Molecular Ecology (2006) **15**, 1095–1108 doi: 10.1111/j.1365-294X.2006.02857.x

Genetic structure of sibling butterfly species affected by *Wolbachia* infection sweep: evolutionary and biogeographical implications

SATOKO NARITA,* MASASHI NOMURA,* YOSHIOMI KATO† and TAKEMA FUKATSU‡
*Faculty of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan, †Department of Biology, International Christian University, Tokyo 181-8585, Japan, ‡Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

Abstract

It was recently recognized that in Japan, the common yellow butterfly, *Eurema hecabe*, consists of two sibling species, which have been unnamed yet and tentatively called yellow (Y) type and brown (B) type. We investigated the diversity of nuclear and mitochondrial genes in Japanese populations of Y type and B type of *E. hecabe*. The phylogeny based on nuclear genes agreed with the distinction between Y type and B type, which had been also supported by a wide array of biological data. However, the phylogeny based on mitochondrial genes did not reflect the distinction. PCR survey of *Wolbachia* revealed that B-type populations were all infected while Y-type populations contained both infected and uninfected individuals. A single genotype of *Wolbachia*, which was inferred to be a CI-inducing strain from their *wsp* gene sequence, was prevalent in these populations. Notably, the mitochondrial phylogeny was in perfect agreement with the pattern of *Wolbachia* infection, suggesting that the *Wolbachia* infection had affected the mitochondrial genetic structure of the host insects. Probably, the *Wolbachia* strain and the associated mitochondrial genomes have been occasionally introduced from B-type populations to Y-type populations through migration and subsequent interspecific hybridization, and CI-driven population sweep has been spreading the *Wolbachia* strain and the particular mitochondrial haplotypes, which originated from B-type populations, into Y-type populations. On the basis of these results together with the geological and biogeographical knowledge of the Japanese Archipelago, we proposed an evolutionary hypothesis on the invasion and spread of *Wolbachia* infection in B-type and Y-type of *E. hecabe*.

Keywords: biogeography, *Eurema hecabe*, mitochondrial genetic structure, population sweep, sibling species, *Wolbachia*

Received 7 September 2005; revision accepted 22 November 2005

Introduction

Geographical variations across populations within a species have often provided interesting research subjects in the field of evolutionary biology. Such variations, which can be morphological, physiological, behavioural, ecological and/or genetic ones, may reflect adaptation of the populations to local environments as well as evolutionary history of the populations. Close inspection of such intraspecific variations has sometimes revealed that the 'species' actually contain several closely related multiple species, so-called 'sibling species' or 'cryptic species', that are difficult to distinguish morphologically from each other, at least without skilful eyes (Futuyma 1997; Coyne & Orr 2004; Hebert *et al*. 2004).

The common yellow butterfly, *Eurema hecabe* (L.) (Pieridae: Lepidoptera), is widely distributed in the Oriental tropics and adjacent regions, including temperate mainlands and subtropical southwestern islands of Japan (Yata 1989). This butterfly is multivoltine and known for its seasonal polyphenism in wing-colour patterns. For example, the upperside black distal border of the forewing is wide in the summer morph but narrow or absent in the autumn

Correspondence: Takema Fukatsu, Fax: +81-29-861-6080;
E-mail: t-fukatsu@aist.go.jp

morph, which is determined by photoperiod and temperature during development (Kato & Sano 1987). For a long time, Japanese populations of *E. hecabe* had been thought to comprise a single species. However, Kato & Handa (1992) found that temperate populations and subtropical populations of the butterfly differed in the expression of the polyphenism in response to photoperiod and temperature. Following the discovery, it has been demonstrated that temperate populations and subtropical populations of *E. hecabe* are distinct in a number of traits; different use of host plants (Kato *et al*. 1992), slightly different coloration of wing fringe (Kato 1999), different allelic frequencies of allozymes (Nomura & Kato 1993), and premating reproductive isolation based on the choice by females though hybridization could occur under rearing conditions (Kato 2000b; Kobayashi *et al*. 2001). These data consistently and strongly suggest that the temperate populations with yellowish wing fringe (Y type) and the subtropical populations with brownish wing fringe (B type) constitute closely related but distinct biological species, respectively, although they have not yet been given formal names (Kato & Yata 2005). On account of the above-listed biological knowledge and the common occurrence across the Japanese Archipelago, Y type and B type of *E. hecabe* can be regarded as good model system to investigate the evolutionary aspects of closely related species such as speciation process, adaptation to local environments, biogeographical history, and others. In addition, *Wolbachia* infection was recently reported from Japanese populations of *E. hecabe*, where at least two *Wolbachia* strains, one causing cytoplasmic incompatibility and prevalent while the other causing feminization and much less frequent, were identified (Hiroki *et al*. 2002, 2004).

Members of the genus *Wolbachia* constitute a group of rickettsial endocellular bacteria, belonging to the α-subdivision of the *Proteobacteria*, and are commonly found among insects and other arthropods. In the arthropod hosts, *Wolbachia* endosymbionts often cause a variety of reproductive alterations, including cytoplasmic incompatibility (CI), parthenogenesis induction, feminization of genetic males, male killing, etc. Considering that *Wolbachia* endosymbionts are maternally inherited through host generations by transovarial transmission, these reproductive phenotypes effectively increase the frequency of infected females in the host populations, often at the expense of the host fitness. By causing such reproductive manipulations in a selfish manner, *Wolbachia* endosymbionts are able to rapidly spread their infection in the host populations, which has been often referred to as '*Wolbachia* sweep' (O'Neill *et al*. 1997; Werren 1997; Stouthamer *et al*. 1999; Bourtzis & Miller 2003).

Previous theoretical and empirical studies have suggested several possibilities as to how *Wolbachia* endosymbionts can affect the evolution of closely related host species. One possibility is that *Wolbachia* infection may facilitate the speciation of the host arthropods. In closely related parasitoid wasps of the genus *Nasonia*, it was suggested that the *Wolbachia*-induced bidirectional CI preceded the evolution of other forms of reproductive isolation (Bordenstein *et al*. 2001). Another possibility is that *Wolbachia* infection may affect the genetic structure and diversity of the host arthropods, particularly those of maternally inherited elements like mitochondria. Owing to the action of CI or other reproductive phenotypes, even a small number of *Wolbachia*-infected individuals that invaded a previously uninfected population can lead to rapid spread and fixation of the infection (Turelli & Hoffmann 1991; Turelli *et al*. 1992). In association with the *Wolbachia* sweep, a small number of mitochondrial haplotypes of the founder individuals are also spread in the population, whereby mitochondrial diversity of the population will be significantly reduced. When interspecific hybridization involves, *Wolbachia* infection may facilitate introgression of mitochondrial genomes between closely related species. For example, decrease of mitochondrial diversity associated with *Wolbachia* infection has been reported from *Drosophila simulans* (Ballard *et al*. 1996; Dean *et al*. 2003). Association between *Wolbachia* infection and particular mitochondrial haplotypes has been known from a chrysomelid beetle (Keller *et al*. 2004) and fire ants (Shoemaker *et al*. 2003). Interspecific mitochondrial introgression putatively promoted by *Wolbachia* infection has been identified in *Acraea* butterflies (Jiggins 2003). The genetic structure of Y type and B type and their *Wolbachia* infection in Japanese populations of *E. hecabe* are of great interest in these contexts.

In this study, we investigated the diversity of nuclear and mitochondrial genes in Japanese populations of Y type and B type of *E. hecabe*. The phylogeny based on nuclear genes agreed with the distinction between Y type and B type. However, the phylogeny based on mitochondrial genes did not reflect the distinction. We found that the mitochondrial phylogeny was in perfect agreement with the pattern of *Wolbachia* infection, suggesting that the *Wolbachia* infection has affected the mitochondrial genetic structure of the host insects. On the basis of these results together with the geological and biogeographical knowledge of the Japanese Archipelago, we proposed an evolutionary hypothesis on the invasion and spread of *Wolbachia* infection in Y type and B type of *E. hecabe*.

Materials and methods

Insects

In total, 62 fresh adult females of *Eurema hecabe* were collected from 27 geographical localities in Japan (Table 1, Fig. 1). Two individuals were collected from every locality except Honjo, Matsudo, Okinawa Island and Iriomote

Table 1 Butterfly samples used in this study

Locality (wing fringe type)*		Infection status†	Accession no. of nucleotide sequence			
			ND5	*16S rRNA*	*EF1-α*	*Tpi*
Eurema hecabe						
1 Sendai, Miyagi	(Y)	–	AB194755	AB194745	AB194749	AB231163
	(Y)	–	AB194755	AB194745	AB194749	AB231163
2 Nigata, Nigata	(Y)	+	AB194760	AB194743	AB194749	AB231171
	(Y)	+	AB194760	AB194743	AB194749	AB231171
3 Hitachi, Ibaraki	(Y)	+	AB194761	AB194746	AB194749	AB231169
						AB231170
	(Y)	+	AB194761	AB194746	AB194749	—
4 Honjo, Saitama	(Y)	+	AB194761	AB194746	AB194749	AB231167
	(Y)	+	AB194761	AB194746	AB194749	—
	(Y)	–	AB194758	AB194745	AB194749	AB231168
						AB231168
	(Y)	–	AB194758	AB194745	AB194749	—
5 Matsudo, Chiba	(Y)	+	AB194761	AB194746	AB194749	AB231164
	(Y)	+	AB194761	AB194746	AB194749	—
	(Y)	–	AB194757	AB194745	AB194749	AB231165
	(Y)	–	AB194757	AB194745	AB194749	—
6 Takao, Tokyo	(Y)	–	AB194756	AB194745	AB194749	AB231166
	(Y)	–	AB194756	AB194745	AB194749	—
7 Kametomi, Yamanashi	(Y)	+	AB194760	AB194743	AB194749	AB231172
	(Y)	+	AB194760	AB194743	AB194749	—
8 Omi, ShigaOmi, Shiga	(Y)	+	AB194760	AB194743	AB194749	AB231174
						AB231175
	(Y)	+	AB194760	AB194743	AB194752	—
9 Nara, Nara	(Y)	+	AB194761	AB194743	AB194749	AB231178
	(Y)	+	AB194761	AB194743	AB194749	—
10 Ibaragi, Osaka	(Y)	+	AB194761	AB194743	AB194749	AB231173
	(Y)	+	AB194761	AB194743	AB194749	—
11 Koya, Wakayama	(Y)	+	AB194761	AB194743	AB194749	AB231176
						AB231177
	(Y)	+	AB194761	AB194743	AB194754	—
12 Okayama, Okayama	(Y)	+	AB194761	AB194743	AB194749	AB231179
						AB231180
	(Y)	+	AB194761	AB194743	AB194749	—
13 Matsue, Shimane	(Y)	+	AB194761	AB194743	AB194749	AB231181
						AB231182
	(Y)	+	AB194761	AB194743	AB194749	—
14 Kagawa, Kagawa	(Y)	+	AB194761	AB194743	AB194749	AB231183
	(Y)	+	AB194761	AB194743	AB194749	—
15 Tsushima Is., Nagasaki	(Y)	–	AB194761	AB194745	AB194749	AB231188
	(Y)	–	AB194759	AB194745	AB194753	AB231189
	(Y)	–				AB231190
16 Fukuoka, Fukuoka	(Y)	+	AB194761	AB194743	AB194749	AB231184
						AB231185
	(Y)	+	AB194761	AB194743	AB194749	—
17 Kumamoto, Kumamoto	(Y)	+	AB194761	AB194743	AB194749	AB231186
	(Y)	+	AB194761	AB194743	AB194751	AB231187
18 Kagoshima, Kagoshima	(Y)	+	AB194761	AB194743	AB194749	AB231191
	(Y)	+	AB194761	AB194743	AB194749	—
19 Tanegashima Is., Kagoshima	(Y)	+	AB194762	AB194748	AB194749	AB231192
	(Y)	+	AB194762	AB194748	AB194749	—
20 Yakushima Is., Kagoshima	(Y)	+	AB194762	AB194748	AB194749	AB231193
	(Y)	+	AB194762	AB194748	AB194749	—

Table 1 *Continued*

Locality (wing fringe type)*		Infection status†	Accession no. of nucleotide sequence			
			ND5	*16S rRNA*	*EF1-α*	*Tpi*
21 Okinawa Is., Okinawa	(Y)	+	AB194763	AB194747	AB194749	AB231194
	(Y)	+	AB194763	AB194747	AB194749	AB231194
	(Y)	+	AB194763	AB194747	AB194750	AB231195
			AB194763	AB194747	AB194750	AB231196
	(B)					AB231197
	(B)	+	—	—	—	AB231198
	(B)	+				AB231199
22 Kume Is., Okinawa	(B)	+	AB194763	AB194743	AB194750	AB231200
						AB231201
	(B)	+	AB194763	AB194743	AB194750	AB231202
23I shigaki Is., Okinawa	(B)	+	AB194761	AB194743	AB194750	AB231205
	(B)	+	AB194761	AB194743	AB194750	—
24 Taketomi Is., Okinawa	(B)	+	AB194761	AB194743	AB194750	—
	(B)	+	AB194761	AB194743	AB194750	—
25 Yonaguni Is., Okinawa	(B)	+	AB194761	AB194743	AB194750	AB231203
	(B)	+	AB194761	AB194743	AB194750	AB231204
26 Hateruma Is., Okinawa	(B)	+	AB194764	AB194744	AB194750	AB231206
						AB231207
	(B)	+	AB194764	AB194744	AB194750	AB231208
27 Iriomote Is., Okinawa	(B)	+	AB194761	AB194743	AB194750	AB231209
						AB231210
	(B)	+	AB194761	AB194743	AB194750	—
	(B)	+	AB194761	AB194743	AB194750	—
	(B)	+	AB194761	AB194743	AB194750	—
Eurema blanda						
Iriomote Is., Okinawa		–	AB211194	AB211195	AB211196	AB231211

*Wing fringe type: B, brown type; Y, yellow type.
†*Wolbachia* infection status: +, infected; –, uninfected.

Island, where four or five individuals each were collected. Field-collected adults were brought to the laboratory and stored at –20 °C until DNA extraction. The butterflies were classified into two types, yellow (Y) or brown (B), according to the fringe colour of forewings (Kato 1999, 2000a). The identification of Y type and B type was unequivocal for the fresh butterflies, although it was sometimes obscure for old butterflies whose wing fringe had been worn. The wing specimens were kept as vouchers. *Eurema blanda* collected from Iriomote Island (Table 1) was used as outgroup. Dissected thoracic muscles were subjected to DNA extraction by using the DNeasy Tissue Kit (QIAGEN).

PCR and sequencing

The nuclear *Tpi* gene, encoding triose phosphate isomerase, is located on the Z chromosome in lepidopteran species (Turner & Sheppard 1979). A segment of the gene containing a highly variable intron was amplified by using the primers (5′-GGTCACTCTGAAAGGAGAACCACTTT-3′) and (5′-CACAACATTTGCCCAGTTGTTGCCAA-3′) (Jiggins *et al*. 2001). The polymerase chain reaction (PCR) temperature profile was 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 2 min and 72 °C for 2 min, and finally 72 °C for 7 min. The PCR products were purified using the PCR Purification Kit (QIAGEN), ligated to the pGEM-T-easy vector (Promega), and transformed into *Escherichia coli* JM109 competent cells. Inserted plasmids were cloned and extracted from the transformants, and then subjected to DNA sequencing by using the ABI PRISM BigDye™ terminator chemistry and the ABI 3100 capillary sequencer (Applied Biosystems).

The autosomal nuclear *EF1-α* gene, encoding elongation factor 1α for eukaryotic translation process, was amplified by using the primers EFS599 (5′-CCGGTTTGAACTCAGATCATGT-3′) and EFA923 (5′-CGCCTGTTTAACAAAAACAT-3′) (designed by B. Normark). The PCR temperature profile was 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 2 min and 72 °C for 2 min, and finally 72 °C for 7 min. The PCR products were purified using the PCR

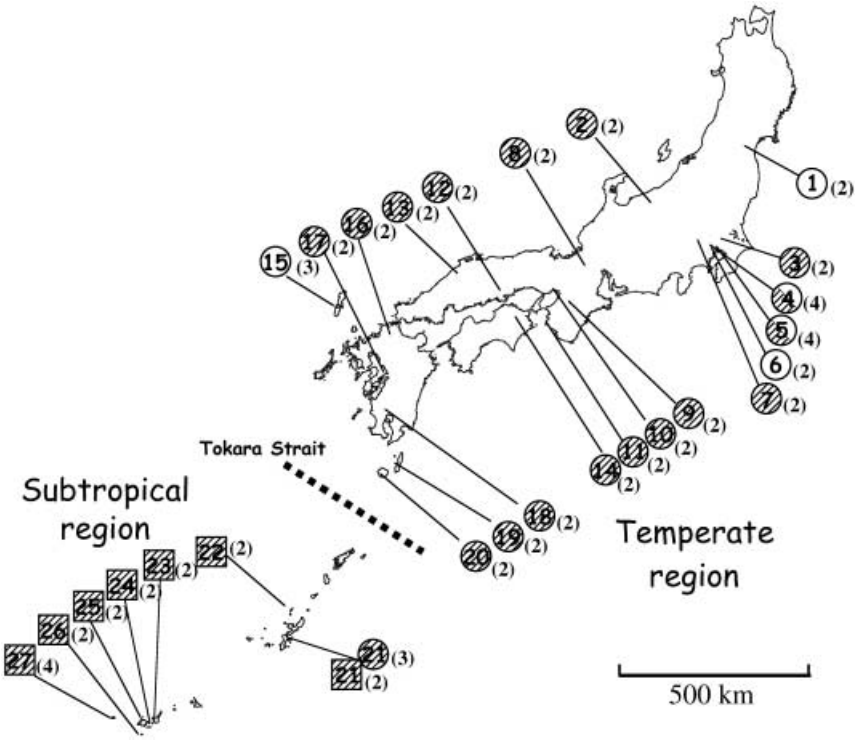


Fig. 1 Collection localities of *Eurema hecabe* used in this study. Locality numbers reflect those in Table 1. Sample sizes are shown in parentheses. Circle, Y-type population; square, B-type population; shaded, all specimens infected with *Wolbachia*; half-shaded, a part of specimens infected with *Wolbachia*; no shade, *Wolbachia* infection not detected. Y type and B type were not found at the same locality except for Okinawa Island (locality no. 21).

Purification Kit (QIAGEN) and directly subjected to DNA sequencing. However, some samples that showed ambiguous signals were subjected to the cloning procedure as described above.

The mitochondrial *ND5* gene, encoding NADH dehydrogenase subunit 5, was amplified by using the primers V1 (5′-CCTGTTTCTCTGCTTTAGTTTAGTTCA-3′) and A1 (5′-AATATDAGGTATAAATCATAC-3′) (Yagi *et al*. 1999). The mitochondrial *16S rRNA* gene, encoding large subunit ribosomal RNA, was amplified by the primers LR-J-12887 (5′-CCGGTTTGAACTCAGATCATGT-3′) and LR-N-13398 (5′-CGCCTGTTTAACAAAAACAT-3′) (Simon *et al*. 1994). The PCR temperature profile for the mitochondrial genes was 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 46 °C for 2 min and 72 °C for 2 min, and finally 72 °C for 7 min. The PCR products were purified by using the PCR Purification Kit and directly subjected to DNA sequencing.

The nucleotide sequences of *Tpi*, *EF1-α*, *ND5* and *16S rRNA* genes from *E. hecabe* and *E. blanda* were deposited in the DDBJ/EMBL/GenBank databases. Accession numbers are listed in Table 1.

Phylogenetic analyses

Nucleotide sequences were aligned by using the program package CLUSTAL_X (Thompson *et al*. 1997). The sequence data of *Tpi*, *EF1-α*, *ND5* and *16S rRNA* genes were analysed separately. Phylogenetic trees were constructed by the maximum-likelihood (ML), the neighbour-joining (NJ) and the maximum-parsimony (MP) methods by using the program package PAUP version 4.0b10 (Swofford 2001). To visualize genealogical relationships and possible population substructure, networks on the basis of the sequence data were constructed by the statistical parsimony algorithm (Templeton *et al*. 1992) implemented in the program package TCS version 1.13 (Clement *et al*. 2000). The TCS program calculates the minimal number of mutational steps by which the sequences can be joined with > 95% confidence.

Detection and identification of Wolbachia

PCR detection of *Wolbachia* infection was performed by using the primers (5′-TTGTAGCCTGCTATGGTATAACT-3′) and (5′-GAATAGGTATGATTTTCATGT-3′) for the 16S rRNA gene (O'Neill *et al*. 1992), and the primers 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and 691R (5′-AAAAATTAAACGCTACTCCA-3′) for the *wsp* gene (Zhou *et al*. 1998). Infection with *Wolbachia* of either the 'A' or 'B' supergroup was differentiated by specific PCR targeting the *ftsZ* gene: the primers ftsZAdf (5′-CTCAAGCACTAGAAAAGTCG-3′) and ftsZAdr (5′-TTAGCTCCTTCGCTTACGTG-3′) for the A supergroup, and the primers ftsZBf (5′-CCGATGCTCAAGGGTTAGAG-3′) and ftsZBr (5′-CCACTTAACTGTTTGGTTTG-3′) for the B supergroup (Werren *et al*. 1995). By using these *ftsZ*

primer sets, A super group and B super group of *Wolbachia* were identified by presence/absence of the PCR products. For characterization of *Wolbachia* strains in *E. hecabe*, single butterflies were chosen to represent different mitochondrial haplotypes. A 0.6-kb segment of *wsp* gene was amplified from these samples by using the primers 81F and 691R, and the PCR products were cloned and subjected to DNA sequencing. The nucleotide sequences of *wsp* gene of *Wolbachia* sp. from *E. hecabe* were deposited in the DDBJ/EMBL/GenBank databases under accession nos AB210826–AB210831. The sequence data were manually aligned with published *Wolbachia* sequences from other insects. Phylogenetic trees were constructed by the ML, NJ and MP methods by using the PAUP 4.0b10.

Statistical tests for reduced mitochondrial genetic diversity

In an attempt to statistically detect reduced mitochondrial genetic diversity in populations of *E. hecabe*, which can occur in association with *Wolbachia* sweep, the HKA test (Hudson *et al*. 1987) was performed by using the program packages DNASP version 4.0 (Rozas *et al*. 2003) and HKA (written by Jody Hey; http://lifesci.rutgers.edu/~heylab/ProgramsandData/Programs/HKA/HKA_Documentation.htm) on the basis of the sequence data of mitochondrial (*ND5*, *16S RDNA*) and nuclear (*Tpi*, *EF1-α*) genes. The mitochondrial *ND5* data and *16S rRNA* data were combined because of their genetic linkage. In the analysis, the effective population sizes of the mitochondrial gene (*ND5* + *16S rRNA*), the Z chromosomal gene (*Tpi*) and the autosomal gene (*EF1-α*) were corrected by using the ratio of 1:3:4. Statistical significance was evaluated by 10 000 coalescent simulations. To calculate interspecific divergence values, *E. blanda* was used as outgroup.

Results

Occurrence of yellow and brown types of Eurema hecabe *across the Japanese Archipelago*

In total, 62 adult females of *Eurema hecabe* from 27 geographical localities in Japan were collected (Table 1, Fig. 1). The samples from northeastern regions including the Honshu mainland (locality nos 1–13), the Shikoku mainland (no. 14), the Kyushu mainland (nos 16–18), Tsushima Island (no. 15), Tanegashima Island (no. 19) and Yakushima Island (no. 20), where the climate is mostly temperate, were all Y type. On the other hand, the samples from southwestern regions including Kume Island (no. 22), Ishigaki Island (no. 23), Taketomi Island (no. 24), Yonaguni Island (no. 25), Hateruma Island (no. 26) and Iriomote Island (no. 27), where the climate is subtropical, were all B type. Only in Okinawa Island (no. 21), both Y type and B type were found, as had been reported (Kato 1999, 2000a). Other than these samples, we had observed many more individuals of adult males and females of *E. hecabe*, which consistently agreed with the distribution patterns of only Y type in northeastern regions, only B type in southwestern islands, and both Y type and B type in Okinawa Island.

Phylogeny of nuclear gene agreed with yellow and brown types of E. hecabe

In total, 42 butterfly samples were subjected to cloning and sequencing of the *Tpi* gene. Four clones for each of the samples were sequenced, whereby 168 sequences of 517–582 bp were obtained and aligned. The 457-bp alignment, from which aligned nucleotide sites containing gaps had been excluded, were polymorphic in 184 sites, representing 42 haplotypes. Each of the four sequences from single samples constituted one or two haplotypes. On the phylogeny, the haplotypes were split into two discrete monophyletic groups. One group with 29 haplotypes consisted of all Y type, whereas the other group with 13 haplotypes consisted of all B type (Fig. 2).

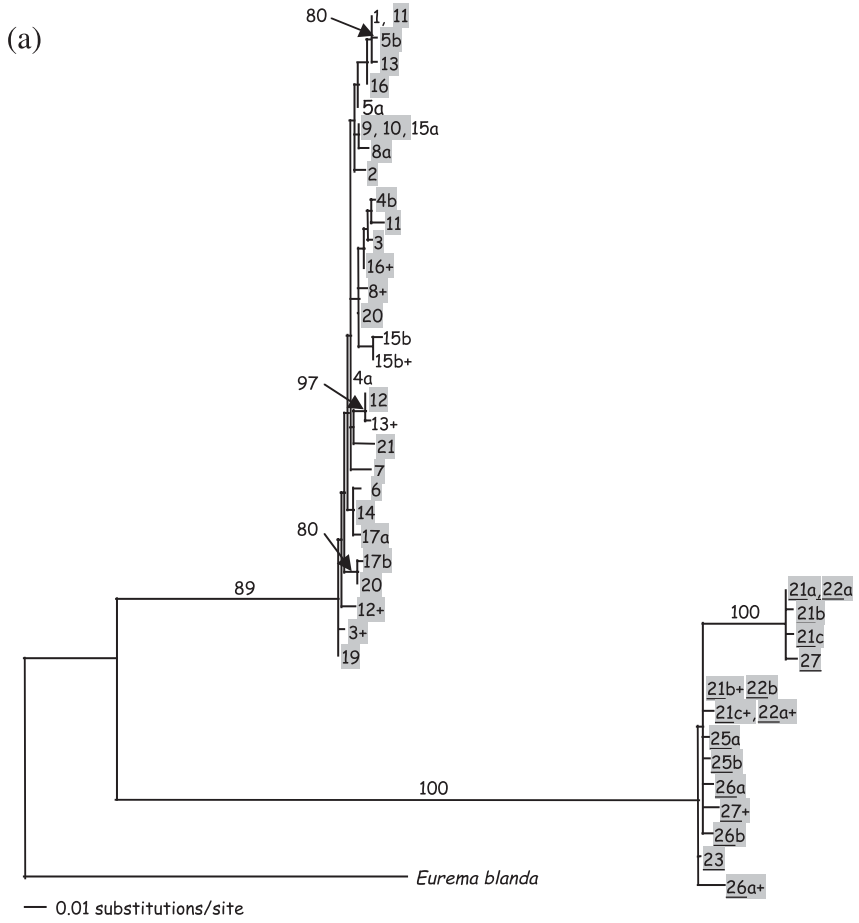
The 62 butterflies sequenced for the *EF1-α* gene, 286 bp in size, were polymorphic in five nucleotide sites, constituting six haplotypes that could be linked to each other by single substitutions. Three samples (from populations 8, 11 and 17) were heterozygotes in which both the alleles were subjected to the analysis. One of the haplotypes was morphologically all B type, while the other five haplotypes were all Y type (Fig. 3).

Thus, the data of the nuclear genes were in good agreement with the morphological (Kato 1999), ecological (Kato *et al*. 1992), physiological (Kato & Handa 1992), reproductive (Kato 2000b; Kobayashi *et al*. 2001) and allozyme (Nomura & Kato 1993) data that had supported the differentiation of Y type and B type in *E. hecabe*.

Phylogeny of mitochondrial genes disagreed with yellow and brown types of E. hecabe

The 62 butterflies sequenced for the *ND5* gene, 716 bp in size, were polymorphic in 26 nucleotide sites, including 24 parsimoniously informative sites and constituting 10 haplotypes. On the phylogeny, the haplotypes were split into two discrete monophyletic groups. One group with five haplotypes (from populations 1, 4, 5, 6 and 15) consisted of all Y type, whereas the other group with five haplotypes contained both Y type and B type (Fig. 4).

The 62 individuals sequenced for the *16S rRNA* gene, 439–441 bp in size, were polymorphic in eight positions, which were all parsimoniously informative and constituting six haplotypes. On the phylogeny, a basal haplotype, which was from populations 1, 4, 5, 6 and 15, represented



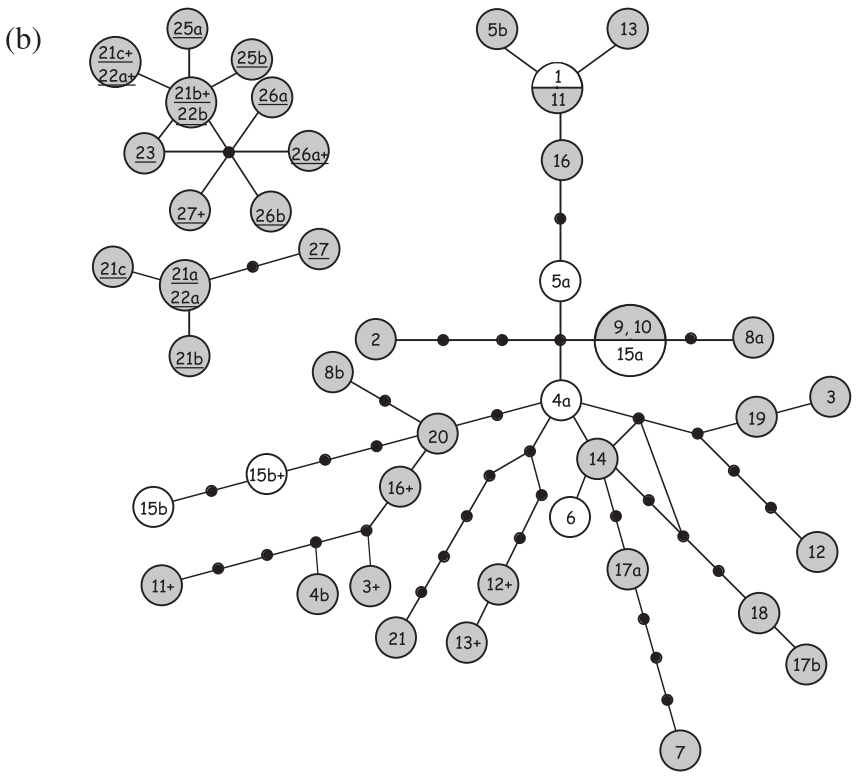


Fig. 2 (a) Maximum-likelihood phylogeny on the basis of the nuclear *Tpi* gene sequences (457 aligned nucleotide sites) of *Eurema hecabe*. A 50% majority-rule consensus bootstrap tree is shown, with bootstrap values over 50% at the nodes. Neighbour-joining and maximum-parsimony trees gave substantially the same topologies (data not shown). (b) Parsimony network on the basis of the *Tpi* gene sequences (457 bp). A network with 95% connection limit is shown, where the size of each of the circles reflects the number of individuals with each of the haplotypes, respectively. In the figures, the sequences are labelled according to the locality numbers shown in Table 1 and Fig. 1. The letters (a or b) next to the locality numbers indicate different sequences from different individuals collected at the same locality, and the plus signs (+) next to the locality numbers indicate different sequences from the same individuals. Underlined numbers indicate B-type butterflies, while the others are Y-type ones. Shaded numbers indicate *Wolbachia*-infected individuals, whereas the others are uninfected ones.

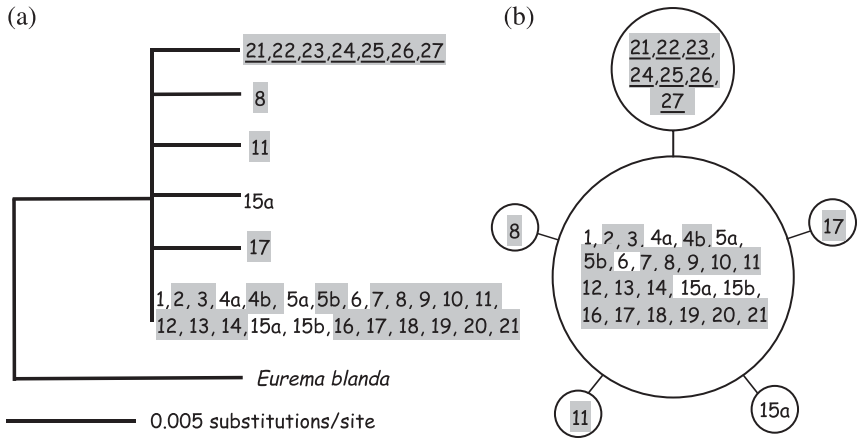


Fig. 3 (a) Maximum-likelihood phylogeny on the basis of the nuclear *EF1-α* gene sequences (286 aligned nucleotide sites) of *Eurema hecabe*. A 50% majority-rule consensus bootstrap tree is shown, although bootstrap values are less than 50% for all nodes in this tree. Neighbour-joining and maximum-parsimony trees gave substantially the same topologies (data not shown). (b) Parsimony network on the basis of the *EF1-α* gene sequences. A network with 95% connection limit is shown, where the size of each of the circles reflects the number of individuals with each of the haplotypes, respectively. In the figures, the sequences are labelled as in Fig. 2.

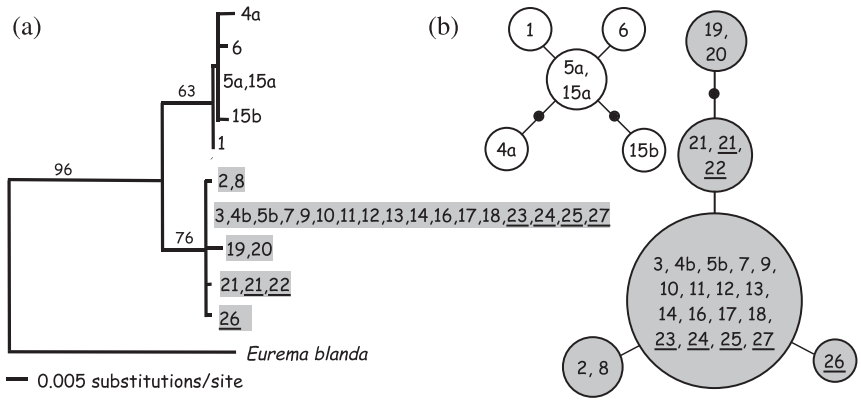


Fig. 4 (a) Maximum-likelihood phylogeny on the basis of the mitochondrial *ND5* gene sequences (716 aligned nucleotide sites) of *Eurema hecabe*. A 50% majority-rule consensus bootstrap tree is shown, with bootstrap values over 50% at the nodes. Neighbour-joining and maximum-parsimony trees gave substantially the same topologies (data not shown). (b) Parsimony network on the basis of the *ND5* sequences. A network with 95% connection limit is shown, where the size of each of the circles reflects the number of individuals with each of the haplotypes, respectively. In the figures, the sequences are labelled as in Fig. 2.

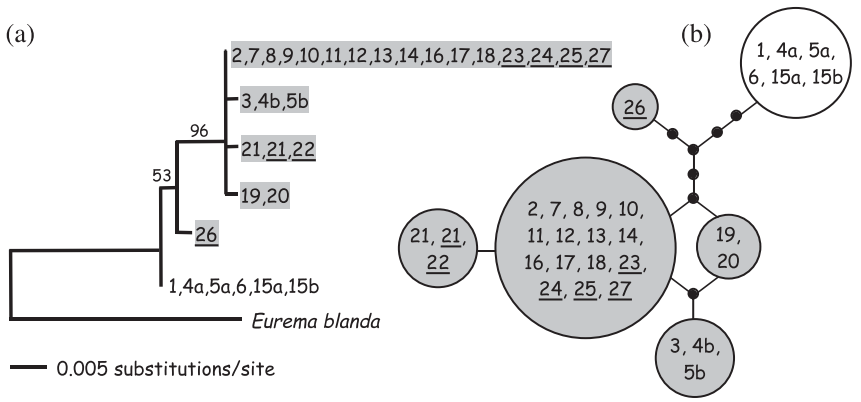


Fig. 5 (a) Maximum-likelihood phylogeny on the basis of the mitochondrial *16S rRNA* gene sequences (441 aligned nucleotide sites) of *Eurema hecabe*. A 50% majority-rule consensus bootstrap tree is shown, with bootstrap values over 50% at the nodes. Neighbour-joining and maximum-parsimony trees gave substantially the same topologies (data not shown). (b) Parsimony network on the basis of the *16S rRNA* sequences. A network with 95% connection limit is shown, where the size of each of the circles reflects the number of individuals with each of the haplotypes, respectively. In the figures, the sequences are labelled as in Fig. 2.

all Y type, whereas the other four haplotypes, which constituted a clade, contained both Y type and B type (Fig. 5).

These results indicated that the phylogeny of the mitochondrial genes disagreed with the phylogeny of the nuclear genes, and consequently disagreed with the morphological, ecological, reproductive and allozyme differentiation of Y type and B type in *E. hecabe*.

A single genotype of Wolbachia *was prevailing in southwestern populations of yellow and brown types of* E. hecabe

Of 62 butterflies examined by diagnostic PCR, 54 (87%) were *Wolbachia* positive (Table 1). Specific PCR targeting *ftsZ* identified these *Wolbachia* all in the B supergroup.

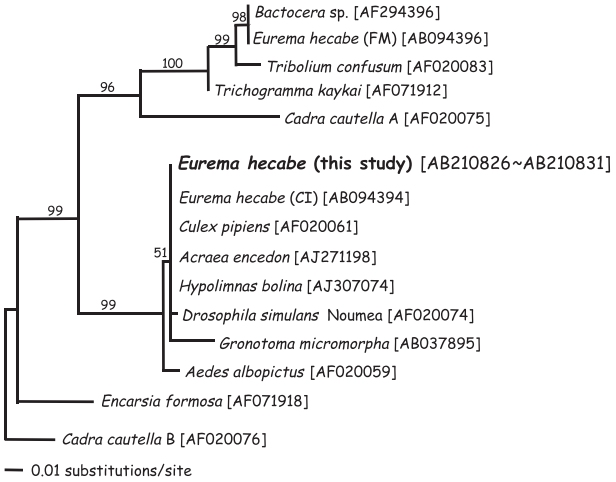


Fig. 6 Maximum-likelihood phylogeny on the basis of the *wsp* gene sequences of *Wolbachia* endosymbionts from *Eurema hecabe* and other insects. A 50% majority-rule consensus bootstrap tree is shown, with bootstrap values over 50% at the nodes. Neighbour-joining and maximum-parsimony trees gave substantially the same topologies (data not shown). Scientific names of the host insects are shown. In brackets are nucleotide sequence accession numbers. In parentheses are reproductive phenotypes (CI, cytoplasmic incompatibility; FM, feminization) caused by the *Wolbachia* strains in *E. hecabe*.

The *wsp* sequences (585 bp) from six individuals, which represented six distinct mitochondrial haplotypes (5b, 8, 9, 20, 21 and 26), were completely identical to each other and placed in the B supergroup in the *Wolbachia* phylogeny (Fig. 6). The *wsp* sequences were identical to the sequence of a CI-inducing *Wolbachia* strain previously identified from *E. hecabe* (Hiroki *et al*. 2004).

When the data of *Wolbachia* infection were mapped on the butterfly populations (Fig. 1), southwestern populations were all infected. All B-type butterflies were infected, whereas Y-type butterflies contained infected and uninfected individuals. A few populations with uninfected individuals were marginally located in northeastern regions (nos 1, 4, 5 and 6 in Fig. 1) and in an isolated island (no. 15 in Fig. 1).

These results suggested that a single genotype of *Wolbachia* prevailed in southwestern populations of Y type and B type of *E. hecabe*.

Wolbachia *infection was coincident with a particular group of mitochondrial haplotypes*

When the data of *Wolbachia* infection were mapped on the phylogeny of the mitochondrial genes, a striking pattern emerged. The mitochondrial haplotypes were consistently split into two groups, one all infected and the other all uninfected (Figs 4 and 5). The infected group contained both Y-type and B-type butterflies whereas the uninfected group consisted of only Y-type butterflies. These results indicated that the *Wolbachia* infection in *E. hecabe* did not reflect the distinction of Y type and B type, but was perfectly coincident with a particular group of mitochondrial haplotypes.

Table 2 The HKA test for reduction in mitochondrial genetic diversity among yellow type and brown type populations of *Eurema hecabe*

	Brown (all infected)			Yellow (uninfected)			Yellow (infected)		
	mtDNA	Nuclear DNA		mtDNA	Nuclear DNA		mtDNA	Nuclear DNA	
	ND5 +16S	*Tpi*	*EF1-α*	*ND5 +16S*	*Tpi*	*EF1-α*	*ND5 +16S*	*Tpi*	*EF1-α*
Intraspecific polymorphism									
No. of samples (No. of alleles)	16 (16)	11 (22)	16 (32)	12 (12)	7 (14)	12 (24)	36(36)	23(46)	36(72)
Segregating sites (obs*)	7	39	0	7	16	1	6	46	3
Segregating sites (exp†)‡	7.18/5.87	38.82	1.13	4.65/6.71	18.35	1.12	10.01/7.19	41.99	1.81
Total number of sites§	1157	496	286	1157	496	286	1157	496	286
Interspecific divergence									
No. of differences (obs*)	91.00	155.00	5.00	89.00	119.00	4.00	92.00	118.00	4.00
No. of differences (exp†)‡	90.82/92.13	155.18	3.87	91.35/89.29	116.65	3.88	87.99/90.81	122.01	5.19
Sum of deviation¶		0.0028	1.4728		0.8327	0.0831		1.1579	1.0836
P value**		0.96	0.22		0.36	0.77		0.28	0.30

*Observed value.
†Expected value.
‡In the mitochondrial DNA columns, value expected on the basis of *Tpi* data/value expected on the basis of *EF1-α* data is shown.
§Nucleotide sites containing alignment gaps were excluded.
¶The effective population sizes of the mitochondrial gene (*ND5 +16S rRNA*), the Z-chromosomal gene (*Tpi*) and the autosomal gene (*EF1-α*) were corrected.
**Proportion of the values in the coalescent simulation that were greater than the observed value.

Statistical test for reduced mitochondrial genetic diversity

To statistically evaluate the extent of nuclear–mitochondrial genetic divergence in *E. hecabe* populations, we performed the HKA test, which compares polymorphism and divergence at two or more unlinked genetic loci, by using the sequence data of the mitochondrial and nuclear genes from Y-type and B-type populations. Neither of B-type individuals (all infected), uninfected Y-type individuals nor infected Y-type individuals exhibited significant reduction in their mitochondrial genetic diversity compared to their nuclear genes, *Tpi* and *EF1-α* (Table 2).

Discussion

Morphological, ecological, physiological, reproductive and genetic lines of evidence have consistently suggested that Y type and B type of *Eurema hecabe* constitute biologically distinct groups, so-called sibling species. Our data of nuclear genes, *Tpi* and *EF1-α*, also reflected the distinction of Y type and B type (Figs 2 and 3), confirming the conventional view.

Unexpectedly, however, our data of mitochondrial genes, *ND5* and *16S rRNA*, contradicted the above-listed lines of evidence. Namely, the phylogeny based on the mitochondrial genes did not agree with the distinction of Y type and B type (Figs 4–6). The discrepancy between the nuclear and mitochondrial genes must be, if possible, reconciled and interpreted by some naturally occurring mechanisms.

There are several mechanisms that may cause such discrepancy of nuclear and mitochondrial genealogies between closely related species. One mechanism is stochastic lineage sorting of ancestral mitochondrial DNA polymorphisms (Brower *et al*. 1996; Page & Charleston 1998). Introgression of mitochondrial DNA through interspecific hybridization can be another possible mechanism (Brower 1994; Dowling & Secor 1997). Alternatively, if a particular mitochondrial haplotype is associated with a significant selective advantage, the haplotype will rapidly spread in the populations of the species and significantly bias the genetic structure (Rand 2001). It should be also noted that *Wolbachia* endosymbionts, which cause CI or other reproductive phenotypes, can facilitate such discrepancy of nuclear and mitochondrial genealogies, by driving the spread of associated mitochondrial genomes, such as those introgressed through interspecific hybridization (Jiggins 2003).

Strikingly, the phylogeny of the mitochondrial genes was in perfect agreement with the pattern of *Wolbachia* infection (Figs 4 and 5), suggesting that the mitochondrial genetic structure of the host insects may be strongly affected by the *Wolbachia* infection. It was reported that CI-inducing *Wolbachia* is prevalent in *E. hecabe* populations (Hiroki *et al*. 2004), and the *Wolbachia* endosymbionts identified in this study were certainly inferred to be CI-inducing type on the basis of their *wsp* gene sequence (Fig. 6). Thus, CI-driven population sweep of mitochondrial DNA is a candidate mechanism responsible for the phylogenetic pattern, while the possibility cannot be excluded that less frequent feminizing *Wolbachia* strain (Hiroki *et al*. 2002, 2004) may also contribute to the process to a lesser extent.

Y-type butterflies and B-type butterflies were mostly allopatric in Japan, and all B-type butterflies were infected whereas Y-type butterflies contained both infected and uninfected individuals (Fig. 1). Mitochondrial haplotypes of *Wolbachia*-infected Y-type butterflies were in common with those of B-type butterflies and distinct from those of uninfected Y-type butterflies (Figs 4 and 5). The uninfected butterflies were found only in marginal Y-type populations, which were geographically distant from B-type populations (Fig. 1). *Wolbachia* endosymbionts from Y-type populations were genetically indistinguishable from those from B-type populations on the basis of a fast-evolving gene *wsp* (Fig. 6). All these patterns are best explained by the scenario that the *Wolbachia*-associated mitochondrial haplotypes had been originally present in B-type populations, were introgressed from B-type populations into Y-type populations, and have been spreading in Y-type populations by hitch-hiking the *Wolbachia* sweep.

How many times has the invasion of the *Wolbachia* strain from B-type populations to Y-type populations occurred? On the basis of genetic and ecological lines of evidence, we infer that the *Wolbachia* invasions have probably occurred repeatedly. Multiple mitochondrial haplotypes were identified in the Y-type butterflies infected with the *Wolbachia* strain (Figs 4 and 5), which is suggestive of multiple invasions from different sources. In Japan, strayer butterflies from subtropical areas have been constantly recorded in summer and autumn, which are carried by a southwesterly seasonal wind (Nakasuji 1988). Thus, it appears plausible that a certain number of *Wolbachia*-infected B-type butterflies are constantly supplied to Y-type populations by the seasonal wind, which presupposes the putative repeated invasions.

If the host populations have recently experienced a *Wolbachia*-driven population sweep, their mitochondrial DNA may display reduced polymorphism compared to nuclear loci that are not affected by the sweep. Contrary to the expectation, however, the HKA test detected no significant reduction in their mitochondrial genetic diversity compared with their nuclear genes, which was not only in uninfected Y-type individuals but also in infected Y-type individuals and in all-infected B-type populations (Table 2). There are several possibilities as to why reduced mitochondrial genetic diversity was not observed in these populations in spite of the fixed infection with *Wolbachia*. One possibility is that since the evolutionary rate of mitochondrial genes is fast, the reduced mitochondrial genetic diversity in association with an ancient *Wolbachia* sweep

has been eroded by accumulated mutations. This scenario may apply to the all-infected B-type populations where the *Wolbachia* infection was estimated to be ancient. Another possibility is that repeated invasions and introgressions of *Wolbachia*-infected insects from different sources have cancelled out the reduced mitochondrial genetic diversity associated with the *Wolbachia* sweeps. This scenario appears to account for the situation in the infected Y-type individuals as argued above.

When did the population sweeps of the *Wolbachia* strain and associated mitochondrial haplotypes in Y-type populations occur? B type and Y type of *E. hecabe* were not only phenotypically but also genetically differentiated on the basis of nuclear genes (Figs 2 and 3). In contrast, on the basis of a fast-evolving gene *wsp* (Zhou *et al*. 1998), the majority of *Wolbachia* endosymbionts in Japanese populations of *E. hecabe* were of substantially identical genotype (Fig. 6). These results suggest that the spread of the *Wolbachia* strain is probably a very recent event in comparison with the divergence of Y type and B type of *E. hecabe*.

How could the *Wolbachia* strain and associated mitochondrial haplotypes be introgressed from B-type populations into Y-type populations in spite of the species barrier? On the basis of morphological, ecological, physiological, reproductive and genetic lines of evidence, Y type and B type of *E. hecabe* are certainly regarded as closely related but distinct biological species. In Okinawa Island (see no. 21 in Fig. 1), Y-type butterflies and B-type butterflies occur sympatrically, differ in use of host plants and ecological niches, and do not hybridize in the wild (Kato *et al*. 1992; Kato 1999, 2000a). Mating experiments in the laboratory revealed the presence of premating reproductive isolation between Y type and B type, mainly based on the choice by females (Kato 2000b; Kobayashi *et al*. 2001). However, it was also observed that copulation between Y type and B type sometimes occurred under rearing conditions, particularly when young female was involved in the mating (Kobayashi *et al*. 2001). The hybrid offspring were viable, although they often suffer higher mortality and reduced fitness probably due to postmating reproductive barriers (Kato, unpublished). We suppose that such hybridization events have occurred only rarely in the wild, but the incidence over evolutionary time might be sufficient to affect the genetic structure of the butterfly populations. Alternatively, such hybridization events might have occurred in the past when reproductive isolation between Y type and B type was not so established as it is.

On the basis of these results, arguments and circumstances together with the geological and biogeographical knowledge of the Japanese Archipelago, we propose an evolutionary hypothesis on the invasion and spread of *Wolbachia* infection in B type and Y type of *E. hecabe*, which parsimoniously account for these patterns in a consistent framework (Fig. 7). The ancestor of *E. hecabe* inhabited the ancient Japan (Fig. 7a). Tectonic movements and water-level shifts due to glacial activities formed the Tokara Strait, known as the Watase's line, an important zoogeographical line that vicariates the Japanese fauna of terrestrial vertebrates and invertebrates into the Palaearctic subregion and the Oriental subregion (Ujiié 1990; Ota 1998) (Fig. 7b, c). Following the vicariance, the mainland population and the southwestern population independently accumulated mutations and differentiated into Y type and B type, respectively (Fig. 7d). Probably during the differentiation process, a CI-inducing *Wolbachia* strain invaded the southwestern B-type population (Fig. 7e), which soon led to fixation of the infection (Fig. 7f). After the formation of the present Japanese Archipelago, which includes formation of many isolated islands in the southwestern part, the *Wolbachia* infection and the southwestern B-type mitochondrial haplotypes were occasionally introduced to the mainland Y-type population by migration of infected female butterflies to the mainland and rare successful hybridization with resident male butterflies (Fig. 7g). To date, the *Wolbachia* infection and the associated mitochondrial haplotypes have been spreading in the mainland Y-type population and approaching to fixation (Fig. 7h).

Interestingly, the hypothetical model appears to be compatible with the formation process of the Japanese Archipelago that was constructed on the basis of a wide array of geological and biogeographical lines of evidence (reviewed in Ota 1998). Over 20 million years ago (Ma), the ancient Japan consisted of a landmass (Fig. 7a). The Tokara Strait, which separated the southwestern and mainland parts of Japan, was formed 2–20 Ma (Fig. 7b, c). The southwestern part started to divide into many islands 0.1–1.5 Ma (Fig. 7g). Conceivably, Y type and B type of *E. hecabe* diverged after the formation of the Tokara Strait. The genetic distance (*D*) of *ND5* between the two groups (see Fig. 4a), which is likely to reflect the divergence, was calculated to be $D = 0.02795 \pm 0.00515$ by using the Kimura 2-parameter method (Kimura 1980). The molecular evolutionary rate of *ND5* in *Parnassius* butterflies was estimated to be 0.01D per 0.75 million years (Yagi *et al*. 2001). By extrapolating the evolutionary rate, the divergence of Y type and B type was calculated to have occurred around 2.1 Ma, which agreed with the nearest estimated age of the Tokara Strait formation.

Of course, it should be kept in mind that the above hypothesis might be too simplified to explain the complex evolutionary processes that had actually occurred. For example, Y-type butterflies co-occur with B-type butterflies in Okinawa Island (see Fig. 1). A recent survey also revealed several sporadic occurrences of Y-type butterflies in other southwestern islands (Kato & Yata 2005). These patterns could be accounted for by assuming occasional migration of the butterflies in an opposite direction. In the above hypothesis, the speciation of Y type and B type was

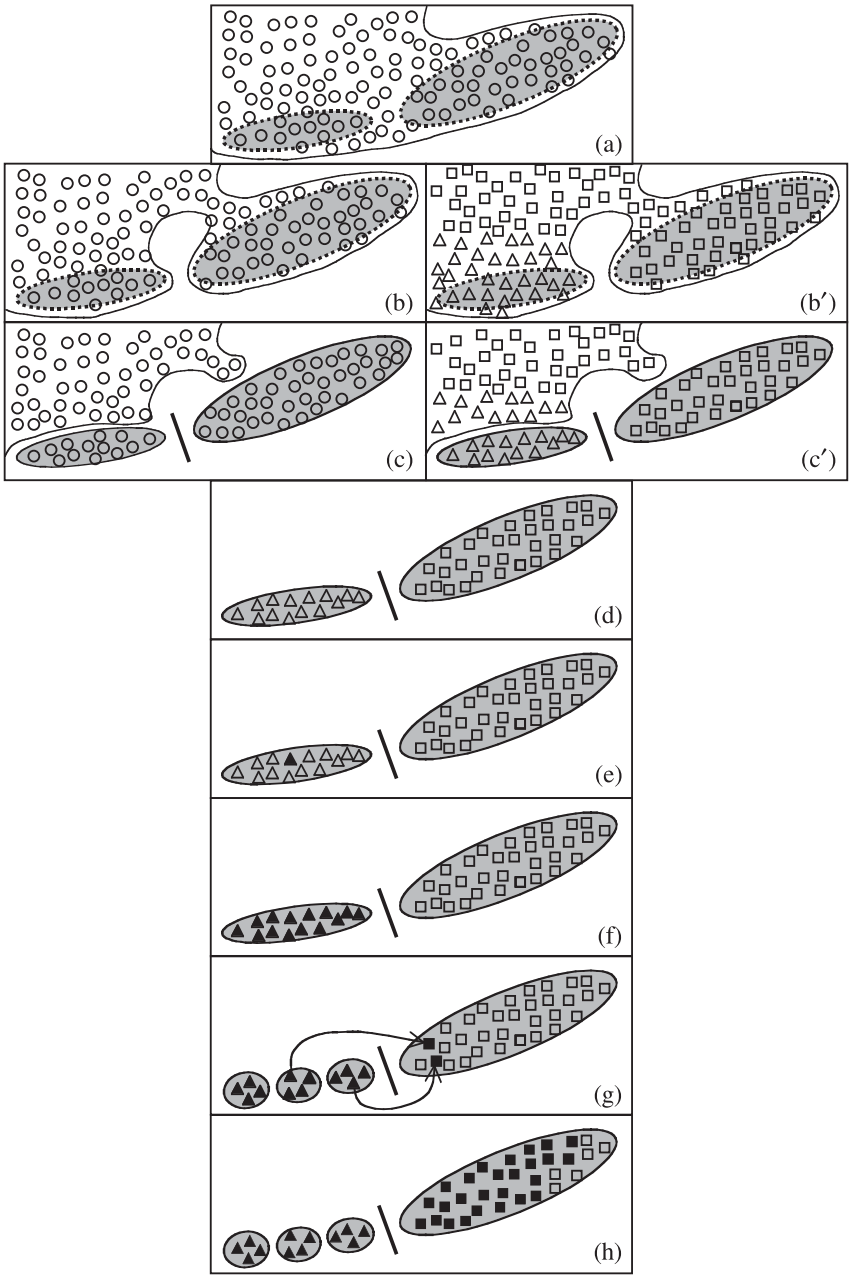


Fig. 7 An evolutionary hypothesis on the invasion and spread of *Wolbachia* infection in Y-type and B-type populations of *Eurema hecabe* in the formation process of the Japanese Archipelago. For details, refer to the text. Circles, squares and triangles indicate the speciation process of Y type and B type, and solid ones indicate *Wolbachia* infection. Shaded areas indicate the areas of the ancient Japanese Archipelago. Bold lines indicate the Tokara strait.

assumed to have occurred in the Japanese Archipelago. However, it is also conceivable that Y type and B type had already diverged in the Eurasian continent (Fig. 7b′), and Y type migrated from the north of ancient Japan while B type from the south (Fig. 7c′). The current geographical distribution of Y type and B type in temperate and subtropical regions, respectively, might be attributed not only to their evolutionary history but also to their physiological adaptation to the environments. To address which of these scenarios can best explain the evolutionary process of *E. hecabe* populations, wider sampling and molecular phylogenetic analysis of Y-type and B-type butterflies from Japan and adjacent Asian countries will be needed.

Recently, it has been reported that several Japanese butterflies are spreading their distribution range northwards, probably in association with the global warming (Parmesan *et al*. 1999; Yoshio & Ishii 2001; Yoshio 2002). Considering that B-type butterflies are more adapted to warm climate than Y-type butterflies (Kato & Handa 1992; Kato *et al*. 1992), the dynamics of phenotype, genotype and *Wolbachia* infection in Japanese populations of *E. hecabe* are also of great interest in this context.

Acknowledgements

We would like to thank M. Hiroki, D. Kageyama, K. Miura and O. Yata for helpful comments and suggestions on the manuscript, and S. Cho, K. Matsukura, A. Mochizuki, H. Sakamoto, T. Sato and H. Wago for samples of *E. hecabe*. S.N. was supported by the Japan Society for the Promotion of Science (JSPS) fellowship for Young Scientists.

References

Ballard JWO, Hatzidakis OJ, Karr TL, Kreitman M (1996) Reduced variation in *Drosophila simulans* mtDNA. *Genetics*, **144**, 1519–1528.

Bordenstein SR, O'Hara FP, Werren JH (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*, **409**, 707–710.

Bourtzis K, Miller T (2003) *Insect Symbiosis*. CRC Press, Boca Raton, Florida.

Brower AVZ (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceedings of the National Academy of Sciences, USA*, **91**, 6491–6495.

Brower AVZ, DeSalle R, Volger A (1996) Gene trees, species trees, and systematics: a cladistic perspective. *Annual Review of Ecology and Systematics*, **27**, 423–450.

Clement M, Posada D, Grandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Dean MD, Ballard KJ, Glass A, Ballard JWO (2003) Influence of two *Wolbachia* strains on population structure of east African *Drosophila simulans*. *Genetics*, **165**, 1959–1969.

Dowling TE, Secor CL (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593–619.

Futuyma D (1997) *Evolutionary Biology*, 3rd edn. Sinauer Associates, Sunderland, Massachusetts.

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences, USA*, **101**, 14812–14817.

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

Hiroki M, Tagami Y, Miura K, Kato Y (2004) Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **271**, 1751–1755.

Hudson RR, Kreitman M, Aguade M (1987) A test of neutral molecular evolution based on nucleotide data. *Genetics*, **116**, 153–159.

Jiggins FM (2003) Male-killing *Wolbachia* and mitochondrial DNA: selective sweep, hybrid introgression and parasite population dynamics. *Genetics*, **164**, 5–12.

Jiggins CD, Linares M, Naisbit RE, Salazar C, Yang ZH, Mallet J (2001) Sex-linked hybrid sterility in a butterfly. *Evolution*, **55**, 1631–1638.

Kato Y (1999) Fringe colour, seasonal morph and host-plant use of the pierid butterfly *Eurema hecabe* (L.) (Lepidoptera, Pieridae) on Okinawa-jima Island. *Transactions of the Lepidopterological Society of Japan*, **50**, 111–121 (in Japanese with English summary).

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

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

Kato Y, Handa H (1992) Seasonal polyphenism in a subtropical population of *Eurema hecabe* (Lepidoptera, Pieridae). *Japanese Journal of Entomology*, **60**, 305–318.

Kato Y, Sano M (1987) Role of photoperiod and temperature in seasonal morph determination of the butterfly *Eurema hecabe*. *Physiological Entomology*, **13**, 417–423.

Kato Y, Yata O (2005) Geographic distribution and taxonomical status of two types of *Eurema hecabe* (L.) (Lepidoptera, Pieridae) in south-western Japan and Taiwan. *Transactions of the Lepidopterological Society of Japan*, **56**, 171–183 (in Japanese with English summary).

Kato Y, Hiroki M, Handa H (1992) Interpopulation variation in adaptation of *Eurema hecabe* (Lepidoptera, Pieridae) to host plant. *Japanese Journal of Entomology*, **60**, 749–759.

Keller GP, Windsor DM, Saucedo JM, Werren H (2004) Reproductive effects and geographical distributions of two *Wolbachia* strains infecting the Neotropical beetle, *Chelymorpha alternans* Boh. (Chrysomelidae, Cassidinae). *Molecular Ecology*, **13**, 2405–2420.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

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

Nakasuji F (1988) Migration of butterflies and its evolutionary adaptation. *Special Bulletin of Lepidopterological Society of Japan*, **6**, 211–249. (in Japanese with English summary).

Nomura M, Kato Y (1993) Allozyme variation in *Eurema hecabe*. *The Nature and Insects*, **28**, 15–18 (in Japanese).

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

O'Neill SL, Hoffmann AA, Werren JH (1997) *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press, New York.

Ota H (1998) Geographic patterns of endemism and speciation in amphibians and reptiles of the Ryukyu archipelago, Japan, with special reference to their paleogeographical implications. *Researches on Population Ecology*, **40**, 189–204.

Page RDM, Charleston MA (1998) Tree within tree: phylogeny and historical associations. *Trends in Ecology & Evolution*, **13**, 356–359.

Parmesan C, Ryrholm N, Stefanescu C *et al.* (1999) Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature*, **399**, 579–583.

Rand DM (2001) The units of selection on mitochondrial DNA. *Annual Review of Ecology and Systematics*, **32**, 415–448.

Rozas J, Sánchez-Delbarrio JC, Messeguer X, Rozas R (2003) DNASP: DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.

Shoemaker DD, Keller G, Ross KG (2003) Effects of *Wolbachia* on mtDNA variation in two fire ant species. *Molecular Ecology*, **12**, 1757–1771.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primer. *Annals of the Entomological Society of America*, **87**, 651–701.

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

Swofford DL (2001) PAUP*: *Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4.0. Sinauer Associates, Sunderland, Massachusetts.

Templeton AR, Grandall KA, Sing GF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data: cladogram estimation. *Genetics*, **132**, 619–633.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876–4882.

Turelli M, Hoffmann AA (1991) Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature*, **353**, 440–442.

Turelli M, Hoffman AA, mcKechnie SW (1992) Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. *Genetics*, **132**, 713–723.

Turner JRG, Sheppard PM (1979) Absence of crossing-over in female butterflies (*Heliconius*). *Heredity*, **34**, 265–269.

Ujiié H (1990) Geological history of the Ryukyu Island Arc. In: *Nature of Okinawa; Geomorphology and Geology* (ed. Ujiié H), pp. 251–255. Hirugisha, Naha (in Japanese).

Werren JH (1997) Biology of *Wolbachia*. *Annual Review of Entomology*, **42**, 587–607.

Werren JH, Windsor D, Guo L (1995) Distribution of *Wolbachia* among Neotropical arthropods. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **262**, 197–204.

Yagi T, Sasaki G, Takebe H (1999) Phylogeny of Japanese papilionid butterflies inferred from nucleotide sequences of the mitochondrial *ND5* gene. *Journal of Molecular Evolution*, **48**, 42–48.

Yagi T, Kato T, Chichvarkhin A, Shinkawa T, Omoto K (2001) Molecular phylogeny of butterflies *Parnassius glacialis* and *P. stubbendorfit* at variation localities in East Asia. *Genes and Genetic System*, **76**, 229–234.

Yata O (1989) A revision of the Old World species of the genus *Eurema* Hübner (Lepidoptera, Pieridae) I. Phylogeny and zoogeography of the subgenus *Terias* Swainson and description of the subgenus *Eurema* Hübner. *Bulletin of the Kitakyushu Museum of Natural History*, **9**, 1–103.

Yoshio M (2002) Northward invasions of butterflies and climatic warming. *The Nature and Insects*, **37**, 4–7 (in Japanese).

Yoshio M, Ishii M (2001) A phenological study on northward invasion of the great mormon, *Papilio memnon* L. (Lepidoptera: Papilionidae). *Japanese Journal of Ecology*, **51**, 125–130 (in Japanese).

Zhou W, Rousset F, O'Neill S (1998) Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **265**, 509–515.

This work was carried out at the Chiba University as a part of the dissertation research of Satoko Narita, on the effects of *Wolbachia* infection in *Eurema* butterflies.
