REVIEW ARTICLE

Molecular analysis of sex chromosome-linked mutants in the silkworm *Bombyx mori*

TSUGURU FUJII[1], HIROAKI ABE[2] and TORU SHIMADA[1]*

[1]*Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan*
[2]*Department of Biological Production, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho 3-5-8, Fuchu, Tokyo 183-8509, Japan*

Abstract

In *Bombyx mori*, the W chromosome determines the female sex. A few W chromosome-linked mutations that cause masculinization of the female genitalia have been found. In female antennae of a recently isolated mutant, both female-type and male-type *Bmdsx* mRNAs were expressed, and *BmOr1* (bombykol receptor) and *BmOr3* (bombykal receptor), which are predominantly expressed in the antennae of male moths, were expressed about 50 times more abundantly in the antennae of mutant females than in those of normal females. These mutants are valuable resources for the molecular analysis of the sex-determination system. Besides the *Fem* gene, the quantitative egg size-determining gene *Esd* is thought to be present on the W chromosome, based on the observation that ZWW triploid moths produce larger eggs than ZZW triploids. The most recently updated *B. mori* genome assembly comprises 20.5 Mb of Z chromosome sequence. Using these sequence data, responsible genes or candidate genes for four Z-linked mutants have been reported. The *od* (distinct oily) and *spli* (soft and pliable) are caused by mutation in *BmBLOS2* and *Bmacj6*, respectively. *Bmap* is a candidate gene for *Vg* (vestigial). Similarly, *Bmprm* is a candidate gene for *Md* (muscle dystrophy), causing abnormal development of indirect flight muscle.

[Fujii T., Abe H. and Shimada T. 2010 Molecular analysis of sex chromosome-linked mutants in the silkworm *Bombyx mori*. *J. Genet*. **89**, 365–374]

Introduction: sex chromosomes of *Bombyx mori*

In Lepidoptera, sex is determined by female heterogamety. Primitive non-ditrysian Lepidoptera have a Z0/ZZ sex chromosome system, while the ZW/ZZ sex chromosome system is common in Ditrysia, a group of advanced Lepidoptera. Therefore, it has been speculated that the W chromosome first appeared with the origin of the ditrysian clade of Lepidoptera (Traut and Marec 1996). There are two hypotheses to account for the origin of the W chromosome. In the first of these, a free homologue of an autosome is believed to have fused with the Z chromosome, which is then transmitted as a W chromosome (Traut and Marec 1996). The second hypothesis is the *de novo* formation of the W chromosome based on the supernumerary B chromosome, which provides a pairing partner for the univalent Z chromosome in the Z0 female (Lukhtanov 2000). In female lepidopterans, meiosis is achiasmatic, and there is no crossing over between the W chromosome and the corresponding Z chromosome (Traut 1977). Recombinational isolation of the W chromosome in Lepidoptera causes its differentiation from the Z chromosome regardless of its origin.

The silkworm *B. mori* has a W chromosome. In this species, AAA : ZZ aneuploid individuals develop as males, whereas AAA : ZZW triploids develop as females (Hasimoto 1933). This indicates that the W chromosome of *B. mori* has a dominant female-determining gene (*Fem*), because these polyploid (AAA : ZZ and AAA : ZZW) individuals have the same A : Z ratio (Hasimoto 1933). So far, 12 W-chromosome-specific PCR markers have been identified in *B. mori* (Abe *et al*. 2005). Sequence analysis of bacterial artificial chromosome (BAC) clones containing W-specific

*For correspondence. E-mail: toru@ss.ab.a.u-tokyo.ac.jp.

Keywords. W chromosome; Z chromosome; morphological traits; mutants.

PCR markers (W-BACs) has revealed that they are composed of many long terminal repeat (LTR) retrotransposons, non-LTR retrotransposons, retroposons, DNA transposons and their derivatives (Abe *et al*. 2005). In genomic *in situ* hybridization (CGH) studies, the complete *B. mori* W chromosome was labelled equally by female and male genomic DNA probes (Traut *et al*. 1999). These authors concluded that the W chromosome of *B. mori* is composed of common repetitive elements that accumulate predominantly in the W chromosome. Abe *et al*. (2008) reported that a large part of the W chromosome is not used for sex determination in *B. mori*, because the T(W;2)*Y*-abe chromosome lacks 11 of 12 W-specific RAPD markers, (T(W;2)*Y*-abe chromosome contains only W-Rikishi marker) but the sexual phenotype of Z/T(W;2)*Y*-abe individuals is that of the normal female.

Many Z chromosome-linked traits in Lepidoptera have been identified in crossing experiments using closely related species (Sperling 1994; Prowell 1998). In *B. mori*, 18 Mendelian mutations whose phenotypes are visible in the egg, larva, and adult have been mapped on the Z chromosome (Banno *et al*. 2005). In 2004, draft sequences of the male (Z/Z) *B. mori* genome using 3-fold and 5.9-fold whole-genome shotgun (WGS) sequencing were reported by Mita *et al*. (2004) and Xia *et al*. (2004), respectively. Recently, these two data sets were re-assembled and 87% of the genomic sequences were anchored to 28 chromosomes (The International Silkworm Genome Consortium 2008) using a high resolution, single-nucleotide polymorphism linkage map (Yamamoto *et al*. 2006). In the new assembly, the Z chromosome was assigned five scaffolds composed of 20.4-Mb sequences. The availability of this newly assembled sequence database (Build2) makes it possible to characterize the genes responsible for each mutation in terms of their nucleotide sequence.

In this article, we review genetic studies on visible W-linked traits. We first discuss mutants of *Fem* that cause masculinization of the female, and we then consider a putative egg size-determining gene on the W chromosome. The male killing factor on the W chromosome has been reviewed by Abe *et al*. (2010) and will therefore not be considered here. We also review the molecular analysis of visible Z-linked mutant traits (*Vg, spli, Md*, and *od*).

W-linked morphological traits

Masculinization caused by mutation in the Fem gene

In *Drosophila, doublesex* (*dsx*) is key gene acting at the downstream end of the sex-differentiation cascade (Saccone *et al*. 2002). As in *Drosophila*, in *B. mori* the primary transcript of *doublesex* (*Bmdsx*) is alternatively spliced in males and females to yield sex-specific mRNAs that encode a male-specific (BmDSXM) and a female-specific polypeptide (BmDSXF). *Bmdsx* may play an essential role in silkworm sexual development because masculinization of the female genital organs was observed in transgenic female silkworms that expressed male-type *Bmdsx* mRNA (Suzuki *et al*. 2005). Recently, Suzuki *et al*. (2008) reported that *BmPSI*, a *Bombyx* homologue of *P*-element somatic inhibitor (PSI), is required for male-specific splicing of the *Bmdsx*. However, *BmPSI* is located on an autosome, and there is no difference in the expression level of *BmPSI* between males and females. Therefore, it remains to be elucidated exactly how the *Fem* gene on the W chromosome controls the splicing pattern of *Bmdsx*.

In *B. mori*, mutations causing feminization or masculinization have not been isolated on the autosomes or the Z chromosome. On the other hand, an intersex phenotype found to be caused by a mutation of the female-determining gene (*Fem*) (Hirokawa 1995). Unfertilised eggs of *B. mori* treated with hot water (46°C for 2 or 18 min) develop parthenogenetically (reviewed in Strunnikov 1995). During a genetic study of a parthenogenetic line, Hirokawa (1995) found female moths with abnormal genitalia. In normal females, the eighth abdominal segment is almost completely degenerate and lacks scaly hairs, whereas in mutant females the eighth abdominal segment developed well with scaly hairs. In addition to this mutant phenotype, chitinous structures are seen in the abnormal females. The progeny of mutant females crossed with normal males consisted of mutant females and normal males in the ratio 1:1. Based on these results, Hirokawa (1995) speculated that the masculinization was caused by a mutation of the female-determining gene (*Fem*) on the W chromosome. The mutation was designated *Isx* (*Intersex*).

Recently, Fujii *et al*. (2009a) isolated an abnormal line called KG, generating masculinized females. They concluded that masculinization is caused by a mutation of the *Fem* gene on the W chromosome, because masculinized females appeared among crosses between females of the abnormal line and males of the standard silkworm strain, p50T, while masculinized females did not appear in crosses between p50T females and males of the mutant line. Figure 1 illustrates the newly isolated masculinization mutant. Phenotypic abnormalities of the external genitalia of the new mutant line are similar to those of the *Isx* mutant (Hirokawa 1995) and transgenic female silkworms expressing male-type *Bmdsx* (Suzuki *et al*. 2005). The phenotypic abnormalities of the internal genitalia in the new mutant and transgenic female silkworms expressing male-type *Bmdsx* are similar in that both often lack eggs at the basement of the ovarioles (figure 2). Using RT-PCR and quantitative real-time PCR analyses, Fujii *et al*. (2009a) showed that (i) both female-type and male-type *Bmdsx* mRNAs are expressed in the KG line (figure 3); and (ii) *BmOr1* (bombykol receptor) and *BmOr3* (bombykal receptor), which are predominantly expressed in the antennae of normal male moths, were expressed about 50 times more abundantly in the antennae of mutant females than in those of normal females (figure 4). These masculinization mutations on the W chromosome are useful resources for the analysis of the mechanism of sex determination in *B. mori*.

Effect of sex chromosome constitution on the size of eggs produced by polyploid females

In *B. mori*, polyploid individuals with different sex chromosome constitutions can be induced by artificial treatments such as exposure to low temperature or hot water (reviewed in Strunnikov 1995). In *B. mori*, polyploidy affects egg size. Eggs deposited by ZZWW tetraploid females are larger than that deposited by ZZW triploid and ZW diploid females (reviewed in Tazima 1964). However, it is not clear why doubling the chromosome set induces such changes.

In crossing experiments between ZZWW tetraploid females and ZZ diploid males, Hasimoto (1933) found ZZ-type triploid (♂), ZZW triploid (♀) and ZZWW-type triploid (♀) individuals in the ratio 1 : 4 : 1. In another experiment, Kawamura (1988) identified ZWW individuals in filial triploids of ZZWW tetraploids. She crossed *sch*/*sch* diploid males with +/+/W/W tetraploid females and identified *sch* larvae in addition to $+^{sch}$ larvae (*sch* : $+^{sch}$ = 1 : 80). *sch* is a Z-linked mutant in which newly hatched larvae are reddish brown, while the wild type is black. Moreover, *sch* moths produced

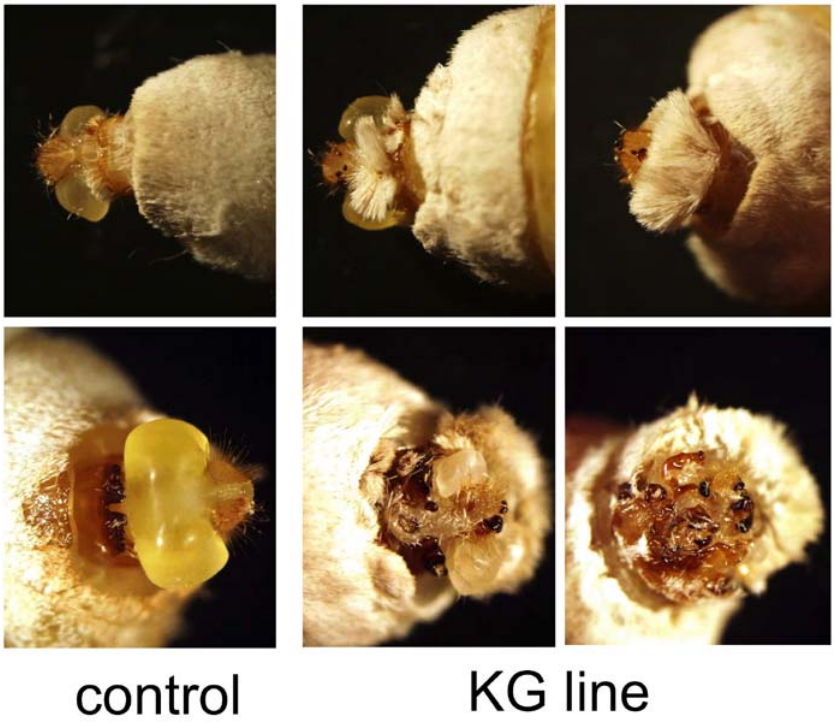

Figure 1. External genitalia of a p50T female (control) and masculinized female moths of the KG line. Masculinization is characterized by the development of the eighth abdominal segment with scaly hairs, formation of some chitinous structures and degeneration of the pheromone glands.

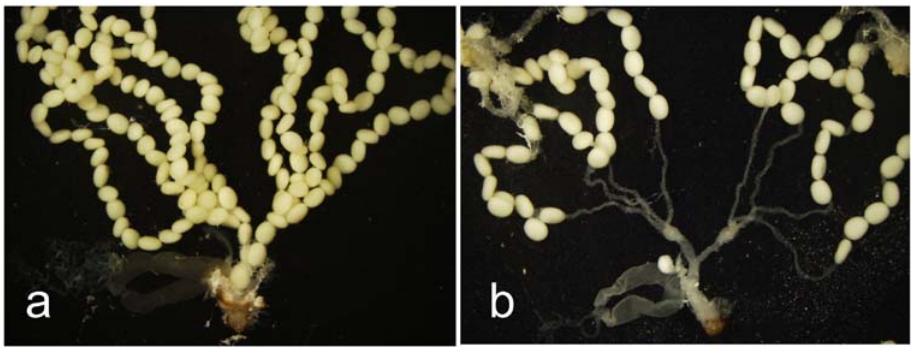

Figure 2. Ovaries of (a) p50T female moth (control), and (b) a masculinized female moth of the KG line. KG females often lack eggs at the basement of the ovarioles.

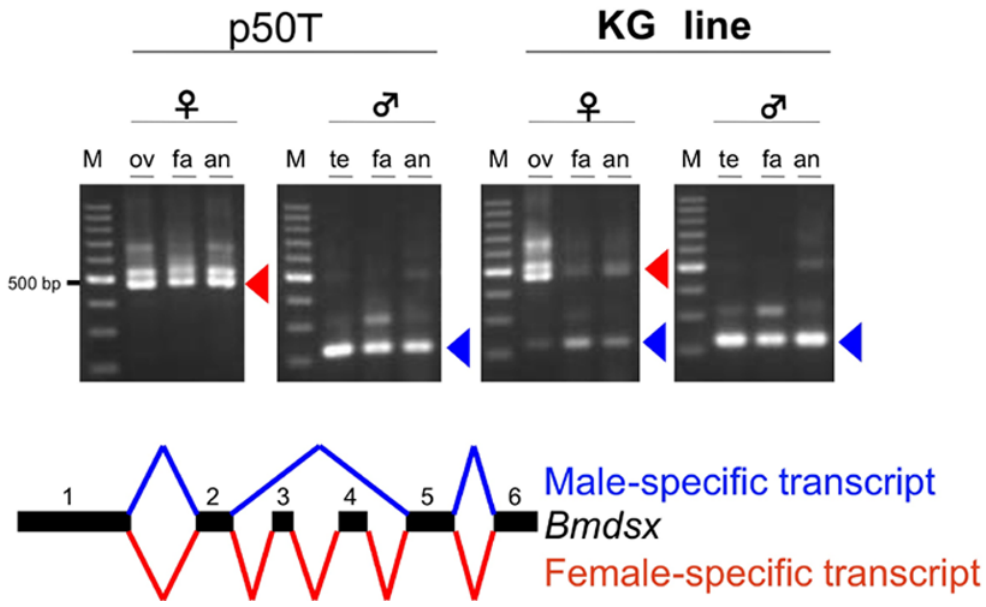

Figure 3. RT-PCR analysis of the splicing pattern of *Bmdsx*. ov, ovary; te, testis; fa, fat body; an, antenna. RNA was extracted from the ovary, testis, and fat body of a fifth-instar larva, and from the antennae of 10 moths. The primer set OP-dsx2/OP-dsx rev1 (Ohbayashi *et al*. 2001) was used. Red arrowheads indicates male-specific *Bmdsx*. Blue arrowheads indicates female-specific *Bmdsx*. M, molecular size marker (100-bp ladder).

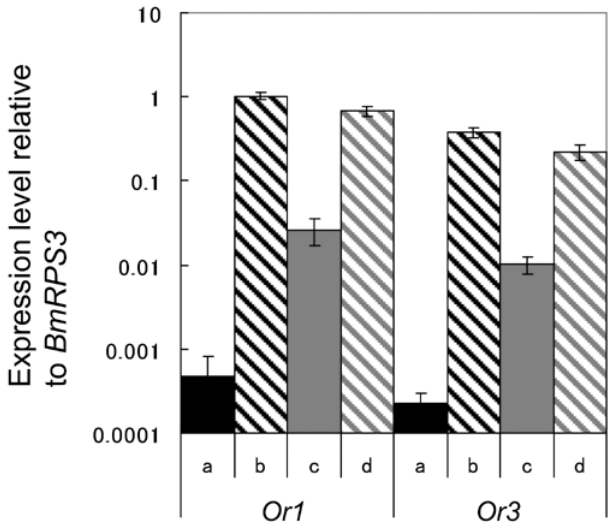

Figure 4. Quantitative real-time PCR analysis of odorant receptor genes in moth antennae. a, p50T female; b, p50T male; c, KG line female; d, KG line male. Each sample consisted of 10 individuals. a, c, $n = 4$; b, d, $n = 3$. Error bars indicate standard deviation ranges. *Or1* encodes the bombykol receptor and *Or3* the bombykal receptor. The same primer sets as Wanner *et al*. (2007) were used for the amplification of *Or1, Or3* and *BmRPS3*.

giant eggs similar to those produced by ZZWW tetraploids, whereas $+^{sch}$ moths did not produce giant eggs (table 1). These observations were explained as follows: (i) the sex chromosome constitutions of *sch* and $+^{sch}$ moths are ZWW and ZZW, respectively; (ii) there is a quantitative egg size-determining gene, *Esd*, on the W chromosome.

Table 1. Effect of sex chromosome constitution on the size of eggs produced by polyploid females of *Bombyx mori*.

Autosome sets	Sex chromosomes	Size of eggs	Author
	ZZW	Normal*	Kawamura 1998
3A	ZWW	Giant	Kawamura 1998
AAA	ZW	Unknows	
	ZZWW	Giant	Kawamura 1998
4A	ZZZW	Normal	Hirokawa 1992
AAAA	ZZW	Unknown	

Normal indicates eggs as large as that of AA:ZW diploid females.
*AAA:ZZW triploid deposites irregular-shared eggs besides normal size eggs.

Interesting results have been obtained on the effect of sex chromosome constitution on egg size. During the induction of ZZZW tetraploid by the hot water treatment of eggs deposited by ZZW triploid females crossed to ZZ diploid males, Hirokawa (1995) identified exceptional males in addition to ZZZW tetraploid females. In the next generation, by crossing these males with diploid females, he obtained triploid females that produced giant eggs. From a genetic analysis, Hirokawa (1995) proposed two hypotheses to explain these phenomena. First, large egg size may be attributable to the W chromosome lacking the *Fem* gene (Df(W) *Fem*). He postulated that ZZZDf(W) *Fem* tetraploid

males were generated and that ZWDf(W) *Fem* triploid females produced giant eggs similar to those of ZWW triploid females. An alternative hypothesis is that generation of the ZZ0-type egg pronucleus (elimination of the W chromosome) resulted in the production of ZZZ0-type tetraploid males and that the ZWO-type triploid females deposited large eggs.

In conclusion, the presence of *Esd* on the W chromosome was not confirmed because the size of the eggs produced by polyploid individuals may be affected by the egg-size reducing factor on the Z chromosome. Therefore, the size of the eggs produced by ZW0-type triploid and ZZW0-type tetraploid should be studied (table 1). Production of eggs of normal size by these polyploids would support Kawamura's hypothesis. On the other hand, the production of giant eggs by these polyploids would indicate the presence of an egg-size-reducing factor on the Z chromosome. Unfortunately, no one has succeeded in inducing such polyploid individuals (AAA : ZW0 and AAAA : ZZW0).

Z-linked morphological traits

Structural analysis of the deleted Z chromosome using PCR markers

In *B. mori*, many Z-chromosome deletions (proximal and distal ends, and middle part) have been induced by exposure to X-rays (reviewed in Tazima 1964). Heterozygous males having a deletion of either of the Z chromosomes are viable, whereas females with a Z-chromosome deletion die during embryogenesis because of the lack of Z-linked genes. Eggs with embryonic lethal factors are useful for the structural analysis of the deleted Z chromosome. The breakpoints of the deleted Z chromosomes can be determined using the DNA extracted from embryonic lethal eggs with the help of PCR markers of the Z chromosome. Fujii *et al*. (2006, 2007, 2008) determined the breakpoints of deleted Z chromosomes, such as Z^{Vg}, Z_1, Z_3 and $+^{pe}Z_4$, as described below (figure 5). These deleted Z chromosomes can be used for the deletion mapping of the Z-linked morphological traits controlled by *Vg*, *spli*, *Md* and *od*.

Vg (vestigial)

More than 10 wing development-related mutants are known in *B. mori* (Banno *et al*. 2005). A responsible gene has been clarified for only one of them: *fl*. The wing-deficient phenotype of *fl* homozygotes is caused by a defect in *Bmfringe*, encoding Fng glycosyltransferase, which regulates the Notch signalling pathway (Sato *et al*. 2008). *Vg* is an X-ray-induced dominant gene that causes a vestigial phenotype of the wings (figure 6). Individuals of genotype $+^{Vg}od/Vg$ always exhibit

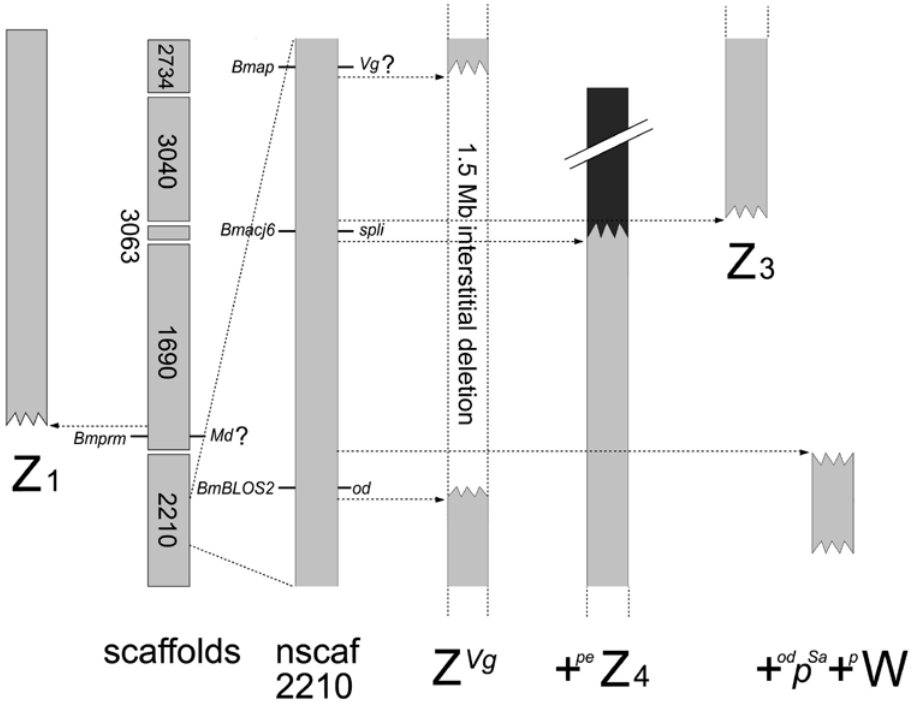

Figure 5. Schematic illustration of deleted Z chromosomes. Five scaffolds (nscaf 2734, 2.0 Mb; nscaf 3040, 4.8 Mb; nscaf 3063, 0.5 Mb; nscaf 1690, 8.0 Mb; nscaf 2210, 5.1 Mb) were assigned on the Z chromosome (The International Silkworm Genome Consortium 2008). The black region represents the chromosome 5 region of the $+^{pe}Z_4$ chromosome. Only the Z chromosomal region is shown on the $+^{od}p^{Sa+p}$W chromosome.

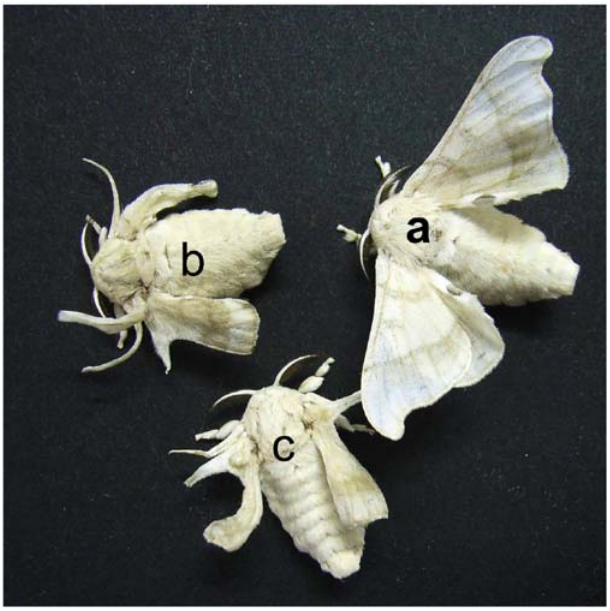

Figure 6. Wing phenotype of *Vg* moths. (a) Control (+/+); (b, c) *Vg*/+.

the *od* phenotype because the Z chromosome containing the *Vg* gene (Z^{Vg} chromosome) lacks the *od* locus (1–49.6) (Tazima 1944). Therefore, a terminal deletion may include the *od* locus in Z chromosomes bearing the *Vg* mutation.

Fujii *et al*. (2008) determined the breakpoint of the Z^{Vg} chromosome using WGS sequence data and embryonic lethal eggs with a Z^{Vg}/W sex chromosomal constitution. They did not identify a terminal deletion but a 1.5-Mb-interstitial deletion on the Z^{Vg} chromosome. Moreover, they succeeded in obtaining a fragment containing a breakpoint junction. Sequencing of the fragment resulted in the precise identification of the proximal and distal breakpoints. Neither breakpoint disrupted the computationally predicted genes on the Z chromosome. However, *Bombyx* has an orthologue of *apterous* (*ap*) in *Drosophila* near the distal breakpoint of the 1.5-Mb-interstitial deletion (Fujii *et al*. 2008).

In *Drosophila*, Apterous is a LIM-homeodomain protein responsible for the establishment of dorsal and ventral compartments in the developing wing. Expression of *apterous* is restricted to the dorsal compartment of the wing disc (Cohen *et al*. 1992; Diaz-Benjumea and Cohen 1993). Ectopic expression of *apterous* causes a severe wing defect (Rincon-Limas *et al*. 2000). As in *Drosophila*, in *Precis coenia* (Lepidoptera: Nymphalidae) the wing disc is divided into dorsal and ventral domains by expression of *apterous* (Carroll *et al*. 1994). The 1.5-Mb deletion at the 5′-flanking region of the *Bombyx* orthologue of *apterous* probably causes its ectopic expression, leading to the vestigial phenotype.

spli (soft and pliable)

In *B. mori*, there are several mutants related to the hardness or softness of the larval body. For example, the body of *sk* (stick, 4–25.8) mutant larvae is firm to the touch, while that of *spli* (soft and pliable) mutants is extremely soft and pliable. It is presumed that *spli* larvae have a defect in the central nervous system or in a brain-controlled body wall muscle response (Murakami and Ohnuma 1978). The *spli* phenotype was first identified in the F_1 generation of $+^{pe}$ males generated in a cross between Z/T(W;5)$+^{pe}$, *pe/pe* and Z/Z, *pe/pe*, moths. In *pe* homozygotes, the pigment in the serosa cells is light orange and the compound eyes are pink. $+^{pe}$ males were presumed to have a chromosome 5 fragment bearing a $+^{pe}$ locus (5–0.0) that was dissociated from the T(W;5)$+^{pe}$ chromosome.

Fujii *et al*. (2010a) performed genetic and cytogenetic analyses of the Z chromosome in the n41 strain (*spli/spli*, *spli*/W). They found that the Z chromosome was broken between the *sch* (1–21.5) and *od* (1–49.6) loci to form two fragments (Z_3 and Z_4) (figure 6). They also found a chromosomal fragment bearing a $+^{pe}$ locus, which was dissociated from the T(W;5)$+^{pe}$ chromosome, fused to a Z chromosome fragment bearing the $+^{od}$ locus ($+^{pe}Z_4$) (figure 6). It was confirmed that the fused chromosome comprised chromosome 5 and the Z chromosome by fluorescence *in situ* hybridization using BAC clones as probes. Breakpoints of Z_3 and Z_4 fragments were determined using Z-chromosome PCR markers. In conclusion, (i) there is no overlapping region between Z_3 and Z_4; and (ii) a region of less than 96 kb, which contains the *Bombyx* homologue of *Drosophila acj6* (*Bmacj6*), is absent in the Z chromosome of the n41 strain.

Acj6 is a POU transcription factor of class IV, homologous to vertebrate Brn3 family members, which perform essential functions in the differentiation of specific neuronal cell types (Treacy *et al*. 1991; Clyne *et al*. 1999a). In *Drosophila, acj6* mutant was found by the Bump assay to isolate flies with reduced mobility from an EMS-treated population (Clyne *et al*. 1999a). The mobility phenotypes associated with *acj6* mutations are thought to reflect functional defects in the central nervous system that regulates locomotor function (Certel *et al*. 2000). Like *acj6* mutants, *spli* larvae are less active than wild-type insects (which move very slowly), which is consistent with the fact that *Bmacj6* is the gene responsible for the *spli* phenotype (Fujii *et al*. 2010a).

In *Drosophila*, the *acj6* mutant was first isolated during behavioural screening for mutations lacking odorant responses (Ayer and Carlson 1991). In addition to the locomotor defect, *acj6* mutants also showed olfactory defects. In insects with a null mutation of *acj6*, some olfactory receptor neurons in the maxillary palp responded normally to odours, some did not respond to odours and some showed changes in odour specificity (Clyne *et al*. 1999a). In another study, Clyne *et al*. (1999b) reported that a subset of odour receptor genes did not expressed in *acj6* null mutants.

Murakami and Ohnuma (1978) observed that normal *B. mori* males flapped their wings vigorously in a mating dance in response to females, while *spli* males did not show such a response, even though these males were able to flutter their

wings when suspended in the air. The sex pheromone in *B. mori* is bombykol (Butenandt *et al*. 1959). Fujii *et al*. (2009b) confirmed that *spli* male moths are less sensitive to bombykol than normal males. This suggests that (i) *Bmacj6* functions in the olfactory system in a manner similar to that seen in *Drosophila;* and (ii) the *spli* mutant can be used for the study of olfaction in *B. mori*.

Md (muscle dystrophy)

Both males and females of *B. mandarina*, the closest wild ancestor of *B. mori*, can fly. *B. mori* has lost the ability to fly during the process of domestication, although *B. mori* males vibrate their wings when they are exposed to the sex pheromone from females. Wing vibration plays an essential role in determining the final location of the female, because it draws pheromone-containing air past the male's antennae, enabling the insect to find its way to the source of the pheromone (Obara 1979). In *B. mori*, a mutant (*Md*, muscle dystrophy) related to the wing-vibrating ability of the male was reported.

In the course of dissociation experiments on the T(W:3)*Ze* chromosome, Fujii *et al*. (2007) identified deleted Z chromosomes, which they designated as Z_1 and Ze^WZ_2 (figure 6). These were generated by a breakage event between *sch* (1–21.5) and $+^{od}$ (1–49.6) loci. The Z_1 chromosome has *sch*, while the Ze^WZ_2 chromosome has $+^{od}$. The Ze^WZ_2 chromosome has an additional chromosomal fragment dissociated from the T(W:3)*Ze* chromosome. Interestingly, Z/Z_1 Ze^WZ_2 males can flap their wings, whereas Z/Z_1 males cannot because of indirect flight muscle dystrophy (Fujii *et al*. 2007). This observation indicates that the flapless phenotype is not caused by the dominant mutation on the Z_1 chromosome. In *Drosophila*, the genes that encode major myofibrillar proteins, such as actin 88F, tropomyosin 2, troponin I, troponin T, myosin light chain 2 and myosin heavy chain, are haploinsufficient for flight. Haploidy for these genes results in dominant flightlessness. Therefore, it was presumed that the muscle-related dose-sensitive gene is located on the deleted region of the Z_1 chromosome (the Z_2 region of the Z chromosome) (Fujii *et al*. 2007).

In *Drosophila*, about 20 myofibrillar proteins have been reported (Vigoreaux 2001). The genes encoding *Bombyx* homologues of three *Drosophila* myofibrillar proteins (kettin, projectin, and titin) are located on the Z_1 region of the Z chromosome (Koike *et al*. 2003; Fujii *et al*. 2007). Fujii *et al*. (2008) searched the 14,623 computationally predicted genes of *B. mori* (The International Silkworm Genome Consortium 2008) which encode other *Bombyx* homologues of *Drosophila* myofibrillar proteins. They found that the *Bombyx* homologues of *paramyosin* (*prm*), *stretchin-MLCK*, *flightin*, and ADP/ATP translocase are Z-linked. The Z_1 chromosome contains the gene for the *Bombyx* homologue of flightin, while it lacks those for paramyosin, stretchin-MLCK, and ADP/ATP translocase.

The F_1 generation produced by crossing *spli* females (W/Z3 $+^{pe}Z_4$) with normal (Z/Z) males contains Z/Z_3 males because of nondisjunction between the W chromosome and $+^{pe}Z_4$ during female meiosis (Fujii *et al*. 2010a). Z/Z_3 males can flap their wings (T. Fujii, unpublished data). The Z_3 chromosome lacks the genes for *stretchin-MLCK* and ADP/ATP translocase. These two genes are therefore not haploinsufficient for flight, and the candidate gene for the muscle dystrophy phenotype caused by the Z_1 chromosome is *prm*, although *prm* does not mutate to cause dominant flightlessness in *Drosophila*.

od (distinct oily)

The normal larval skin of *B. mori* is opaque and white because of the accumulation of uric acid granules in the epidermis. However, there are many mutants (so-called oily mutants) whose larval skin is transparent and similar to oiled paper. The mutant genes responsible for these oily skin characters are distributed in distinct loci in up to 18 linkage groups. Oily mutants can be classified into two types. One type is caused by the inability to synthesize uric acid. The other results from the lack of accumulation of uric acid in the epidermis. *od* (1–49.6, distinct translucent) is an oily mutant of the latter type (Tamura and Sakate 1983).

$+^{od}p^{Sa} + p$W is a W chromosome to which a short fragment of the Z chromosome containing the *od* locus has been translocated (Tazima 1948; Sahara *et al*. 2003). To locate the *od* locus, Fujii *et al*. (2008) determined the length of the Z chromosomal region using embryonic lethal eggs with the $Z_1/ + {}^{od}p^{Sa} + p$W constitution. They showed that the region is less than 0.6-Mb long. Of this sequence, the 0.2-Mb region overlaps between the 1.5-Mb deleted region on the Z^{Vg} chromosome and the Z-chromosome region on the $+^{od}p^{Sa} + p$W chromosome (figure 6). Accordingly, the *od* locus was localized to the 0.2-Mb region. Fujii *et al*. (2008) identified a gene, *BmBLOS2*, whose mRNA was absent from the 0.2-Mb region in *od* homozygotes. Sequencing of *BmBLOS2* revealed that exons 1, 2, and 3 of *BmBLOS2* were deleted in the *od* strain. The deletion was presumed to be generated by intrachromosomal recombination between two transposable elements, Bm1 (Adams *et al*. 1986).

BLOS2 is one of eight subunits of the lysosome-related organelles complex 1 (BLOC-1). In humans, a complex of four proteins—AP-3 (adaptor protein 3), BLOC-1 (biogenesis of lysosome-related organelles complex 1), BLOC-2 and BLOC-3—is responsible for a genetic disease, the Hermansky–Pudlak syndrome (HPS) (Wei 2006). HPS consists of several genetically different recessive disorders which share the clinical manifestation of hypopigmentation and a platelet storage pool deficiency due to abnormal vesicle trafficking to lysosomes and related organelles, such as melanosomes and platelet-dense granules (Wei 2006). Electron microscopy revealed that *od* cells contain fewer urate granules, which are smaller and more rounded than those in normal cells (Tamura and Akai 1990). The translucent phe-

notype of *od* larvae is rescued in transgenic silkworms expressing wild-type *BmBLOS2* (figure 7) (Fujii *et al*. 2010b). These results suggest that (i) *BmBLOS2* is the gene responsible for *od*, and (ii) a function of *BmBLOS2* is the biogenesis of uric acid granules. In *Drosophila*, orthologues of the genes that are mutated in human HPS include nine eye pigmentation genes related to the biogenesis of eye pigment granules (Falcon-Perez *et al*. 2007). This suggests the possibility that *Bombyx* orthologues of HPS genes are responsible for other oily mutants with defects in uric granules.

As mentioned in section *Vg* (vestigial), the Z chromosome of the *spli* strain is divided into two parts: Z_3 and $+^{pe}Z_4$. Fujii *et al*. (2010b) found that there were transparent patches in the white integument of od/Z_3 $+^{pe}Z_4$ larvae. They concluded that sporadic loss of the $+^{pe}Z_4$ fragment, which carries the $+^{od}$ locus, results in the generation of mottled *od* larvae. Fujii *et al*. (2010b) found that Z^{Vg}/Z_3 $+^{pe}Z_4$ larvae exhibited a mottled, translucent integument similar to that of od/Z_3 $+^{pe}Z_4$ larvae. The Z^{Vg} chromosome lacks the *od* locus (Tazima 1944). Therefore, Fujii *et al*. (2010b) concluded that the mottled translucent phenotype observed in Z^{Vg}/Z_3 $+^{pe}Z_4$ larvae is not a novel mutant but a mottled variant of *od* caused by sporadic loss of the $+^{pe}Z_4$ chromosome during cell division.

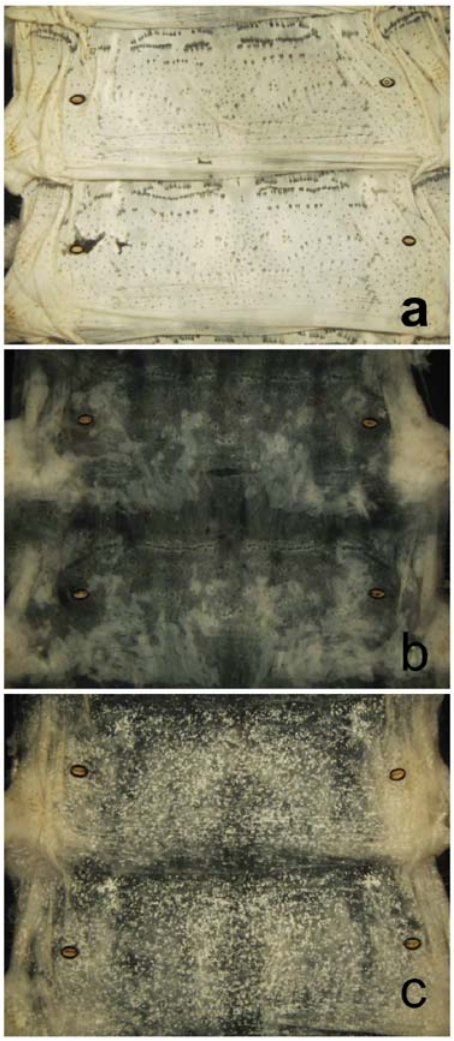

Figure 7. Larval phenotype of transgenic silkworms. Larval integuments were dissected and photographed. a, $od/+^{od}$. b, *od*/W. c, *od*/W, *A3-GAL4*/+, *UAS-BmBLOS2*/+.

Conclusion

Although the W chromosome may carry the gene that determines femaleness, this gene has not yet been cloned. The W-linked mutant in the KG line, which exhibits a masculinized phenotype, is an important resource in the search for this gene because it affects the sex-specific expression of *Bmdsx*, a downstream sex determinant. Since it is only the W-Rikishi region that is retained in the T(W;2)*Y*-abe chromosome, which is able to determine femaleness, a precise sequence-level comparison between the mutant and normal silkworms should be undertaken.

Generally, Z chromosome-linked genes are not dosage compensated in *Bombyx*; their expression is biased towards males (Suzuki *et al*. 1998, 1999; Koike *et al*. 2003). The Z chromosome is enriched in testis-specific genes (Arunkumar *et al*. 2009) and two genes coding for the pheromone receptors *Or1* and *Or3* are located on the Z chromosome (Sakurai *et al*. 2004). Cloning and molecular characterization of the genes on the Z chromosome which affect male-specific functions, will be helpful in enabling us to understand how much the Z chromosome contributes to sexual dimorphism, in addition to the contribution of the W chromosome to sex determination.

References

Abe H., Seki M., Ohbayashi F., Tanaka N., Yamashita J., Fujii T. *et al*. 2005 Partial deletions of the W chromosome due to reciprocal translocation in the silkworm *Bombyx mori. Insect Mol. Biol.* **14**, 339–352.

Abe H., Fujii T., Tanaka N., Yokoyama T., Kakehashi H., Ajimura M. *et al*. 2008 Identification of the female-determining region of the W chromosome in *Bombyx mori. Genetica* **133**, 269–282.

Abe H., Fujii T. and Shimada T. 2010 Sex chromosomes and sex determination in *Bombyx mori.* In *Molecular biology and genetics of the Lepidoptera* (ed. M. R. Goldsmith and F. Marec), pp. 65–87. CRC Press, Boca Raton, USA.

Adams D. S., Eickbush T. H., Herrera R. J. and Lizardi P. M. 1986 A highly reiterated family of transcribed oligo(A)-terminated, interspersed DNA elements in the genome of *Bombyx mori. J. Mol. Biol.* **187**, 465–478.

Arunkumar K. P., Mita K. and Nagaraju J. 2009 The silkworm Z chromosome is enriched in testis-specific genes. *Genetics* **182**, 493–501.

Ayer Jr R. K. and Carlson J. 1991 *acj6:* A gene affecting olfactory physiology and behavior in *Drosophila. Proc. Natl. Acad. Sci. USA* **88**, 5467–5471.

Banno Y., Fujii H., Kawaguchi Y., Yamamoto K., Nishikawa K., Nishizaka A. *et al*. 2005 *A guide to the silkworm mutants: gene name and gene symbol.* Kyusyu University, Fukuoka, Japan.

Butenandt V. A., Beckmann R., Stamm D. and Hecker E. 1959 Über den Sexual-Lockstoff des Seidenspinners *Bombyx mori.* Reindarstellung und Konstitution. *Z. Naturforsch.* **14**, 283–284.

Carroll S. B., Gates J., Keys D. N., Paddock S. W., Panganiban G. E., Selegue J. E. and Williams J. A. 1994 Pattern formation and eyespot determination in butterfly wings. *Science* **265**, 109–114.

Certel S. J., Clyne P. J., Carlson J. R. and Johnson W. A. 2000 Regulation of central neuron synaptic targeting by the *Drosophila* POU protein, Acj6. *Development* **127**, 2395–2405.

Clyne P. J., Certel S. J., de Bruyne M., Zaslavsky L., Johnson W. A. and Carlson J. R. 1999a The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POUdomain transcription factor. *Neuron* **22**, 339–347.

Clyne P. J., Warr C. G., Freeman M. R., Lessing D., Kim J. and Carlson J. R. 1999b A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila. Neuron* **22**, 327–338.

Cohen B., McGuffin M. E., Pfeifle C., Segal D. and Cohen S. M. 1992 *apterous:* a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev.* **6**, 715–729.

Diaz-Benjumea F. J. and Cohen S. M. 1993 Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila. Cell* **75**, 741–752.

Falcon-Perez J. M., Romero-Calderon R., Brooks E. S., Krantz D. E. and Dell'Angelica E. C. 2007 The *Drosophila* pigmentation gene *pink (p)* encodes a homologue of human Hermansky–Pudlak syndrome 5 (HPS5). *Traffic* **8**, 154–168.

Fujii T., Tanaka N., Yokoyama T., Ninaki O., Oshiki T., Ohnuma A. *et al*. 2006 The female-killing chromosome of the silkworm, *Bombyx mori*, was generated by translocation between the Z and W chromosomes. *Genetica* **127**, 253–265.

Fujii T., Yokoyama T., Ninagi O., Kakehashi K., Obara Y., Nenoi M. *et al*. 2007 Isolation and characterization of sex chromosome rearrangements generating male muscle dystrophy and female abnormal oogenesis in the silkworm, *Bombyx mori. Genetica* **130**, 267–280.

Fujii T., Abe H., Katsuma S., Mita K. and Shimada T. 2008 Mapping of sex-linked genes onto the genome sequences using various aberrations of the Z chromosome in *Bombyx mori. Insect Biochem. Mol. Biol.* **38**, 1072–1079.

Fujii T., Daimon T., Abe H., Katsuma S. and Shimada T. 2009a Newly identified mutation of the W-linked female determinant gene causing masculinization of the female in the silkworm, *Bombyx mori*. Proceedings of the 60th Meeting of the Japanese Society of Sericulture Science Kanto Branch, Tokyo, Japan, pp. 22.

Fujii T., Fujii T., Abe H., Ohnuma, A., Katsuma S. and Shimada T. 2009b Defect of responses to the sex pheromone observed in the males of Z-linked mutant, *spli* and *Bt*. In Proceedings of the 60th Meeting of the Japanese Society of Applied Entomology and Zoology, pp. 106.

Fujii T., Daimon T., Uchino K., Banno Y., Katsuma S., Sezutsu H. *et al*. 2010a Transgenic analysis of the *BmBLOS2* governing the translucency of larval integument of the silkworm, *Bombyx mori. Insect Mol. Biol*. (doi:10.1111/j.1365-2583.2010.01020.x).

Fujii T., Kuwazaki S., Yamamoto K., Abe H., Ohnuma A., Katsuma S. *et al*. 2010b Identification and molecular characterization of a sex chromosome rearrangement causing a soft and pliable (*spli*) larval body phenotype in the silkworm, *Bombyx mori. Genome* **53**, 45–54.

Hasimoto H. 1933 The role of the W-chromosome in the sex determination of *Bombyx mori. Jpn. J. Genet.* **8**, 245–247 (in Japanese).

Hirokawa M. 1992 Induction of ZZZW sex-chromosome type tetraploid strain by treatment with hot-water of inseminated eggs laid by triploid female moths in the silkworm, *Bombyx mori. J. Seric. Sci. Jpn.* **61**, 59–65 (in Japanese).

Hirokawa M. 1995 Studies on sex control in the silkworm, *Bombyx mori. Bull. Fukushima Seric. Exp. Sta.* **28**, 1–104 (in Japanese with English summary).

International Silkworm Genome Consortium. 2008 The genome of a lepidopteran model insect, the silkworm *Bombyx mori. Insect Biochem. Mol. Biol.* **38**, 1036–1045.

Kawamura N. 1988 The egg size determining gene, *Esd*, is a unique morphological marker on the W chromosome of *Bombyx mori. Genetica* **76**, 195–201.

Koike Y., Mita K., Suzuki M.G., Maeda S., Abe H., Osoegawa K. *et al*. 2003 Genomic sequence of a 320-kb segment of the Z chromosome of *Bombyx mori* containing a kettin ortholog. *Mol. Genet. Genomics* **269**, 137–149.

Lukhtanov V. A. 2000 Sex chromatin and sex chromosome systems in non-ditrysian Lepidoptera (Insecta), *J. Zool. Syst. Evol.* Res. **38**, 73–79.

Mita K., Kasahara M., Sasaki S., Nagayasu Y., Yamada T., Kanamori H. *et al*. 2004 The genome sequence of silkworm, *Bombyx mori. DNA Res.* **11**, 27–35

Murakami A. and Ohnuma A. 1978 Genetic analysis of a mutant showing abnormal behavior in the silkworm. *Ann. Rep. Nati. Inst. Genet. Jap.* **28**, 99–100.

Obara Y. 1979 *Bombyx mori* mating dance: an essential in locating the female. *Appl. Entomol. Zool.* **14**,130–132.

Ohbayashi F., Suzuki M. G., Mita K., Okano K. and Shimada T. 2001 A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori. Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **128**, 145–158.

Prowell D. P. 1998 Sex linkage and speciation in Lepidoptera. In *Endless forms: species and speciation* (ed. S. Berlocher and D. Howard). Oxford University Press, New York, USA.

Rincón-Limas D.E., Lu C.H., Canal I. and Botas J. 2000 The level of DLDB/CHIP controls the activity of the LIM homeodomain protein Apterous: evidence for a functional tetramer complex *in vivo. EMBO J.* **19**, 2602–2614.

Saccone G., Pane A. and Polito L. C. 2002 Sex determination in flies, fruitflies and butterflies. *Genetica* **116**, 15–23.

Sahara K., Yoshido A., Kawamura N., Ohnuma A., Abe H., Mita K. *et al*. 2003 W-derived BAC probes as a new tool for identi cation of the W chromosome and its aberrations in *Bombyx mori. Chromosoma* **112**, 48–55.

Sakurai T., Nakagawa T., Mitsuno H., Mori H., Endo Y., Tanoue S. *et al*. 2004 Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori. Proc. Natl. Acad. Sci. USA* **101**, 16653–16658.

Sato K., Matsunaga T. M., Futahashi R., Kojima T., Mita K., Banno Y. and Fujiwara H. 2008 Positional cloning of a *Bombyx* wingless locus *flügellos (fl)* reveals a crucial role for fringe that is specific for wing morphogenesis. *Genetics* **179**, 875–885.

Sperling F. 1994 Sex-linked genes and species differences in Lepidoptera. *Can. Entomol.* **126**, 807–818.

Strunnikov V. A. 1995 *Control over reproduction, sex and heterosis of the silkworm.* Harwood Academic Publishers, Moscow, Russia.

Suzuki M. G., Shimada T. and Kobayashi M. 1998 Absence of dosage compensation at the transcription level of a sex-linked gene in a female heterogametic insect, *Bombyx mori. Heredity* **81**, 275–283.

Suzuki M. G., Shimada T. and Kobayashi M. 1999 *Bm kettin*, homologue of the *Drosophila kettin* gene, is located on the Z chromo-

some in *Bombyx mori* and is not dosage compensated. *Heredity* **82**, 170–179

Suzuki M. G., Funaguma S., Kanda T., Tamura T. and Shimada T. 2005 Role of the male BmDSX protein in the sexual differentiation of *Bombyx mori. Evol. Dev.* **7**, 58–68.

Suzuki M. G., Imanishi S., Dohmae N., Nishimura T. and Shimada T. and Matsumoto S. 2008 Establishment of a novel *in vivo* sex-specific splicing assay system to identify a trans-acting factor that negatively regulates splicing of *Bombyx mori dsx* female exons. *Mol. Cell Biol.* **28**, 333–343.

Tamura T. and Sakate S. 1983 Relationship between expression of oily character and uric acid incorporation in the larval integument of various oily mutants of the silkworm, *Bombyx mori. Bull. Seric. Exp. Stn.* **28**, 719–740 (in Japanese).

Tamura T. and Akai H. 1990 Comparative ultrastructure of larval hypodermal cell in normal and oily *Bombyx* mutant. *Cytologia* **55**, 519–530.

Tazima Y. 1944 Studies on chromosome aberrations in the silkworm. II. Translocation involving second and W-chromosomes. *Bull. Seric. Exp. Stn.* **12**, 109–181 (in Japanese).

Tazima Y. 1948 Translocation of the Z chromosome to the W chromosome of the silkworm, *Bombyx mori.* In Oguma Commemoration Volume on Cytology and Genetics, pp. 88–91 (in Japanese).

Tazima Y. 1964 *The genetics of the silkworm*. Academic press, London, UK.

Traut W. 1977 A study of recombination, formation of chiasmata and synaptonemal complexes in female and male meiosis of *Ephestia kuehniella* (Lepidoptera). *Genetica* **47**, 135–142.

Traut W. and Marec F. 1996 Sex chromatin in Lepidoptera. *Quart. Rev. Biol.* **71**, 239–256.

Traut W., Sahara K., Otto T. D. and Marec F. 1999 Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. *Chromosoma* **108**, 173–180.

Treacy M. N., He X. and Rosenfeld M. G. 1991 I-POU: a POU domain protein that inhibits neuron-specific gene activation. *Nature* **350**, 577–584.

Vigoreaux J. O. 2001 Genetics of the *Drosophila* flight muscle myofibril: a window into the biology of complex systems. *BioEssays* **23**, 1047–1063

Wanner K. W., Anderson A. R., Trowell S. C., Theilmann D. A., Robertson H. M. and Newcomb R. D. 2007 Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori. Insect Mol. Biol.* **16**, 107–119.

Wei M. L. 2006 Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. *Pigment Cell Res.* **19**, 19–42.

Xia Q., Zhou Z., Lu C., Cheng D., Dai F., Li B. *et al*. 2004 A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). *Science* **306**, 1937–1940.

Yamamoto K., Narukawa J., Kadono-Okuda K., Nohata J., Sasanuma M., Suetsugu Y. *et al*. 2006 Construction of a single nucleotide polymorphism linkage map for the silkworm, *Bombyx mori*, based on bacterial artificial chromosome end sequences. *Genetics* **173**, 151–161.

Received 5 December 2009, in revised form 25 April 2010; accepted 26 April 2010
Published on the Web: 6 September 2010