) for two different times (3 and 7 days) and then transferred to 25°C. Emerging adults were checked for infection with *Cardinium* and *Wolbachia* by using PCR. Offspring from adults that were found to be *Cardinium*-infected and *Wolbachia*-free were allowed to mate.

Crossing experiments

To determine the effects of *Cardinium* infection, we carried out crossing experiments between infected and tetracycline-treated cured mites in all *Cardinium*-infected species. A crossing pair was denoted as the female/male strain and antibiotic-cured colonies were designated as 'Es^-'. For example, 'Es^+/Es^-' denotes a cross between a *Cardinium*-infected female and an antibiotic-cured male in *E. suginamensis*. Single females in the teleiochrysalis stage (the final immature stage) obtained from each stock culture were transferred onto a small leaf disc (*ca* 4 cm^2) of the appropriate plant together with an adult male (1- to 5-day-old) from either the same or a different culture. Males were removed 2 days after emergence of the adult females. Each female was allowed to lay eggs for 5 days after oviposition started and was then removed. This ensured that only young mites were used. Eggs on leaf discs were checked daily to determine eclosion rates from eggs, survival rates at the immature stages and sex ratio (percentage of females). PCR was carried out for all females and males used in the crossing experiments to confirm that the expected combinations were achieved in the pairs. Data from the pairs that did not achieve the expected combination were discarded. All experiments were carried out at 25°C and L:D = 16:8.

Results

Sequence and phylogenetic relationships

Nucleotide sequences of the 16S rDNA genes amplified from the seven populations showed little variation, with 97.7–100% similarity. The phylogenetic tree based on the 16S rDNA genes clearly indicated that the microorganisms in the spider mites were *Cardinium*, forming a monophyletic group with other *Cardinium*. The *Cardinium* from the spider mite populations seemed to be closely related to the symbionts from other mite and tick species in the phylogenetic tree (Figure 1). Three populations of *T. urticae* had identical sequences, whereas the other four populations had unique sequences, indicating that they harbored different strains of *Cardinium*.

The effects of *Cardinium* infection on spider mites

The only population to show any reproductive abnormality in the crossing experiments was the *E. suginamensis* population, which showed CI.

Eotetranychus suginamensis: The population of *E. suginamensis* showed unidirectional CI when penicillin-cured females were crossed with infected males (Es^-/Es^+) (Table 2). This cross was carried out twice with similar results. The hatchability of eggs and the number of F_1 females in the Es^-/Es^+ cross were significantly lower than those of the other crosses (Es^+/Es^+, Es^+/Es^- and Es^-/Es^-). However, the overall lower number of eggs hatching and the overall lower number of F_1 females produced per pair are underestimated owing to extensive variation between pairs. The hatchability (Figure 2) and proportion of F_1 females (Figure 3) varied greatly in the Es^-/Es^+ cross, but not so much in the other three crosses. Five pairs of the Es^-/Es^+ cross produced no F_2 females, indicating a strong CI effect by *Cardinium*. Conversely, Es^+/Es^+, Es^+/Es^- and Es^-/Es^- never showed much lower values in hatchability and proportion of females.

Oligonychus ilicis: The population of *O. ilicis* showed no sex distortion (data not shown). No reduction in egg

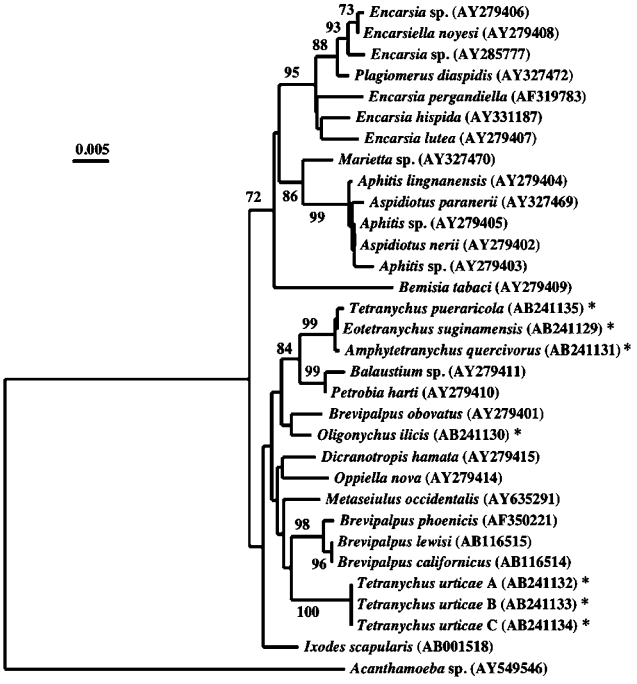

Figure 1 Phylogenetic tree based on 16S rDNA sequences of *Cardinium*, constructed by using a neighbor-joining procedure in CLUSTAL X. Each *Cardinium* is shown by its host name. Numbers on the nodes indicate bootstrap values (%). *T. urticae* A, *T. urticae* B and *T. urticae* C are populations collected at Nanae (Hokkaido), Matsukawa (Nagano) and Iida (Nagano), respectively. Asterisks designate symbiont sequences obtained in this study.

Table 2 Compatibility of crosses between *Cardinium*-infected (Es^+) and antibiotic-treated (Es^-) colonies of the Taiwanese population of *Eotetranychus suginamensis*

Cross	N[a]	*No. of eggs/female*	*Hatchability (%)*	*Survival rate in immature stages (%)*	*No. of F_1 females*	*No. of F_1 males*
Female×Male						
$Es^+ \times Es^+$	29	20.9±0.62 ab	96.5±0.90 b	98.1±0.66 b	13.1±0.47 b	6.7±0.38 abc
$Es^+ \times Es^-$	23	20.3±0.73 ab	96.4±0.81 b	98.6±0.53 b	13.1±0.59 b	6.1±0.33 ab
$Es^- \times Es^+$(1)[b]	18	22.9±1.03 b	70.9±5.06 a	91.4±1.96 a	7.2±1.55 a	8.3±0.62 c
$Es^- \times Es^+$(2)	16	19.0±1.13 a	76.1±5.51 a	98.6±0.78 b	6.4±1.06 a	7.6±0.50 bc
$Es^- \times Es^-$	21	18.6±0.46 a	96.3±1.03 b	95.8±1.06 ab	11.6±0.52 b	5.6±0.30 a
$F_{4,102}$ [c]		3.748**	18.255***	5.533***	13.623***	4.646**

[a]Number of pairs tested.
[b]Numerals in parentheses indicate the number of trials.
[c]Means (±SE) differ significantly at $P<0.01$ (**) and $P<0.001$ (***) (ANOVA). Values in a column followed by different letters are significantly different at $P<0.05$ (Tukey HSD test). The number of eggs per female and the number of F_1 offspring were ln-transformed and hatchability and survival rate were arcsine-root transformed before ANOVA.

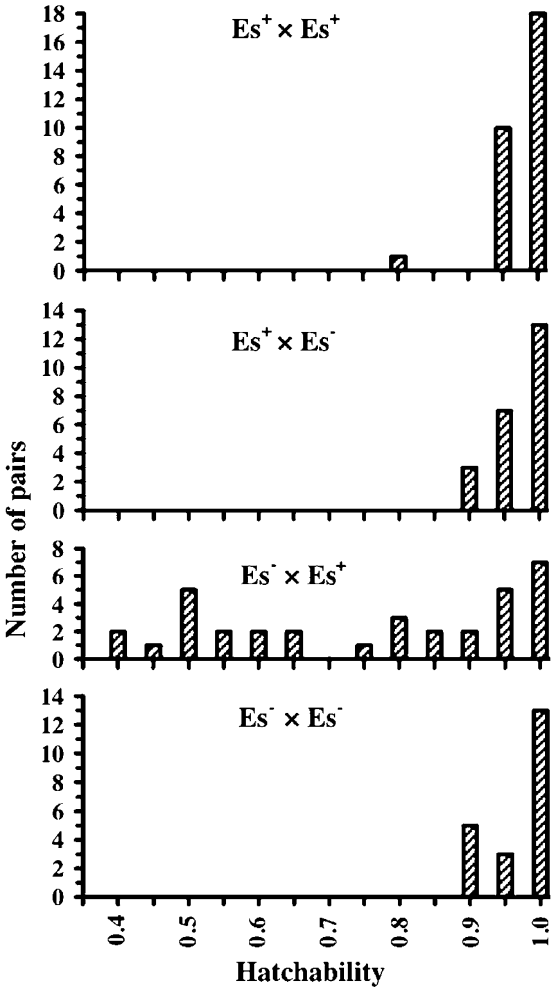

Figure 2 Egg hatchability of the F_1 offspring produced by crosses between *Cardinium*-eliminated (Es^-) and *Cardinium*-infected (Es^+) individuals of *E. suginamensis*. See also Table 2.

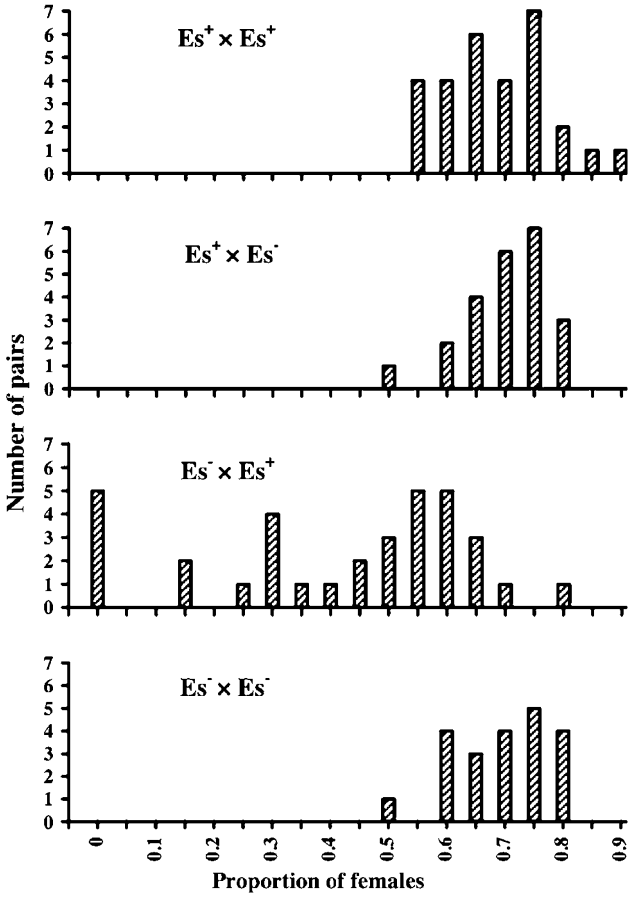

Figure 3 Sex ratio (proportion of females) in F_1 offspring produced by the crosses between *Cardinium*-eliminated (Es^-) and *Cardinium*-infected (Es^+) individuals of *E. suginamensis*. See also Table 2.

hatchability (91.8±2.33 to 96.5±1.62, mean±s.e.m.) was observed among the four combinations ($F_{3,52}=0.938$, $P>0.05$, ANOVA). The Oi^-/Oi^+ cross resulted in a slightly reduced proportion of females (66.2±2.15; $F_{3,52}=6.403$, $P<0.001$, ANOVA) among the four combinations, but it was not significantly different from that in the Oi^-/Oi^- cross (68.3±1.40; $P>0.05$, Tukey HSD test), indicating that *Cardinium* apparently did not affect host reproduction.

Amphitetranychus quercivorus: A *Cardinium*-free colony was established by treatment with penicillin, and four combinations of crosses were observed between

Cardinium-infected and *Cardinium*-free colonies. No differences ($P>0.05$, ANOVA) were observed in hatchability ($F_{3,52}=2.427$; 96.8 ± 1.07 to 99.4 ± 0.61) and survival rate at immature stages ($F_{3,52}=1.161$; 89.0 ± 1.61 to 93.8 ± 1.55) among the four crosses (data not shown). The sex ratios between the Aq^+/Aq^- cross (80.7 ± 1.94) and the Aq^-/Aq^+ cross (71.7 ± 2.50) were significantly different ($F_{3,52}=3.159$, $P<0.05$), but values in these two crosses did not differ from those in the Aq^+/Aq^+ (78.2 ± 1.88) and Aq^-/Aq^- (74.2 ± 2.43) crosses ($P>0.05$, Tukey HSD test). These results suggest that *Cardinium* in this population did not have the perceptible ability to manipulate the host sex ratio.

Tetranychus urticae (red form): Members from the original three infected populations were crossed with antibiotic-cured colonies (data not given). Four combinations of crosses in the A (Nanae) and B (Matsukawa) populations showed no significant differences ($P>0.05$) in egg hatchability (98.9 ± 0.64 to 100 ± 0.00, $F_{3,45}=1.545$, for A; and 99.3 ± 0.45 to 99.7 ± 0.35, $F_{3,44}=0.192$, for B), survival rate at the immature stages (96.7 ± 1.02 to 99.8 ± 0.24, $F_{3,45}=2.430$, for A; and 99.1 ± 0.59 to 99.7 ± 0.31, $F_{3,44}=0.384$, for B) and sex ratio (72.3 ± 2.03 to 75.9 ± 2.26, $F_{3,45}=0.153$, for A; and 76.2 ± 1.46 to 82.1 ± 1.66, $F_{3,44}=2.524$, for B). In the C (Iida) population, hatchability ($F_{3,48}=4.674$, $P<0.001$; 96.9 ± 0.96 to 100 ± 0.00) was significantly different among the four combinations, because all eggs in the C^+/C^- cross hatched. However, no male-biased sex ratio was observed among the four combinations ($F_{3,48}=1.104$, $P>0.05$). Thus, we observed no reproductive abnormalities in the three populations of *T. urticae*.

Tetranychus puerariicola: The population of *T. puerariicola* harbored infection with both *Cardinium* and *Wolbachia*. Bacteria-free colonies, in which both bacteria species were eliminated from the host, were established by tetracycline treatment. Four combinations of crosses were observed between members from the original population and those from bacteria-free colonies. Egg hatchability and survival rate at immature stages were not significantly different ($P>0.05$, Tukey HSD test) among the four combinations. The sex ratio was different between the Tp^+/Tp^- and Tp^-/Tp^- crosses, but the values were similar ($P>0.05$) to those of the Tp^+/Tp^+ and Tp^-/Tp^+ crosses (first group in Table 3). These results suggest that no reproductive abnormalities occurred when *T. puerariicola* was infected with the two bacteria simultaneously.

Culturing the *T. puerariicola* strain at 35°C for 3 or 7 days completely eliminated *Wolbachia*, but not *Cardinium* (Figure 4). *Cardinium* populations tolerated heat up to 40°C for 7 days, with several exceptions. *Cardinium* infection rates tested by PCR after heat treatment in females were 27/30 (90%) at 35°C for 7 days, 28/30 (93.3%) at 40°C for 3 days and 24/30 (80%) at 40°C for 7 days. We used a colony treated at 35°C for the 7 days in the experiments. In crosses between mites of *Cardinium*-infected and *Cardinium*-free colonies, no significant differences ($P>0.05$, ANOVA) were found in egg hatchability, survival rate at immature stages and sex ratio of the F_1 generation (second group in Table 3), indicating that *Cardinium* did not independently cause any sex distortion.

Wolbachia-infected and *Cardinium*-free colonies were established by penicillin treatment, and four combinations of crosses were observed between *Wolbachia*-infected and *Wolbachia*-free colonies. No significant

Table 3 Compatibility of crosses between *Cardinium–Wolbachia* double-infected (Tp^{CW+}) and antibiotic-treated (Tp^{CW-}) colonies, crosses between *Cardinium*-infected (Tp^{C+}) and antibiotic-treated (Tp^{C-}) colonies and crosses between *Wolbachia*-infected (Tp^{W+}) and antibiotic-treated (Tp^{W-}) colonies in the Hitachiohta population of *Tetranychus puerariicola*

Cross	N[a]	*No. of eggs/female*	*Hatchability (%)*	*Survival rate in immature stages (%)*	*% Female offspring*
Female × Male					
Cardinium-Wolbachia (double-infected)					
$Tp^{CW+}\times Tp^{CW+}$	14	38.2 ± 1.90	97.4 ± 0.71 a	96.6 ± 0.68	78.7 ± 0.81 ab
$Tp^{CW+}\times Tp^{CW-}$	21	41.8 ± 1.31	96.1 ± 0.97 a	93.9 ± 1.03	78.0 ± 0.52 a
$Tp^{CW-}\times Tp^{CW+}$	31	41.0 ± 1.13	94.3 ± 0.85 a	95.8 ± 0.80	78.9 ± 0.83 ab
$Tp^{CW-}\times Tp^{CW-}$	27	41.0 ± 1.00	97.3 ± 0.83 a	95.0 ± 0.91	81.0 ± 0.61 b
$F_{3,89}$ [b]		1.131 NS	2.960*	0.726 NS	3.221*
Cardinium-infected (Wolbachia were eliminated by heat treatment)					
$Tp^{C+}\times Tp^{C+}$	21	42.8 ± 1.48	93.4 ± 1.01	96.2 ± 1.01	84.2 ± 0.79
$Tp^{C+}\times Tp^{C-}$	22	44.6 ± 0.82	94.9 ± 0.97	95.6 ± 0.97	84.7 ± 0.45
$Tp^{C-}\times Tp^{C+}$	23	43.2 ± 1.11	96.8 ± 0.89	96.2 ± 0.99	84.1 ± 0.65
$Tp^{C-}\times Tp^{C-}$	22	43.7 ± 1.19	96.1 ± 1.04	96.9 ± 0.99	84.8 ± 0.63
$F_{3,84}$		0.551 NS	2.384 NS	0.578 NS	0.328 NS
Wolbachia-infected (Cardinium were eliminated by penicillin treatment)					
$Tp^{W+}\times Tp^{W+}$	25	41.9 ± 1.30	95.2 ± 0.98	94.2 ± 1.33	82.6 ± 0.86
$Tp^{W+}\times Tp^{W-}$	24	42.1 ± 1.05	94.2 ± 1.06	96.1 ± 0.90	82.1 ± 0.84
$Tp^{W-}\times Tp^{W+}$	20	45.2 ± 0.67	95.6 ± 0.96	95.6 ± 1.39	84.0 ± 0.70
$Tp^{W-}\times Tp^{W-}$	23	45.0 ± 0.81	92.4 ± 1.13	96.2 ± 1.08	81.4 ± 0.94
$F_{3,88}$		2.148 NS	1.964 NS	0.457 NS	1.469 NS

[a]Number of pairs tested.
[b]Means (±SE) differ significantly at $P<0.05$ (*) (ANOVA); NS, not significant at the 5% level. Values in a column followed by the same letters are not significantly different at the 5% level (Tukey HSD test).

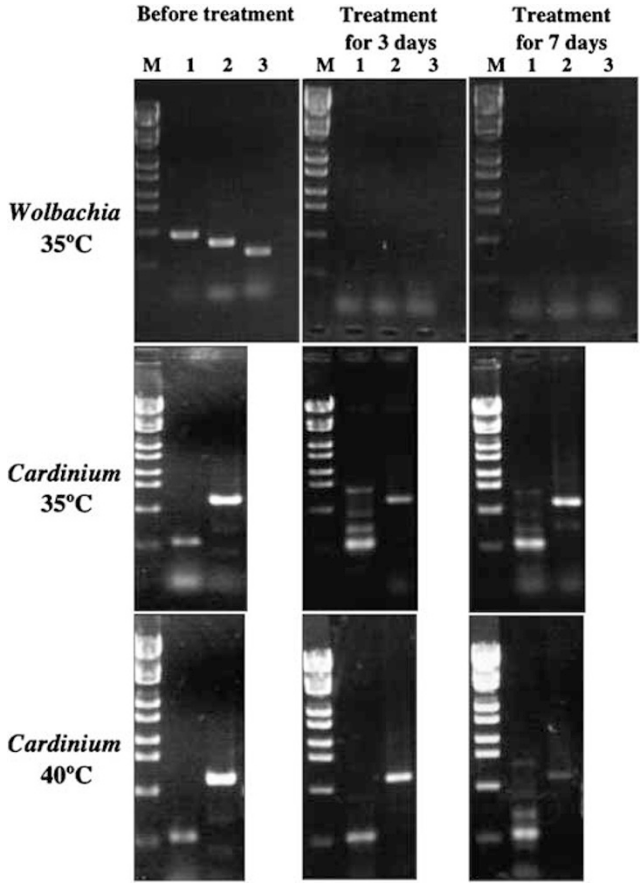

Figure 4 PCR assay showing the effects of 3- and 7-day heat treatment on *Wolbachia* and *Cardinium* infection in *T. pueraricola*. *Wolbachia* was sensitive and *Cardinium* was resistant to heat. Lanes for *Wolbachia*: M, marker (*λ-Eco*T14I digest); 1, 16S rDNA; 2, *fts*Z; 3, *wsp*. Lanes for *Cardinium*: M, marker; 1, 16S rDNA amplified by CLO-f1/CLO-r1; 2, 16S rDNA amplified by fD1/CLO-r2.

differences ($P > 0.05$, ANOVA) were found in egg hatchability, survival rate at immature stages or sex ratio among the four crosses (third group in Table 3), showing that *Wolbachia* by itself did not affect the sex ratio of *T. pueraricola*.

Discussion

Phylogenetic analysis of the 16S rDNA sequences clearly revealed that the bacteria in the seven populations of spider mites belonged to *Cardinium* and that similar *Cardinium* strains were harbored by all known acarian species. The 16S rDNA sequence of *Cardinium* infecting *E. suginamensis* had 98.1% similarity to the symbionts in the tick *I. scapularis* (Kurtti *et al*, 1996), in which *Cardinium* was first described. The sequence also had 96.2% similarity to the symbiont in the parasitic wasp *Encarsia pergandiella* (Hunter *et al*, 2003), in which CI was first observed.

Cardinium induced CI in one out of the seven populations of spider mites examined in this study. This is the second instance of a bacterial symbiont other than *Wolbachia* that can induce CI. The other known instance is *Cardinium* in the parasitoid wasp *E. pergandiella* (Hunter *et al*, 2003). *Cardinium*, as well as *Wolbachia* in a number of mite species (Breeuwer, 1997; Gotoh *et al*, 2003, 2005), did not have a pronounced effect on crosses in *E. suginamensis*. However, five pairs of *E. suginamensis* produced no F_1 females, showing a severe CI effect. Females in the incompatible crosses produced female offspring (daughters) but the number of daughters produced was 25–29% less than the number of daughters produced by females in the compatible crosses. The reduced number of daughters was mainly the result of the death of female eggs; that is, the hatchability of eggs produced by females in these crosses was 20–26% less than the hatchability of eggs produced by females in the compatible crosses. The egg hatchability observed in the incompatible crosses was slightly higher in *Cardinium*-infected *E. suginamensis* (71–76%) than in spider mites infected with *Wolbachia* (56–99%) (Gotoh *et al*, 2003, 2005). However, a reduction of hatchability was not always observed in the incompatible crosses caused by *Wolbachia* infections, even in the same combination. In some crosses, a reduction of hatchability occurred in the first trial, but not in the second trial (Gotoh *et al*, 2005). Therefore, it is unclear whether *Cardinium* infection always results in reduced hatchability, because this study dealt with only one example. In the present study, we used 1- to 5-day-old males for crossing experiments. We may need to examine further the effects of male age on incompatibility level by comparing the 1- and 5-day-old males, because older males usually express less CI than younger ones (Hoffmann *et al*, 1986). In the six populations of the four species, no reproductive abnormalities, such as a female ratio less than 50%, were observed in the F_1 generation in our experimental conditions. Vala *et al* (2000) reported that *Wolbachia* infection causes a more severe hybrid breakdown phenotype in the F_2 generation of a cross between uninfected females and infected males than in the F_1 generation. The possible effects of *Cardinium* in the F_2 generation are untested and could not be ruled out in our study.

In this study, the Sapporo population of *A. quercivorus* was infected with the non-CI strains of *Cardinium*. Our previous study shows that the Sapporo (43° N) females were incompatible with the Tsukuba (36° N) males, which resulted in low egg hatchability and a male-biased sex ratio, whereas the reciprocal crosses were compatible and produced normal progeny with a female-biased sex ratio (Gotoh *et al*, 1995; *A. quercivorus* was referred to as *Tetranychus quercivorus*). In that study, 12 types of antibiotic, including tetracycline hydrochloride, were used to treat the Sapporo females, but they were not effective in restoring the compatibility of the two populations (Gotoh *et al*, 1995). *Wolbachia* also did not infect the Sapporo and Tsukuba populations (Gotoh *et al*, 2003). We first found *Cardinium* in *A. quercivorus* and no other symbiotic bacteria were detected. These results clearly show that unidirectional reproductive incompatibility between the Sapporo and Tsukuba populations of *A. quercivorus* is not due to intracellular bacteria such as *Cardinium* and *Wolbachia*.

An interesting aspect in the present study is that the *Cardinium* strain responsible for CI in *E. suginamensis* is closely related to the non-CI strains found in *T. pueraricola* and *A. quercivorus*, based on the 16S rDNA tree. A similar phenomenon was found in *Wolbachia* strains infecting spider mites. On the basis of *wsp* (*Wolbachia* surface protein) gene sequences, *Wolbachia* in

Panonychus mori Yokoyama had an identical sequence to those in *T. urticae* (green form), but CI occurred only in the former (Gotoh *et al*, 2003, 2005). Phylogenetic similarity of symbionts is not related to their phenotypes due to one, or a combination of, the following three factors. First, symbiont genotype is different, and a gene involved in reproductive alteration might have a mutation in its sequence. Second, the host species have different genetic backgrounds. Third, symbiont density in the host animals is different, which seems to be caused by host–symbiont interaction and results in different phenotypes. The second and third factors seem to be under the control of some other factors. Future studies in which a CI-causing *Cardinium* strain is transferred to a *Cardinium*-uninfected host or to a host harboring non-CI *Cardinium* might further clarify this interesting biological difference.

Infections of both *Cardinium* and *Wolbachia* in the same host are known in four *Aphytis* species (Hymenoptera) and four mite species (Acari) (Weeks *et al*, 2003; Zchori-Fein and Perlman, 2004), although it is unknown whether the symbionts infecting these arthropod species induce reproductive abnormalities. Infections of both *Cardinium* and *Wolbachia* have not so far been found in spider mites but were found in *T. pueraricola*. *Cardinium* were sensitive to tetracycline, rifampicin, penicillin G, ampicillin and chloramphenicol antibiotics (Morimoto *et al*, 2006). Tetracycline and rifampicin were effective against *Wolbachia,* but penicillin G was less effective (Fenollar *et al*, 2003). Therefore, we used penicillin G for elimination of *Cardinium* alone and tetracycline for elimination of both *Cardinium* and *Wolbachia*. Heat treatment is sometimes used for elimination of symbionts from host arthropods. In this study, it successfully eliminated only *Wolbachia* from *T. pueraricola*, creating a population infected with only *Cardinium* (Figure 4). No abnormalities were observed in the double-infected population or in populations infected with either one of the symbionts. However, selective elimination of symbionts will help in the analysis of reproductive abnormalities in double-infected populations or species.

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