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Strong cytoplasmic incompatibility and high vertical transmission rate can explain the high frequencies of *Wolbachia* infection in Japanese populations of *Colias erate poliographus* (Lepidoptera: Pieridae)

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Abstract

Wolbachia, belonging to *Alphaproteobacteria*, is ubiquitously found in arthropods and filarial nematodes, and is known to manipulate the reproduction of its hosts in various ways, such as feminization, male killing, induction of parthenogenesis or induction of cytoplasmic incompatibility. We found that the *Wolbachia* infection frequencies of the butterfly *Colias erate poliographus* were high (85.7–100%) in seven Japanese populations. Crossing experiments and rearing revealed that the *Wolbachia* strain exhibited strong cytoplasmic incompatibility and perfect vertical transmission in *C. erate poliographus*. Moreover, a comparison of the survival rates between infected and cured broods suggested that *Wolbachia* infection had beneficial effects on host fitness. Our findings suggested that the high infection frequencies in Japanese populations have been accomplished by these advantageous traits of the *Wolbachia* strain. Furthermore, the multilocus sequence typing (MLST) scheme revealed that the *Wolbachia* in *C. erate poliographus* is a novel strain (ST141), belonging to supergroup B.

Keywords: *Wolbachia*, cytoplasmic incompatibility, *Colias erate poliographus*, perfect vertical transmission, MLST

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Introduction

The reproductive systems of arthropod hosts are often manipulated by endosymbiotic bacteria, such as *Spiroplasma*, *Rickettsia*, *Wolbachia*, *Arsenophonus* and *Cardinium* (O'Neill *et al*., 1997; Bourtzis & Miller, 2003, 2006). Among these, *Wolbachia* are particularly focused upon due to their high prevalence in arthropod hosts and the various types of reproductive aberrations they induce (Werren *et al*., 1995; Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000). *Wolbachia* strains can extensively manipulate host reproduction by inducing parthenogenesis, feminizing genetic males, killing male embryos or causing cytoplasmic incompatibility between gametes (O'Neill *et al*., 1997; Hiroki *et al*., 2002, 2004; Bourtzis & Miller, 2003; Narita *et al*., 2007a). The most

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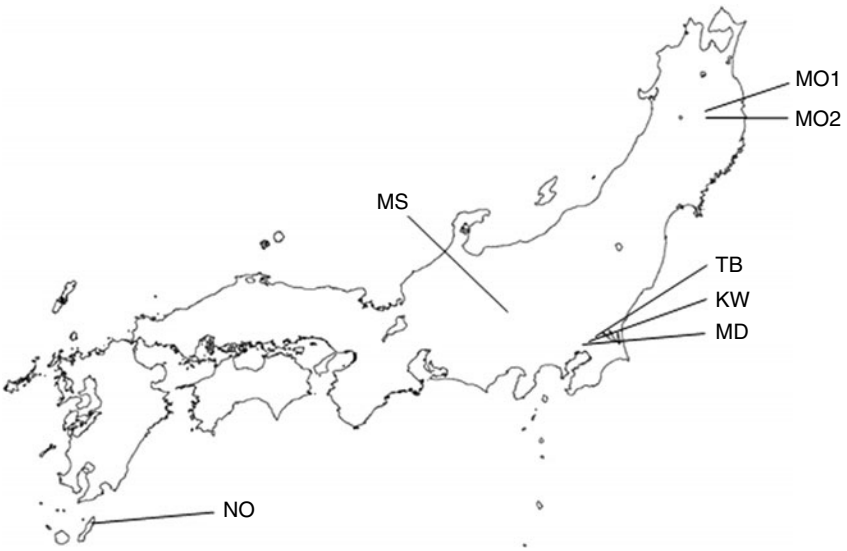

Fig. 1. Collection localities of *C. erate poliographus* in Japan. MO, Morioka; MS, Minamisaku; TB, Tsukuba; KW, Kashiwa; MD, Matsudo; NO, Nishinoomote.

common type of *Wolbachia*-induced reproductive manipulation is cytoplasmic incompatibility. Cytoplasmic incompatibility results in embryonic mortality for mating between insects of the same species with differing *Wolbachia* infection statuses (Bourtzis *et al*., 1998; Bourtzis & Miller, 2003) and can be either unidirectional or bidirectional. Unidirectional cytoplasmic incompatibility is typically expressed when an infected male mates with an uninfected female. The reciprocal mating is fully compatible, as are matings between infected individuals. Bidirectional cytoplasmic incompatibility usually occurs in matings between infected individuals harboring different strains of *Wolbachia* (Bourtzis & Miller, 2003).

The pale clouded yellow butterfly *Colias erate poliographus* Motschulsky (Lepidoptera: Pieridae) is distributed in Far East Russia, Sakhalin, the Korean Peninsula, China and Japan. A previous survey of *Wolbachia* infection among lepidopteran insects in Japanese populations revealed that ten out of 11 *C. erate poliographus* individuals examined were infected with *Wolbachia* (Tagami & Miura, 2004). However, the biological effects of *Wolbachia* infection on *C. erate poliographus*, such as reproductive manipulations or fitness effects, remained to be examined.

In the present study, we examined (i) the infection frequencies of other Japanese populations of *C. erate poliographus*, (ii) the type of *Wolbachia*-inducing reproductive manipulation in *C. erate poliographus*, (iii) the vertical transmission efficiency of the *Wolbachia* strain in its host *C. erate poliographus* and (iv) the fitness effects of *Wolbachia* infection on the survival rate and development period of *C. erate poliographus*. Furthermore, a multilocus sequencing typing (MLST) analysis (Baldo *et al*., 2006) and phylogenetic analysis were employed to characterize the *Wolbachia* strain.

Materials and methods

Field sampling

In 2007, adult individuals of *C. erate poliographus* were collected at seven geographic locations in Japan, namely Morioka (MO1 and MO2), Minamisaku (MS), Tsukuba (TB), Kashiwa (KW), Matsudo (MD) and Nishinoomote (NO) (fig. 1, table 1).

Diagnostic PCR

Leg tissues from each adult butterfly were crushed using plastic pestles in 0.5-ml tubes containing 10 µl of proteinase K (20 mg ml^{-1}). Following addition of 190 µl of STE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl),

Table 1. *Wolbachia* infection status of *C. erate poliographus* collected at seven geographic locations in Japan.

Locality symbol	Location	Date of sampling in 2007	Females	Males	Female ratio	Infection frequency (%)
MO1	Tamayama, Morioka, Iwate Prefecture	Jul. 21	4	18	0.18	95.5
MO2	Nishine, Shizukuishi, Iwate Prefecture	Jul. 21	4	4	0.50	100
MS	Minamisaku, Nagano Prefecture	Sep. 4	2	5	0.29	85.7
TB	Tsukuba, Ibaraki Prefecture	Aug. 24	2	10	0.17	91.7
KW	Kashiwa, Chiba Prefecture	Mar. 20; May 5	2	10	0.17	100
MD	Matsudo, Chiba Prefecture	Mar. 28; Apr. 27; Sep. 27	2	8	0.20	100
NO	Nishinoomote, Kagoshima Prefecture	Jun. 3	8	10	0.44	100

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Table 2. Hatching rates of eggs produced by four crossing combinations of *Wolbachia*-infected and cured *C. erate poliographus*.

Mother	Father	Brood	No. of eggs	Egg hatching rate (%)
Infected	Infected	C1	66	77.3
		C2	129	58.9
Infected	Cured	A1	258	65.9
		A2	199	47.7
Cured	Infected	B1	351	0
		B2	129	0
		B3	176	0
Cured	Cured	D1	142	68.3
		D2	243	27.1

the samples were sequentially incubated at 55°C for 35 min and 95°C for 5 min. After centrifugation at 13,000 rpm for 1 min, the supernatants were subjected to diagnostic PCR. Compared to other tissues, such as abdomen and thorax, legs are more effectual for the simple preparation of DNA since they contain fewer amounts of substances (e.g. pigments or fat) that can inhibit PCR. Reliability of *Wolbachia* detection using leg tissues has been proven by Narita *et al*. (2007b), which compared *Wolbachia* infection status in various tissues (legs, ovaries, testes, Malpighian tubules and fat body) in *Wolbachia*-infected butterflies.

PCR detection of *Wolbachia* infection was performed using primers groEfl (5′-TTG TAG CCT GCT ATG GTA TAA CT-3′) and groErl (5′-GAA TAG GTA TGA TTT TCA TGT-3′) for the *groE* gene (Masui *et al*., 1997) and primers wsp81F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and 691R (5′-AAA AAT TAA ACG CTA CTC CA-3′) for the *wsp* gene (Zhou *et al*., 1998).

To confirm that DNA was properly extracted, the host mitochondrial cytoplasmic c oxidase I (*COI*) gene was amplified using the primer set COI-321F (5′-GAT TTT TTG GAC ATC CTG AAG-3′) and COI-689R (5′-CTA AAA TTA CTC CTG TTA ATC C-3′) (Narita *et al*., 2007b) in the same samples. PCR amplifications were conducted under the following temperature profile: 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by incubation at 72°C for 7 min. Samples in which the *COI* gene failed to be amplified were excluded from the analysis.

Cytoplasmic incompatibility

A total of eight naturally infected female butterflies collected from Morioka (MO1 and MO2) were individually allowed to oviposit on the leaves of the white clover *Trifolium repens* L. in plastic cups. Hatched larvae derived from each mother were separated into two groups. Individuals in the first group were mass-reared in plastic cases (10–20 individuals per case) on fresh leaves of *T. repens* in a laboratory at 25°C under a long-day regimen (16 h light : 8 h dark). The emerged adults were kept in plastic cups and fed with 10% sucrose solution. These individuals were referred to as untreated individuals (infected individuals) and used for crossing experiments.

Individuals in the second group were individually fed with an artificial diet (Kato & Sakakura, 1994) containing 0.05% tetracycline hydrochloride from the 1st larval stage until pupation. The emerged adults were kept in plastic cups and fed with 10% sucrose solution. These individuals were referred to as treated individuals and used in crossing experiments.

Treated and untreated males and females were crossed in all four possible combinations. All crosses were between non-siblings. The hatching rates of the resulting eggs were recorded.

To check whether the females used in crossing were fertilized, the female bursa copulatrix was dissected after oviposition, and the presence or absence of spermatophores was examined.

Survival rate, development period and sex ratio

The larvae derived from each crossing were reared individually on an artificial diet containing cut leaves of the white clover *T. repens*. Since no cannibalism was possible, this individual rearing allowed us to obtain precise data for the sex ratios at the adult stage, survival rates during the larval stages and pupal stage, and the development times during the larval stages and pupal stage.

Adults were distinguished as males and females by their wing colors and their abdominal tip morphologies.

The development period data were subjected to statistical analyses using the software R ver. 2.4.0 (R Development Core Team, 2005). Since some of the data sets did not exhibit normal and/or homogeneous variance, we adopted a generalized linear model (McCullagh & Nelder, 1989) for Gaussian, inverse Gaussian, gamma and negative binomial distributions, which were selected according to the Akaike information criterion.

MLST analysis of Wolbachia

Thoracic muscles were dissected from the mother of brood C1 and father of brood B1 and stored at −20°C until DNA extraction. DNA was extracted using a DNeasy Tissue Kit™ (QIAGEN). These two infected butterflies were fully characterized by MLST and WSP analyses. The *wsp* gene and the five MLST genes (*coxA*, *gatB*, *hcpA*, *ftsZ* and *fbpA*) were sequenced using standard protocols (Baldo *et al*., 2006).

The host and strain information have been submitted to the MLST database (http://pubmlst.org/wolbachia/).

The sequence data were aligned with published *Wolbachia* sequences from other insects. Likelihood-ratio tests were performed using MODELTEST VERSION 3.06 (Posada & Crandall, 2001) to determine the models of evolution with the best fit for each gene and the concatenated MLST data. Phylogenetic trees were constructed by the maximum likelihood and maximum parsimony methods using PAUP 4.0b10 (Swofford, 2001). Bootstrap support for clades was evaluated using 250 (maximum likelihood method) or 1000 (maximum parsimony method) pseudoreplicates, and the 50% majority rule bootstrap was applied.

Results

Infection frequencies of Wolbachia *in seven local populations*

To investigate the infection frequencies of *Wolbachia* in seven local populations (fig. 1), diagnostic PCR was performed on all the collected butterflies ($n = 79$). The infection frequencies in all seven local populations were extremely high. Butterflies collected from Morioka (MO2), Kashiwa (KW), Matsudo (MD) and Nishinoomote (NO) were all infected with *Wolbachia*. On the other hand, some butterflies

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Table 3. Survival rates of offspring produced by four crossing combinations of *Wolbachia*-infected and cured *C. erate poliographus*.

Mother	Father	Brood	No. of larvae	Survival rate of larval stages (pupae/hatched larvae)	No. of pupae	Survival rate of pupal stage (adults/pupae)	No. of adults	No. of adults females : males	Female ratio
Infected	Infected	C1	51	0.88	45	0.96	43	20 : 23	0.47
		C2	67	0.82	55	0.95	52	26 : 26	0.50
Infected	Cured	A1	77	0.80	62	1	62	26 : 36	0.42
		A2	40	0.68	27	1	27	15 : 12	0.56
Cured	Infected	B1	0	–	–	–	–	–	–
		B2	0	–	–	–	–	–	–
		B3	0	–	–	–	–	–	–
Cured	Cured	D1	86	0.62	53	0.89	47	25 : 22	0.53
		D2	49	0.58	28	1	28	13 : 15	0.46

Table 4. Development periods of offspring produced by four crossing combinations of *Wolbachia*-infected and cured *C. erate poliographus*.

Mother	Father	Brood	Development period of larval stages (*n*)*	Development period of pupal stage (*n*)*
Infected	Infected	C1	16.4 ± 2.1 (51)	7.6 ± 1.2 (45)
		C2	18.1 ± 1.2 (67)	6.9 ± 1.1 (55)
Infected	Cured	A1	22.4 ± 1.4 (77)	7.2 ± 1.2 (62)
		A2	22.0 ± 1.2 (40)	7.1 ± 1.1 (27)
Cured	Infected	B1	–	–
		B2	–	–
		B3	–	–
Cured	Cured	D1	19.1 ± 2.6 (86)	7.0 ± 1.0 (53)
		D2	18.4 ± 2.7 (49)	6.9 ± 1.6 (28)

* Mean (days) ± standard deviation.

collected from Morioka (MO1), Minamisaku (MS) and Tsukuba (TB) were not infected with *Wolbachia* (table 1).

Strong cytoplasmic incompatibility caused by Wolbachia

To examine whether *Wolbachia* induced cytoplasmic incompatibility in *C. erate poliographus*, all four possible crossing combinations were performed between infected and cured parents (table 2). When crossings were performed between cured females and infected males, none of the 656 eggs hatched (broods B1, B2 and B3). In contrast, when the other three crossing combinations were performed, large numbers of the eggs hatched (egg hatching rate: 27.1–77.3%).

After oviposition, we dissected the females used for crossing and confirmed that they were all fertilized by examining the presence of spermatophores in the bursa copulatrix. These data suggest that *Wolbachia* causes strong cytoplasmic incompatibility in *C. erate poliographus*.

Survival rates and sex ratios

We compared the survival rates of the larval stages and pupal stage among the broods. The survival rates of the larval stages in broods D1 and D2 were significantly lower than those in broods A1, A2, C1 and C2 (table 3; $P < 0.001$, Fisher's exact probability test), while the survival rates of the pupal stage did not differ significantly among the broods.

Table 5. Proportions of infected individuals among offspring produced by *Wolbachia*-infected and cured *C. erate poliographus*.

Mother	Father	Brood	Percentage of infected females (*n*)	Percentage of infected males (*n*)
Infected	Infected	C1	100 (15)	100 (14)
		C2	100 (22)	100 (24)
Infected	Cured	A1	100 (26)	100 (30)
		A2	100 (13)	100 (12)

The sex ratios in all six broods (C1, C2, A1, A2, D1 and D2) did not deviate significantly from 1 : 1 (table 3; $P > 0.05$, Fisher's exact probability test). These results indicate that sex ratio distortions, such as feminization or male killing, do not occur in *C. erate poliographus* infected with *Wolbachia*.

Development period

We compared the development periods of the larval stages and pupal stage among the broods. The development periods of the larval stages in broods A1 and A2 were significantly longer than those in broods C1, C2, D1 and D2 (table 4; $P < 0.001$, generalized linear model), while the development periods of the pupal stage did not differ significantly among the broods.

Vertical transmission rates

When adult butterflies emerged, we examined the presence or absence of *Wolbachia* in their legs by diagnostic PCR. All butterflies ($n = 156$) derived from four broods (C1, C2, A1 and A2) were infected with *Wolbachia* (table 5).

Characterization of the Wolbachia *strain*

Two *Wolbachia*-infected adults (the mother of brood C1 and father of brood B1) were subjected to PCR amplification of the *wsp* gene and five MLST genes (*coxA*, *gatB*, *hcpA*, *ftsZ* and *fbpA*) and the DNA sequences were determined. The nucleotide sequences of the MLST genes and *WSP* gene of *Wolbachia* from *C. erate poliographus* have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB436683-AB436694, respectively. The *wsp* gene sequences of the *Wolbachia* strain were identical with those of the *Wolbachia* strains from *C. erate poliographus* reported by Tagami & Miura (2004).

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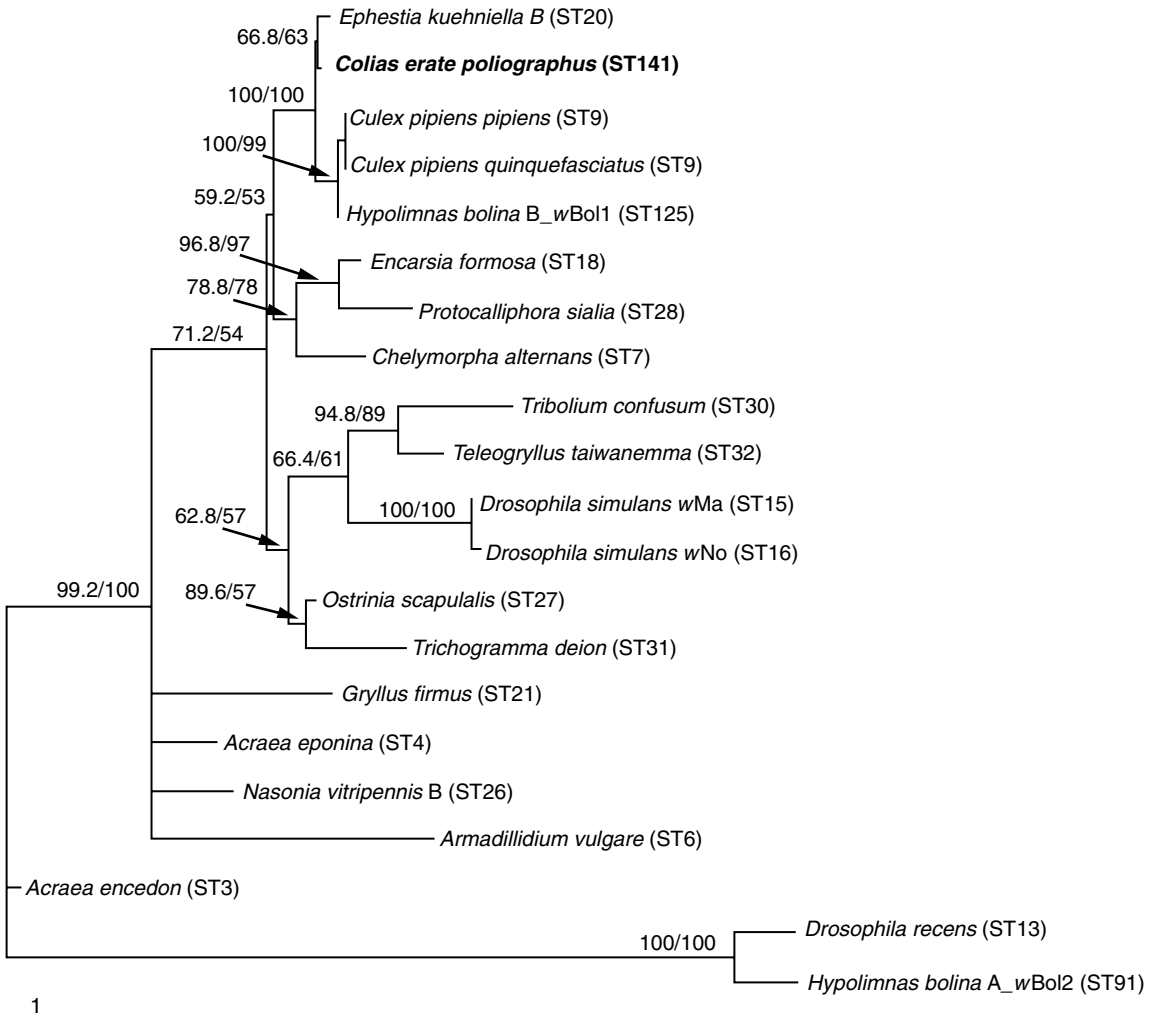

Fig. 2. Maximum likelihood phylogeny using the GTR+I+G model based on the concatenated data set for the five MLST loci of *Wolbachia* from *C. erate poliographus* and the 17 other sequence types (STs) belonging to supergroup B (2079 bp). The tree was rooted with *Wolbachia* in supergroup A (ST13 and ST91). Branches supported by bootstrap probabilities of less than 50% in the maximum likelihood or maximum parsimony method were collapsed. The maximum likelihood (left) and maximum parsimony (right) bootstrap values of >50% are shown for each node.

According to the MLST scheme, the *Wolbachia* in *C. erate poliographus* is a novel sequence type (ST141) and belongs to supergroup B. Regarding the phylogeny, this *Wolbachia* strain formed a monophyletic group with the cytoplasmic-incompatibility-inducing *Wolbachia* strains found in the pyralid moth *Ephestia kuehniella* (ST20) and the mosquito *Culex pipiens* (ST9) and the male-killing *Wolbachia* strain found in the nymphalid butterfly *Hypolimnas bolina* (ST125), which was supported by high bootstrap probabilities (100% in both the maximum likelihood and maximum parsimony methods) (fig. 2).

Discussion

Type of Wolbachia*-induced reproductive manipulation in* C. erate poliographus

Wolbachia are known to manipulate the reproduction of their hosts in various ways, such as induction of parthenogenesis, feminization, male killing or induction of cytoplasmic incompatibility. We examined the type of *Wolbachia*-induced reproductive manipulation by crossing experiments and rearing of *C. erate poliographus*.

Thelytokous parthenogenesis is a phenomenon in which females produce exclusively female offspring without fertilization, and is only known in haplodiploid insect groups, such as Hymenoptera and Thysanoptera (Stouthamer, 1997; Arakaki *et al*., 2001). In the present study, male-derived spermatophores were present in both infected and antibiotic-treated mothers of nine broods. Thus, the possibility of parthenogenesis induction by *Wolbachia* infection is extremely low in *C. erate poliographus*.

Feminization is a phenomenon in which inherently genetic males are phenotypically changed into females, and is only known in the woodlice *Armadillidium* and the butterfly *Eurema hecabe* (Rigaud, 1997; Rigaud *et al*., 1997; Hiroki *et al*., 2002, 2004; Narita *et al*., 2007a). Male killing is a widely occurring phenomenon in insects in which male progeny are selectively killed (Hurst & Jiggins, 2000).

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If feminization or male killing by *Wolbachia* infection occurred in *C. erate poliographus*, the sex ratio of infected broods would be female-biased. In this study, the sex ratios of all broods were 1 : 1 irrespective of their *Wolbachia* infection status, thus excluding the possibility of feminization or male killing.

Cytoplasmic incompatibility is typically expressed when an infected male mates with an uninfected female. The underlying mechanism of cytoplasmic incompatibility is considered to be a modification-rescue system. In other words, a *Wolbachia* strain in males modifies the sperm so as to kill the offspring during embryogenesis. If the same *Wolbachia* strain is also possessed by females, the offspring will be rescued by removal of the modification (Werren, 1997; Bourtzis & Miller, 2003; Poinsot *et al*., 2003). Cytoplasmic incompatibility is the most common type of host manipulation cased by *Wolbachia* (Bourtzis & Miller, 2003). The hatching rates of eggs produced by such incompatible crossings are low. In this study, we examined all four possible crossing combinations of *Wolbachia*-infected and cured *C. erate poliographus*. Among them, complete suppression of egg hatching was only observed in one combination (between cured females and infected males), which is a typical phenomenon of strong cytoplasmic incompatibility.

We further found that the cytoplasmic-incompatibility-inducing *Wolbachia* strain in *C. erate poliographus* is a novel sequence type (ST141) by using the MLST scheme. The monophyly of *Wolbachia* strains in *C. erate poliographus* (ST141), *Ephestia kueniella* (ST20), *Culex pipiens* (ST9) and *Hypolimnas bolina* (ST125) was supported by high bootstrap probabilities. Unfortunately, these findings provide us with very little information regarding the evolutionary origin of these *Wolbachia* strains at present. Future discoveries of novel *Wolbachia* strains in this clade may allow us to infer some historical processes of horizontal transfer of *Wolbachia*.

Fitness effect of Wolbachia *infection on* C. erate poliographus

A number of studies have investigated the fitness effects of *Wolbachia* infection and variously reported a positive effect (Vavre *et al*., 1999; Dobson *et al*., 2002; Fry & Rand, 2002) or a negative or lack of effect (Hoffmann & Turelli, 1988; Giordano *et al*., 1995; Johanowicz & Hoy, 1999; Bordenstein & Werren, 2000; Harcombe & Hoffmann, 2004). In this study, we investigated the fitness effect of *Wolbachia* infection on *C. erate poliographus* from the data for the survival rates and growth rates (development periods). In *C. erate poliographus*, the survival rates during the larval stages were significantly higher in broods produced by infected mothers than in broods produced by cured mothers, although there was no significant difference in the growth rates. These results imply that the *Wolbachia* have beneficial effects on their larval hosts. However, we must remain cautious about this finding because the parents were treated with antibiotics, which might have had negative maternal effects on their progeny.

How the high prevalence of Wolbachia *infection is accomplished in natural populations of* C. erate poliographus

For vertically transmitted endosymbionts like *Wolbachia*, vertical transmission efficiency is one of the most important factors for successful maintenance in the host populations (Hoffmann *et al*., 1990, 1998; Turelli & Hoffmann, 1995; Werren, 1997).

We found that the vertical transmission rates of *Wolbachia* were 100% in *C. erate poliographus*. Furthermore, this *Wolbachia* strain was found to cause strong cytoplasmic incompatibility in *C. erate poliographus*.

It has been reported that, due to the effect of the strong cytoplasmic incompatibility induced by *Wolbachia*, even small numbers of *Wolbachia*-infected individuals invading previously uninfected populations led to rapid spreading and fixation of infection in a Californian population of *Drosophila simulans* (Turelli & Hoffmann, 1991; Turelli *et al*., 1992), a Japanese population of *Laodelphax striatellus* (Hoshizaki & Shimada, 1995) and a Japanese population of *Eurema hecabe* (Hiroki *et al*., 2005). In a similar way, the strong cytoplasmic incompatibility, high vertical transmission rate and possible beneficial effects on their hosts revealed in the present study may explain the extremely high frequencies of *Wolbachia* infection in Japanese populations of *C. erate poliographus*.

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