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ORIGINAL ARTICLE

Male death resulting from hybridization between subspecies of the gypsy moth, *Lymantria dispar*

Y Higashiura[1], H Yamaguchi[1], M Ishihara[2], N Ono[1], H Tsukagoshi[1,4], S Yokobori[1], S Tokishita[1], H Yamagata[1] and T Fukatsu[3]

[1]School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan; [2]Biological Laboratory, Hyogo College of Medicine, Hyogo, Japan and [3]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Ibaraki, Japan

We explored the origin of all-female broods resulting from male death in a Hokkaido population of *Lymantria dispar* through genetic crosses based on the earlier experiments done by Goldschmidt and by testing for the presence of endosymbionts that are known to cause male killing in some insect species. The mitochondrial DNA haplotypes of the all-female broods in Hokkaido were different from those of normal Hokkaido females and were the same as those widely distributed in Asia, including Tokyo (TK). Goldschmidt obtained all-female broods through backcrossing, that is, F1 females obtained by a cross between TK females (*L. dispar japonica*) and Hokkaido males (*L. dispar praeterea*) mated with Hokkaido males. He also obtained all-male broods by mating Hokkaido females with TK males. Goldschmidt inferred that female- and male-determining factors were weakest in the Hokkaido subspecies and stronger in the Honshu (TK)

subspecies. According to his theory, the females of all-female broods mated with Honshu males should produce normal sex-ratio broods, whereas weaker Hokkaido sexes would be expected to disappear in F1 or F2 generations after crossing with the Honshu subspecies. We confirmed both of Goldschmidt's results: in the case of all-female broods mated with Honshu males, normal sex-ratio broods were produced, but we obtained only all-female broods in the Goldschmidt backcross and obtained an all-male brood in the F1 generation of a Hokkaido female crossed with a TK male. We found no endosymbionts in all-female broods by 4,′6-diamidino-2-phenylindole (DAPI) staining. Therefore, the all-female broods observed in *L. dispar* are caused by some incompatibilities between Honshu and Hokkaido subspecies.
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Introduction

Sexual reproduction is almost universal as a means of reproduction in a wide range of organisms and has the advantage of allowing gene exchanges to purge detrimental mutations and/or rapidly evolving parasites (Maynard Smith, 1978; Hamilton *et al.*, 1990). Moreover, a two-sex system is generally used by most sexual organisms and has evolutionary advantages in numerous situations (Iwasa and Sasaki, 1987). Despite this, sex determination mechanisms are diverse and can differ even in closely related species (Peichel *et al.*, 2004). In *Drosophila melanogaster*, sex is determined by the ratio of the chromosome number (X) to the number of the autosomal set (A) (Bridges, 1921). Animals with an X/A ratio of 1 become female, and those with an X/A ratio of 0.5 become male. In the housefly, *Musca domestica*, however, a male-determining factor (abbreviated as 'M') on the Y chromosome is critical for development

into a male (Hediger *et al.*, 1998). Without the 'M' factor, a female-determining factor (abbreviated as 'F') functions maternally, such that the animal develops as a female (Dübendorfer and Hediger, 1998).

Goldschmidt (1934) proposed the theory of sex determination of *Lymantria dispar* after studying hybrids among Japanese local races. In *L. dispar*, the heterogametic sex is female (ZW) and the homogametic sex is male (ZZ). Goldschmidt (1955) hypothesized that the female sex-determining factor, 'F', is a maternally inherited cytoplasmic factor and that the male sex-determining factor, 'M', is located on the Z chromosome (see Appendix; Figure 4). Goldschmidt (1934) suggested that different races of *L. dispar* possess female- and male-determining factors of different strengths. Within each race these factors are well balanced, but in inter-race crosses changes in the relative levels of these factors can generate intersex individuals or even cause sex reversal. Goldschmidt (1934) inferred that the female- and male-determining factors were weakest in Hokkaido subspecies (*L. dispar praeterea*) and stronger in Honshu (Tokyo (TK)) subspecies (*L. dispar japonica*). Goldschmidt (1934) obtained all-male broods from a cross between Hokkaido females and Honshu males. Inversely, Goldschmidt (1930) obtained all-female broods of *L. dispar* by backcrossing, that is, when F1 females resulting from a cross between Honshu females and Hokkaido males were

mated with Hokkaido males (Appendix Table A1). The results of these crosses indicate that presumably weaker Hokkaido sexes disappear in the F1 or the backcrossed F2 generation when crossed with the other sex from the Honshu subspecies. The production of all-female or all-male broods is thus interesting in the context of sex determination in *L. dispar*.

Higashiura *et al*. (1999) found all-female broods owing to male death in *L. dispar* at Bibai (BB) in Hokkaido, Japan, and maternal inheritance of this trait. Higashiura *et al*. (1999) also observed six all-female broods out of 66 egg batches in the next generation in the same 0.2-ha forest, indicating the persistence of all-female broods. Clarke and Ford (1982) re-examined crosses between Hokkaido females and Honshu males and obtained all-male broods as Goldschmidt (1934) indicated. However, the characteristics of all-female broods obtained by the Goldschmidt backcross have not been re-examined.

If the Goldschmidt backcross indeed generates all-female broods in Hokkaido *L. dispar*, mitochondrial DNA (mtDNA) analysis of normal and all-female broods could reveal the maternal origin of the all-female broods. Bogdanowicz *et al*. (2000) described mtDNA variation among worldwide populations of *L. dispar* and resolved the haplotypes into four groups: (1) Okinawa, Japan, (2) Hokkaido, Japan, (3) Honshu and Kyushu, Japan, and mainland Asia, and (4) Europe, Tunisia, and North America. To test whether normal and all-female broods of Hokkaido *L. dispar* have different origins, we analysed mtDNA haplotypes in both broods. We also re-examined the Goldschmidt backcross that produces all-female broods at the backcrossed F2 generation. If the hypothesized weaker male sex of Hokkaido *L. dispar* indeed disappears in the Goldschmidt backcross, then females of the all-female broods mated with the hypothesized strong Honshu male should produce normal broods. In this study, we present the results of such crosses, which show that the origin of all-female broods is the hybridization between Honshu and Hokkaido subspecies.

At present, all-female broods are known to arise from selective male killing at egg or larval stages, particularly in arthropods (Majerus, 2003). Several microbial symbionts, including organisms in the genera *Wolbachia*, *Spiroplasma*, *Rickettsia* and *Arsenophonus*, are known to produce all-female broods in their host insects, as cytoplasmic elements are inherited only matrilineally (Hurst *et al*., 1997, 2003; Majerus, 2003). To test whether certain bacteria are present in all-female broods, diagnostic polymerase chain reaction (PCR) was carried out on the larvae of all-female broods of Hokkaido *L. dispar* using primers that amplify fragments of genes found in the above-mentioned bacteria and in eubacteria in general. As endosymbiont bacteria show up as bright flecks in the cytoplasm by 4′,6-diamidino-2-phenylindole (DAPI) staining, we also stained the eggs of all-female broods of Hokkaido *L. dispar* with DAPI to test whether certain symbionts were involved in the production of the all-female broods. Given that microsporidians are commonly found in lepidopteran insects (Agnew *et al*., 2003), we would expect DAPI staining to reveal both prokaryotic and eukaryotic endosymbionts in all-female broods.

The aims of this study were (i) to test whether microbial symbionts are involved in the production of all-female broods by diagnostic PCR and DAPI staining

and (ii) to test whether all-female broods of *L. dispar* originate from hybridization between Honshu and Hokkaido subspecies by mtDNA analyses and the Goldschmidt backcross. This information will be useful in characterizing the molecular mechanisms of sex determination in *L. dispar*.

Materials and methods

Study species

Inoue (1982) phenotypically classified *L. dispar* of Japan into five subspecies: *L. dispar praeterea* Kardakoff for Hokkaido populations, *L. dispar japonica* (Motschulsky) for the mainland (Honshu, Shikoku and Kyushu) populations (Figure 1) and three other island subspecies. All of these subspecies are univoltine. Schintlmeister (2004) treated the Hokkaido populations as *L. dispar dispar*, the same as mainland Asian and European populations. However, we adopted Inoue's classifications in this paper, because Hokkaido mtDNA haplotypes are quite different from Asian haplotypes, showing about 2% mtDNA sequence divergence (Bogdanowicz *et al*., 2000). Pogue and Schaefer (2007) reported that the distribution of *L. dispar japonica* is in Honshu, Shikoku and Kyushu, and extends north into parts of Hokkaido. They also treated Hokkaido populations as a distinct species, *L. umbrosa* (Butler). The type locality of the lectotype male for the revised species, *L. umbrosa*, is the most southwestern region of Hokkaido, or Hakodate (Figure 1). The distributions of the two species in Hokkaido are unclear. Therefore, we did not adopt the classifications described by Pogue and Schaefer (2007).

In Hokkaido, larvae pupate in July and August. Adults emerge, mate and deposit eggs in August and September. As each female lays its full egg complement in only one mass containing 100–900 eggs, it is possible to recognize the properties of the original female by rearing eggs from individual masses. Eggs diapause during winter and hatch in the following May. Larvae feed on leaves of the Japanese larch *Larix leptolepis*, the Japanese birch *Betula platyphylla* and many other broad-leaved trees.

Sample collection and rearing of Hokkaido *L. dispar*

We collected egg masses at BB (Figure 1) and Shikaoi (SK) in Hokkaido, Japan. The collection sites were two *Betula platyphylla* forests (designated *Betula*-1 and *Betula*-2), *Larix leptolepis* forest at BB and one *Betula platyphylla* forest at SK. We collected and analysed seven egg masses from the *Larix* forest at BB in September 1999, six egg masses from *Betula*-1 forest at BB in October 1999 and 2001, 41 egg masses from *Betula*-2 forest at BB in October between 1999 and 2002, 11 egg masses at SK in November 2000 and 3 egg masses at Niikappu (NP) in December 2002. After collection, the egg masses were kept individually in a cold room at 0 °C until May of the following year. Then, about 100 eggs from each of the egg masses were placed in an incubator at 20 °C. After hatching, 20 first instar larvae from each of the egg masses were frozen in liquid nitrogen and stored at −80 °C for mtDNA analysis. The remaining larvae were reared to pupation on an artificial diet (product #F9642B, Bio-Serv, Frenchtown, NJ, USA). We counted unhatched
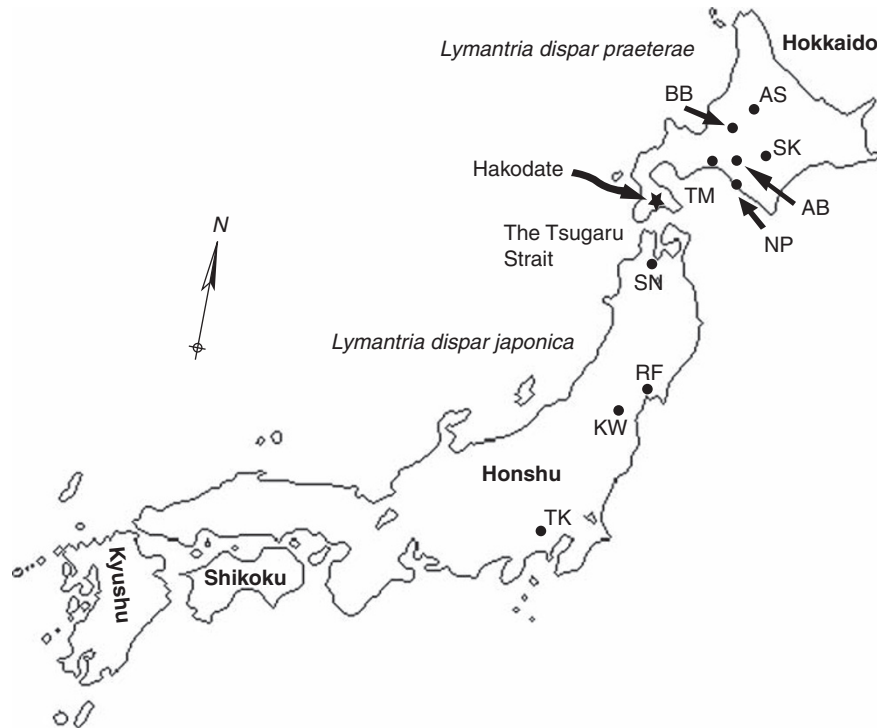
Figure 1 Map showing collection sites for egg masses of *L. dispar* used in this study. The arrow indicates North. Bibai (BB) (43°17′N, 141°51′E), Shikaoi (SK) (43°06′N, 142°57′E), Abira (AB) (42°45′N, 141°49′E), Niikappu (NP) (42°24′N, 142°14′E), Tomakomai (TM) (42°38′N, 141°37′E) and Asahikawa (AS) (43°49′N, 142°19′E) are in Hokkaido, Japan. Sannai (SN) (40°48′N, 140°40′E), Rifu (RF) (38°19′N, 140°56′E), Kawasaki (KW) (38°10′N, 140°40′E) and Tokyo (TK) (35°38′N, 139°23′E) are in Honshu, Japan. *L. dispar praeterae* is distributed in Hokkaido, Japan. *L. dispar japonica* is distributed in Honshu, Shikoku and Kyushu, Japan (Inoue, 1982). The Tsugaru Strait divides these two subspecies into their distributions. Hakodate, Hokkaido, is the type locality of *L. umbrosa* (Pogue and Schaefer, 2007).

eggs in some of the egg masses to obtain egg-hatch rates. Sex ratios among the pupae were recorded for each of the egg masses. As egg-hatch rates were <60% in all-female (male death) broods in the previous study (Higashiura *et al*., 1999), we regarded egg masses showing egg-hatch rates of 70% or more as normal sex-ratio broods without rearing.

PCR detection of bacterial symbionts
For PCR detection of bacterial symbionts, we used larvae hatched from three and two egg masses of all-female broods and normal broods, respectively, collected from *Betula*-2 forest at BB, Hokkaido, in October 1999. All of these broods were subjected to mtDNA sequencing as described in the next section. Five first instar larvae preserved in 99% ethanol from each of the egg masses were individually subjected to DNA extraction using a QIAamp Tissue Mini Kit (Qiagen, Hildeu, Germany). The purified DNA was eluted with 200 μl of TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM ethylenediaminetetraacetic acid). For detection of potentially male-killing bacterial symbionts, diagnostic PCR analysis was conducted as described (Tsuchida *et al*., 2002). The following primers were used: ftsF and ftsR for the *ftsZ* gene of *Wolbachia* spp., 16SA1 and TKSSspR for the 16S rRNA gene of *Spiroplasma* spp., 16SA1 and Rick16SR for the 16S rRNA gene of *Rickettsia* spp., 16SA1 and Ars16SR for the 16S rRNA gene of *Arsenophonus* spp., 16SA1 and 16SB1 for the 16S rRNA gene of general eubacteria, and MtrA1 and MtrB1 for the mitochondrial rRNA gene of the host insect (Appendix Table A2). For positive control reactions, DNA samples

from *Callosobruchus chinensis* (Coleoptera: Bruchidae) infected with *Wolbachia* sp., *Drosophila melanogaster* (Diptera: Drosophilidae) infected with *Spiroplasma* sp., *Nephotettix cincticeps* (Homoptera: Deltocephalidae) infected with *Rickettsia* sp. and *Diaphorina citri* (Homoptera: Psyllidae) infected with *Arsenophonus* sp. were also subjected to diagnostic PCR.

DAPI staining of embryos to detect endosymbionts
We collected the eggs of an all-female brood obtained by crossing a female of an all-female brood (44 females and 0 males) with a male of a normal brood (14 females and 16 males) collected at Abira (AB) in October 2005. As a control, we obtained eggs of normal broods by crossing individuals from two normal broods (a mother from a brood containing 18 females and 16 males, and a father from a brood containing 20 females and 17 males) collected at BB in October 2006. Eggs were collected from both all-female and normal sex-ratio broods at 3 days after oviposition. The collected eggs from each brood were treated with 50% (v/v) aqueous bleach solution for 30 s to 3 min. After washing with phosphate-buffered saline (PBS), eggs were fixed with Carnoy's fixative for 3 h in a siliconized tube at 4 °C. Subsequently, eggs were placed in PBS Tween for 15 min, and were treated with 50% aqueous bleach solution for 1 min before the surgical procedure. The chorion and vitelline membranes were removed with tungsten needles (cat. #500134, World Precision Instruments, Sarasota, FL, USA) under a microscope in PBS Tween solution. The dechorinated eggs were transferred to PBS Tween solution in another siliconized tube. A volume of

1 ml of DAPI solution (1 mg ml^{-1}) was added and the mixture was incubated for 5 min. The stained eggs were observed under a fluorescence microscope using an ultraviolet filter.

As a positive control, *Callosobruchus chinensis* embryos infected with *Wolbachia* (strain jC) (Kondo *et al.*, 2002) were stained using DAPI solution. We also evaluated *C. chinensis* without *Wolbachia* obtained by tetracycline treatment (Kondo *et al.*, 2002). *C. chinensis* eggs were collected within 24 h after oviposition. The eggs were submerged in Carnoy's fixative with adzuki beans, and then detached from the beans. After removing the beans from the fixative, the eggs were fixed in a siliconized tube for 2 h at 4 °C. Eggs were rehydrated in PBS Tween solution for 15 min and stained with DAPI solution for 5 min. The observational method for stained *C. chinensis* eggs was the same as that used for *L. dispar* eggs.

mtDNA sequencing
In all, 5 or 10 frozen first instar larvae from each of the broods were ground with a pestle in a pre-chilled mortar and subjected to total DNA extraction using a QIAamp DNA Mini kit (Qiagen). In all, 1075 bp of mtDNA segment encoding the parts of three genes, cytochrome *c* oxidases I and II and nicotinamide adenine dinucleotide dehydrogenase subunit 1, was amplified by PCR from 2 ng of template DNA. The gene-specific primers have been described previously (Bogdanowicz *et al.*, 2000) (Appendix Table A2). PCR (40 cycles of 94 °C for 0 s, 48 °C for 0 s and 72 °C for 15 s) was carried out using a Rapid Cycler (Idaho Technology, Salt Lake City, UT, USA). The amplified products were directly subjected to DNA sequencing using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA sequences determined in this study have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers AB244647–244657 and AB244665. Pairwise sequence-based distances among the studied mtDNA haplotypes (a total of 1075 base pairs across the three genes) were estimated using PAUP* 4.0b10 (Swofford, 2003) under the Hasegawa, Kishino and Yano (HKY) model (Hasegawa *et al.*, 1985).

Crossing experiments
We collected two egg masses at Sannai (SN), Aomori in northern Honshu, Japan, in November 2001. The mtDNA haplotypes of these larvae were determined using the protocol described above. Hatched larvae were reared to adulthood and sexed in July 2002. The females were crossed with males from egg masses collected from *Betula*-2 at BB, Hokkaido, in October 2001. The offspring were reared to adulthood and sexed in July 2003. The F1 females were backcrossed with males with Hokkaido mtDNA haplotypes from egg masses of normal broods collected at NP, AB and Tomakomai (TM), Hokkaido, Japan in December 2002. The backcrossed F2 offspring were reared to adulthood and sexed in July 2004.

Females of all-female broods emerged from egg masses collected from *Betula*-2 at BB, Hokkaido, in October 2001. In July 2002, they were crossed either with (one) males from Honshu egg masses or (two) males from BB, Hokkaido; the latter cross served as a positive control. The Honshu egg masses were collected

in November 2001 at Rifu (RF), Kawasaki (KW) and also at SN. Larvae from these crosses were reared to pupation and sexed in July 2003. An artificial diet (Bio-Serv; product #F9642B) was used for rearing.

A Hokkaido female that emerged from an egg mass collected at Asahikawa (AS) in April 2006 was crossed with a TK male from an egg mass collected at TK in October 2005. The offspring were reared and sexed in July 2007.

The exact *P*-value for the binomial proportion test (hereafter abbreviated as the binomial test) (SAS Institute, 1999) is used to analyse the egg hatch rate and sex ratio data.

Results

Detection of endosymbionts
Diagnostic PCR analysis of 15 larvae from three all-female broods of *L. dispar* detected no *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Arsenophonus* or general eubacteria. These symbionts were also not detected in the 10 larvae from two normal sex-ratio broods. A mitochondrial gene of the host insect was successfully amplified from all of the samples, indicating that the DNA extraction was successful. Each positive control sample gave specific PCR products of the expected sizes for each bacterium and general eubacteria, confirming the specificity of the PCR (data not shown).

No differences were detected between *L. dispar* embryos of all-female and normal broods by DAPI staining (Figures 2a and b). However, in *C. chinensis* embryos infected with *Wolbachia*, we were able to detect a cytoplasm containing large amounts of *Wolbachia* (Figure 2c). In embryos without *Wolbachia*, which were obtained through tetracycline treatment, we found a single type of nucleus, lacking all apparent endosymbionts (Figure 2d).

mtDNA sequences of all-female and normal sex-ratio broods of Hokkaido *L. dispar*
The mtDNA haplotypes of the all-female broods were found to be quite different from those of the normal sex-ratio broods (Figure 3). Pairwise mtDNA sequence divergence values from comparisons of the normal broods ranged from 0 to 0.0028 (substitution per site). The haplotype H1 in Figure 3 had been identified previously and was included in the Hokkaido haplotypes reported by Bogdanowicz *et al.* (2000). Nevertheless, the mtDNA haplotype (A1) of the all-female broods was the most common among those seen in Honshu (Japan), Kyushu (Japan) and mainland Asia (Bogdanowicz *et al.*, 2000). Pairwise mtDNA sequence divergence values from comparisons of the all-female broods ranged from 0 to 0.0019. However, the sequence divergence between the normal and the all-female broods ranged from 0.016 to 0.019.

The 18 all-female broods shown in Figure 3 contained no males at all. In one brood of the minimum rearing number shown in Figure 3, the sex ratio was 6:0 (females:males) ($P(1:1) = 0.03$: the binomial test). In one brood of the maximum rearing number shown in Figure 3, the sex ratio was 29:0 ($P(1:1) < 0.001$: the binomial test). The egg-hatch rates of the all-female broods were between 38 and 58%, indicating that all-female production was probably because of male death.
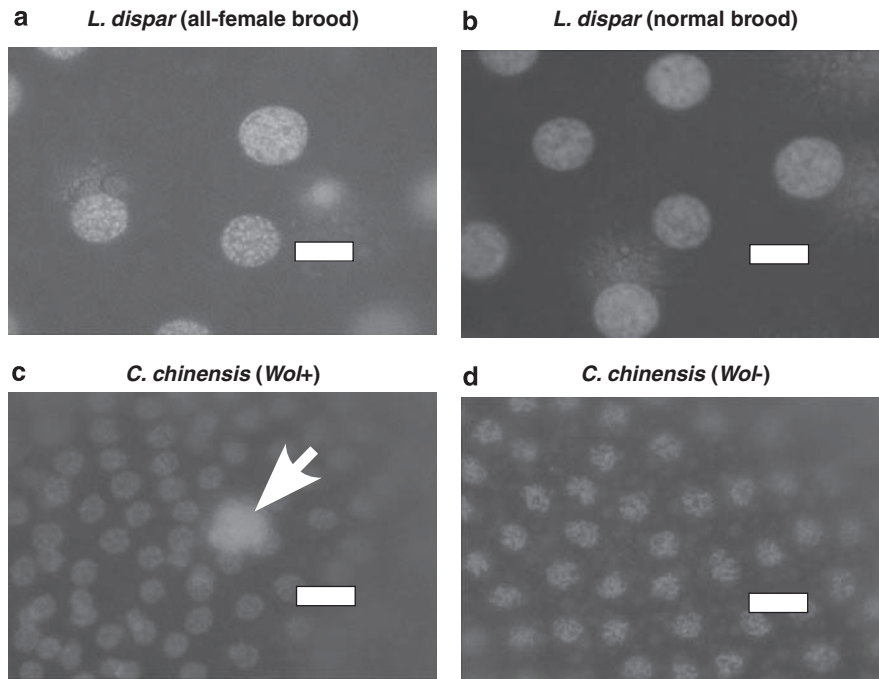
a *L. dispar* (all-female brood)   **b** *L. dispar* (normal brood)

c *C. chinensis* (*Wol+*)   **d** *C. chinensis* (*Wol-*)

Figure 2 Eggs stained with DAPI solution and viewed under a fluorescence microscope using an ultraviolet filter to detect endosymbionts. As DAPI stains nuclei exclusively, we observe a cytoplasm containing many parasitic bacteria. The length of each white bar represents 20 μm. (**a**) An embryo deposited by a female from an all-female brood of *Lymantria dispar* at 3 days after oviposition. (**b**) An embryo from a normal sex-ratio brood of *L. dispar* at three days after oviposition. (**c**) An embryo of *Callosobruchus chinensis* infected with *Wolbachia* (strain jC) (Kondo *et al*., 2002) within 24 h after oviposition. The arrow shows cytoplasm with abundant bacteria. (**d**) An embryo of *C. chinensis* without *Wolbachia* obtained by tetracycline treatment.

Locality Tree Species	Number	Haplotype	COI (378) 1857	1929	1962	1998	2007	2020	2034	2133	2142	2148	COII (345) 3433	3436	3451	3523	3724	3739	ND1 (329) + others (23) 12372	12382	12406	12408	12428	12480	12507	12603	12627	12642	12681
All-female broods BB *Larix*	3	A1	T	T	T	G	A	G	A	T	A	T	A	T	G	T	T	T	A	A	A	A	A	G	T	C	T	T	C
BB *Betula* -1	1	A2	T	T	T	G	A	G	A	T	A	T	A	T	G	T	T	T	A	A	A	A	A	G	T	T	T	T	C
BB *Betula* -2	11	A1	T	T	T	G	A	G	A	T	A	T	A	T	G	T	T	T	A	A	A	A	A	G	T	C	T	T	C
BB *Betula* -2	1	A9	T	T	T	G	A	G	A	T	A	T	A	T	G	T	T	T	A	A	A	A	A	G	T	C	T	C	C
SK *Betula*	2	A1	T	T	T	G	A	G	A	T	A	T	A	T	G	T	T	T	A	A	A	A	A	G	T	C	T	T	C
Normal sex ratio 1:1 BB *Larix*	2	H1	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	A	A	T	A	C	C	T	T	T
BB *Larix*	1	H10	C	A	C	A	G	**A**	G	C	A	C	G	C	G	C	C	C	A	A	A	A	T	A	C	C	T	T	T
BB *Larix*	1	H11	C	A	C	A	G	G	G	C	G	C	G	C	G	C	C	C	G	A	A	A	T	A	C	C	T	T	T
BB *Betula* -1	5	H1	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	A	A	T	A	C	C	T	T	T
BB *Betula* -2	1	H12	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	**G**	A	T	A	C	C	**A**	T	T
BB *Betula* -2	1	H13	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	**G**	A	A	T	A	C	C	T	T	T
BB *Betula* -2	1	H14	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	A	G	T	A	C	C	T	T	T
BB *Betula* -2	26	H1	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	A	A	T	A	C	C	T	T	T
SK *Betula*	9	H1	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	A	A	T	A	C	C	T	T	T
NP *Betula*	1	H1	C	A	C	A	G	G	G	C	A	C	G	C	G	C	C	C	A	A	A	A	T	A	C	C	T	T	T
NP *Betula*	2	H6	C	A	C	A	G	G	G	C	A	C	G	C	A	C	C	C	A	A	A	A	T	A	C	C	T	T	T

Figure 3 Variable sites of three genes, cytochrome *c* oxidases I and II (COI and COII), and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1), in the mtDNA of (the upper) all-female broods and (the lower) normal sex-ratio broods of *Lymantria dispar*, in Hokkaido, Japan. There are 17 fixed differences between all-female broods and normal sex ratio broods. These fixed different sites are shaded in grey. The 4–5-digit numbers in the upper column indicate the nucleotide position corresponding to the mtDNA sequence of *Drosophila yakuba* (Clary and Wolstenholme, 1985). 'Number' indicates the number of egg masses with the given haplotype among the egg masses collected at the locality. 'A1' is the same as the most common haplotype (28 out of 44 individuals) in Russia, China, Japan and Korea, and 'H1' is the same as the most common haplotype (3 out of 5 individuals) in Hokkaido, Japan (Bogdanowicz *et al*., 2000). Non-synonymous substitutions for A1 or H1 are boxed. BB, Bibai; NP, Niikappu; SK, Shikaoi (Figure 1).

The 50 normal sex-ratio broods in Figure 3 had more than 70% egg-hatch rate, or had a sex ratio of 1:1 (for example, the most skewed ratio of one normal brood in Figure 3, in which females:males = 12:4; $P(1:1) = 0.08$: the binomial test).

Crossing experiments

We used five normal sex-ratio broods of Honshu *L. dispar* in this study (Table 1). The mtDNA haplotype of all of these broods was A1, an Asian type distributed through Asia, including Japan, south of Hokkaido (Bogdanowicz *et al.*, 2000). Of 15 broods of Hokkaido *L. dispar* used for crossing experiments in this study, 11 broods exhibited

normal sex ratios near 1:1, whereas 4 broods consisted of all females. Egg-hatch rates were $\geqslant 67\%$ in the normal broods and $\leqslant 56\%$ in the all-female broods, indicating that all-female production was probably because of male death. In the Hokkaido *L. dispar*, the mtDNA haplotypes of the normal broods were H1 or H6, which are haplotypes that are found only in Hokkaido.

When the Honshu females and the Hokkaido males shown in Table 2, both of which came from broods with normal sex ratios, were crossed, the F1 offspring exhibited normal sex ratios. However, when F1 females were again crossed with Hokkaido males, the back-crossed F2 offspring were entirely female. Two series of independent experiments gave the same results (Table 2).

Table 1 Characteristics of *Lymantria dispar* broods used for crossing experiments shown in Tables 2–4

Brood location and number (mtDNA haplotype)[a]	Island (Japan)	Collection time	Egg-hatch rate	No. of females	No. of males	P-value[b]
(a) Normal sex-ratio broods						
TK (A1)	Honshu	October 2005	67%	22	19	0.755
SN 1 (A1)	Honshu	November 2001		24	39	0.077
SN 2 (A1)	Honshu	November 2001		31	31	1.000
KW (A1)	Honshu	November 2001		6	8	0.791
RF (A1)	Honshu	November 2001		18	22	0.636
NP 1 (H6)	Hokkaido	December 2002		8	16	0.152
NP 2 (H1)	Hokkaido	December 2002		6	12	0.238
NP 3 (H6)	Hokkaido	December 2002		43	31	0.201
AB 4 (H1)	Hokkaido	December 2002		38	36	0.908
AB 6 (H1)	Hokkaido	December 2002		31	22	0.272
TM 7 (H1)	Hokkaido	December 2002		4	4	1.000
BB 1 (H1)	Hokkaido	October 2001	97%	6	7	1.000
BB 2 (H1)	Hokkaido	October 2001	100%	10	15	0.424
BB 7 (H1)	Hokkaido	October 2001	100%	14	11	0.690
BB 8 (H1)	Hokkaido	October 2001	95%	8	10	0.815
AS (H1)	Hokkaido	April 2006	99%	27	38	0.215
(b) All-female broods						
BB 3 (A1)	Hokkaido	October 2001	48%	14	0	<0.001
BB 4 (A1)	Hokkaido	October 2001	49%	13	0	<0.001
BB 5 (A1)	Hokkaido	October 2001	56%	27	0	<0.001
BB 6 (A1)	Hokkaido	October 2001	50%	11	0	<0.001

Abbreviations: AS, Asahikawa; BB, Bibai; KW, Kawasaki; mtDNA, mitochondrial DNA; NP, Niikappu; RF, Rifu; SN, Sannai; TK, Tokyo; TM, Tomakomai (Figure 1).
[a]A1 haplotype of mtDNA is distributed in Asia, and H1 is distributed only in Hokkaido (Bogdanowicz *et al.*, 2000).
[b]Probability of 1:1 sex ratio (the binomial test).

Table 2 Re-evaluation of Goldschmidt's backcross result

Parents		Number of F1 offspring		Backcross male (mtDNA haplotype)	Results of backcrosses	
Female (mtDNA haplotype)	Male (mtDNA haplotype)	No. of females	No. of males		No. of females	No. of males
SN 1 (A1)[a]	BB 1 (H1)[a]	64	63	NP 2 (H1)	60[b]	0
				NP 3 (H6)[a]	19[b]	0
				AB 4 (H1)	18[b]	0
				AB 6 (H1)	53[b]	0
				TM 7 (H1)	42[b]	0
SN 1 (A1)	BB 2 (H1)	33	28	NP 1 (H6)	48[b]	0
				NP 2 (H1)	28[b]	0
				NP 3 (H6)	45[b]	0
				AB 4 (H1)	25[b]	0

Abbreviations: AB, Abira; BB, Bibai; mtDNA, mitochondrial DNA; NP, Niikappu; SN, Sannai; TM, Tomakomai (Figure 1).
[a]A1 haplotype of mtDNA is distributed through Asia, and H1 and H6 are distributed only in Hokkaido (Bogdanowicz *et al.*, 2000). The mtDNA haplotypes are shown in Figure 3.
[b]The sex ratio is significantly different from 1:1 at $P < 0.001$ (the binomial test).

Table 3 Crossing of the all-female broods and the Hokkaido males or the Honshu males

Females of all-female broods at Bibai, Hokkaido (mtDNA haplotypes)	Mated males (mtDNA haplotypes)	F1 offspring	
		No. of females	No. of males
(a) Crosses of all-female broods and Hokkaido males			
BB 3 (A1)	BB 1 (H1)	16[a]	0
BB 4 (A1)	BB 1 (H1)	12[a]	0
BB 5 (A1)	BB 7 (H1)	11[a]	0
BB 6 (A1)	BB 2 (H1)	15[a]	0
BB 6 (A1)	BB 8 (H1)	24[a]	0
(b) Crosses of all-female broods and Honshu males			
BB 3 (A1)	SN 1 (A1)	8	5
BB 4 (A1)	SN 2 (A1)	14	16
BB 5 (A1)	SN 1 (A1)	7	6
BB 6 (A1)	KW (A1)	11	8
BB 6 (A1)	RF (A1)	23	19

Abbreviations: BB, Bibai; KW, Kawasaki; mtDNA, mitochondrial DNA; RF, Rifu; SN, Sannai (Figure 1).
[a]The mtDNA haplotypes are shown in Figure 3.
[b]The sex ratio is significantly different from 1:1 at $P < 0.001$ (the binomial test).

Table 4 Results of an Asahikawa (AS), Hokkaido, female mated with a Tokyo (TK), Honshu, male (mitochondrial DNA (mtDNA) haplotype is shown in Figure 3)

Female (mtDNA haplotype)	Male (mtDNA haplotype)	Progeny		Egg hatch rate
		No. of females	No. of males	
AS (H1)	TK (A1)	0	35[a]	48/61 (79%)[b]

[a]The sex ratio is significantly different from 1:1 at $P < 0.001$ (the binomial test).
[b]The egg hatch rate is significantly different from 50% at $P < 0.0001$ (the binomial test).

These results are in perfect agreement with Goldschmidt's finding of all-female brood production in the hybridization crosses between Honshu females (*L. dispar japonica*) and Hokkaido males (*L. dispar praeterea*) (Goldschmidt, 1930; Appendix Table A1).

When Hokkaido females from all-female broods were mated with Hokkaido males, their offspring were all females in five replicates (Table 3a). These results confirm the result of Higashiura *et al*. (1999), indicating that the all-female broods produce all-female broods when females of these broods are mated with Hokkaido males.

When Hokkaido females of the all-female broods were mated with Honshu males, however, the offspring exhibited normal sex ratios of approximately 1:1 in five replicates (Table 3b).

We confirmed that an AS, Hokkaido, female mated with a TK, Honshu male produced an all-male brood. The egg hatch rate of the brood was 79%, which is significantly higher than 50% ($P < 0.0001$: the binomial test; Table 4).

Discussion

Higashiura *et al*. (1999) found all-female broods in the Hokkaido population of *L. dispar* and showed that all-female broods mated with Hokkaido males produce all-female broods. Male killing caused by cytoplasmic bacterial symbionts has been reported in a variety of insects (Hurst *et al*., 1997, 2003; Majerus, 2003). Although we analysed the infection status of all-female and normal broods using PCR tests specific for *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Arsenophonus* and general eubacteria, we found no bacteria in either all-female or normal broods of *L. dispar* larvae in Hokkaido. Moreover, we obtained cytological evidence showing that all-female broods of *L. dispar* do not have obvious prokaryotic or eukaryotic endosymbionts that might cause male killing, as we found no differences in DAPI staining between the embryos of the all-female brood and normal sex ratio. In contrast, the cytoplasm of *Callosobruchus chinensis* was stained by DAPI because of its high content of bacteria, or *Wolbachia*. These results suggest that male death in *L. dispar* may be caused by something other than endosymbionts, but do not exclude the possibility of viral influences. An RNA virus is reported to cause selective male-killing in the oriental tea tortrix, *Homona magnanima* (Nakanishi *et al*., 2008).

The mtDNA analysis of *L. dispar* performed in this study revealed the different origins of the all-female broods and normal sex-ratio broods found in the same populations in Hokkaido, Japan. The normal sex-ratio broods possessed Hokkaido mtDNA haplotypes, whereas the all-female broods had Asian (including Honshu) mtDNA haplotypes. Here we must consider the results of Goldschmidt's backcross (1930), that is, F1 females resulting from a cross between Honshu, Japan, females (*L. dispar japonica*) and Hokkaido males (*L. dispar praeterea*) mated with Hokkaido males, as an explanation for the all-female broods observed in Hokkaido.

We successfully reproduced Goldschmidt's result by backcrossing Honshu females with Hokkaido males, both of which were obtained from normal broods, and found that the backcrossed F2 progeny were entirely female (Figure 4; Appendix Equations (A1) and (A2)). On the other hand, when Hokkaido females from all-female broods were crossed with Honshu males, the offspring exhibited normal 1:1 sex ratios (Appendix Equation (A3)). The results of the crosses conform to the prediction made by Goldschmidt's theory. The all-female broods observed in a small fraction of Hokkaido females may have originated from hybridization between Honshu females and Hokkaido males. These all-female broods produce all-female broods as long as the females of these broods are mated with Hokkaido males.

The origin of the Honshu females found in Hokkaido is not clear. Bogdanowicz *et al*. (2000) found that one of six analysed Hokkaido individuals carried an Asian mtDNA haplotype and proposed that this female may be 'either a migrant from elsewhere in Asia or an individual of mixed ancestry'. We are currently investigating the distribution of mtDNA haplotypes throughout Hokkaido.

The reciprocal hybridization crosses between *L. dispar* individuals give the opposite results, producing all-male broods (Figure 4; Appendix Equation (A4); Goldschmidt, 1934). Clarke and Ford (1982) reassessed this cross, asking whether Hokkaido females mated with Honshu males would produce all-male progeny, including both sex-reversed males and normal males. Goldschmidt (1934) thought that F1 females of this cross had the
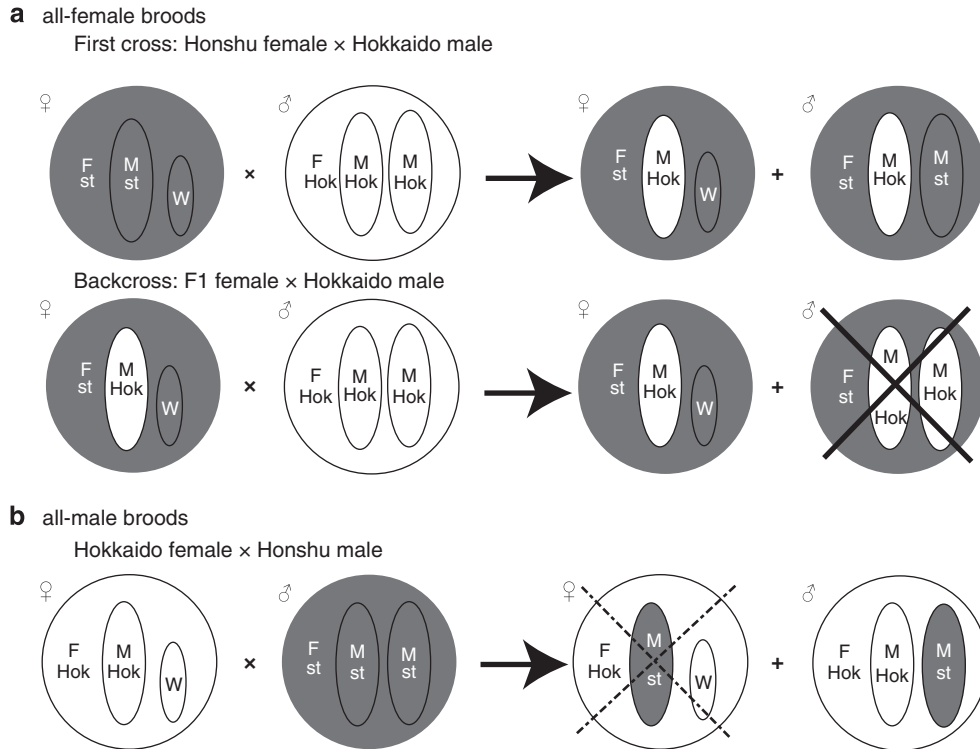
Figure 4 Results of hybridization crosses in *Lymantria dispar* according to Goldschmidt's sex determination theory (Goldschmidt, 1934). Circles indicate the cytoplasm holding the female determinant factor (F). Large ovals indicate the Z chromosome holding the male determinant factor (M). 'F' and 'M' indicate sex determinant factors, not the chromosome. Small ovals indicate W chromosome. Open and grey symbols, circles and ovals, show weak and strong sex determinants, respectively. Female is ZW and male is ZZ. Honshu individuals have strong sex determinants indicated by 'st', and are represented by $(F_{st}) M_{st} W$ for females, and $(F_{st}) M_{st} M_{st}$ for males (parentheses indicate a cytoplasmic factor). Hokkaido individuals possess the weakest sex determinants, and are represented by $(F_{Hok}) M_{Hok} W$ for females, and $(F_{Hok}) M_{Hok} M_{Hok}$ for males. **(a)** All-female broods obtained by backcrossing, that is, F1 females of a cross between strong Honshu females (*L. dispar japonica*) and weak Hokkaido (Hok) males (*L. dispar praeterea*) mated with Hokkaido males. Lethality is indicated by the cross bar. **(b)** All-male broods obtained by crosses between weak Hokkaido females and strong Honshu males. The dashed cross bar indicates sex-reversed female, or male.

weakest female and strong male sex determinants and would be transformed into males. Clarke and Ford (1982), however, found that all of the phenotypically male hybrids lacked the heterochromatic W chromosome found in normal females. They stated that 'the excess of males in Goldschmidt's classic cross is due to the Haldane (1922) effect of genic imbalance and not to sex reversal'. We obtained an all-male brood by crossing a Hokkaido female with a TK male. The egg-hatching rate of the brood was significantly > 50%. This implies that either incompatibilities may manifest before or after oviposition. However, Goldschmidt (1934) likely took the high hatching rate of all-male broods into consideration, as his work mentions the different survival rates of sex-reversed males and females. The survival rate and the presence of the heterochromatic W chromosome during larval stages of such all-male broods should be studied further, as Clarke and Ford (1982) may have evaluated the presence of the heterochromatic W chromosome in late-instar larvae, after the female larvae had died.

Hornett *et al*. (2006) proposed the existence of a suppressor gene against the male-killing trait caused by *Wolbachia* in the Southeast Asian and Polynesian nymphalid butterfly *Hypolimnas bolina*. Polynesian females infected with the bacteria exhibited the male-killing trait; however, Southeast Asian females produced 1:1 sex ratio progenies even when infected with the bacteria. Hornett *et al*. (2006) concluded that zygotic suppression is controlled at a single, genetically dominant locus because they obtained 1:1, 2:1 and 4:1 sex ratio progenies in the F1, backcrossed F2 and backcrossed F3 generations of Southeast Asian females (backcrossed with Polynesian males). However, in *L. dispar*, the presence of a suppressor gene only on the Z chromosomes of the Honshu and Asian backgrounds may be able to produce entirely all-female broods in the backcross, but we found no obvious symbionts.

Goldschmidt's theory of sex determination is based on the hypothesis that different races of *L. dispar* possess female- and male-determining factors of different strength (Goldschmidt, 1934). The balance within each race and the imbalance of sex-determining strength between different races can be considered to be the outcome of a coevolutionary 'arms race' between the sexes in a recent theory of sexual conflict (Arnqvist and Rowe, 2005). This theory is based on the presence of antagonistic interactions between females and males, for example, mating frequencies. At the species-specific level, these coevolutionary 'arms races' between the sexes are hidden by continuous adaptation and counter-adaptation, but they propel the evolution of the mating system in a group of insects (Arnqvist and Rowe, 2002). In *L. dispar*, the 'arms races' between the sexes are hidden in race-specific crosses, but the outcome of the conflict may occur in crosses between sexual races. The sex-determining genes reflect the outcome of this

sexual conflict (Arnqvist and Rowe, 2005) in the manner indicated by Goldschmidt's theory of sex determination.

Goldschmidt (1934) pointed out that 'an intersex is an individual which starts development with its original, gametic sex, but changes sex during development'. He regarded the timing of this change, or turning point, to be important and formulated the time law of intersexuality. Dietrich (2003) pointed out that 'Goldschmidt was attempting a synthesis of evolutionary and developmental biology similar to the synthesis that has been emerging during the past 15 years (known as evolutionary developmental biology, or evo-devo)'. As shown in the results of the inter-race crosses in this paper, his theory should be re-evaluated, at least for *L. dispar*. Goldschmidt (1955) assumed that the 'F' factor on the W chromosome was the maternally inherited cytoplasmic factor. In insects such as the blowfly, hessian fly, fungus gnat and house fly, maternal factors are involved in female sex determination (Ullerich, 1984; Inoue and Hiroyoshi, 1986; Stuart and Hatchett, 1991; de Saint Phalle and Sullivan, 1996; Dübendorfer and Hediger, 1998; Sánchez and Perondini, 1999). On the basis of the results presented here, we suggest that the molecular mechanisms of male or female mortality may be revealed by crosses between different races with different sex strengths. Moreover, it is expected that the research investigating these molecular mechanisms will clarify the sexual determinants of *L. dispar* in the near future.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix

A sex determination theory in *Lymantria dispar* by Goldschmidt (1934).

Goldschmidt (1934) proposed that the sex of *L. dispar* is determined by a quantitative balance between female and male sex determinants. He inferred that the strength of sex determinants is the same within each locality, but is different between localities. He divided Japanese *L. dispar* into eight races. He defined intersex as genetic females or males exhibiting intermediate phenotypes. The intersex does not occur in matings between insects in the same locality. However, it may occur when the balance of the strength of the sexual determinants collapses, as in matings between insects from different localities. Mosbacher (1975) confirmed the existence of the intersex by examining the shapes of forewing scales and antenna hairs in hybridization crosses of *L. dispar*. In his hypothesis (Goldschmidt, 1955), the female sex determinant (F) is located on the W chromosome, and each egg contains a W chromosome at least until meiosis, as the female is heterogametic, or ZW. Thus, each oocyte can express the female determinant on the W chromosome as a cytoplasmic factor, or a maternal factor. Goldschmidt (1955) assumed that the male sex determinant (M) is located on the Z chromosome and that it functions as a zygotic factor. In his notation, (F)MM denotes a ZZ male, two Ms from the two Z chromosomes and one F cytoplasmically inherited from the mother (parentheses indicate a cytoplasmic factor), whereas (F)MW denotes a ZW female, one M from the one Z chromosome and one F from the mother, with W indicating the presence of a W chromosome. (F)MM individuals develop into males because two Ms are stronger than one F, and (F)MW individuals become females because one M is weaker than one F. In hybridization, we must consider the different strength of sex determinants in individuals from each location. We focus on the Hokkaido and Honshu populations. The sex determinants of Hokkaido *L. dispar* (*L. d. praeterea*) for both sexes are inferred to be weakest among those investigated by Goldschmidt (1934), whereas those of Honshu *L. dispar* (*L. d. japonica*) are stronger. Other subspecies possess sex determinants of intermediate intensities; however, all of them are stronger than those of Hokkaido *L. dispar*. Given that the sex determinants of the Hokkaido *L. dispar* are denoted as F_{Hok} and M_{Hok} and those of the Honshu *L.*

dispar as F_{st} and M_{st}, the cross between a Honshu female and a Hokkaido male can be represented as follows:

$$(F_{\text{st}}) M_{\text{st}} W \times (F_{\text{Hok}}) M_{\text{Hok}} M_{\text{Hok}}$$
$$= (F_{\text{st}}) M_{\text{Hok}} W + (F_{\text{st}}) M_{\text{st}} M_{\text{Hok}} \qquad (A1)$$

In the F1 generation, $[(F_{\text{st}}) M_{\text{Hok}}W]$ individuals become females because the female determinant (F_{st}) is stronger than the male determinant (M_{Hok}); this outcome is compatible with the genetic sex (Figure 4). $[(F_{\text{st}}) M_{\text{st}} M_{\text{Hok}}]$ individuals become males because M_{st} plus M_{Hok} is still stronger than F_{st}. The backcross between the F1 female and the Hokkaido male can be represented as follows:

$$(F_{\text{st}}) M_{\text{Hok}} W \times (F_{\text{Hok}}) M_{\text{Hok}} M_{\text{Hok}}$$
$$= (F_{\text{st}}) M_{\text{Hok}} W + (F_{\text{st}}) M_{\text{Hok}} M_{\text{Hok}} \qquad (A2)$$

In the backcrossed F2 generation, $[(F_{\text{st}}) M_{\text{Hok}}W]$ individuals become females. However, it is hypothesized that $[(F_{\text{st}}) M_{\text{Hok}} M_{\text{Hok}}]$ individuals (that is, extreme intersexual males) develop into females, because M_{Hok} plus M_{Hok} is weaker than F_{st}. Goldschmidt (1934) thought that females produced through sex reversal rarely survived. He stated that 'it has to be added that the viability of the higher grades of intersexes is lowered and that therefore their number (of sex reversal females, that is, males) is smaller than expected'. In fact, he presents many examples of all-female broods produced by such weak males (Hokkaido males) mated with F1 females of a cross between many Japanese Honshu females and Hokkaido males (Appendix Table A1).

If all-female broods in Hokkaido occur in this manner, then the females of all-female broods mated with strong males or Honshu males may produce offspring in a normal sex ratio, according to Goldschmidt's theory (Goldschmidt, 1934), as follows:

$$(F_{\text{st}}) M_{\text{Hok}} W \times (F_{\text{st}}) M_{\text{st}} M_{\text{st}}$$
$$= (F_{\text{st}}) M_{\text{st}} W + (F_{\text{st}}) M_{\text{st}} M_{\text{Hok}} \qquad (A3)$$

These males are heterozygous normal males (Goldschmidt, 1934), and are the same as the F1 hybrid males shown in Equation (A1).

Reciprocal hybridization crosses of *L. dispar* give reverse results, producing all-male broods (Goldschmidt, 1934). In this case, Hokkaido females mated with Honshu males would produce all-male broods including both sex-reversed males and normal males (Figure 4).

Table A1 Goldschmidt's results from backcrossing *Lymantria dispar* from different regions with Hokkaido males, i.e., F1 females of a cross between Honshu females (*L. d. japonica*) and Hokkaido males (*L. d. praeterea*) mated with Hokkaido males

(Honshu females × Hokkaido males) × Hokkaido males	No. of females	No. of males
(Tokyo × Hokkaido) × Hokkaido	976	1
(Fukui × Hokkaido) × Hokkaido	57	0
(Tsu × Hokkaido) × Hokkaido	6	0
(Kyoto × Hokkaido) × Hokkaido	49	0
(Wakayama × Hokkaido) × Hokkaido	21	0
(Kanazawa × Hokkaido) × Hokkaido	13	0
(Biwa-Lake × Hokkaido) × Hokkaido	22	0

After Tables 1, 3, 4, and 5 in Goldschmidt (1930).
Tokyo (35°41′ N, 139°45′ E), Fukui (36°03′ N, 136°13′ E), Tsu (34°43′ N, 136°3′ E), Kyoto (35°00′ N, 135°46′ E), Wakayama (34°14′ N, 135°1′ E), Kanazawa (36°33′ N, 136°39′ E), and Biwa-Lake (35°7′ N, 136°5′ E).

Table A2 Sequencing primers used in this study

Symbiont or Lymantria dispar gene	Primer name	Primer sequence (5′ to 3′)	References
Wolbachia spp.	ftsF	GTATGCCGATTGCAGAGCTTG	Kondo *et al*. (2002)
Wolbachia spp.	ftsR	GCCATGAGTATTCACTTGGCT	Kondo *et al*. (2002)
Spiroplasma spp.	TKSSspR	TAGCCGTGGCTTTCTGGTAA	Tsuchida *et al*. (2002)
Rickettsia spp.	Rick16SR	CATCCATCAGCGATAAATCTTTC	Tsuchida *et al*. (2002)
Arsenophonus spp.	Ars16SR	TTAGCTCCGGAGGCCACAGT	Tsuchida *et al*. (2002)
Spiroplasma spp., *Rickettsia* spp., *Arsenophonus* spp. and general eubacteria	16SA1	AGAGTTTGATCMTGGCTCAG	Tsuchida *et al*. (2002)
General eubacteria	16SB1	TACGGYTACCTTGTTACGACTT	Tsuchida *et al*. (2002)
Mitochondrial rDNA of *L. dispar*	MtrA1	AAWAAACTAGGATTAGATACCCTA	Fukatsu *et al*. (2001)
Mitochondrial rDNA of *L. dispar*	MtrB1	TCTTAATYCAACATCGAGGTCGCAA	Fukatsu *et al*. (2001)
Forward primer for CO I of *L. dispar*	COI-F	GGATCACCTGATATAGCATTCCC	Bogdanowicz *et al*. (2000)
reverse primer for CO I of *L. dispar*	COI-R	CCCGGTAAAATTAAAATATAAACTTC	Bogdanowicz *et al*. (2000)
Forward primer for CO II of *L. dispar*	COII-F	TAATTTGAACTATYTTACCIGC	Bogdanowicz *et al*. (2000)
Reverse primer for CO II of *L. diapar*	COII-R	GAGACCATTACTTGCTTTCAGTCATCT	Bogdanowicz *et al*. (2000)
Forward primer for ND 1 of *L. dispar*	ND1-F	TAGAATTAGAAGATCAACCAGC	Bogdanowicz *et al*. (2000)
Reverse primer for ND 1 of *L. diapar*	ND1-R	ATGGATTTACTATTTTGGCAG	Bogdanowicz *et al*. (2000)

Abbreviations: CO I, cytochrome *c* oxidases I; CO II, cytochrome *c* oxidases II; ND 1, nicotinamide adenine dinucleotide dehydrogenase subunit 1.

This cross can be shown as follows:

$$(F_{\text{Hok}}) M_{\text{Hok}} W \times (F_{\text{st}}) M_{\text{st}} M_{\text{st}}$$
$$= (F_{\text{Hok}}) M_{\text{st}} W + (F_{\text{Hok}}) M_{\text{st}} M_{\text{Hok}} \qquad (A4)$$

Goldschmidt (1934) thought that these F1 females had the weakest (F_{Hok}) female and strong male (M_{st}) sex determinants, and were transformed into males.