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Population genetic structure and *Wolbachia* infection in an endangered butterfly, *Zizina emelina* (Lepidoptera, Lycaenidae), in Japan

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Abstract

Zizina emelina (de l'Orza) is listed on Japan's Red Data List as an endangered species because of loss of its principal food plant and habitat. We compared parts of the mitochondrial and nuclear genes of this species to investigate the level of genetic differentiation among the 14 extant populations. We also examined infection of the butterfly with the bacterium *Wolbachia* to clarify the bacterium's effects on the host population's genetic structure. Mitochondrial and nuclear DNA analyses revealed that haplotype composition differed significantly among most of the populations, and the fixation index F_{ST} was positively correlated with geographic distance. In addition, we found three strains of *Wolbachia*, one of which was a male killer; these strains were prevalent in several populations. There was linkage between some host mitochondrial haplotypes and the three *Wolbachia* strains, although no significant differences were found in a comparison of host mitochondrial genetic diversity with nuclear genetic diversity in *Wolbachia*-infected or -uninfected populations. These genetic analyses and *Wolbachia* infection findings show that *Z. emelina* has little migratory activity and that little gene flow occurs among the current populations.

Keywords: genetic structure, male killing, mitochondrial DNA, *Wolbachia*, *Zizina emelina*

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Introduction

The distributions of animal and plant species are extremely variable in time and space (e.g., Hewitt, 1996, 2000; Taberlet *et al*., 1998). Studies of geographic differentiation have revealed genetic patterns resulting from historical and contemporary demographic and evolutionary processes (Avise, 1994, 2000). The present-day distribution of genotypes is partly the result of climate-influenced changes in species distributions (Nichols & Hewitt, 1994; Schmitt & Müller, 2007; Saitoh *et al*., 2008; Dvořáková *et al*., 2010; Šmídová *et al*., 2011; Hucka *et al*., 2012). Information on genotype distribution will be much more effective for the conservation of endangered species than that on only physical distribution, especially in terms of identifying units for conservation and designing management plans (Moritz, 1994; Meffe & Carroll, 1997; Primack, 2004).

The lycaenid butterfly *Zizina emelina* (de l'Orza) (previously *Zizina otis emelina*) (Lepidoptera, Lycaenidae) is distributed in Honshu, Shikoku, and Kyushu in mainland Japan and

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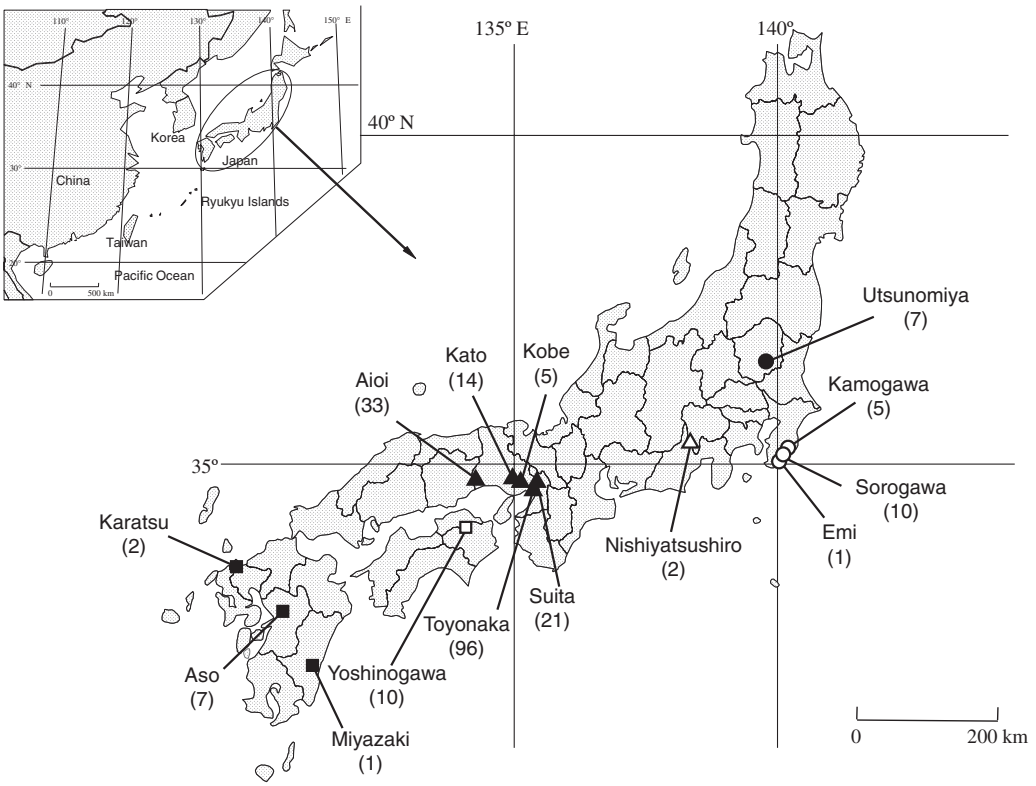

Fig. 1. Sites in Japan where *Z. emelina* was collected. Numbers of individuals collected are given in parentheses. Six regions, defined to separate the distributions by distance, are indicated by open circles (region 1), a closed circle (region 2), an open triangle (region 3), closed triangles (region 4), an open rectangle (region 5), and closed rectangles (region 6).

inhabits sunny grasslands of early successional stages, such as those of seashores, riverbanks, and farmland levees (Fukuda *et al*., 1984; Yago *et al*., 2008). This species uses the bird's-foot trefoil, *Lotus japonicus* (Fabaceae), as a larval food plant (Fukuda *et al*., 1984). The abundance of this plant, and thus the habitat of *Z. emelina*, is decreasing because of lack of mowing due to cessation of traditional rural land uses and because of the covering of river embankments with concrete (Nakamura, 2003; Sunose & Eda, 2003; Ishii, 2009; Mano & Fujii, 2009). *Zizina emelina* is listed on the Red Data List of Japan (Ministry of Environment, Japan, 2006, 2012) as Threatened IB. Recently, more than 40 small habitats of this species were found in and around Osaka International Airport in northern Osaka Prefecture, central Japan (Minohara *et al*., 2007; Ishii *et al*., 2008). At these sites there were few primary host plants (*L. japonicus*), and *Z. emelina* was using white clover, *Trifolium repens* (Fabaceae), or Japanese clover, *Kummerowia striata* (Fabaceae) (Minohara *et al*., 2007). Although Yago *et al*. (2008) have investigated the molecular systematics and biogeography of the genus *Zizina* globally, to our knowledge the genetic structure of populations of *Z. emelina* has not been examined.

Recently, Sakamoto *et al*. (2010, 2011) found that the Toyonaka population near Osaka International Airport was infected with two strains of *Wolbachia*, *w*EmeTn1 and *w*EmeTn2. *Wolbachia* is a maternally inherited bacterium that is widely distributed among various groups of arthropods (Werren *et al*., 1995; Werren, 1997). It causes a variety of reproductive alterations, including cytoplasmic incompatibility (Hoffmann *et al*., 1990; Turelli & Hoffmann, 1995; Poinsot *et al*., 2003), parthenogenesis induction (Stouthamer *et al*., 1990; Weeks & Breeuwer, 2001), feminization of genetic males (Rigaud *et al*., 1991; Hiroki *et al*., 2002; Negri *et al*., 2006), and male killing (Hurst *et al*., 1999; Fialho & Stevens, 2000). Maternal transmission means that these bacteria are genetically linked to the mitochondrial genome; *Wolbachia* affects the mitochondrial genetic structures of lepidopteran host species (Jiggins, 2003; Narita *et al*., 2006). In *Z. emelina*, one of the *Wolbachia* strains, *w*EmeTn2, induces male killing, and infection with both the *w*EmeTn1 and the *w*EmeTn2 strains of *Wolbachia* may have some effect on genetic structure (Sakamoto *et al*., 2011).

We collected male and female adults from *Z. emelina* populations, in which the main larval food plants differed. To accurately delineate units of conservation for *Z. emelina* and augment our geological and biogeographic knowledge of the historical differentiation of its populations, we examined the effects of *Wolbachia* infection on host genetic structure and evaluated the mitochondrial and nuclear DNA of the host populations.

Sampling sites

From 2001 to 2012, adults of *Z. emelina* were collected from 14 populations over a large area of Japan (fig. 1, table 1). Populations were considered to come from the same region when they were less than 150 km apart and were not separated by sea. There were six main regions (fig. 1). The land use at 14 sites is shown in table 1. Although *Z. emelina* usually uses *L. japonicus*, some populations do not. The Kamogawa, Sorogawa, and Emi populations use mainly *Trifolium repens* and *L. japonicus* (Suzuki, 2007). *Zizina emelina* in the Suita

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Table 1. Individuals of *Z. emelina* collected at 14 geographic locations in Japan.

Location	Main larval food plant	Landscape	Date of sampling	Females	Males
Kamogawa, Chiba	*L. japonicus*, *T. repens*[1]	Meadow	Sep. 2007	5	0
Sorogawa, Chiba	*L. japonicus*, *T. repens*[1]	Bank of paddy field or pond	Sep. and Nov. 2007	10	0
Emi, Chiba	*L. japonicus*, *T. repens*[1]	Seashore	Nov. 2007	1	0
Utsunomiya, Tochigi	*L. japonicus*[2]	Riverbank	Sep. 2011	5	2
Nishiyatsushiro, Yamanashi	*L. japonicus*[2]	Riverbank	Oct. 2011	1	1
Suita, Osaka	*L. corniculatus*[3]	Green buffer zone along railroad	Jul. and Aug. 2006 Apr. 2007	14	7
Toyonaka, Osaka	*T. repens*[3]	Green buffer zone in and around airport	Aug. Sep. Oct. and Nov. 2004 Apr. Oct. and Nov. 2007 Jun. Jul. and Aug. 2009	62	33
Kobe, Hyogo	*L. japonicus*[4]	Bank of paddy field or pond	Aug. and Oct. 2006	2	3
Kato, Hyogo	*L. japonicus*[4]	Bank of paddy field or pond	Jul. 2007 Aug. 2010	10	4
Aioi, Hyogo	*L. japonicus*[4]	Bank of paddy field or pond	Oct. 2006 Jul. 2007 Aug. 2010	17	16
Yoshinogawa, Tokushima	*L. japonicus*[2]	Riverbank	Jul. 2012	10	0
Miyazaki, Miyazaki	*L. japonicus*[2]	Riverbank	Nov. 2011	1	0
Aso, Kumamoto	*L. japonicus*[2]	Meadow, campsite	Sep. 2010	6	1
Karatsu, Saga	*L. japonicus*[2]	Castle site	Jun. 2001	0	2
			Total	144	69

[1]Suzuki (2007).
[2]This study.
[3]Minohara *et al.* (2007).
[4]Takei (2005).
L.: *Lotus*, *T.*: *Trifolium.*

population uses mainly *Lotus corniculatus*, and those in the Toyonaka population use mainly *T. repens* (Minohara *et al.*, 2007; Ishii *et al.*, 2008). The others use *L. japonicus* (Takei, 2005; this study); in these remaining populations there have been no reports of the use of *T. repens*, even though *T. repens* is commonly seen in their habitats.

Materials and methods

DNA extraction

Total DNA was extracted from the legs and thoracic muscles of field-collected adults. The tissues from one adult were placed in a 1.5-ml plastic tube with 180 µl lysis buffer and proteinase K, incubated at 56°C for 2 h or longer, and subjected to DNA purification with a DNeasy Tissue kit (Qiagen, Valencia, CA, USA). Total DNA was eluted with 50 µl of elution buffer.

PCR and sequencing

Detection and identification of *Wolbachia* were performed with the primers 81F (5′-TGGTCCAATAAGTGATGAAGAAC-3′) and 691R (5′-AAAAATTAAACGCTACTCCA-3′) for *wsp* (Braig *et al.*, 1998) and the primers ftsZBf (5′-CCGATGCTCAAGCGTTAGAG-3′) and ftsZBr (5′-CCACTTAACTCTTTCGTTTG-3′) (Werren *et al.*, 1995) or FtsZFT2 (5′-GAAGGTGTGCGACGTATGCG-3′) and FtsZRTB2 (5′-ACTCTTTCGTTTGTTTGCTCAGTTG-3′ (Wenseleers *et al.*, 1998) for *ftsZ*. The cycles for *wsp* were as follows: an initial 30-s exposure at 94°C, followed by 40 cycles each at 94°C for 30s, 55°C for 30s, and 75°C for 120s, with a final extension at 72°C for 120s. The cycles for *ftsZ* were as follows: an initial 300-s exposure at 94°C, followed by 45 cycles each at 94°C for 30s, 60°C for 60s, and 72°C for 120s, with a final extension at 72°C for 120s. The positive PCR products were purified and sequenced with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA).

The mitochondrial *ND5* gene, which encodes NADH dehydrogenase subunit 5, was amplified using the primers V1 (5′-CCTGTTTCTGCTTTAGTTCA-3′) (Yagi *et al.*, 1999) and KA1L (5′-GTTCTAATATAAGGTATAAATCATAT-3′) (Saigusa *et al.*, 2001; Yago *et al.*, 2008; Ohshima *et al.*, 2010). The PCR temperature profile for *ND5* was an initial 60-s exposure at 94°C, followed by 30 cycles each at 94°C for 60s, 45°C for 60s, and 72°C for 120s. The mitochondrial *COI* gene, which encodes cytochrome oxidase subunit I, was amplified using the primers LCO1490 (5′-GGTCAACA AATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). The PCR temperature profile for *COI* was an initial 120-s exposure at 94°C, followed by 40 cycles each at 94°C for 15 s, 52°C for 30s, and 72°C for 60s, with a final extension at 72°C for 300s. The PCR products were purified and then sequenced with an ABI PRISM 3100 Genetic Analyzer.

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Table 2. Rates of *Wolbachia* infection in populations of *Z. emelina*.

Population	*Wolbachia* strain	Males	Females	Total
Kamogawa	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	5 (100%)	5 (100%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	0 (0%)	0 (0%)	0 (0%)
	Total	*n*=0	*n*=5	*n*=5
Sorogawa	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	2 (20%)	2 (20%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	0 (0.0%)	8 (80%)	8 (80%)
	Total	*n*=0	*n*=10	*n*=10
Emi	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	1 (100%)	1 (100%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	0 (0%)	0 (0%)	0 (0%)
	Total	*n*=0	*n*=1	*n*=1
Utsunomiya	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	2 (100%)	4 (80%)	6 (86%)
	Uninfected	0 (0%)	1 (20%)	1 (14%)
	Total	*n*=2	*n*=5	*n*=7
Nishiyatsushiro	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	1 (100%)	0 (0%)	1 (50%)
	Uninfected	0 (0%)	1 (100%)	1 (50%)
	Total	*n*=1	*n*=1	*n*=2
Suita	*w*EmeTn1	6 (86%)	9 (65%)	15 (71%)
	*w*EmeTn2	0 (0.0%)	2 (14%)	2 (10%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	1 (14%)	3 (21%)	4 (19%)
	Total	*n*=7	*n*=14	*n*=21
Toyonaka[1]	*w*EmeTn1	24 (73%)	34 (55%)	58 (61%)
	*w*EmeTn2	0 (0%)	14 (23%)	14 (15%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	9 (27%)	14 (23%)	23 (24%)
	Total	*n*=33	*n*=62	*n*=95
Kobe	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	3 (100%)	2 (100%)	5 (100%)
	Total	*n*=3	*n*=2	*n*=5
Kato	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	4 (100%)	10 (100%)	14 (100%)
	Total	*n*=4	*n*=10	*n*=14
Aioi	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	16 (100%)	17 (100%)	33 (100%)
	Total	*n*=16	*n*=17	*n*=33
Yoshinogawa	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	0 (0%)	10 (100%)	10 (100%)
	Total	*n*=0	*n*=10	*n*=10
Miyazaki	*w*EmeTn1	0 (0%)	0 (0%)	0 (0%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	0 (0%)	1 (100%)	1 (100%)
	Total	*n*=0	*n*=1	*n*=1
Aso	*w*EmeTn1	1 (0%)	4 (0%)	5 (71%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)

Continued

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Table 2. (*Cont.*)

Population	*Wolbachia* strain	Males	Females	Total
	Uninfected	0 (0%)	2 (100%)	2 (29%)
	Total	*n*=0	*n*=6	*n*=7
Karatsu	*w*EmeTn1	1 (50%)	0 (0%)	1 (50%)
	*w*EmeTn2	0 (0%)	0 (0%)	0 (0%)
	*w*EmeNy1	0 (0%)	0 (0%)	0 (0%)
	Uninfected	1 (50%)	0 (0%)	1 (50%)
	Total	*n*=2	*n*=0	*n*=2

[1]Includes the data from Sakamoto *et al.* (2011).

Table 3. *Wolbachia* infection of female adults of *Z. emelina* collected from Kamogawa, Sorogawa, Emi, Suita, Kato, and Aioi in 2007, and egg hatchability and sex ratio of their offspring. Number of individuals are in parentheses.

Site	Mother				Offspring			
	Individual number	*Wolbachia* infection	Egg hatchability	Viability	Male	Female	Female rate	*P*
Kamogawa	K8	*w*EmeTn2	0.70 (44)	0.77 (31)	0	24	1.00	***
	K9	*w*EmeTn2	0.68 (22)	0.87 (15)	0	13	1.00	***
Sorogawa	K1	Uninfected	0.75 (33)	1.00 (20)	10	10	0.50	ns
	K4	Uninfected	0.88 (64)	0.86 (35)	15	15	0.50	ns
	K11	Uninfected	0.87 (38)	0.81 (31)	15	10	0.40	ns
	K22	Uninfected	0.96 (83)	0.86 (58)	32	18	0.34	*
	K23	Uninfected	0.91 (65)	0.88 (58)	26	25	0.49	ns
Emi	K24	*w*EmeTn2	0.40 (30)	1.00 (11)	0	11	1.00	***
Suita	P6	*w*EmeTn1	1.00 (70)	ns	21	14	0.40	ns
	P7	Uninfected	1.00 (37)	ns	18	10	0.36	ns
	P9	*w*EmeTn1	1.00 (54)	ns	18	13	0.42	ns
	P10	Uninfected	0.97 (29)	ns	14	10	0.42	ns
	P11	*w*EmeTn1	0.98 (59)	ns	20	23	0.54	ns
	P12	*w*EmeTn1	1.00 (10)	ns	3	4	0.57	ns
	P13	*w*EmeTn1	1.00 (59)	ns	21	18	0.46	ns
Kato	Y4	Uninfected	0.89 (28)	ns	4	7	0.64	ns
	Y5	Uninfected	0.86 (14)	ns	2	5	0.71	ns
	Y6	Uninfected	0.94 (31)	ns	6	8	0.57	ns
	Y7	Uninfected	1.00 (45)	ns	7	10	0.58	ns
Aioi	A1	Uninfected	0.79 (29)	ns	5	12	0.71	ns
	A2	Uninfected	0.95 (56)	ns	11	11	0.50	ns

*P<0.05; *** P<0.001; [ns]P>0.05.

In lepidopteran species the nuclear *Tpi* gene, which encodes triose phosphate isomerase, is located on the Z chromosome (Logsden *et al.*, 1995). A segment of the gene containing a highly variable intron was amplified using the primers (5′-GGTCACTCTGAAAGGAGAACCATCTT-3′) and (5′-CACAACATTTGCCCAGTTGTTGCCAA-3′) (Jiggins *et al.*, 2001) and sequenced. Primers TpiZif (5′-AGAAAGACGAATTGGTTGCTGA-3′) and TpiZir (5′-TGGTAATAGGGCTTTAGTCTG-3′) for precise amplification in *Z. emelina* were designed from the *Tpi* nucleotide sequences obtained using the method described above. The cycles were as follows: an initial 60-s exposure at 95°C, followed by 35 cycles each at 95°C for 60s, 54°C for 60s, and 72°C for 30s, with a final extension at 72°C for 300s. All samples were screened using the new primers and sequenced. The nucleotide sequences of *ND5*, *COI*, and *Tpi* from *Z. emelina* were deposited in the DDBJ/EMBL/GenBank databases.

Phylogenetic and statistical analyses

Phylogenetic trees were constructed using the maximum-likelihood method and the programme package PAUP* 4.0b10 (Swofford, 2002). For maximum-likelihood analyses, we applied best-fit models (*ND5+COI*: TrN+I;*Tpi*: HKY) selected using the Akaike information criterion (Akaike, 1974) in Modeltest 3.7 (Posada & Crandall, 1998). The robustness of the branches was tested by bootstrap analyses with 1000 replications as part of the maximum-likelihood method. To visualize genealogical relationships and potential population substructures, networks were constructed on the basis of the sequence data using the statistical parsimony algorithm (Templeton *et al.*, 1992) implemented in the software package TCS version 1.21 (Clement *et al.*, 2000). The TCS program calculates the minimum number of mutational steps by which sequences can be joined with >95% confidence.

To estimate the variation attributable to differences among populations, analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed with Arlequin 3.5 (Excoffier & Lischer, 2010). Potential isolation among populations and regions was tested by estimating the pairwise fixation index F_{ST} by haplotype permutations among populations and regions (10,000 replicates), as implemented in Arlequin 3.5. F_{ST} was calculated only for populations and regions in which the sample size was at least

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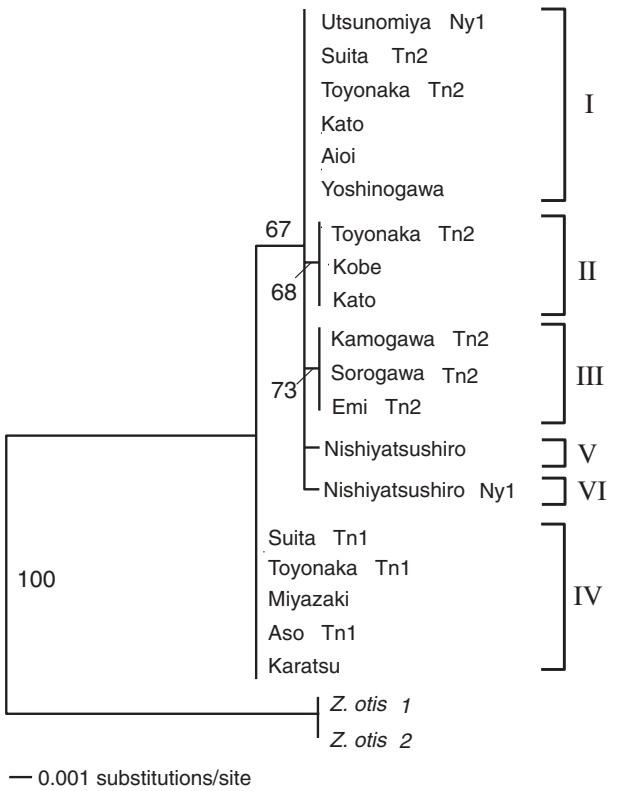

Fig. 2. Maximum-likelihood phylogeny based on the mitochondrial *ND5+COI* gene sequences (1490 aligned nucleotide sites) of *Z. emelina*. Bootstrap values of <50% are not shown. Roman numerals (I–VI) indicate the different haplotypes. *Zizina otis* was used as an outgroup. *Wolbachia* strains are indicated after the population names. Tn1, *w*EmeTn1; Tn2, *w*EmeTn2; Ny1, *w*EmeNy1.

10 individuals. To test for isolation by distance, the correlation between genetic and geographical distances was assessed by the regression of F_{ST} on the geographic distance (km).

In an attempt to statistically detect reduced mitochondrial genetic diversity compared with nuclear genetic diversity in populations of *Z. emelina*, the Hudson–Kreitman–Aguadé (HKA) test (Hudson *et al.*, 1987) was performed with the software packages DnaSP version 5.10 (Rozas *et al.*, 2003) and HKA (written by JodyHey; http://genfaculty.rutgers.edu/hey/software#HKA) on the basis of the sequence data of mitochondrial (*ND5* and *COI*) and nuclear (*Tpi*) genes. The mitochondrial *ND5* and *COI* data were combined because of their genetic linkage. In the analysis, the effective population sizes of the mitochondrial gene (*ND5+COI*) and the Z chromosomal gene (*Tpi*) were corrected using a ratio of 1:3. To calculate interspecific divergence values, *Z. otis* was used as an outgroup. Because six haplotypes, including two haplotypes with deletions (or insertions), were found in *Tpi*, gaps in the sequence alignment were removed and the remaining sequences were used for the molecular analyses.

Rearing experiment

Eggs were obtained from field-collected females of *Z. emelina* in six populations. Hatching larvae were enumerated to determine hatchability and then reared to the adult stage to examine sex ratio. The rearing experiment was performed as described previously (Sakamoto *et al.*, 2011).

Results

Rates of infection by three strains of Wolbachia

Examination of 99 adults by PCR assay using the *wsp* and *ftsZ* primers revealed that nine populations – Kamogawa, Sorogawa, Emi, Utsnomiya, Nishiyatsushiro, Suita, Toyonaka, Aso, and Karatsu – were infected with *Wolbachia* (table 2). The infection rates of the populations varied from 20 to 100%. The Kobe, Kato, Aioi, Yoshinogawa, and Miyazaki populations were uninfected. Although because of recombination the *wsp* sequences did not necessarily accurately reflect the genetic relationships, phylogenetic analysis of the *wsp* and *ftsZ* sequences revealed that there were three strains of *Wolbachia*. Two of the three strains had sequences identical to those of *w*EmeTn1 and *w*EmeTn2 found previously in *Z. emelina* (Sakamoto *et al.*, 2011). The other was a new strain of *Wolbachia*, *w*EmeNy1. It had both *wsp* and *ftsZ* gene sequences identical to those found in *Acraea encedon* (AJ271199), *Hypolimnas bolina* (AB167399), *Phyllonorycter quinnata* (AJ005887), *Parornix devoniella* (AJ005888) and *Cnaphalocrocis medinalis* (HQ336508).

In the Suita population (n=21), 71% of individuals were infected with strain *w*EmeTn1 and 10% were infected with *w*EmeTn2; no individuals were infected with the two strains simultaneously. In the other populations, all infected individuals were infected with either of the two strain of *Wolbachia*; rates of infection with each strain differed among populations (table 2).

Offspring sex ratio

Broods including more than five offspring that reached adulthood were used for analyses of sex ratio and hatchability. All three *w*EmeTn2-infected females (K8, K9, and K24) produced only female offspring (P<0.001 by binomial test), whereas no uninfected or *w*EmeTn1-infected females produced female-biased broods (P>0.05 by binomial test; table 3).

Mitochondrial and nuclear DNA

In the mitochondrial DNA analysis, the 147 individuals sequenced for the 832-bp *ND5* gene were polymorphic at four nucleotide sites, constituting four haplotypes (GenBank: AB714583–AB714594). The individuals sequenced for the 658-bp *COI* gene were polymorphic at three positions, constituting four haplotypes (GenBank: AB714595–AB714606). The combined *ND5* and *COI* sequences constituted six haplotypes (arbitrarily named I–VI; fig. 2). Haplotype networks were produced according to population (fig. 3a). The Toyonaka population had the most haplotypes (I, II, and IV), and all of the other eight populations from Suita westward (Suita, Kobe, Kato, Aioi, Yoshinogawa, Miyazaki, Aso, and Karatsu) had one or two haplotypes in common with those of the Toyonaka population. Haplotype III was found only in the three eastern most populations (Kamogawa, Sorogawa, and Emi), and haplotypes V and VI were found only in Nishiyatsushiro, which was relatively isolated from the other populations. Nucleotide diversities were less than 0.002 (table 4). AMOVA revealed a significant genetic structure among the populations and regions tested (i.e., those in

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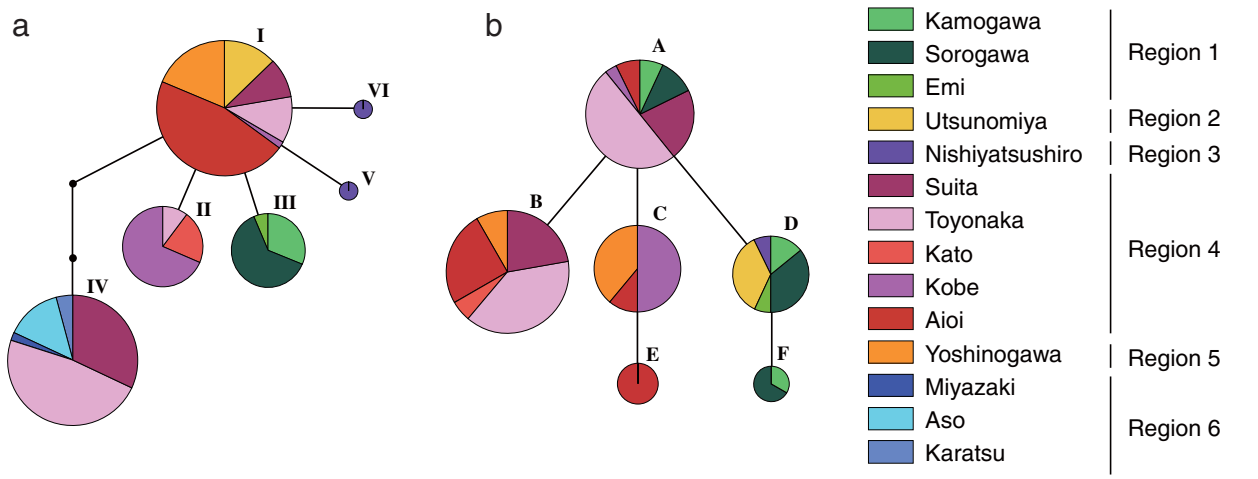

Fig. 3. Haplotype networks for populations, based on the (a) mitochondrial and (b) nuclear gene sequences of *Z. emelina*. A network with 95% connection limit is shown; the size of each circle reflects the number of individuals with each of the haplotypes. Each haplotype is coloured according to the proportion of individuals in each population (shown in the colour key at right).

Table 4. Genetic diversity of mitochondrial haplotypes within populations of *Z. emelina*.

Population	No. of individuals (No. of alleles)	Nucleotide diversity	Haplotype	Haplotype diversity
Kamogawa	3	0.000000	III	0.000
Sorogawa	10	0.000000	III	0.000
Emi	1	0.000000	III	0.000
Utsunomiya	7	0.000000	I	0.000
Nishiyatsushiro	2	0.001342	V, VI	1.000
Suita	21	0.000767	I, IV	0.381
Toyonaka	40	0.000726	I, II, IV	0.344
Kobe	4	0.000000	II	0.000
Kato	14	0.000096	I, II	0.143
Aioi	25	0.000000	I	0.000
Yoshinogawa	10	0.000000	I	0.000
Miyazaki	1	0.000000	IV	0.000
Aso	7	0.000000	IV	0.000
Karatsu	2	0.000000	IV	0.000
Total	147	–	6	–

Table 5. Analysis of molecular variance of mitochondrial and nuclear haplotypes in *Z. emelina*.

	d.f.	Sum of squares	Variance of component	%	F_{ST}	P^1
Frequency and number of different bases						
Mitochondrial DNA (*ND5+COI*)						
Among populations	5	75.24	0.78	72.68	0.73	<0.001
Among individuals within populations	114	33.46	0.29	27.32		
Total	119	108.63	1.07			
Among regions	3	30.58	0.56	44.68	0.45	<0.001
Among individuals within regions	122	85.24	0.70	55.32		
Total	125	115.82	1.26			
Nuclear DNA (*Tpi*)						
Among populations	5	18.28	0.23	43.04	0.43	<0.001
Among individuals within populations	83	25.75	0.31	56.96		
Total	88	44.02				
Among regions	2	12.27	0.27	41.27	0.41	<0.001
Among individuals within regions	94	37.13	0.39	58.73		
Total	96	49.40				

[1]After 10,000 random permutations.

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Table 6. Population pairwise F_{ST} values based on the frequency and number of different bases in *Z. emelina*.

	Sorogawa	Suita	Toyonaka	Kato	Aioi	Yoshinogawa
Sorogawa	–	0.773***	0.765***	0.956***	1.000***	1.000***
Suita	0.606 ***	–	−0.031	0.774***	0.768***	0.681***
Toyonaka	0.583***	−0.046	–	0.758***	0.739***	0.680***
Kato	0.792***	0.737***	0.701***	–	0.945***	0.910***
Aioi	0.504***	0.086	0.116*	0.420**	–	0.000
Yoshinogawa	0.588***	0.421**	0.439***	0.115	0.088	–

*P<0.05, **P<0.01, ***P< 0.001, after 100,000 random permutations.
Upper right: mitochondrial DNA.
Lower left: nuclear DNA.

Table 7. Regional pairwise F_{ST} values based on the frequency and number of different bases in *Z. emelina*.

	Region 1	Region 4	Region 5	Region 6
Region 1	–	0.473***	1.000***	1.000***
Region 4	0.476***	–	0.223***	0.372***
Region 5	0.611***	0.203**	–	1.000***
Region 6	NA	NA	NA	–

*P< 0.05, **P<0.01, ***P<0.001, after 100,000 random permutations.
Upper right: mitochondrial DNA.
Lower left: nuclear DNA.
NA: not applicable.

which the sample size was at least 10 individuals; P<0.001; table 5). Genetic differentiation, as determined by F_{ST}, was also significant among all pairs of populations (P<0.05) except for two, namely Suita–Toyonaka and Aioi–Yoshinogawa (table 6). Genetic differentiation among all pairs of regions was also significant (P<0.001; table 7). Comparison of the haplotype results with those for infection status (fig. 4a) revealed that individuals infected with *w*EmeTn1 had haplotype IV, *w*EmeTn2-infected individuals had haplotype I, II, or III, and *w*EmeNy1-infected individuals had haplotype I or V.

In the nuclear DNA analysis a total of 103 female adult samples were subjected to sequencing of *Tpi*; 355–360 bp were obtained and aligned, representing six haplotypes (arbitrarily named A–F; GenBank: AB714607–AB714624; table 8). The 355-bp alignment, from which aligned nucleotide sites containing gaps had been excluded, was polymorphic at three sites, representing four haplotypes (arbitrarily named A to D; fig. 5). The six haplotypes in the haplotype network were analysed (fig. 3b); two gaps (of four nucleotides and one nucleotide) were considered to represent a fifth character state. The Aioi population had the most haplotypes (A, B, C, and E), and haplotype E was found only in the Aioi population. Haplotype A was found throughout the study area, although not in all populations. Haplotypes B and C occurred in some of the populations from Suita westward, and haplotypes D and F occurred in some of the populations from Nishiyatsushiro eastward. AMOVA revealed a significant nuclear genetic structure among the six populations and three regions tested (P <0.001; table 5). Genetic differentiation (F_{ST}) was also significant among all pairs of populations (P<0.05), with the exception of four, namely Suita–Toyonaka, Suita–Aioi, Kato-Yoshinogawa, and Aioi–Yoshinogawa (table 6). Genetic differentiation was also significant among all pairs of regions tested (P<0.01; table 7). Nuclear haplotypes tended to be shared between regions, whereas mitochondrial haplotypes were not (fig. 3). Nuclear DNA haplotype was not associated with *Wolbachia* infection (fig. 4b).

The relationship that we obtained between F_{ST} value and geographic distance as a result of the mitochondrial and nuclear DNA analyses showed that the degree of genetic differentiation was positively correlated with geographic distance: the farther apart the sets of populations were, the lower their decrease in gene flow (fig. 6). Each coefficient of determination in the logarithmic regression was higher than that in the linear regression in the case of both mitochondrial and nuclear DNA. No significant difference was found between mitochondrial genetic diversity and nuclear genetic diversity in *Wolbachia*-infected or -uninfected populations by HKA test (P>0.05; table 9).

DISCUSSION

Wolbachia *and sex ratio distortion*

An understanding of reproductive manipulation by *Wolbachia* is important to any discussion of the genetic diversity of *Z. emelina*. Sakamoto *et al*. (2011) revealed that the presence of *w*EmeTn2 is associated with the death of male *Z. emelina* in the Toyonaka population. We also found *w*EmeTn2-infected females in three populations, Kamogawa, Sorogawa, and Emi, and they produced only female offspring. This strain was therefore also likely responsible for killing males in these populations. Sakamoto *et al*. (2011) found that a high rate of infection with *w*EmeTn1 did not induce sex ratio distortion in the Toyonaka population. We found individuals infected with *w*EmeTn1 in the Suita population; similarly, there was no sex ratio distortion. Individuals in the Utsunomiya and Nishiyatsushiro populations were infected with a new strain of *Wolbachia*, *w*EmeNy1. Although this *Wolbachia* is identical, in terms of both *wsp* and *ftsZ*, to those known to cause male killing in *A. encedon* and *H. bolina*, we found *w*EmeNy1-infected males of *Z. emelina* in the field. Therefore, *w*EmeNy1 is not likely to kill or feminize its male hosts.

Genetic diversity of Z. emelina *in Japan and effects of* Wolbachia *on diversity*

We expected the extent of among population differentiation to be associated with species movement, dispersal ability, and degree of isolation, depending on the amount of gene flow; previous observations support these predictions (Hastings & Harrison, 1994; Hamrick & Godt, 1996). Mitochondrial and nuclear markers revealed different

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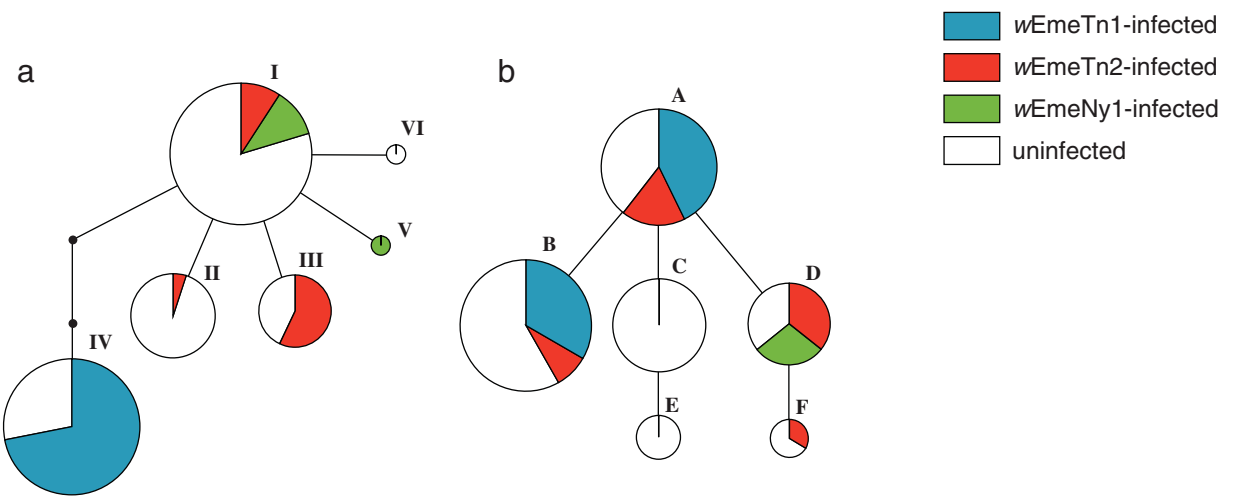

Fig. 4. Haplotype networks and *Wolbachia* infection, based on the (a) mitochondrial and (b) nuclear gene sequences of *Z. emelina*. A network with 95% connection limit is shown; the size of each circle reflects the number of individuals with each of the haplotypes. Each haplotype is coloured according to the proportion of individuals classified by *Wolbachia* infection status (shown in the colour key at right).

Table 8. Genetic diversity of nuclear haplotypes within populations of *Z. emelina*.

	No. of individuals (no. of alleles)[1]	Nucleotide diversity	Haplotype	Haplotype diversity
Kamogawa	5 (5)	0.001690	A, D (+F)	0.600
Sorogawa	10 (10)	0.001315	A, D (+F)	0.467
Emi	1 (1)	0.000000	D	0.000
Utsunomiya	4 (4)	0.000000	D	0.000
Nishiyatsushiro	1 (1)	0.000000	D	0.000
Suita	14 (14)	0.001486	A, B	0.528
Toyonaka	28 (28)	0.001461	A, B	0.519
Kobe	2 (2)	0.000000	B	0.000
Kato	10 (10)	0.000563	A, C	0.200
Aioi	17 (17)	0.002858	A, B, C (+E)	0.618
Yoshinogawa	10 (10)	0.002629	B, C	0.467
Total	94 (94)	–	4	–

[1]Number of individuals with each haplotype.

patterns of genetic structure in *Z. emelina*. In our data, mitochondrial haplotypes – unlike nuclear haplotypes – tended not to be shared among regions. This finding can be explained first in terms of population size. Because of genetic drift, small and bottlenecked populations have low levels of genetic diversity (Bonnell & Selander, 1974; O'Brien *et al.*, 1983; Ellegren *et al.*, 1996; Groombridge *et al.*, 2000). Because the mitochondrial genome has a smaller effective population size than that of an average nuclear locus, the rate of genetic drift is increased in mtDNA (Fay & Wu, 1999). Therefore, especially in the mtDNA of *Z. emelina*, single haplotypes were observed in populations and regions.

Second, the relative lack of sharing of mitochondrial haplotypes among regions can be explained in terms of the lower dispersal probabilities of females: males of *Z. emelina*, unlike females, patrol to find a mating partner (Sakamoto *et al.*, unpublished observations). Moreover, a highly biased sex ratio may lead to higher dispersal rates and trigger the evolution of sex-specific dispersal (Leturque & Rousset, 2003; Bonte *et al.*, 2009): the female-biased sex ratio induced by male killers can thus induce higher rates of male dispersal in *Z. emelina*.

Third, selective sweep by *Wolbachia* could reduce mtDNA diversity. Theoretical studies have shown that the presence of male killers should markedly reduce mitochondrial diversity, because the original mitochondrial DNA lineages in the uninfected hosts will ultimately be lost and replaced by haplotypes associated with the symbiont (Johnstone & Hurst, 1996). There has been a recent selective sweep of the mitochondrial DNA within populations of *A. encedon*, in which the benefit of infection of females with male killers was increased by fitness compensation via resource reallocation in the larval period (Jiggins, 2003). Our examination of the distribution of the *Wolbachia* genotypes identified among the mitochondrial haplotypes (fig. 4a) revealed linkages between some haplotypes and *Wolbachia* strains. Haplotype I was associated with *w*EmeTn2 and *w*EmeNy1, and each strain was found in different populations. Additionally, *w*EmeTn2 was associated with three different haplotypes, which were not sympatric, indicating that horizontal transmission has occurred infrequently in the past and that *w*EmeTn2 infection is old, probably predating the emergence of several mitochondrial haplotypes. In contrast, particular nuclear DNA

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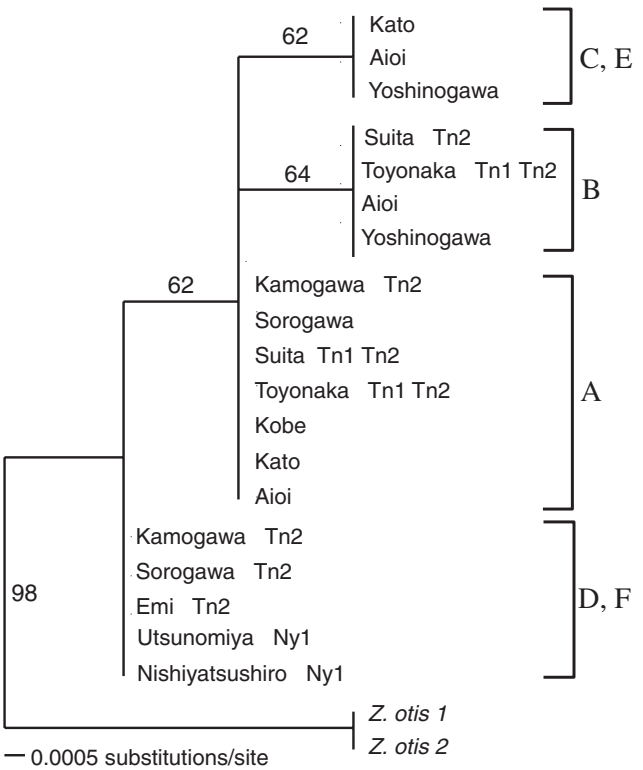

Fig. 5. Maximum-likelihood phylogeny based on the nuclear *Tpi* gene sequences (355 aligned nucleotide sites) of *Z. emelina*. Bootstrap values of <50% are not shown. Letters (A–F) indicate the different haplotypes (see text). *Zizina otis* was used as an outgroup. *Wolbachia* strains are indicated after the population names. Tn1, *w*EmeTn1; Tn2, *w*EmeTn2; Ny1, *w*EmeNy1.

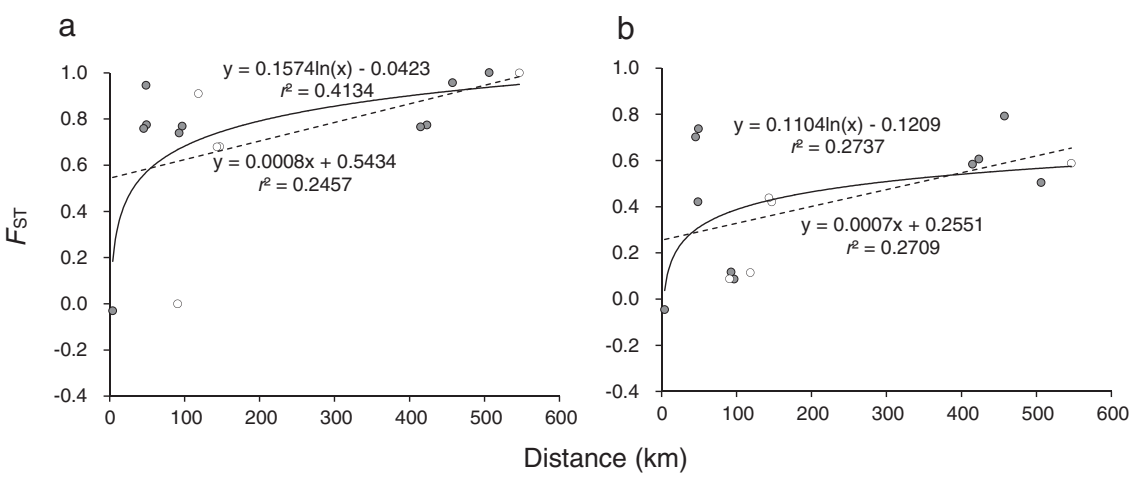

Fig. 6. Relationships between genetic differentiation (F_{ST}) and geographic distance among populations of *Z. emelina* in Honshu and Shikoku, Japan. Geographic distance was measured as the straight-line distance between sites. Each point represents a pair of populations. Solid line, logarithmic regression between F_{ST} and distance; dashed line, linear regression between F_{ST} and distance. Closed circles, separated by sea; open circles, not separated by sea (a) mitochondrial genetic differentiation; (b) nuclear genetic differentiation

haplotypes were not associated with infection with particular *Wolbachia* strains (fig. 4b). The HKA test detected no significant difference in mitochondrial genetic diversity compared with nuclear genetic diversity in infected or uninfected populations (table 9). Although the results of the HKA test did not make it clear whether mtDNA diversity was decreased, our results reveal that the dynamics of *Wolbachia* affect the mitochondrial haplotype structure of *Z. emelina*.

Those populations with relatively high genetic variation were the Toyonaka one (for mtDNA) and the Aioi one (for nuclear DNA), both of which belonged to region 4; genetic

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Table 9. Results of HKA testing for mitochondrial and Z-chromosome polymorphism within *Z. emelina*, and divergence between *Z. emelina* and *Zizina otis*.

	Total individuals		Kamogawa–Sorogawa–Emi (infected with *w*EmeTn2)		Toyonaka (infected with *w*EmeTn1 and *w*EmeTn2)		Aioi (uninfected)	
	mtDNA *ND5+COI*	Nuclear DNA *Tpi*	mtDNA *ND5+COI*	Nuclear DNA *Tpi*	mtDNA *ND5+COI*	Nuclear DNA *Tpi*	mtDNA *ND5+COI*	Nuclear DNA *Tpi*
Intraspecific polymorphism								
No. of alleles	141	103	13	16	40	28	25	17
Segregating sites (obs[1])	7	3	0	1	4	1	0	2
Segregating sites (exp[2])	6.55	3.45	0.64	0.36	3.43	1.57	1.22	0.78
Total number of sites[3]	1490	352	1490	352	1490	352	1490	352
Interspecific divergence								
No. of differences (obs[1])	28.68	6.30	30.00	4.63	27.45	5.50	29.00	5.47
No. of differences (exp[2])	29.13	5.85	29.36	5.26	28.02	4.93	27.78	6.69
Sum of deviation[4]		0.11		1.74		0.32		3.10
P value		0.74		0.18		0.57		0.08

[1]Observed value.
[2]Expected value.
[3]Nucleotide sites containing alignment gaps were excluded.
[4]The effective mitochondrial gene (*ND5+COI*) and Z-chromosomal gene (*Tpi*) population sizes were corrected.

diversity was relatively well maintained in these habitats. Furthermore, the TCS networks revealed central haplotypes (haplotype I in the case of mitochondrial DNA and haplotype A in the case of nuclear DNA) that were shared by most populations. These are possible common ancestors. The above-mentioned results suggested that region 4 was the centre of genetic diversity for this butterfly. However, our maximum-likelihood phylogenetic trees based on mitochondrial and nuclear DNAs did not entirely support the ancestry inferred from the TCS results. Further analyses – of outgroups, sibling species and subspecies – are needed to reveal the historical patterns of distribution of *Z. emelina* in Japan.

Yago *et al.* (2008) examined the molecular systematics and biogeography of the genus *Zizina* worldwide. They found five haplotypes of *ND5* in *Z. emelina* in Japan, and the combination of haplotypes in each population was the same as that in our study. A large and highly significant number of endangered populations and species have low levels of genetic variation compared with those of related, non-endangered species (Frankham, 1995). The number of haplotypes of *ND5* or *Tpi* in *Z. emelina* is lower than that in other butterflies (e.g., Yoshio, 2005; Nakatani *et al.*, 2006; Narita *et al.*, 2006). This lower number could be indicative of extinction risk, although it is difficult to compare genetic diversities because of differences in ecological and historical conditions.

We used F_{ST} analysis to reveal the genetic differentiation among populations and among regions. F_{ST} and species migration, or dispersal ability, are strongly correlated (Frankham *et al.*, 2002). Given that we defined population differentiation as occurring when two populations or regions did not share haplotypes, or as supported by F_{ST} value if they shared haplotypes, we considered that there were eight groups in our Japanese study area, namely Kamogawa–Sorogawa–Emi, Utsunomiya, Nishiyatsushiro, Suita–Toyonaka, Kato–Kobe, Aioi, Yoshinogawa, and Miyazaki–Aso–Karatsu (1 and 3). Therefore, gene flow among *Z. emelina* populations has been highly limited: the butterfly has low levels of migratory activity and lives in small, fragmented populations.

The degree of genetic differentiation is correlated with geographic distance among populations in many animal species (e.g., Forbes & Hogg, 1999; Haig *et al.*, 2001). In *Z. emelina*, there was a correlation between F_{ST} and geographic distance, with a relatively high coefficient of determination in the logarithmic regression. Ibrahim *et al.* (1996) demonstrated spatial patterns using three different forms of dispersal. A stepping-stone model (Ibrahim *et al.*, 1996), in which only migration to adjacent populations is allowed, can explain the migratory pattern of *Z. emelina*. We conclude that *Z. emelina* has little migratory activity and its populations are not continuous, because very little gene flow occurs beyond a certain distance. From these results, together with geological and biogeographic knowledge of climatic change and processes of formation of the Japanese Archipelago, we can propose an evolutionary hypothesis for *Z. emelina*. In several refugia, populations of some species might have been pushed towards lower latitudes or altitudes with more suitable habitats during the cool glacial periods (Saitoh *et al.*, 2008; Ikeda *et al.*, 2009; Jeratthitikul *et al.*, 2013). The most likely scenario is a split of *Z. emelina* populations into mainly genetic lineages, resulting in the formation of each specific genotype frequency through the glacial period. Later, *Z. emelina* would have expanded its range of habitats northward and fragmented during the postglacial warming period.

These results give valuable information about the conservation of this endangered butterfly. Populations that have

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significant divergence of allele frequencies at mitochondrial and nuclear loci are regarded as management units (Moritz, 1994). The eight groups should be treated as conservation units *in Z. emelina*, because each group has accumulated mutations leading to evolutionary distinctiveness, as supported by the F_{ST} values. Our results also have another implication for conservation management: they suggest that the presence of the male killer *w*EmeTn2 has complex effects on this butterfly. The presence of the male killer could lead to the avoidance of inbreeding depression, but it could also lead to host extinction because of a shortage of males or reduce host genetic diversity, especially in small populations (Johnstone & Hurst, 1996; Hurst & Jiggins, 2000; Jiggins *et al*., 2000). Additionally, it cannot be denied that *w*EmeTn1 and *w*EmeNy1 had some effects on the host.

The presence of *Wolbachia* may place the conservation of *Z. emelina* populations at risk. For this reason, we think that diagnosing the presence or absence of *Wolbachia* infection is important in conserving *Z. emelina*, as is the case in other endangered butterflies (Nice *et al*., 2009; Ritter *et al*., 2013). In conservation management we should avoid introducing infected individuals into uninfected populations. Our results show that the presence or absence of infection is clearly distinguishable between very close populations of *Z. emelina* that are, say, tens of kilometres apart and have limited gene flow. Therefore, for endangered butterflies such as *Z. emelina*, whose populations are fragmented and isolated (Tscharntke *et al*., 2002; Nakamura, 2010), we recommend that the conservation units be chosen particularly carefully.

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