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Variation in the prevalence of cytoplasmic incompatibility-inducing *Wolbachia* in the butterfly *Eurema hecabe* across the Japanese archipelago

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ABSTRACT

Question: Is the prevalence of cytoplasmic incompatibility-inducing *Wolbachia* spreading throughout butterfly populations?

Hypothesis: *Wolbachia* are self-promoting intracellular microbes and the infection spreads easily among host populations.

Organism: Japanese pierid butterfly (*Eurema hecabe*).

Field sites: Thirty-two haphazardly selected local populations throughout the Japanese archipelago.

Methods: We surveyed *Wolbachia* infection from 1997 to 2000 using diagnostic PCR. An inter-population cross-experiment was performed to detect cytoplasmic incompatibility in 1992, 1999 and 2000.

Conclusions: High *Wolbachia* frequencies were detected in southern populations, but no infection was found in the northern parts of Japan. Cytoplasmic incompatibility-inducing *Wolbachia* was found in the two main types of host (brown and yellow), which are reproductively isolated. The invasion of cytoplasmic incompatibility-inducing *Wolbachia* into the central Japanese populations has occurred within the last decade as shown by chronological data. Our data fit well with standard models of *Wolbachia* dynamics.

Keywords: cytoplasmic incompatibility, *Eurema hecabe*, reproductive isolation, speciation, *Wolbachia*.

INTRODUCTION

Manipulations of host reproduction by intracellular symbionts are well-documented among various organisms (Werren and Beukeboom, 1998). One widely known example of such selfish symbionts is the bacteria *Wolbachia* (for reviews, see Werren, 1997; Stouthamer *et al.*, 1999), a genus of the alpha-proteobacteria which is widely distributed among arthropods and filarian nematodes (Bandi *et al.*, 1999; Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000; Casiraghi *et al.*, 2001). The

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transmission of these microorganisms is done maternally through the cytoplasm of the egg, not via sperm. In many arthropod species, *Wolbachia* is known to alter host reproduction by inducing cytoplasmic incompatibility, feminization of genetic males, parthenogenesis, or male-killing. All induced alterations can be interpreted as beneficial to *Wolbachia* because they enhance its (maternal) transmission (Werren and O'Neill, 1997).

Cytoplasmic incompatibility is a mating incompatibility between uninfected eggs and sperm originating from infected males (for a review, see Hoffmann and Turelli, 1997). As a consequence, uninfected female hosts have a reduced fecundity when they mate with infected males. The mechanism of cytoplasmic incompatibility has been interpreted as a 'modification-rescue' model (Werren, 1997). *Wolbachia* modify the sperm of infected males during spermatogenesis. The same or a similar *Wolbachia* strain must be present in the ova after fertilization to rescue the zygote and allow normal development. However, if *Wolbachia* is not present in the egg, modified sperm cause an abnormal first mitotic division resulting in the death of the zygote (O'Neill and Karr, 1990; Reed and Werren, 1995).

Different cytoplasmic incompatibility-inducing *Wolbachia* strains can cause bidirectional incompatibility (Breeuwer and Werren, 1990). If different populations are infected with different cytoplasmic incompatible strains, this can lead to postzygotic isolation between them (Breeuwer and Werren, 1990). It was therefore proposed that *Wolbachia* could promote speciation in its hosts (Laven, 1967; Werren, 1998). This idea is much debated (Hurst and Schilthuizen, 1998; Bordenstein, 2003). However, recent empirical and theoretical studies support the view that *Wolbachia* can promote speciation (Shoemaker *et al.*, 1999; Bordenstein *et al.*, 2001; Telschow *et al.*, 2002).

Basic models show that cytoplasmic incompatiblity-inducing *Wolbachia* can spread into a single panmictic population (Caspari and Watson, 1959; Fine, 1978; Hoffmann and Turelli, 1997). Although the prevalence of cytoplasmic incompatiblity-inducing *Wolbachia* is known in many organisms, the progressive change in prevalence in natural populations over time has been reported only for two insect species (Turelli and Hoffmann, 1991; Hoshizaki and Shimada, 1995).

The pierid butterfly *Eurema hecabe* is common in most parts of Japan. Two distinct types are known that differ in colour (brown and yellow), larval food plant use and seasonal wing morph response (Kato, 1999, 2000a). Populations of the brown type occur only in the subtropical regions of the Ryukyu Islands, whereas the yellow type is found in both the temperate regions of the main island of Japan and the subtropics. On subtropical Okinawa-jima Island, both types occur sympatrically. These sympatric populations are partially prezygotic isolated by female mate discrimination (Kobayashi *et al.*, 2001) as well as postzygotic isolated by hybrid breakdown (M. Hiroki, unpublished data).

The yellow type of *E. hecabe* in Okinawa is infected with two different *Wolbachia* strains – one infection causes feminization, the other cytoplasmic incompatibility (Hiroki *et al.*, 2002, 2004). Interestingly, the sympatrically occurring brown type is infected with the same cytoplasmic incompatiblity-inducing *Wolbachia* strain but not with the feminization strain (Hiroki *et al.*, 2004). *Wolbachia*-mediated mitochondrial introgression across species was also demonstrated in closely related *Drosophila simulans* and *D. mauritania* (Rousset and Solignac, 1995). The *Wolbachia* infection pattern in *E. hecabe* might play an important role in genetic divergence between the yellow and the brown type. Investigations of *Wolbachia* prevalence in many local populations of *E. hecabe*, including detection of cytoplasmic incompatibility, are therefore necessary for understanding the evolutionary pathway of *E. hecabe* diversity.

In this study, we initially assess the variation in *Wolbachia* prevalence in *E. hecabe* throughout the Japanese archipelago by diagnostic PCR. Next, we examine whether infect-

ing *Wolbachia* causes cytoplasmic incompatibility or not. The population dynamics of *Wolbachia* is analysed using the mathematical model of Hoffmann and Turelli (1997). Finally, we discuss the evolutionary consequences of *Wolbachia* infections upon host diversity.

MATERIALS AND METHODS

Insects

Adult butterflies of *E. hecabe* were collected from 1992 to 2000. Sampling sites and sample numbers used for the *Wolbachia* prevalence survey (performed between 1997 and 2000) are summarized in Table 1.

Cross-experiments

To determine whether infecting *Wolbachia* causes cytoplasmic incompatibility, breeding experiments were performed among four populations (Hitachi and Mitaka for the yellow type, Ishigaki and Okinawa for the brown type). Matings between populations are easily achieved because there is no prezygotic barrier between allopatric populations (Kato, 2000b).

Isofemale lines were established from the females collected in 1992, 1999 and 2000. Larvae were reared under 25°C and 16 h light/8 h dark conditions with fresh leaves of their host plant (*Lespedeza cuneata* for yellow type larvae and *Ormocarpum cochinchinense* for brown type larvae), or with an artificial diet based on *Albizia julibrissin* leaf powder (Kato and Sakakura, 1994) for both types. Detection of *Wolbachia* in the samples collected in 1999 and 2000 was performed during the *Wolbachia* prevalence survey described below, but was not performed for the samples collected in 1992. To remove infecting *Wolbachia*, larvae were fed on an artificial diet containing tetracycline hydrochloride (0.6 $mg \cdot g^{-1}$ diet powder) throughout the larval stage.

Virgin females and males were crossed within a week of adult eclosion. Combinations of crosses are summarized in Table 2. Mated females were kept individually in plastic cups and allowed to oviposit on their host plant for several days. Eggs deposited were counted daily, and the ratio of hatched eggs was determined for 3 days after oviposition.

Detection of *Wolbachia* by PCR

Samples of DNA were prepared from fresh reproductive tissues of adult butterflies by STE boiling methods (O'Neill *et al.*, 1992). *Wolbachia* was detected by PCR using the *Wolbachia ftsZ* gene-specific primer set, ftsZf1 and ftsZr1 (Werren *et al.*, 1995). To maximize detection efficiency, we carried out PCR under low annealing temperatures: 30 s at 94°C, 30 s at 46°C and 2 min at 72°C per cycle for 30 cycles. Parts of products ($N = 34$) were 100-fold diluted and were quality checked with ftsZBf-ftsZBr primers, which amplifies the inner region of the *ftsZ* gene (Werren *et al.*, 1995) under an annealing temperature of 55°C for 12 cycles. All were successfully amplified, and non-specific amplification by low annealing temperature was thereby avoided. Each sample was checked in duplicate, and when the product was absent in both trials, these insects were regarded as uninfected. Positive control of DNA preparation was checked using 12S rDNA insect-specific primers (O'Neill *et al.*, 1992).

Table 1. List of sampling sites with latitude and sample size (locations are aligned from north to south)

	Locality (prefecture)	Latitude (N)	Sample size			
			1997	1998	1999	2000
Yellow type						
1	Sendai (MIYAGI)	37°75′			19	
2	Yamagata (YAMAGATA)	37°74′			26	
3	Muikamachi (NIIGATA)	36°58′			20	20
4	Kanazawa (ISHIKAWA)	36°55′			15	
5	Shioya (TOCHIGI)	36°52′			9	5
6	Sakae (NAGANO)	36°50′				7
7	Hitachi (IBARAKI)	36°41′			15	9
8	Kawachi (TOCHIGI)	36°37′			10	
9	Ogawa (IBARAKI)	36°36′			16	23
10	Suzaka (NAGANO)	36°33′			15	
11	Fujioka (GUNMA)	36°11′				11
12	Tsukuba (IBARAKI)	36°10′			8	
13	Nagawa (NAGANO)	36°04′		8		
14	Mitsukaido (IBARAKI)	36°01′			6	
15	Niiza (SAITAMA)	35°46′			12	
16	Mitaka (TOKYO)	35°42′		33	22	45
17	Kofu (YAMANASHI)	35°35′		14		
18	Matsue (SHIMANE)	35°30′			5	
19	Nagakute (AICHI)	35°16′			18	
20	Hikone (SHIGA)	35°14′		15		
21	Suita (OSAKA)	34°49′			21	
22	Hamamatsu (SHIZUOKA)	34°42′			7	
23	Toba (MIE)	34°30′			7	
24	Kochi (KOCHI)	33°33′			18	
25	Beppu (OOITA)	33°19′			6	
26	Matsuyama (EHIME)	32°57′			27	
27	Kagshiama (KAGOSHIMA)	31°10′			33	
28	Tokunoshima (KAGOSHIMA)	27°50′			5	15
29	Okinoerabu (KAGOSHIMA)	27°36′			8	
30	Yoron (KAGOSHIMA)	27°01′			16	
31	Okinawa (OKINAWA)	26°42′	14		9	8
Brown type						
32	Okinawa (OKINAWA)	26°42′			21	
33	Ishigaki (OKINAWA)	24°20′	2	5	9	11

Estimation of vertical transmission rate

To estimate the parameters affecting the frequency of *Wolbachia* infection, vertical transmission rate was examined. Efficiency of vertical transmission at 25°C was examined for ten matrilines: three were of the yellow type (Mitaka) and seven of the brown type (Okinawa and Ishigaki). From each matriline, broods were reared on an artificial diet under 25°C.

Table 2. Egg hatchability in cross-experiments within/between populations

Population and type/year	Crosses (females × males)	Number of crosses	Mean hatchability (range)
A. Mitaka (Ye), Ishigaki (Br)			
1991	ISH × MIT	5	0.75 (0.64–0.97)
	MIT × ISH	5	0
2000	$ISH^W \times MIT^W$	5	0.67 (0.64–0.88)
	$MIT^W \times ISH^W$	8	0.60 (0.49–0.82)
B. Ishigaki (Br)			
2000	$ISH^W \times ISH^W$	11	0.68 (0.31–0.86)
	$ISH^W \times ISH^T$	5	0.42 (0.07–0.73)
	$ISH^T \times ISH^W$	5	0
	$ISH^T \times ISH^T$	7	0.60 (0.25–0.85)
C. Mitaka (Ye)			
2000	$MIT^W \times MIT^W$	9	0.52 (0.23–0.73)
	$MIT^T \times MIT^W$	6	0
D. Okinawa (Br)			
1999	$OKI^W \times OKI^W$	12	0.61 (0.00–0.89)
	$OKI^W \times OKI^T$	4	0.41 (0.19–0.74)
	$OKI^T \times OKI^W$	5	0
	$OKI^T \times OKI^T$	7	0.58 (0.13–1.00)
E. Hitachi (Ye), Okinawa (Br)			
1999	$HIT^N \times OKI^W$	3	0
	$HIT^N \times OKI^T$	3	0.48 (0.34–0.65)

Note: Ishigaki (ISH) and Okinawa (OKI); brown typ (Br). Mitaka (MIT) and Hitachi (HIT); yellow type (Ye). W = *Wolbachia* PCR positive. N = *Wolbachia* PCR negative. T = tetracycline treatment. No superscript = infection status was not examined.

The vertical transmission efficiency under high temperature was also examined because imperfect maternal transmission under high temperature is known in some species (Rigaud *et al.*, 1997). Broods of one matriline from the yellow type and two from the brown type were reared at 30°C. After adult eclosion, template DNA preparation and detection of *Wolbachia* infection by PCR was carried out as described above.

RESULTS

Infection of *Wolbachia* in local populations

The distribution of *Wolbachia* infection is summarized in Fig. 1. Infection of *Wolbachia* was found in populations of both types. For brown type populations (e.g. Ishigaki and Okinawa), the *Wolbachia* infection rate was very high. In contrast, the frequency of *Wolbachia* infection differed among the yellow type populations, and was high in southern populations and low or non-existent in northern ones.

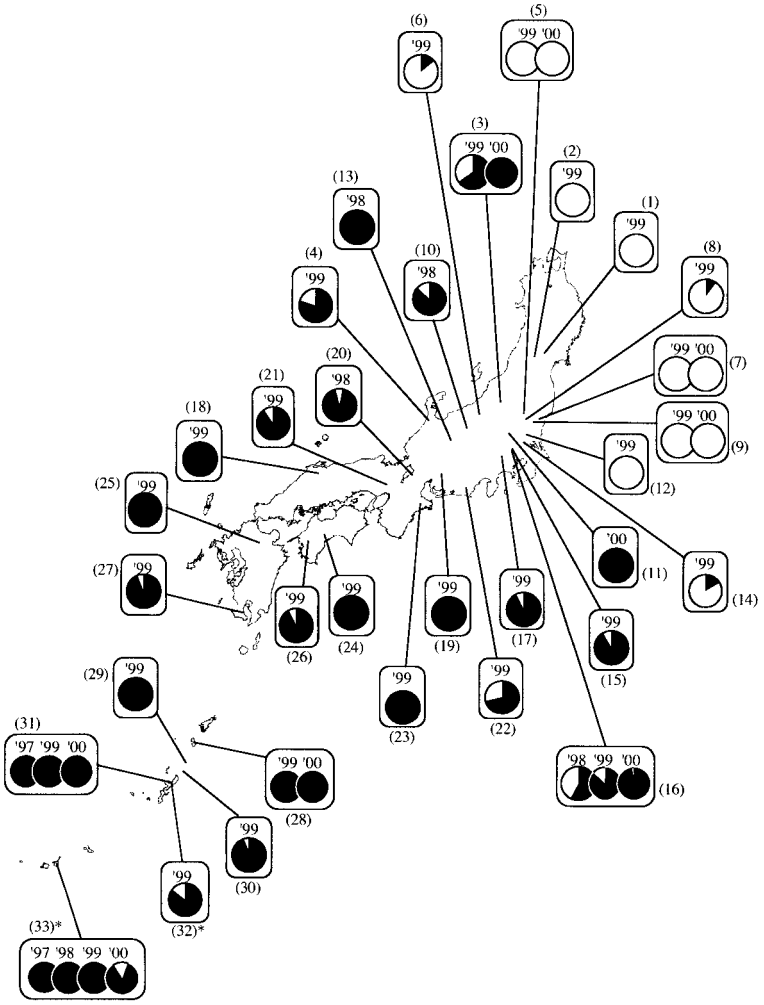

Fig. 1. Distribution of *Wolbachia* infection in the Japanese archipelago. Numbers in parentheses represent the sampling sites shown in Table 1. The black portion of the pie graph shows the proportion of *Wolbachia* infection. Numbers above pie graphs show sampling year. * Shows the brown type populations.

At seven localities, *Wolbachia* infection was observed over two or more years. A rapid increase of *Wolbachia* prevalence was found in two populations, Muikamachi and Mitaka. In 2000, the frequency of *Wolbachia* infection reached 100% in both populations. In contrast, infection frequency did not change in the other five populations: *Wolbachia* infection was maintained at high prevalence in Okinawa and Ishigaki, whereas it was absent in northern populations (Shioya, Hitachi and Ogawa) for all years sampled.

For the Mitaka population, a change in frequency was observed on a fine time-scale (Fig. 2). In 1998, infection frequency was relatively low (37.8%) in May, increasing to 73.7% by early August. The infection frequency remained high and increased through subsequent generations, reaching 100% in 2000.

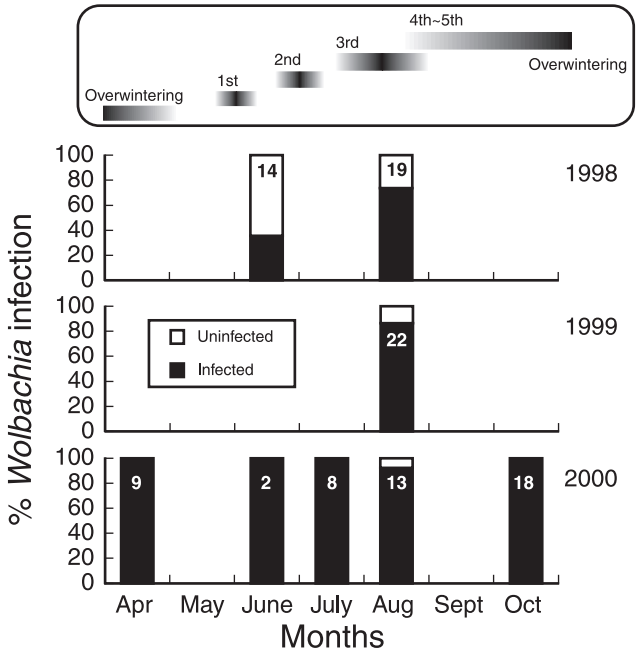

Fig. 2. Seasonal and yearly variation in *Wolbachia* infection frequency in the Mitaka population. Numbers in bar graphs show sample sizes. Schematic above bar graphs shows annual generations of *E. hecabe* estimated from the data of Kato (1986).

Cytoplasmic incompatibility

Between the Ishigaki (the brown type) and the Mitaka (the yellow type) populations (Table 2A), unidirectional incompatibility was found in 1992. No eggs hatched from Mitaka females crossed with Ishigaki males. In 2000, *Wolbachia* was detected in both populations (Fig. 1), and these two populations became reciprocally compatible (Table 2A). On the other hand, unidirectional incompatibility was found between tetracycline-treated and untreated insects from Ishigaki (Table 2B). When tetracycline-treated females from Mitaka were crossed with untreated males of the same population, no eggs hatched (Table 2C).

Wolbachia infection was also found in the brown type population of Okinawa (Fig. 1). Reciprocal crosses of the Okinawa population with and without tetracycline treatment showed that this *Wolbachia* caused cytoplasmic incompatibility, and the intensity of incompatibility was 100% (Table 2D). In contrast, crosses between Okinawa and Hitachi showed that these populations were unidirectionally incompatible (Table 2E). Tetracycline treatment on Okinawa butterflies revealed that this incompatibility was due to *Wolbachia* infection.

Vertical transmission efficiency of *Wolbachia*

The efficiency of maternal transmission was found to be perfect for all matrilines regardless of the host type, population or rearing temperature (Table 3).

Table 3. Intra-brood infection ratio of *Wolbachia* under different thermal conditions

	Intra-brood percentage of *Wolbachia* infection					
Temperature	Mitaka		Okinawa		Ishigaki	
25°C	100	(8)	100	(9)	100	(8)
	100	(20)	100	(9)	100	(6)
	100	(8)	100	(5)	100	(6)
					100	(5)
30°C	100	(6)			100	(12)
					100	(11)

Note: Values indicate within-brood frequency of *Wolbachia* infection for each matriline. Numbers in parentheses indicate sample sizes.

DISCUSSION

Geographical prevalence of *Wolbachia*

As shown in Fig. 1, the pattern of *Wolbachia* prevalence in *E. hecabe* was polymorphic: the infection frequency was high in southern populations but was zero in most northern populations. Intra-population crosses between tetracycline-treated and untreated butterflies in the Ishigaki, Okinawa and Mitaka populations clearly showed that *Wolbachia* infections cause cytoplasmic incompatibility (Table 2). Note that we did not check *Wolbachia* prevalence at Mitaka before 1998. However, in 1992 unidirectional incompatibility was found between the Ishigaki population (brown type) and the Mitaka population (yellow type), indicating that *Wolbachia* was absent in Mitaka at that time (Table 2A). The frequency of *Wolbachia* infection at Mitaka increased during the following years (Fig. 2). In 2000, perfect reciprocal compatibility was found between the Mitaka and Ishigaki populations (Table 2A), suggesting that cytoplasmic incompatibility-inducing *Wolbachia* went to fixation in Mitaka during the last decade. These results suggest that cytoplasmic incompatibility-inducing *Wolbachia* in *E. hecabe* is now spreading from the southwest to the northeast of Japan. This is the third report to document the ongoing prevalence of cytoplasmic incompatibility-inducing *Wolbachia*. To date, the spatial spread of *Wolbachia* has been shown only in *Drosophila simulans* (Turelli and Hoffmann, 1991) and *Laodelphax striatellus* (Hoshizaki and Shimada, 1995).

Previous studies have shown that two types of *Wolbachia* occur in *E. hecabe*, one of which causes feminization and the other cytoplasmic incompatibility (Hiroki *et al.*, 2002, 2004). Interestingly, we could detect a spatial spread only for cytoplasmic incompatibility-inducing *Wolbachia*. Although we reared many matrilines from several locations, female-biased matrilines were only found in Okinawa-jima (unpublished observations). Why feminization *Wolbachia* does not spread in *E. hecabe* is discussed in detail elsewhere (Hiroki *et al.*, 2004).

The maternal transmission efficiency of *Wolbachia* in the laboratory is very high in *E. hecabe* (Table 3). This is in contrast to field observations showing that *Wolbachia* is not at fixation in some populations (Fig 1). One explanation is that *Wolbachia* prevalence is

still increasing in these populations. However, high infection rates in most southwestern populations suggests that this is not true because *Wolbachia* infections can reach a stable state within a short time (Fig. 3). Another possible explanation is that infected individuals sometimes lose *Wolbachia* in natural environments. Although we have not investigated this, there are several factors that could explain a lower prevalance in the field. In our study, larvae were reared under laboratory conditions with an artificial diet. Butterflies in the wild are likely to have worse nutrition and more stress. Furthermore, naturally occurring antibiotics may occur in the wild. All these factors may cause the loss of *Wolbachia* infection. In addition, the transmission efficiency of the bacterium may be dependent on the genotype of its host (Turelli, 1994).

Theoretical interpretation of the dynamics of *Wolbachia* prevalence

Theoretical studies (Caspari and Watson, 1959; Fine, 1978; Hoffmann and Turelli, 1997) have shown that the dynamics of cytoplasmic incompatibility-inducing *Wolbachia* can be described by a simple mathematical model with the parameters cytoplasmic incompatibility intensity, vertical transmission rate and cost of infection. We used this model to analyse the spread of *Wolbachia* in the Mitaka population. Cytoplasmic incompatibility intensity is 100% in all three populations examined (Table 2). Vertical transmission was also 100% for those populations. We could not detect a cost of infection. Using these parameter values and an initial infection frequency of 39.7%, we calculated the expected curve of infection frequency, based on the equation (2.1) of Hoffmann and Turelli (1997):

$$p_{t+1} = p_t (1 - s_f) \Big/ 1 - s_f p_t - s_h p_t (1 - p_t)$$

where p_t shows the infection frequency in generation t, s_h is the relative decrease of uninfected females mated with infected males, and s_f is the cost of infection.

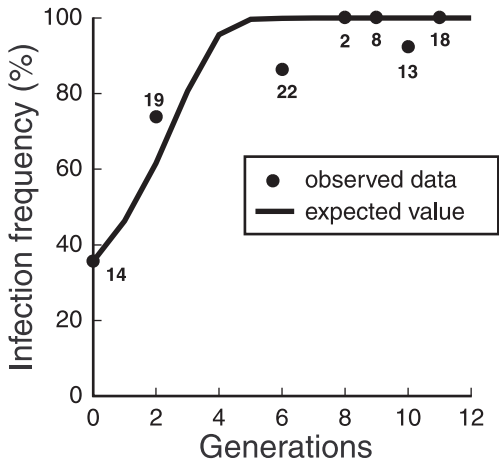

Fig. 3. Expected and observed values of *Wolbachia* infection ratio in the Mitaka population. Expected values were calculated following Hoffmann and Turelli (1997). Parameters: initial infection frequency = 35.7, vertical transmission rate = 1, intensity of cytoplasmic incompatibility = 1, cost of *Wolbachia* infection = 0.

To keep the calculations simple, we assumed that *E. hecabe* has four generations per year, although a partial fifth generation may occur in some years (Kato, 1986). Observed frequencies fitted well to the expected values (Fig. 3). Therefore, we conclude that the increase in prevalence rate is due to the infection of cytoplasmic incompatibility-inducing *Wolbachia* and not to other mechanisms such as genetic drift. We note that there have been no reports to date of the fine-scale dynamics of cytoplasmic incompatibility-inducing *Wolbachia* prevalence in natural populations. Therefore, our results provide important empirical support for the mathematical model.

Does cytoplasmic incompatibility-inducing *Wolbachia* infection affect speciation between two types of *E. hecabe*?

The role of cytoplasmic incompatibility-inducing *Wolbachia* in reproductive isolation between closely related species has been investigated in several studies (Giordano *et al.*, 1997; Hurst and Schilthuizen, 1998; Bordenstein *et al.*, 2001, Telschow *et al.*, 2002). In *E. hecabe*, two types co-exist on several islands which differ in morphology, seasonal adaptation and larval food plant use (Kato, 2000a). In this study, we demonstrated the prevalence of cytoplasmic incompatibility-inducing *Wolbachia* in both host types. The *Wolbachia* strains of these two host types are identical. Therfore, no bidirectional incompatibility is induced and cytoplasmic incompatibility-inducing *Wolbachia* cannot act as a reproductive barrier (Table 2A). This is in contrast to other insect cases where *Wolbachia* plays an important role in reproductive isolation (Giordano *et al.*, 1997; Bordenstein *et al.*, 2001).

The extent of *Wolbachia* prevalence in *E. hecabe* further implies problems for the taxonomic study of this species. Cytoplasmic factors such as mitochondria are associated with the prevalence of cytoplasmic incompatibility-inducing *Wolbachia* (Turelli *et al.*, 1992). Therefore, the reconstruction of host phylogeny using mtDNA is affected by the prevalence of *Wolbachia*. In *E. hecabe*, the ongoing prevalence of *Wolbachia* in the yellow type populations suggests that the prevalence introgressed from the brown type populations, since reproductive isolation is imperfect between these two types (Kobayashi *et al.*, 2001). Thus, it is suspected that the phylogeny of the mtDNA gene may not reflect the phylogenetic differentiation between the brown and yellow type populations of *E. hecabe*. Consequently, mitochondria originating from the brown type might have introgressed into the yellow type populations together with *Wolbachia* infection, as in the case of *Drosophila simulans* complex (Rousset and Solignac, 1995).

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REFERENCES

Bandi, C., Anderson, T.J.C., Genchi, C. and Blaxter, M.L. 1999. Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. Lond. B*, **265**: 2407–2413.

Bordenstein, S.R. 2003. Symbiosis and the origin of species. In *Insect Symbiosis* (K. Bourtzis and T. Miller, eds.), pp. 283–304. New York: CRC Press.

Bordenstein, S.R., O'Hara, F.P. and Werren, J.H. 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*, **409**: 707–710.

Breeuwer, J.A.J. and Werren, J.H. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, **346**: 558–560.

Casiraghi, M., Anderson, T.J.C., Bandi, C., Bazzocchi, C. and Genchi, C. 2001. A phlogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. *Parasitology*, **122**: 93–103.

Caspari, E. and Watson, G.S. 1959. On the evolutionary importance of cytoplasmic sterility in mosquitoes. *Evolution*, **13**: 568–570.

Fine, P.E. 1978. On the dynamics of symbiont-dependent cytoplasmic incompatibility in Culicine mosquitos. *J. Invert. Pathol.*, **30**: 10–18.

Giordano, R., Jackson, J.J. and Robertson, H.M. 1997. The role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. *Proc. Natl. Acad. Sci. USA*, **94**: 11439–11444.

Hiroki, M., Kato, Y., Kamito, T. and Miura, K. 2002. Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae). *Naturwissenschaften*, **89**: 167–170.

Hiroki, M., Tagami, Y., Miura, K. and Kato, Y. 2004. Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*. *Proc. R. Soc. Lond. B*, **271**: 1751–1755.

Hoffmann, A.A. and Turelli, M. 1997. Cytoplasmic incompatibility in insects. In *Influential Passengers* (S.L. O'Neill, A.A. Hoffmann and J.H. Werren, eds.), pp. 42–80. Oxford: Oxford University Press.

Hoshizaki, S. and Shimada, T. 1995. PCR-based detection of *Wolbachia*, cytoplasmic incompatibility microorganisms, infected in natural populations of *Laodelphax striatellus* (Homoptera: Delphacidae) in central Japan: has the distribution of *Wolbachia* spread recently? *Insect Mol. Biol.*, **4**: 237–243.

Hurst, G.D.D. and Schilthuizen, M. 1998. Selfish genetic element and speciation. *Heredity*, **80**: 2–8.

Jeyaprakash, A. and Hoy, M.A. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.*, **9**: 393–405.

Kato, Y. 1986. The prediapause copulation and its significance in the butterfly *Eurema hecabe*. *J. Ethol.*, **4**: 81–90.

Kato, Y. 1999. Fringe color, seasonal morphs and host-plant use of the pierid butterfly *Eurema hecabe* (L) (Lepidoptera, Pieridae) on Okinawa-jima Island. *Trans. Lepid. Soc. Japan*, **50**: 111–121 (in Japanese with English summary).

Kato, Y. 2000a. Overlapping distribution of two groups of butterfly *Eurema hecabe* differing in the expression of seasonal morphs on Okinawa-jima Island. *Zool. Sci.*, **17**: 539–547.

Kato, Y. 2000b. Does mating occur among populations of two types in the butterfly *Eurema hecabe* (L.) (Lepidoptera. Pieridae)? *Trans. Lepid. Soc. Japan*, **52**: 63–66.

Kato, Y. and Sakakura, F. 1994. Artificial diet rearing of *Eurema blanda* and some notes on its host-plant. *Trans. Lepid. Soc. Japan*, **45**: 21–26 (in Japanese with English summary).

Kobayashi, A., Hiroki, M. and Kato, Y. 2001. Sexual isolation between two sympatric types of the butterfly *Eurema hecabe* (L.). *J. Insect Behav.*, **14**: 353–362.

Laven, H. 1967. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature*, **216**: 383–384.

O'Neill, S.L. and Karr, T.L. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature*, **348**: 178–180.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. and Robertson, H.M. 1992. 16S rRNA

phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA*, **89**: 2699–2702.

Reed, K.M. and Werren, J.H. 1995. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Mol. Reprod. Dev.*, **40**: 408–418.

Rigaud, T., Antoine, D., Marcadd, I. and Juchault, P. 1997. The effect of temperature on sex ratio in the isopod *Porcellionides pruinosus*: environmental sex determination or a by-product of cytoplasmic sex determination? *Evol. Ecol.*, **11**: 205–215.

Rousset, F.R. and Solignac, M. 1995. Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex. *Proc. Natl. Acad. Sci. USA*, **92**: 6389–6393.

Shoemaker, D.D., Katju, V. and Jaenike, J. 1999. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria. Evolution*, **53**: 1157–1164.

Stouthamer, R., Breeuwer, J.A.J. and Hurst, G.D.D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.*, **53**: 71–102.

Telschow, A., Hammerstein, P. and Werren, J.H. 2002. The effect of *Wolbachia* on genetic divergence between populations: models with two-way migration. *Am. Nat.*, **160**: S54–S66.

Turelli, M. 1994. Evolution of incompatibility-inducing microbes and hosts. *Evolution*, **48**: 1500–1513.

Turelli, M. and Hoffmann, A.A. 1991. Rapid spread of an inherited incompatibility factor in California *Drosophila. Nature*, **353**: 440–442.

Turelli, M., Hoffmann, A.A. and McKechnie, S.W. 1992. Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. *Genetics*, **132**: 713–723.

Werren, J.H. 1997. Biology of *Wolbachia. Annu. Rev. Entomol.*, **42**: 587–609.

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

Werren, J.H. and Beukeboom, L.W. 1998. Sex determination, sex ratios, and genetic conflict. *Annu. Rev. Ecol. Syst.*, **29**: 233–261.

Werren, J.H. and O'Neill, S.L. 1997. The evolution of heritable symbionts. In *Influential Passengers* (S.L. O'Neill, A.A. Hoffmann and J.H. Werren, eds.), pp. 1–41. Oxford: Oxford University Press.

Werren, J.H. and Windsor, M. 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. Lond. B*, **267**: 1277–1285.

Werren, J.H., Zhang, W. and Guo, L.R. 1995. Evolution and phylogeny of *Wolbachia*: Reproductive parasites of arthropods. *Proc. R. Soc. Lond. B*, **261**: 55–63.