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Genetic mapping of Z chromosome and identification of W chromosome-specific markers in the silkworm, *Bombyx mori*

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In the silkworm, *Bombyx mori*, the female is the heterogametic (ZW) sex and the male is homogametic (ZZ). The female heterogamety is a typical situation in the insect order *Lepidoptera*. Although the W chromosome in silkworm is strongly female determining, no W-linked gene for a morphological character has been found on it. The Z chromosome carries important traits of economic value as well as genes for various phenotypic traits, but only 2% of molecular information based on its relative size is known. Studies conducted so far indicate that the Z-linked genes are not dosage compensated. In the present study, we constructed a genetic map of randomly amplified polymorphic DNA fragments (RAPD), simple sequence repeats (SSR), and fluorescent intersimple sequence repeat PCR (FISSR) markers for the Z chromosome using a backcross mapping population. A total of 16 Z-linked markers were identified, characterized, and mapped using *od*, a recessive trait for translucent skin as an anchor marker yielding a total recombination map of 334.5 cM. The linkage distances obtained suggested that the markers were distributed throughout the Z chromosome. Four RAPD and four SSR markers that were linked to W chromosome were also identified. The proposed mapping approach should be useful to identify and map sex-linked traits in the silkworm. The economic and evolutionary significance of Z- and W-linked genes in silkworm, in particular, and lepidopterans, in general, is discussed.
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Introduction

In many organisms, sex is determined by a set of dimorphic sex chromosomes (X and Y) that are thought to have evolved from an autosome (Ohno, 1967; Bull, 1983; Guttman and Charlesworth, 1998). Sex chromosomes have evolved independently many times, and Y chromosomes lack genetic recombination over most or all of their length (Jegalian and Page, 1998; Charlesworth and Charlesworth, 2000). In *Drosophila melanogaster* and humans, the Y chromosome has lost most of its active genes (Carvalho *et al*, 2001; Lahn *et al*, 2001), and those remaining appear largely to affect male-specific functions. For example, several genes have been identified on the Y chromosome of *D. melanogaster* that are expressed during spermatogenesis and influence fitness primarily through their effect on male reproductive success (Chippindale and Rice, 2001; Carvalho, 2002). Similarly, in humans, apart from the testis-determining factor, the Y chromosome contains only a few genes or gene families, many of which have testes-specific functions (Lahn *et al*, 2001). Recently, Skaletsky *et al* (2003) reported that more than 63 million base pairs (Mb) of the Y chromosome are male-specific, consisting of some 23 Mb of euchromatin and a variable amount of heterochromatin.

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In the domesticated silkworm, *Bombyx mori*, the chromosomal sex determination is reversed: the male is the homogametic sex (ZZ) and the female is heterogametic (ZW). The ZW bivalent is reported to have no crossing over (Sturtevant, 1915; Tazima, 1978). These features have been confirmed in other lepidopteran insects (Traut, 1977), and may prove valuable for the detection of sex-linked characters, and for studying the evolution of sex chromosomes. Female sex in silkworm is determined by the presence of a single W chromosome, regardless of the number of autosomes or Z chromosomes (Hasimoto, 1930); hence, the W chromosome is assumed to carry a primary determinant for femaleness. Using irradiated chromosome fragments, Tazima (1964) confirmed this model of sex determination in *B. mori* and showed that the primary sex determinants are localized at one end of the W chromosome.

Although more than 200 visible mutations have been placed on silkworm linkage maps (Fujii *et al*, 1998), no gene for a morphological character has so far been mapped to the W chromosome. In search of W chromosome-specific markers, Abe *et al* (1995, 1996, 1998a, 2000) and Ohbayashi *et al* (1996) identified five RAPDs. Using these markers for subsequent cloning and sequencing of W-derived BAC clones, they revealed that the W chromosome is largely composed of nested, full-length retrotransposable elements (Abe *et al*, 1998b, 2000, 2001). Owing to their high repetition, these kinds of sequences are difficult to use for chromosome walking, or even for isolation of contigs of interest. Identification of additional

W-linked markers would be useful for establishing a global map to facilitate the assembly of W contigs, and for finding sex-determining gene(s). Further, intensive analysis of the W chromosome of *B. mori* could shed light on its organization and evolution.

The classical linkage map of the Z chromosome of *B. mori* contains 15 morphological traits dispersed over 50 cM (centiMorgans) (Fujii *et al*, 1998). They include a number of important traits of economic value, such as late maturity (*Lm*), which affects voltinism (number of life cycles in a year), moltinism (number of larval moults per life cycle), and quantitative traits such as cocoon weight and cocoon shell weight (Tazima, 1978). In addition, the Z chromosome harbors genes for various phenotypic traits expressed in the egg, larva, and moth, such as Giant egg (*Ge*), Green eggshell color (*Gre*), elongated larval body (*e*), chocolate larval color (*sch*), translucent larval skin *(od)*, and Vestigial wing (*Vg*). Recently, Yasukochi (1998) identified 18 Z-linked RAPD markers, and Koike *et al* (2003) identified 13 additional genes in a contiguous 320 kb walk of the Z-chromosome starting with the sex-linked marker, *Bmkettin*, a homolog of the *D. kettin* muscle protein gene (Suzuki *et al*, 1998, 1999).

Characteristics affecting reproductive isolation and host race formation appear to be predominantly sex linked in many groups of lepidoptera (Sperling, 1994). Thus, analysis of Z-linked genes especially those controlling maturity, diapause, body size, etc. in silkworm will help in evaluating the role of these traits in speciation and evolution. Analysis of the repeat content and the interspersed elements on the Z chromosome and their distribution across the chromosome may help to elucidate dosage compensation for genes that have not previously been investigated (Suzuki *et al*, 1998).

Molecular linkage maps using a variety of markers have been constructed for the silkworm (Nagaraju and Goldsmith, 2002; Goldsmith *et al*, 2005). In the present study, a mapping population was constructed specifically to identify and map sex chromosome-linked markers. Randomly amplified polymorphic DNA fragments (RAPD), simple sequence repeats (SSR), and fluorescent intersimple sequence repeat PCR (FISSR) markers were identified, characterized, and analyzed to construct a molecular linkage map of the Z chromosome.

Materials and methods

Silkworm strains

A silkworm strain that carries a Z-linked mutation, translucent larval skin (*od*), was used as a reference for Z chromosome mapping. Normal and translucent larvae can be unambiguously identified in a segregating population. F_1 hybrids were raised by crossing a diapausing, translucent (Z^{od}) female with a wild-type (Z^{PM}) Pure Mysore male. A backcross population was raised by crossing an F_1 hybrid male to a translucent (Z^{od}) female in order to obtain a recombination map of the Z chromosome (Figure 1).

DNA extraction

All DNA extractions were performed on moths frozen in liquid nitrogen (after oviposition, in the case of females) using the method of Nagaraja and Nagaraju (1995).

RAPD analysis

RAPD analysis was carried out using 560 random primers (OPA to OPZ, OPAA and OPAH kits) obtained from Operon Technologies, Alameda, CA, USA. The amplification of genomic DNA was performed according to Nagaraja and Nagaraju (1995).

SSR analysis

SSR analysis was performed using primer sets for 216 different microsatellite loci derived from a variety of sources: a subgenomic library (Reddy *et al*, 1999), GenBank submissions (http://www.ncbi. nih.gov), expressed sequence tags (ESTs) from SilkBase (http://www.ab.a.u-tokyo.ac.jp/silkbase, Mita *et al*, 2003), Z-linked BAC clones (Koike *et al*, 2003), and whole genome shotgun (WGS) sequence data (http://www.ddbj.nig.ac.jp/whatsnew/040423-e.html, Mita *et al*, 2004). PCR amplification was performed in a 10 µl volume containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.1 U of AmpliTaq Gold (Perkin Elmer), 4 µM primer, and 10–20 ng of genomic DNA. The thermal cycling conditions were as follows: initial denaturation of 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C, and a final extension of 10 min at 72°C. The products were separated on a 3% metaphor agarose (FMC) gel.

FISSR-PCR analysis

A total of 43 FISSR-PCR primers labeled on the 5′ end with TAMRA fluorescent dye were screened and PCR amplification was performed as described in Nagaraju *et al* (2002).

Sequence analysis of Z- and W-specific markers

Z-chromosome linked RAPD, SSR, and FISSR markers and W-specific SSR markers were sequenced using an automated ABI 373 DNA sequencer and analyzed using tools available on the NCBI database. The sequences of RAPD, SSR, and FISSR were deposited in GenBank.

Identification of Z- and W-derived markers

In the backcross population (BC) derived from F_1 (♂) (translucent (*od*) (♀) X Pure Mysore (♂)) × translucent (*od*) (♀), the *od* female Z-linked markers, as expected, appear only in F_1 males and segregate in the ratio of 1:1 among BC females, whereas all BC males carry markers (Table 1). In these crosses, the Pure Mysore strain Z-linked markers will not appear in a sex-specific manner in the F_1 offspring (Table 1), but will segregate in the BC and can only be identified as sex linked by using the reciprocal cross, which was not performed in the present study. Therefore, the BC raised in the present study facilitated identification only of *od* strain Z-linked markers. The markers that were present only in the females of the parents, F_1, and BC were considered as W-linked markers. For autosomal markers, all the F_1 offspring were identical, while backcross males and females showed segregation in the ratio of 1:1 for Pure Mysore-derived markers.

Genotyping

The Z-linked DNA markers derived from the translucent (*od*) strain will appear only in the F_1 males (Table 1). The RAPD and FISSR-PCR Z-linked markers segregated as

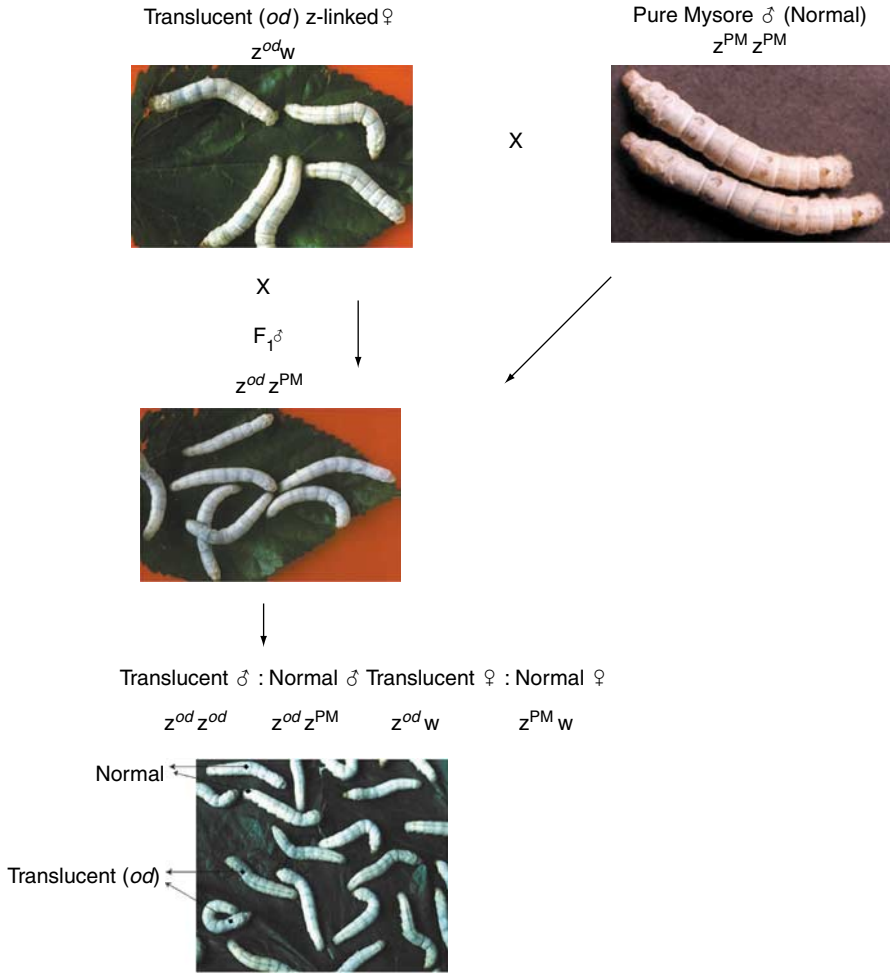

Figure 1 Mating scheme for raising the backcross population for the Z-chromosome mapping study.

Table 1 Expected genotypes and segregation of Z-linked markers in sex chromosomes

Crosses	*Genotypes*		*Translucent (od) derived*				*Pure Mysore derived*	
			Z linked		*W linked*		*Z linked*	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*	*Males*	*Females*
Parental lines	Z^{PM} Z^{PM}	Z^{od} W	–	+	–	+	+	–
F_1	Z^{od} Z^{PM}	Z^{PM} W	+	–	–	+	+	+
BC	Z^{od} Z^{PM}	Z^{PM} W	+	–	–	+	+	+
	Z^{od} Z^{od}	Z^{od} W	+	+	–	+	–	–

Note: *od*, translucent strain; PM, Pure Mysore strain; BC, back cross offspring of F_1 male mated to translucent (*od*) female; '+' indicates the presence and '–' indicates the absence of RAPD/FISSR-PCR/SSR band.

dominant markers and the SSR markers segregated as codominant markers. All markers were scored as present (coded as 1) or absent (coded as 0) in the backcross only if they were present in the *od* strain and in the F_1 males.

Construction of genetic map of Z chromosome

A genetic map of the Z chromosome was constructed based on the segregation of markers in 55 backcross offspring. Goodness of fit to the expected segregation ratio (1:1) of presence or absence of an amplified product at each marker locus was tested in the 40 BC females by Chi-square (χ^2) analysis. The genetic relationship among markers was determined by maximum likelihood (Bailey, 1961) analysis, and the segregation pattern of marker data was analyzed using MAPMAKER version 3.0 with the backcross data as an input file. A minimum LOD score of 3.0 (Log_{10} of the odds ratio) was used for the pair-wise linkage analysis. Genotyping was carried out with the ERROR DETECTION option, and the

recombination values were converted into map distances (in cM) by applying the Kosambi mapping function (Kosambi, 1944).

Results and discussion

Z-linked markers

Out of 560 RAPD primers used in the present study, 13 primers (2.3%) generated Z-linked markers (Table 2). In the backcross females, Z-linked RAPD markers derived from the translucent (*od*) strain should segregate in a ratio of 1:1 in translucent and normal larvae (Figure 2). Out of the 13 Z-linked RAPD markers, the segregation pattern of 10 markers was consistent with a 1:1 ratio (P (χ^2) $\geqslant$0.01), whereas markers OPA-07.1352, OPR-11.1200 OPT-14.583, and TA(CAG)$_4$ deviated slightly from this expected ratio (Table 3).

In the present study, out of the 216 SSR loci that were characterized from different silkworm genomic resources (Prasad *et al*, 2004), two loci, *Bmsat95* [(GA)$_{23}$] and *Bmsat208* [(TA)$_{20}$], showed a Z-linked pattern of inheritance (Table 2) with a segregation that was consistent with the 1:1 ratio (P (χ^2) $\geqslant$0.01) (Table 3). The sequence of *Bmsat95* showed no homology to any of the sequences in the public databases. *Bmsat208* was found to be present in the Z-linked BAC clone 12L3 (Koike *et al*, 2003) in the intergenic region of *Bmtkz*, a *B. mori* tyrosine kinase-like protein, and in *BmubcD4*, – a *B. mori* ubiquitin-conjugating enzyme-like protein (Table 4).

Of 43 FISSR primers anchored either at the 5′ or 3′ end that were tested, only one 5′ anchored primer (TA(CAG)$_4$) showed Z linkage (Table 2). The segregation pattern deviated slightly from a 1:1 ratio (P (χ^2) $\geqslant$0.01) (Table 3). The sequence of the Z-linked product showed

Table 2 Z chromosome-specific RAPD, SSR, and FISSR markers

Locus name	*Primer sequence*	*PCR product size in base pairs (bp)*
OPA-03.736	5′ AGTCAGCCAC 3′	736
OPA-07.1352	5′ GAAACGGGTG 3′	1352
OPA-12.1860	5′ TCGGCGATAG 3′	1860
OPD-18.1714	5′ GAGAGCCAAC 3′	1714
OPF-02.1240	5′ GAGGATCCCT 3′	1240
OPG-06.1254	5′ GTGCCTAACC 3′	1254
OPI-07.1769	5′ CAGCGACAAG 3′	1769
OPR-04.47	5′ CCCGATGCAC 3′	478
OPR-09.1105	5′ TGAGCACGAG 3′	1100
OPR-11.1200	5′ GTAGCCGTCT 3′	1200
OPT-04.992	5′ CACAGAGGGA 3′	992
OPT-14.583	5′ AATGCCGCAG 3′	583
OPU-14.920	5′ TGGGTCCCTC 3′	920
TA(CAG)$_4$	5′ AAATACAGCAGCAGCAG 3′	480
Bmsat208F	ACATGAAATGGGCAAACGACG	176
Bmsat208R	GCTCATATTTGCTTGCCGGTT	
Bmsat95F	ATTGTAACCGATTTGAGAGA	108
Bmsat95R	ATTCGCACAATAAGTTCACT	

Note: Locus name begins with OP (Operon Technologies Inc., Alameda CA, USA) for RAPD markers.

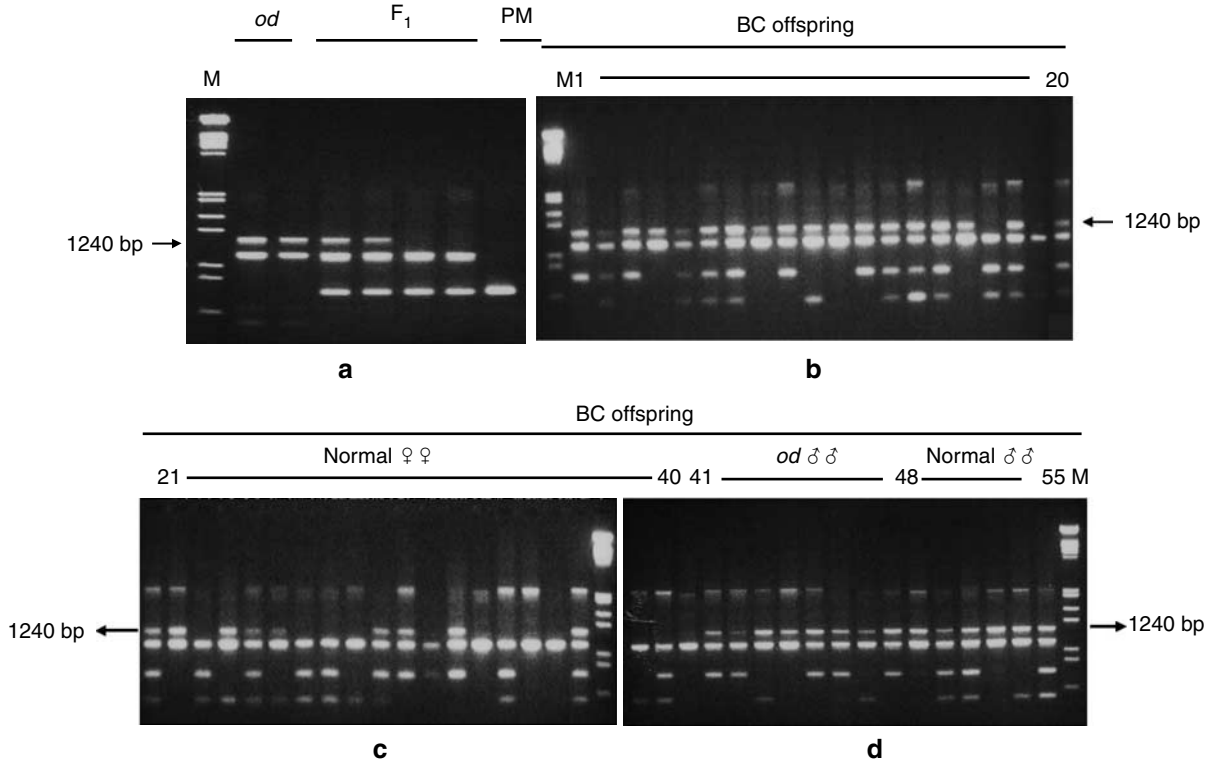

Figure 2 An example of inheritance and segregation of Z-chromosome-specific RAPD markers generated by primer OPF-02.1240. (**a**) Parents: Translucent *(od)* and Pure Mysore and F$_1$ offspring of translucent (*od*) and Pure Mysore; (**b**–**d**) Back cross (BC) offspring of F$_1$ and *od*. The Z-chromosome-specific marker is indicated by an arrow. M: Lambda *Hind*III digest size marker. Lanes 1–55 represent the number of BC offspring used for the analysis.

Table 3 Segregation of Z chromosome-linked markers in BC female progeny

Locus	*Observed*[a]		χ^2
	Translucent (od)	*Normal*	
OPA-03.736	18	10	6.4
OPA-07.1352	18	12	10.0
OPA-12.1860	13	8	0.1
OPD-18.1714	12	13	2.5
OPF-02.1240	16	9	2.5
OPG-06.1254	11	16	4.9
OPI-07.1769	11	12	0.9
OPR-04.47	12	9	0.1
OPR-09.1105	11	13	1.6
OPR-11.1200	18	17	22.5
OPT-04.992	9	8	0.9
OPT-14.583	19	10	8.1
OPU-14.920	18	9	4.9
TA(CAG)4	18	15	16.9
Bmsat208	13	15	6.4
Bmsat95	10	14	1.6
od[b]	143	119	2.0

[a]Out of 55 BC offspring, 40 were females of which half were translucent (*od*) individuals (expected). Segregation of 16 Z-linked markers with *od* phenotype was tested in these individuals.
[b]Segregation of *od* and normal phenotypes as observed in 262 BC offspring.
Note that χ^2-values for none of the Z-linked markers except for OPA-07^{1352}, OPR11^{1200}, OPT-14^{583}, and TA(CAG)$_4$ deviated significantly ($P > 0.01$).

no homology to any of the sequences in the public databases (Table 4).

Construction of Z chromosome genetic map

In the present study, the cumulative segregation data of 16 Z-linked markers of various kinds were used to construct a Z chromosome genetic map. Most of the Z-linked markers were ordered at LOD 3 using the 'ripple' command of MAPMAKER (Figure 3). Map length, distance between the markers and gaps were represented in centiMorgan calculated using the Kosambi mapping function. The LOD score for linkage was less than 3 for the largest gap in the map. The average spacing of the Z-linked markers was 20 cM, and the total map covered approximately 334.5 cM. All the RAPD markers, except OPA-07.1352, showed linkage to the phenotypic translucent (*od*) marker within 55.3 cM. The total length of the map was reduced by 8% from 365 to 334.5 cM when genotyping errors were detected with the MAPMAKER ERROR DETECTION option. The largest reduction was obtained for marker Sat 208 (69%) followed by OPU14 (33%), OPG06, and OPT14 (23%).

The recombination length of the Z chromosome calculated in this study was approximately four times larger than the estimated 80 cM span reported for an RAPD map composed of 18 markers (Yasukochi, 1998), and the 50 cM length of the classical Z chromosome map composed of 15 morphological markers (Fujii *et al*, 1998). The relatively large gaps by the terminal markers have greatly expanded the total map distance reported here, where relatively high recombination rates were observed (Figure 3). Nevertheless, the 16 markers appeared to be well distributed all along the Z chromosome and covered many gaps and unmapped loci in the preexisting maps.

Table 4 Identification of Z-linked markers in *B. mori* Whole Genome Shotgun (WGS) sequence contigs (BLAST results as on Feb 15, 2005)

Marker accession no.	*Markers*	*WGS contig*	*Homology (% Identity, Bit score, and E-value)*	*Genes and provisional function (accession no.)*	*Species*
AY566197	OPU-14.920	BAAB01127123 | contig52248	96%/33 aa, 67.0, 1e-09	AP-1γ (FBgn0030089)	*D. melanogaster*
		BAAB01021218 | contig147849	No homology		
AY566199	OPR-09.1105	BAAB01048734 | contig21903	45%/68 aa, 74.3, 3e-11	CG11851, mannosyltransferase activity	*D. melanogaster*
AY566198	OPG-06.1254	BAAB01209182 | contig9100	51%/199 aa, 198, 6e-49	ENSANGP00000020972 (XM_315297), unknown function	*A. gambiae*
		BAAB01116160 | contig498223	No homology		
AY566201	OPT-04.992	BAAB01106426 | contig476977_483697	33%/941 aa, 520, e-145	Reverse transcriptase Osvaldo retrotransposon (CAB39733.1)	*D. buzzatii*
AY566196	OPF-02.1240	gbAADK01008490.1	No homology		
AY566200	OPT-14.583	BAAB01017829 | contig1401	92%/237nt, 319, 6e-83	Non-LTR retrotransposon and Bm1 retroposon	*B. mori*
AY566202	*Bmsat208*	BAAB01155602.1	100%/136nt, 165.4e-38	*Bombyx mori Bmtitin1, Bmtitin2, Bmmiple, Bmsyx6, BmPM-Scl, Bmtkz, BmubcD4* genes, partial and complete cds (dbjAB090308.2)	*B. mori*
AY566204	TA(CAG)$_4$	gbAADK01006369.1	No homology		
AY566203	*Bmsat95*	gbAADK01032857.1	No homology		

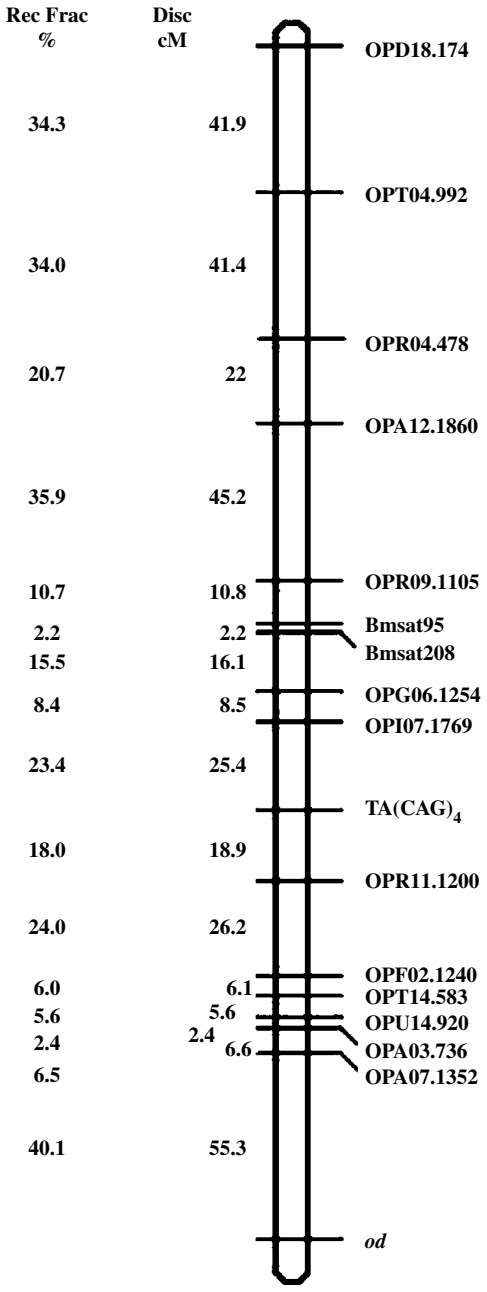

Figure 3 Z-chromosome linkage map based on 16 markers (13 RAPD, two SSR, and one FISSR). The genetic distance calculated between loci is given in cM. The total map covers approximately 334.5 cM.

Similar expansion of map length was observed in *Apis mellifera*, where one of the linkage groups (group I) is more than twice the total length of the *D. melanogaster* genome (Solignac *et al*, 2004). Genotyping errors can also result in a significant increase in recombination length (Brzustowicz *et al*, 1993), but this is unlikely in the present experiments, since the number of genotypes analyzed was few (55) and only reproducible markers that showed the expected pattern of inheritance were scored. Tan and Ma (1998) have theoretically demonstrated that with the addition of new markers, the map length will increase when the marker density is not saturated; conversely, it may decrease when the marker density is saturated. This hypothesis has been supported experimentally for the rice linkage map, which covered 4026.3 cM using 762 markers (Causse *et al*, 1994), but only 1521.6 cM using 2275 markers (Harushima *et al*, 1998). In *A. mellifera*, Solignac *et al* (2004) observed a decrease in the number of linkage groups and unlinked loci with addition of more markers. Tan *et al* (2001) also reported an increased length for a silkworm AFLP map composed of 356 markers compared to the RAPD map containing 1018 markers (Yasukochi, 1998), due to large gaps at several locations. Taking these observations together, it is likely that our Z chromosome map has reached an expanded state and the addition of many more markers would reduce the overall map length.

Sequencing and characterizing of Z-linked markers

Of the 16 Z-linked markers, six RAPD, two SSR, and one FISSR markers were sequenced and analyzed for homology with the nucleotide and protein sequences in public databases, including the silkworm WGS sequence. The markers identified several contigs, the sequences of which revealed interesting features (Table 4). The OPU-14.920 sequence was present in two contigs, namely contig52248 and contig147849. Contig52248 showed homology to a part of the adaptin_N domain of the AP-1γ gene of *D. melanogaster*, which encodes a component of the synaptic vesicle, while contig147849 had no homology with any of the sequences in GenBank. The RAPD OPR-09.1105 was present in contig21903, which showed a stretch of homology to the gene, CG11851, of *D. melanogaster*, which encodes a product with mannosyltransferase activity involved in amino-acid glycosylation. The OPT-14.583 sequence was identified in contig1401, which shared homology with known ubiquitous non-LTR (long terminal repeat) LINE-1 type repetitive sequences and Bm1 elements of *B. mori*. Non-LTR LINE-1-like elements are widely distributed in eucaryotes and comprise approximately 30% of the human genome; a recent study has shown that the X chromosome is rich in non-LTR retrotransposon *Alu* repetitive sequences relative to the Y chromosome and autosomes (Jurka *et al*, 2004). *Bm1* elements are a family of short (130–470 bp) tRNA/U1RNA-derived retroposons or SINEs containing variable 3′ poly (A) tracts, which comprise an estimated 5% of the silkworm genome (Okada *et al*, 1997). The silkworm Z chromosome has double the repetitive elements of autosomes, a trend which is similar to the X chromosomes in mammals and *D. melanogaster*. These elements are implicated in X chromosome inactivation and dosage compensation in these organisms (Bailey *et al*, 2000; Huijser *et al*, 1987). Since the silkworm is reported to lack dosage compensation (Suzuki *et al*, 1999), these observations call for further analysis including many more Z-linked genes. In particular, it may be interesting to evaluate the functional significance of such enriched elements on Z chromosome. Since only 2% of molecular information is available for the Z chromosome (Koike *et al*, 2003), the markers identified in the present study will be particularly useful in the assembly and analysis of Z-linked sequences.

A number of Z-linked characters have significant effects on traits of economic importance for sericulture

(Nakada, 1970; Tazima, 1978). In particular, the so-called maturity genes (early maturity, *lm*; moderate maturity, $+^{Lm}$, and late maturity, *Lm*) are said to be major genes controlling the duration of larval life, body weight, and silk fiber length (Tazima, 1978). Homozygous early maturing larvae (*lm*) are known to make smaller cocoons with short silk fibers, whereas late maturing larvae (*Lm*) usually grow bigger and produce more silk (Morohoshi, 1949). From population studies carried out in the early 1940s, it is known that several genes that control voltinism modulate the effects of *Lm*, shifting expected growth rate, larval span, and consequently body weight (Nagatomo, 1942). These genes are, however, difficult to identify without being able to assign map locations and track them precisely in genetic crosses. Further efforts in the development of a high-density silkworm Z chromosome map may aid in the analysis and positional cloning of such important gene(s).

In many groups of Lepidoptera, genes affecting reproductive isolation are sex linked. Interspecific hybridization of many species of wild silk moths has revealed that many reciprocal crosses result in sterile F_1 offspring (Jolly *et al*, 1969; Nagaraju and Jolly, 1985; Shimada and Kobayashi, 1992). Recent studies in other Lepidoptera have shown that the Z-linked genes associated with mating preferences may play a role in host race formation (Sperling, 1994) and speciation (Iyengar *et al*, 2002). Female F_1 offspring produced in interspecific crosses between two species of sexually dimorphic *Colias* butterflies prefer to mate with males of the paternal species, indicating that Z-linked genes played a role in their speciation (Grula and Taylor, 1980). The Z chromosome analysis in the silk moth, being a genetically and molecularly tractable lepidopteran model system, thus has an important bearing on the understanding of the molecular mechanisms involved in reproductive isolation, host race formation, and body weight differences.

W-linked markers

In the present study, screening with 560 random primers, 216 SSR loci, and 43 FISSR primers resulted in the identification of four RAPD markers and four SSR alleles that were W-specific (Figure 4a–e; see Table 5 for W-specific RAPD and SSR primer sequences). Upon

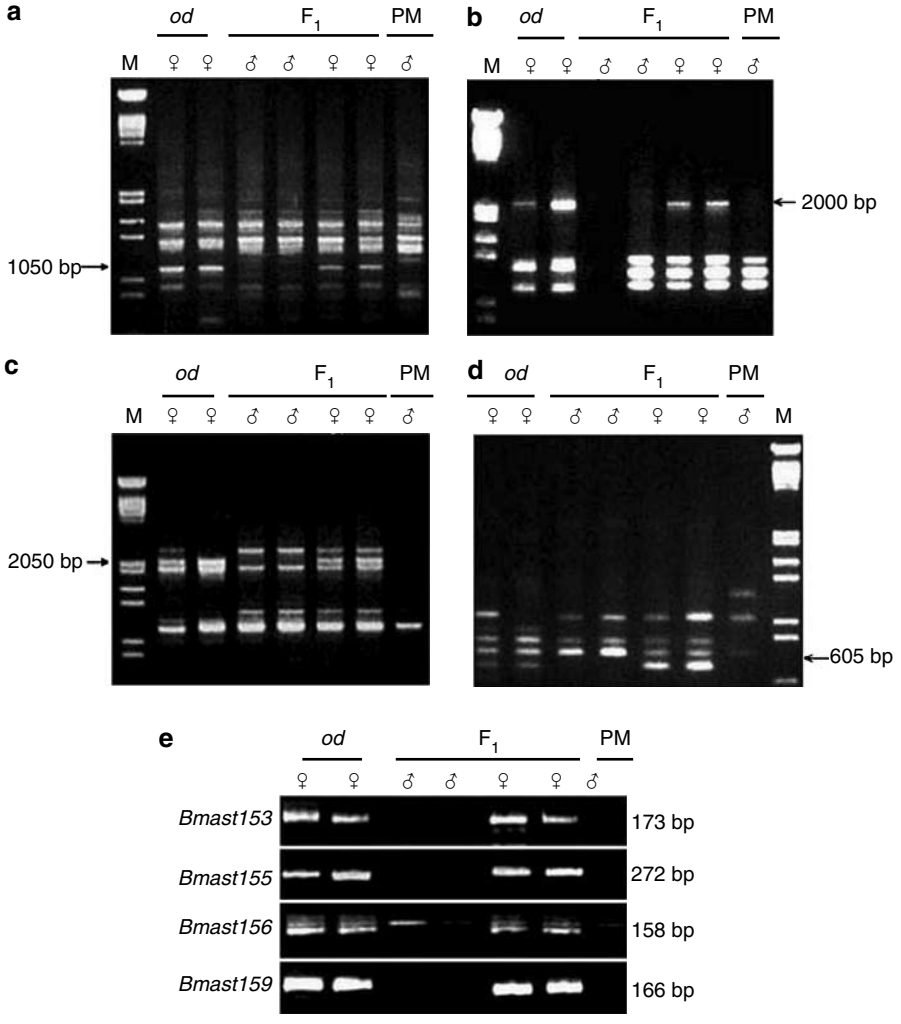

Figure 4 Identification of W chromosome-specific RAPD (**a**–**d**) and SSR (**e**) markers; (**a**) OPA-09, (**b**) OPC-09, (**c**) OPI-18, (**d**) OPM-06, and (**e**) *Bmsat153*, *Bmsat155*, *Bmsat156*, and *Bmsat159* in translucent (*od*) females (♀), F_1 males (♂), F_1 females (♀), and Pure Mysore (PM) male (♂). Arrow indicates W-specific markers. M: Lambda *Hind*III digest size marker.

Table 5 W chromosome-specific RAPD markers and SSR loci

Primers name	*Sequences*	*PCR products size in base pairs (bp)*
OPA-09	5′ GGGTAACGCC 3′	1050
OPC-09	5′ CTCACCGTCC 3′	2000
OPI-18	5′ TGCCCAGCCT 3′	2050
OPM-06	5′ CTGGGCAACT 3′	605
Bmsat*153F*	TGCTGTCGTCTGCTTCCTAA	173
Bmsat153R	CACGGTGCTGACTGTTGTTT	
Bmsat155F	AGGGATGATGGGTAAAGAGC	272
Bmsat155R	GCAGTAGGCATTTGGAAGGAG	
Bmsat156F	CTCCTTATCCATCCGTTT	158
Bmsat*156R*	CTCTCGGATCATAGATACG	
Bmsat159F	ATCTGGTGCTCAAAAACGGA	166
Bmsat159R	CGGAACCAAACAAGAACGAT	

sequencing, we found that the W-linked SSR markers were basically allelic variants of sequences derived from WGS contigs covering 11.3 kb (Table 6). Analysis of the contig sequences revealed the presence of different transposable elements making up approximately 26.5% of the sequence; the remaining sequences showed no homology to any of the sequences in the GenBank database. Contig gi54081682 containing the *Bmsat153* locus harbored Yamato, a *B. mandarina* Pao-like LTR retrotransposon (Abe *et al*, 2001). The *Bmsat155* locus was found to be present in Contig gi54071509, which contained a Bm1 repetitive element and a non-LTR retrotransposon, respectively. Contig gi54079028 identified by *Bmsat156* contained TREST1, a telomeric repeat sequence (Okazaki *et al*, 1993). The present study revealed low abundance of W-specific markers, probably because the W chromosome is composed mainly of retrotransposable elements (Abe *et al*, 1998b, 2000; Sahara *et al*, 2003), which are also found on other chromosomes suggesting that there is very little W-specific DNA. By using polyploid strains, Hasimoto first showed that the W chromosome carries the major determinant for femaleness in the silkworm (Hasimoto, 1930); subsequent studies carried out by Tazima (1941, 1944) and Hasimoto (1948) with irradiated autosomal fragments harboring genes for phenotypic traits translocated to the W chromosome provided further evidence for its female determining role. More than 90% of the characterized W– chromosome-derived sequence is comprised of retrotransposable elements (Abe *et al*, 2000; Sahara *et al*, 2003). This is in contrast to the Z chromosome, where 15.4% of repetitive elements were observed in a contiguous 320 kb sequence (Koike *et al*, 2003). These results indicate that most of the W chromosome is fairly degraded and lacks genetic activity. This presents a similar situation as in the case of Y chromosomes of *D. melanogaster* and mammals (Carvalho *et al*, 2001; Lahn *et al*, 2001).

The RAPD markers identified in the present study will aid in retrieving additional contigs for further analysis and characterization of W chromosome-specific sequences, which will augment the ongoing efforts to identify the putative female determining gene(s). These include a set of sex-limited *Ze* strains that carry reciprocal translocation between Z and the third chromosome, which also contains a small portion of

Table 6 Identification of W-linked markers in *B. mori* Whole genome Shotgun (WGS) sequence contigs (BLAST results as on February 15, 2005)

Marker accession no.	*Marker*	*WGS contig*	*Homology (% Identity, Bit score, and E-value)*	*Identified sequence (accession no.)*
AY566208	*Bmsat153*	gi|54081682|gb|AADK01028030.1	98%/486nt, 658, and 0.0	*B. mandarina* Pao-like LTR retrotransposon Yamato DNA, partial sequence (AB055223)
AY566209	*Bmsat155*	gi|54071509|gb|AADK01038147.1	91%/62nt, 84, and 8e-13 90%/145nt, 168, and 2e-38	*B. mori* Bm1 repetitive DNA element *B. mori* non-LTR retrotransposons on chromosome W
AY566210	*Bmsat156*	gi|54079028|gb|AADK01030684.1	95%/2105nt, 1070, and 0.0	*B. mori* DNA, clone TREST1, partial cds
AY566212	*Bmsat159*	gi|54108487|gb|AADK01001225.1	No homology	

the W chromosome that determines femaleness (Hasimoto, 1953).

Comparative genetic mapping is an effective tool for the study of genome evolution in phylogenetically distant species that represent key stages in insect evolution. Using both physical and genetic methods, orthologous W- and Z chromosome genes of the silkworm can be identified and mapped in other insect species or in other higher order organisms. The Z- and W-linked markers, together with those reported by earlier studies, provide the much needed genetic resources to address these issues.

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