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# W-derived BAC probes as a new tool for identification of the W chromosome and its aberrations in *Bombyx mori*

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**Abstract** We isolated four W chromosome-derived bacterial artificial chromosome (W-BAC) clones from *Bombyx mori* BAC libraries by PCR and used them as probes for fluorescence in situ hybridization (FISH) on chromosome preparations from *B. mori* females. All four W-BAC probes surprisingly highlighted the whole wild-type W sex chromosome and also identified the entire original W-chromosomal region in W chromosome-autosome translocation mutants. This is the first successful identification of a single chromosome by means of BAC-FISH in species with holokinetic chromosomes. Genomic in situ hybridization (GISH) by using female-derived genomic probes highlighted the W chromosome in a similar chromosome-painting manner. Besides the W, hybridization signals of W-BAC probes also occurred in telomeric and/or subtelomeric regions of the autosomes. These signals coincided well with those of female genomic probes except one additional GISH signal that was observed in a large heterochromatin block of one autosome pair. Our results support the opinion that the *B. mori* W chromosome accumulated transposable elements and other repetitive sequences that also occur, but scattered, elsewhere in the respective genome.

## Introduction

Since the first sex chromosome (X) was recognized in the red fire bug, *Pyrrhocoris apterus*, as a conspicuous heterochromatic X-body (Henking 1891), sex chromosomes have been identified in many organisms simply by comparing female and male karyotypes and searching in a light microscope for a morphologically distinguishable (heteromorphic) chromosome pair. Then the sex chromosomes were designated X and Y in systems with male heterogamety (e.g., mammals, flies, etc.) or W and Z in systems with female heterogamety (e.g., birds, reptiles, moths and butterflies, etc.). However, this approach failed to detect sex chromosomes in a number of organisms either because of a lack of morphological markers or, in organisms with small chromosomes, insufficient resolution of light microscopy. In some organisms, these so-called homomorphic sex chromosomes were later recognized by using newly developed staining techniques (e.g., C-banding by Schmid et al. 1979; BrdU-Hoechst-Giemsa technique by Schempp and Schmid 1981) or with the help of sex-linked chromosomal aberrations (e.g., Traut et al. 1990). Advances in molecular cytogenetics enabled researchers to identify sex chromosomes by localizing sex-specific DNA sequences (e.g., a human Y chromosome by Kozuma et al. (1988); a chicken W chromosome by Saitoh and Mizuno (1992)). However, this approach is only available in organisms with sex chromosomes easily distinguishable by standard procedures. There is so far no sex-chromosome specific DNA probes for identification of homomorphic sex chromosomes by in situ hybridization.

A typical example of species with problematic identification of sex chromosomes is the silkworm, *Bombyx mori*, a representative of the insect order Lepidoptera. In spite of a great number of mutant strains, many commercially used strains, and extensive genetical research done since the first practical use of heterosis (Toyama 1906), researchers failed to detect silkworm sex chromosomes till recently (Kawamura and Niino 1991; Traut et al. 1999).

Chromosomes in Lepidoptera are generally small and numerous (most species have diploid chromosome numbers close to 60), uniform in shape and size in mitotic metaphase, and lack primary constrictions. Since no banding technique was found to differentiate the chromosomes, the difficulty of cytogenetic mapping has repeatedly been pointed out in a number of species (e.g., Heckel 1993; Goldsmith 1995). Due to the technical problems mentioned no method for identification of metaphase chromosomes has been established to date. However, chromosome identification in Lepidoptera appears less difficult in the pachytene stage of meiotic prophase I, where

the chromosomes form a haploid number of bivalents that are much longer than mitotic chromosomes and, in addition, display a specific chromomere pattern. For example, the so-called pachytene mapping helped to identify six out of 28 bivalents in *B. mori* oocytes (Traut 1976). In females of some Lepidoptera, the sex chromosome bivalent was recognized by the heterochromatic thread of the W chromosome in the light microscope (Traut and Rathjens 1973; Traut and Marec 1997) or by a specific chromatin structure and delayed synapsis in the electron microscope (Weith and Traut 1980; Wang et al. 1993; Marec and Traut 1994).

In *B. mori* stocks, there are several W-autosome translocation strains either of spontaneous origin or induced by irradiation (listed in Fujii et al. 1998). These mutant strains are also called "sex-limited" strains because they express female specific phenotypes. Using one of the sex-limited strains, the WZ bivalent was identified according to its asymmetric pachytene configuration (Kawamura and Niino 1991).

Recently, Traut et al. (1999) modified comparative genomic hybridization (CGH) for the study of molecular differentiation of sex chromosomes in both the X-Y and W-Z systems. This method makes possible the identification of the Y or W even in species with homomorphic sex chromosomes if they differ sufficiently in their gross DNA composition. In three lepidopteran species including *B. mori*, CGH enabled the authors to visualize the most probable W chromosome not only in pachytene bivalents but also in mitotic metaphase plates.

Here we present a new tool for identification of the W chromosome in Lepidoptera. Recently, bacterial artificial chromosome (BAC) libraries have been constructed in *B. mori* (Wu et al. 1999). By using female-specific primers we selected from the libraries four BAC clones, derived from the W chromosome (W-BAC clones), and used them as probes for fluorescence in situ hybridization (FISH) on chromosome preparations from *B. mori* females of either a normal strain or a sex-limited mutant strain. We also performed in situ hybridization with a total female genomic DNA (GISH; Schwarzacher et al. 1989; Raina and Rani 2001) as a probe. FISH with the W-BAC probes, combined with GISH and morphological attributes such as the W heterochromatin and/or asymmetric sex-chromosome bivalents in oocytes of sex-limited mutants, enabled us conclusive identification of the W chromosome in *B. mori*.

## Materials and methods

### Insect

We used a commercial hybrid strain (Kinsyu-Showa) purchased from Ueda Sanshu Co. (Ueda, Japan), a sex-limited strain ZWII ((T(Z; W; 2; 2)+$^{od}$ W +$^{p}$, $p^{Sa}$, *od*/*od*, *p*/*p*), and $F_1$ hybrids of ZWII females crossed with re9 ($p^{S}$/ $p^{S}$, *re*/*re*) males. In the latter strain and hybrids, the sex of larvae was discriminated according to their phenotype (see below). A genetic survey of the ZWII showed that the W chromosome consists of four elements: the original W, two 2nd chromosome fragments translocated to one end and a short Z fragment to the other end (Tazima, 1944; 1964). The two 2nd chromosome fragments carry the +$^{p}$ and p$^{Sa}$/+$^{Y}$ genes and the Z fragment carries the +$^{od}$ gene (Fig. 1). Female larvae of the ZWII strain have a normal skin pattern with sable color while the male skin is transparent (oily) without marking (plane, *p*). The $F_1$ female larvae express the same marker genes (normal pattern, sable) as their mothers but differ from them by striped skin.

Chromosomes

The ovaries of 1-day to 4-day old 5th instar larvae were dissected in a saline solution and fixed in Carnoy's fluids either ethanol, chloroform and acetic acid (6:3:1) or methanol and acetic acid (3:1). The latter fixative was used, when the ovaries were pretreated in a hypotonic solution (0.075 M KCl). For FISH, chromosome preparations were made as described in Sahara et al. (1999). Cells were dissociated in 60% acetic acid and spread on a heating plate at 55$^{o}$C (Traut 1976). Then the preparations were passed through a graded ethanol series (70%, 80%, 98%) and stored in the freezer until use. For Giemsa staining, chromosome preparations were made following the air-drying method of Takagi (1971).

Selection of W-derived BAC (W-BAC) clones by PCR

From several STS (single tagged sequence) primer sets that amplify the female-specific (W-specific) DNA fragments, we chose a primer set of 5'-CTCCGCCGGTAATCAATGACGTACA-3' (Tail-3-A) and 5'-TGCGCATGCAGTTATTGCATTACACTG-3' (KaLTR-A) (see Abe et al. 1998a; b). This primer set amplified a female-specific 295 bp fragment. We also used another primer set, 5'-ATTCTTGTTCGTCTTGATGATCTAG-3' (new437-A1) and 5'-ATCTTTCGAAGTGAGCACGGAAGCT-3' (new477-B1), designed according to the sequence of a newly found W-RAPD clone. The latter primer set amplified an 801 bp female-specific fragment. Because BAC libraries were constructed using genomic

DNAs of p50 and C108 strains (Wu et al. 1999), the template genomic DNA for PCR was extracted from $F_2$ individuals of the cross between p50 and C108.

Probes and FISH

Chromosomal DNA was isolated according to Blin and Stafford (1976). W-BAC clones were cultured in LB medium containing 20μg/ml of chloramphenicol at 37°C for 16 h, and then the BAC DNA was extracted by a QIAGEN Plasmid Midi kit (QIAGEN K.K. Tokyo, Japan). DNA labeling was done by nick translation using a Nick Translation System (Invitrogen Japan K.K. Tokyo, Japan) with Cy3-dCTP or FluorX-dCTP (Amersham Life Biosciences K.K. Tokyo, Japan).

GISH and BAC-FISH were carried out essentially following the methods described in Sahara et al. (1999) and Traut et al. (1999) with slight modifications. After removal from the freezer, chromosome preparations were passed through the ethanol series and air-dried. Denaturation was done at 72°C for 3 to 4 min in 70% formamide/2xSSC. For one GISH preparation, the probe cocktail contained 3 μg of labeled female DNA, 20 μg of sonicated salmon sperm DNA (Sigma-Aldrich Japan K.K. Tokyo, Japan), and 3 μg of unlabeled sonicated male genomic DNA in 10 μl of hybridization solution (50% formamide, 10% dextran sulfate, 2xSSC). The probe cocktail for BAC-FISH contained 1 μg of labeled W-BAC DNA and 25 μg of sonicated salmon sperm DNA. After hybridization in a moist chamber at 37°C for 3 days, slides were washed at 62°C in 0.1xSSC containing 1% Triton X-100. The slides were then counterstained and mounted in antifade (0.233 g 1,4-diazabicyclo(2.2.2)-octane, 1 ml Tris-HCl, pH 8.0, 9 ml glycerol) containing 0.5 μg/ml of DAPI (4'6-diamidino-2-phenylindole; Sigma-Aldrich Japan K.K. Tokyo, Japan).

Image processing

Black and white images were taken in a Leica DMRE HC fluorescence microscope with a Photometrics CoolSNAP CCD camera through the A, L5 and N2.1 filters of a fluorescence filter set. Pseudocoloring and superimposing of the images were done using Adobe Photoshop, Version 6.0. Routinely red coloring was used for Cy-3, green for FluorX and light blue for DAPI images.

## Results

## Detection of an asymmetric bivalent (WZ)

In early pachytene, no asymmetric bivalents were observed in oocytes of ZWII hybrids. Later, at the beginning of oocyte-nurse cell differentiation, bivalents became thicker. At this stage, one among 28 bivalents displayed an asymmetric configuration (Fig. 2a). The longer chromatid of the bivalent, which was considered to be the W chromosome carrying the Z and 2nd chromosome translocations, twisted around the shorter chromatid (Z) and formed a loop structure at one end (Fig. 2b).

## Sex chromosome identification by GISH (genomic in situ hybridization)

Female genomic probes regularly detected an asymmetric bivalent in ZWII oocytes. Red hybridization signals highlighted almost two thirds of the longer chromatid of the asymmetric bivalent in both the pachytene nuclei (Fig. 3a-e) and early nurse cells (Fig. 3f). The probes also labeled more than a half of one chromosome in mitotic oogonial metaphases (Fig. 3g). These results coincided well with the prediction that the W chromosome of the ZWII strain carries a relatively long autosomal fragment (arrows in Fig. 3; cf. Fig. 1; Tazima 1964). However, we did not observe any unlabeled terminal segment at the opposite end of the W chromosome that would correspond to the translocated fragment of the Z chromosome (cf. Fig. 1). This may indicate that the Z fragment is either too small to be detectable or is located in the autosomal part of the mutant W chromosome, from which it is not recognizable.

## W chromosome detection with W-BAC probes

From BAC libraries of *B. mori* we selected four BAC clones (W-BAC) that amplified female specific DNA fragments by PCR. Two STS primer sets divided the W-BACs into two groups: (i) 5H4C and 18K8H, and (ii) 2K7G and 19L6H (Fig. 4). FISH signals of the 18K8H (Fig. 5b) and 5H4C (Fig. 5c) probes unexpectedly painted the whole W thread of the normal WZ bivalent (Fig. 5a) in the same manner. Also the19L6H (Fig. 5e) and 2K7G (Fig. 5f) probes hybridized to the same regions of the entire WZ bivalent (Fig. 5d).

Genomic and W-BAC (18K8H) probes displayed a similar pattern of hybridization signals on the heterochromatic thread of the normal WZ bivalent (Fig. 6a-c). A W-BAC (2K7G) probe from the other group also hybridized to the normal WZ bivalent in a similar manner as the genomic probe (Fig. 6d-f).

In each pachytene nucleus, scattered and less conspicuous W-BAC signals were observed in some autosome bivalents as well as the Z chromatid, mostly in telomeric and/or subtelomeric regions (Fig. 6g, h). These signals coincided well with the signals from the genomic probe, indicating that some W-chromosome DNA sequences also occur in autosomes. In addition, clear GISH signals were observed in a part of one autosome pair (arrowheads in Fig. 6h) while absent after W-BAC FISH. The GISH-painted autosomal segment was also deeply stained with DAPI (Fig. 6g) but was not located in the nucleolar organizer region (NOR) bivalent (Fig. 6g). We suggest that this segment represents an autosomal block of heterochromatin.

The W chromosome pattern in ZWII females

A representative of the W-BAC probe,18K8H, was used for two-color FISH in ZWII females together with the female genomic probe (GISH). Both the W-BAC probe and genomic probe hybridized to the asymmetric WZ bivalent (Fig. 7a-j). The probes painted almost two thirds of the longer chromatid (see yellowish color of combined red and green signals in Fig. 7b), largely consisting of heterochromatin (Fig. 7a, c, g). Like in the normal WZ bivalent, both GISH (red) and W-BAC (green) signals highlighted identical heterochromatic regions, the original W chromosome (Fig. 7c-j). In mitotic chromosomes, both probes painted a major part of one chromosome (Fig. 7k, l). The painted part varied probably due to different degree of chromosome condensation (Fig. 7m-u). Our results confirmed that the W chromosome in ZWII females carries a relatively long translocation at one end (cf. Fig. 1).

In both meiotic and mitotic chromosomes of the ZWII strain, weak but clear GISH signals were also observed in one autosome pair (arrowheads in Fig. 7b, i). Observations of similar signals in a wild-type nucleus suggest that the heterochromatin block in a pair of autosomes is common in *B. mori*.

## Discussion

Till lately the W chromosome identification in the silkworm, *Bombyx mori*, had been considered difficult, and the W had only been recognized when marked by a conspicuous translocation (Kawamura and Niino 1991). The wild-type W was only recently identified by means of comparative genomic hybridization (CGH) with differently labeled DNA from females and males (Traut et al. 1999). The *Bombyx* W chromosome was highlighted by both DNAs. This prompted us to try an even simpler

approach to identify the W chromosome: GISH with the whole genomic DNA probe from only females. Here we show that the wild-type W chromosome of the silkworm can be identified by several ways besides CGH: (i) according to the DAPI-positive, heterochromatic thread of the W chromosome in the pachytene WZ bivalent, (ii) by FISH with W-derived BAC probes, and (iii) by GISH with female genomic probes. However, the W chromosome heterochromatin (Fig. 6g) is unstable against hypotonic treatment. In pachytene oocytes of pyralid moths, the W heterochromatic thread was discernable either without or with mild treatment in hypotonic solution (Traut et al. 1999; Marec et al. 2001).

Four W-BAC probes, which were divided into two groups by PCR results, surprisingly hybridized to the whole W chromosome of the silkworm in a chromosome painting-like manner. Abe et al. (1998a) reported that transposable elements were preferentially detected in PCR products from the W chromosome. Since the W-BAC clones contain several copies of transposable elements (e.g., Bm1 and BMC1; Table 1) with nested structures (Abe et al. 2000), it appears difficult to confirm their accurate sequence alignment after shotgun sequencing. One W-BAC sequence that we have read (about 90 kb of the 19L6H clone) consisted exclusively of transposable elements (Table 1). A total haploid (n=28) genome size of *B. mori* is about 530 Mb (Gage 1974). Hence the mean size of one chromosome is 18.9 Mb. Whereas the size of W-BAC clones is approximately 170 Kb (Abe et al., unpublished), i.e., about 1% of the putative length of a chromosome. Taken together, our results suggest that each of four W-BAC clones contains homologous regions that are spread over the whole length of the W chromosome. This might have happened by multiplication of transposable elements or other repetitive sequences within the W chromosome, for example, by unequal sister chromatid exchange as suggested for a tandem duplication found in the human Y chromosome (Dechend et al. 2000). Alternatively, the W chromosome might have accumulated multiple copies of transposable elements that were scattered in autosomes, since the non-recombining W is a preferable target of active transposable elements like the Y chromosome, as proposed in *Drosophila* (Steinemann et al. 1995). The scattered hybridization signals in *B. mori* autosomes, observed after W-BAC FISH and GISH in our study, favor the later option. These autosomal signals also support the opinion that the *B. mori* W chromosome accumulated transposable elements and other repetitive sequences that occur elsewhere in the respective genome (Sahara et al. 2003).

In the present study, GISH signals coincided with W-BAC signals except the heterochromatin block in an autosome (Figs. 3, 6, 7, arrowheads). Similar signals from a female genomic probe were observed in a pair of *B. mori* autosomes that also

hybridized with a male-derived genomic probe (Traut et al. 1999). Since the W-BAC did not highlight the same region, we suggest that the large heterochromatin block in a pair of autosomes is an autosome-specific element that probably consists of repetitive sequences (e.g., such as satellite DNA recently described in *Mamestra brassicae* by Mandrioli et al. 2003) or transposable elements other than those accumulated in the W chromosome.

The acquisition of sex-determining function and the suppression of recombination are thought to be prerequisites of sex chromosome differentiation (X-Y or W-Z). Long-term consequences of independent molecular changes without regular corrections by recombination generally provide first molecular and later also morphological differentiation between X and Y or W and Z (Traut 1999). In the silkworm, however, the lengths of the W and Z chromosomes are similar each other, though their molecular differentiation has considerably progressed (Figs. 5, 6; this study). The lack of a morphological differentiation between the sex chromosomes, in spite of the advanced molecular differentiation, might be caused by a rare loss of chromosome compartments in lepidopteran chromosomes due to their holokinetic nature (Wolf 1996).

High-resolution physical maps by means of large insert DNA clones from P1, PAC, BAC or YAC libraries are indispensable for directed sequencing project or finishing stages of shotgun sequence projects (Weier 2001). FISH mapping plays an important role in integrating a cytological map with a physical map. In this respect, our study presents the first successful FISH using BAC probes in Lepidoptera. In *B. mori*, libraries consisting of total 36,864 BACs have been constructed from two strains (Wu et al. 1999). Physical map data, based on RAPD (Proboom 1995; Yasukochi 1998), RFLP (Shi et al. 1995) and mutants (Fujii et al. 1998), are also available in this species. BAC mapping may work well for the sequencing project of other chromosomes but our results show that it is not useful for W chromosome sequencing (the result even show that the W will be resistant to today's sequencing approaches). Here we identified the sex-chromosome bivalent and the W chromosome itself by means of BAC-FISH. The BAC-FISH approach opens the gate for identification of all 28 chromosomes in *B. mori*.

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## Figure legends

**Fig. 1** A putative structure of the W chromosome in the ZWII strain of *Bombyx mori* (from Tazima 1964). Dot column (W) represents the original W chromosome part before translocations occurred. Hatched column (Z) and blank column (2nd) represent translocated Z and two 2nd chromosome fragments, respectively. $+^{Y}$, a standard recessive allele of the yellow blood (*Y*) gene; $+^{p}$, a standard dominant allele of the larval skin gene, plane (*p*); $p^{Sa}$, sable, a co-dominant mutant allele of the *p* gene; $+^{od}$, a standard dominant allele of the oily (*od*) gene

**Fig. 2a, b** Oocyte pachytene bivalents of hybrids between ZWII females and re9 males in *Bombyx mori*, stained with Giemsa solution. **a** Pachytene karyotype (n=28) showing an asymmetric bivalent (arrow), the presumable sex chromosome bivalent. **b** Various patterns of asymmetric synapsis of the sex chromosome bivalent. *Bars* represent 10 μm

**Fig. 3a-g** GISH on spread preparations from ovaries of ZWII females. Chromosomes are counterstained with DAPI (blue). **a** Pachytene complement; strong hybridization signals (red) reveal the original W chromosome part of the asymmetric sex chromosome bivalent, faint signals label a part of an autosome (arrowhead). **b, d** Two representatives of the WZ asymmetric bivalent detected by GISH. **c, e** Schematic drawings of b and d, respectively; red lines correspond to the hybridization signals, blue lines represent the Z chromosome, white lines show translocated 2nd chromosome fragments. **f** Nurse cell chromosome complement. **g** Mitotic chromosomes. Arrows show fragments translocated onto the W. *Bars* represent 10 μm

**Fig. 4a, b** Results of PCR-based screening of W-BAC clones using two primer sets that amplified female specific DNA fragments from genomic DNAs of $F_2$ progeny (parental cross: C108 x p50) in *Bombyx mori*. **a** Electrophoresis of PCR products obtained by the primer set Tail-3-A plus KaLTR-A; DNA fragments of the same size (295 bp) are amplified from two clones, 5H4C and 18K8H. **b** Electrophoresis of PCR products obtained by the primer set new437-A1 plus new477-B1; DNA fragments of the same size (801 bp) are amplified from two clones, 2K7G and 19L6H

**Fig. 5a-f** FISH images of two normal WZ pachytene bivalents of the Kinsyu-Showa *Bombyx mori* strain, painted by W-BAC probes. **a-c** A WZ bivalent stained with DAPI (a), 18K8H probe (b), and 5H4C probe (c). **d-f** A WZ bivalent stained with DAPI (d),

19L6H probe (e), and 2K7G probe (f)

**Fig. 6a-f** W-BAC FISH and GISH images of two normal WZ pachytene bivalents (a-f) and a pachytene oocyte (g, h) from the Kinsyu-Showa *Bombyx mori* strain. **a-c** A WZ bivalent stained with DAPI (a), and painted by 18K8H W-BAC probe (b) and GISH (c). **d-f** A WZ bivalent stained with DAPI (d), and painted by 2K7G W-BAC probe (e) and GISH (f); the heterochromatic thread of the W chromosome seen in (d) corresponds to hybridization signals in (e) and (f). **g** A pachytene complement counterstained with DAPI; note that the autosome bivalent bearing a heterochromatin block, deeply stained with DAPI (arrowhead), differs from the NOR bivalent bearing the nucleolus (N). **h** A superposed image of the same complement with W-BAC (2K7G) (red) and GISH (green) hybridization signals; note that the corresponding signals are observed not only in the W chromatid but also in telomeric and/or subtelomeric regions of several autosome bivalents, whereas the autosomal heterochromatin block (arrowhead) is exclusively highlighted by GISH; also note that all autosomes and the Z-chromosome thread are weakly stained by both GISH and W-BAC probes

**Fig. 7a-i** W-BAC and GISH images of spread chromosome preparations from ZWII females of *Bombyx mori*. **a-j** Pachytene oocyte bivalents; **a** a pachytene complement counterstained with DAPI. **b** a superposed image of the same complement with GISH (red) and W-BAC (18K8H) (green) hybridization signals. **c-j** two representatives of WZ bivalents stained with DAPI (c, g), and painted by GISH (d, h) and a W-BAC probe (18K8H) (e, i). **f, j** schematic drawings of the original W chromosome thread (red), Z (blue), and translocated 2nd chromosome (white). **k-u** Mitotic oogonial chromosomes; **k** a prometaphase mitotic complement counterstained with DAPI. **l** a superposed image of the same complement with GISH (red) and W-BAC (18K8H) (green) hybridization signals. **m-u** Three representatives of W chromosomes stained with DAPI (m, p, s) and their superposed images showing GISH (red) and W-BAC (18K8H) (green) hybridization signals (n, q, t); schematic drawings of the original W chromosome (red) and translocated 2nd chromosome (white) (o, r, u). Arrows indicate either the WZ bivalent (a, b) or the W chromosome (k, l); arrowheads show autosomal heterochromatin blocks that were also painted with genomic probes. *Bar* represents 10 μm (a-l) and 2.5 μm (m-u)

Table 1. Components of approximately 90 kb long region of a W-chromosome derived BAC clone (19L6H) in *Bombyx mori*, revealed by shotgun sequencing.

| Transposable elements | | | | | others |
|---|---|---|---|---|---|
| | retrotransposons | | | | |
| retroposon (Bm1) | non-LTR | | LTR | transposons | |
| | BMC1 | others | | | |
| 2 | 6 | 3 | 4 | 3 | 0 |

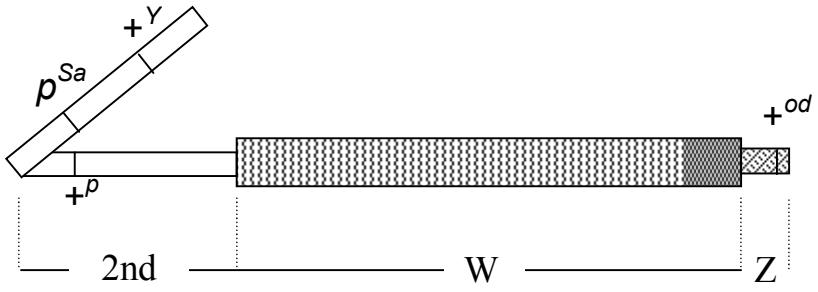


Fig. 1 Sahara et al

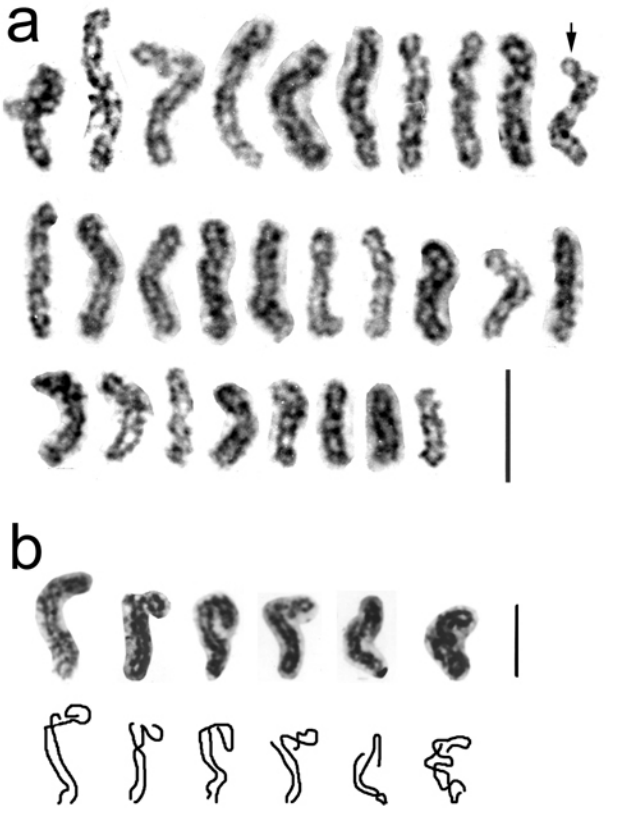


Fig. 2a, b Sahara et al

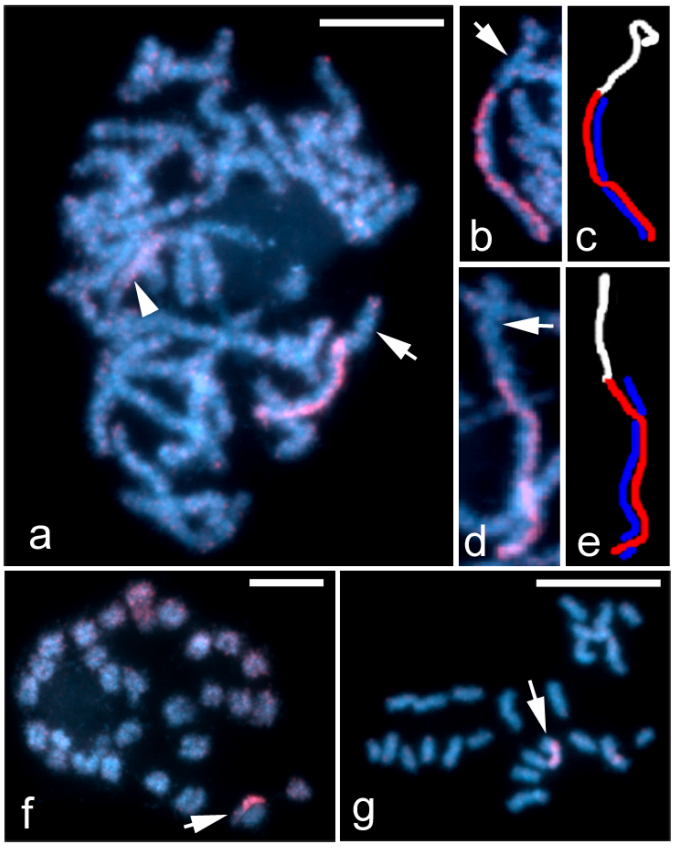


Fig. 3a-g Sahara et al

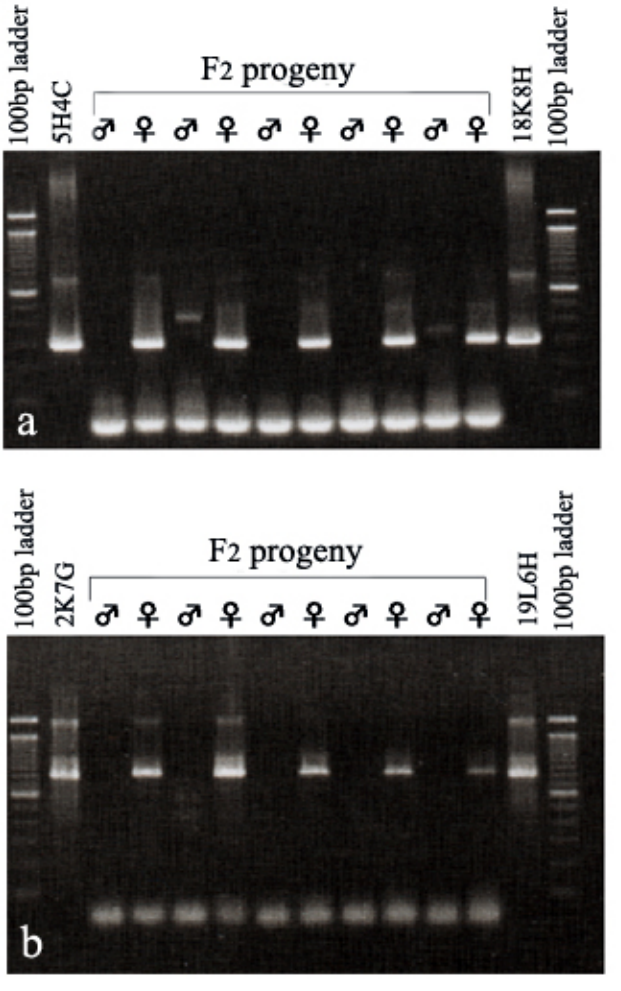


Fig. 4a, b Sahara et al

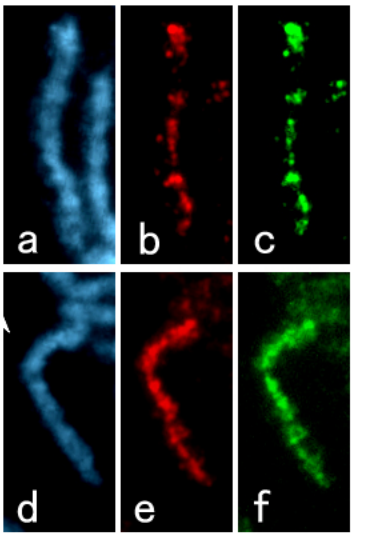


Fig. 5a–f Sahara et al

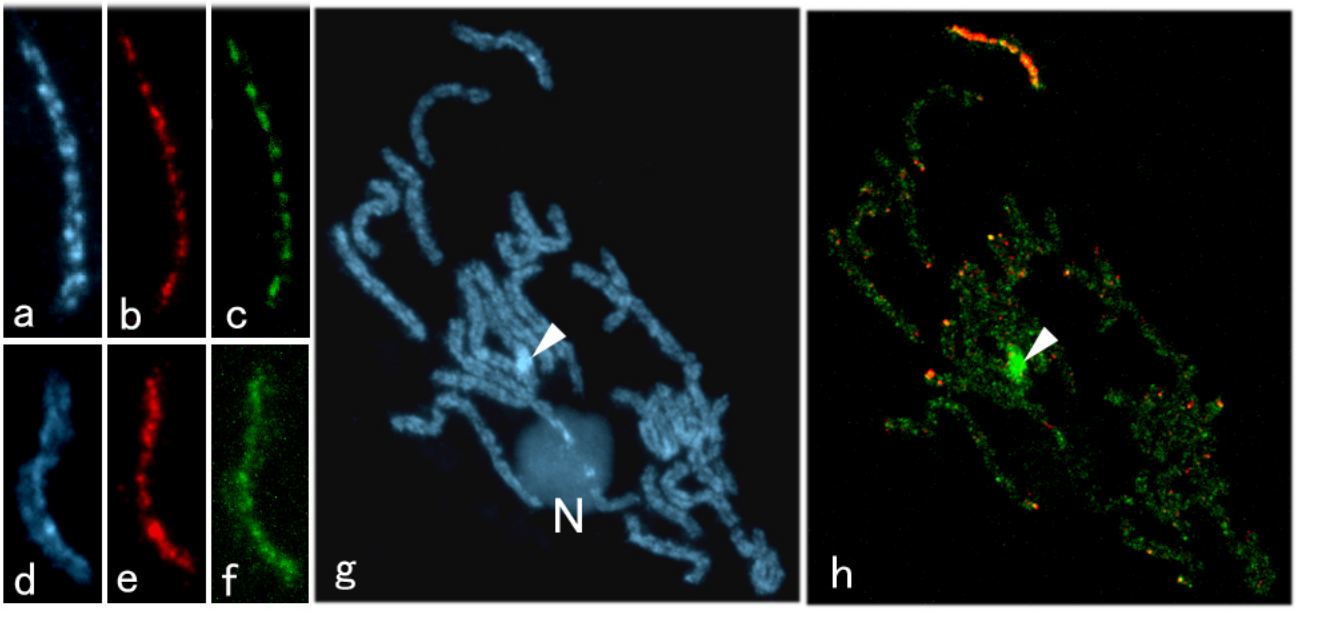


Fig. 6a–h Sahara et al

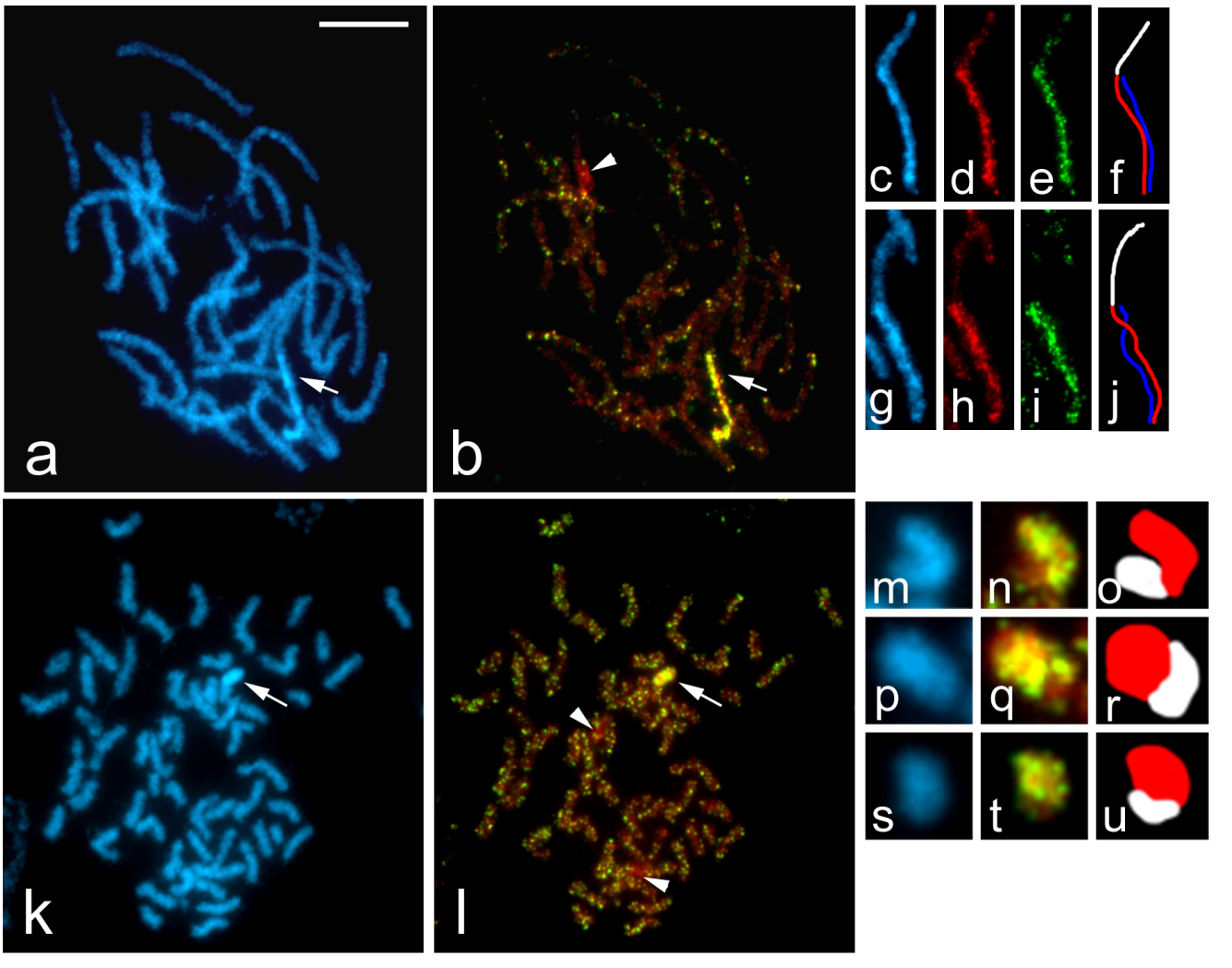


Fig. 7a-u Sahara et al