A history of chromosome identification in *Bombyx mori*

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### Abstract

The silkworm, *Bombyx mori*, is a lepidopteran model with long history of the domesticated insect for silk production, which contributed to human life as well as insect sciences. In chromosome science, the silkworm could be the first record of karyotype count in Lepidoptera. Because of the holokinetic chromosomes, precise chromosome identification and karyotype had been difficult until BAC-FISH (fluorescence in situ hybridization with bacterial chromosome (BAC) DNAs as probes) was applied for the silkworm chromosome analysis. Here we review the research histories for the first *B. mori* karyotype and its contribution for chromosome science and comparative genomics in Lepidoptera.

### Keywords:
- chromosome collinearity
- comparative genomics
- holokinetic chromosome
- karyotype
- Lepidoptera
- silkworm

### Introduction

The silkworm, *Bombyx mori*, is a domesticated moth that is reared for silk production. Japanese sericulture rapidly developed in the Meiji Era, mainly due to political support of new industries. The study of basic genetics, physiology, endocrinology, and anatomy of the silkworm greatly contributed to innovation of the Japanese sericulture. For example, very early genetic studies of K. Toyama on silkworm crosses rediscoved Mendel’s laws of heredity (Toyama 1906). He used the acquired knowledge to hybridize distantly related strains, which improved robustness, silk production and disease resistance. This was the first demonstration of “heterosis” then commonly used for better agricultural productions. K. Toyama has also been one of the first scientists who observed silkworm chromosomes (Toyama 1894). However, he misinterpreted the chromosome number observed (see below). In this review, we focus on the history of chromosome research in *B. mori* because of the first species to achieve a definitive karyotyping among Lepidoptera.

### Correct chromosome counts

After the first record of chromosome count of Lepidoptera (Carnoy 1895 cited in Robinson 1971), more than 1,000 karyotype numbers have been published (e.g. Robinson 1971). Most chromosome counts were determined from squash preparations and/or paraffin sections of spermatocytes. Yatsu (1913) correctly stated that the haploid chromosome number is *n*=28 in *B. mori*, while Toyama (1894) wrongly reported it as the diploid number of *2n*=28. Yatsu (1913) also predicted according to the haploid chromosome number that the mitotic complement consists of *2n*=56 chromosomes. First chromosome micrographs in the silkworm, taken from paraffin-sectioned male meiotic metaphase plates, were published by Kawaguchi (1928). However, it took about half century until the silkworm mitotic karyotype was published. Kawamura (1979) induced mosaic silkworms whose embryos had a mixture of haploid and diploid cells. The haploid and diploid mitotic complements showed the respective 28 and 56 chromosomes.

### Chromosome nature

Moths and butterflies (Lepidoptera) have a special type of chromosomes, the so-called ‘holocentric’ or more precisely ‘holokinetic’ chromosomes. This chromosome type is also characteristic of the silkworm (Murakami and Imai 1974). The term ‘holokinetic’ reflects the fact that these chromosomes do not have the primary constriction (= the centromere) but have a large kinetochore plate which covers a major part of the poleward chromosome surface (Marec 1996). The holokinetic chromosomes also occur in several other groups of insects such as Trichoptera and Hemiptera, in some groups of arachnids and worms, and in some groups of plants (Melters *et al.* 2012).

During meiotic prophase 1 *B. mori* chromosomes form fully paired synaptonemal complexes (SCs). The synopsis of meiotic bivalents starts from both telomeric ends and proceed towards the middle of bivalents, which sometimes results in chromosome interlocking as found by Rasmussen (1976). He proposed a hypothesis that the resolution of chromosome interlocking is associated with double-strand DNA breaks. In female silkworms as well as females of other lepidopteran species, chromosome crossing over does not occur (Sturtevant 1915) and meiosis is thus achiastic. The lack of chiasma for-
mation in female meiosis is substituted by special structures, the modified SCs, to maintain the bivalents until metaphase I and ensure the proper segregation of chromosomes to daughter nuclei. A detailed molecular mechanism of the modified SCs formation has not yet been identified. During chromosome segregation the proteinaceous modified SCs are detached from the chromosomes and their remnants in the equatorial plate, erroneously called ‘elimination chromatin’, can be visualized with some cytological dyes (see Marec 1996, for a detailed review).

**Sex chromosome identification**

Like all Lepidoptera, *B. mori* has a sex chromosome system with female heterogamety; silkworm females have a WZ pair of sex chromosomes, males a ZZ pair. The presence of a single W chromosome is sufficient to determine the female sex in diploids as well as polyploids (Hasimoto 1933; see Sahara et al. 2012). After long searching for the molecular mechanism of sex determination, Kiuchi et al. (2014) finally discovered a small piRNA molecule, the so-called *Fem* piRNA encoded by the W chromosome, which is the primary trigger of female development.

The identification of sex chromosomes using standard cytogenetic techniques was difficult in *B. mori*, although the W chromosome mass called sex-chromatin body, heterochromatin body or W-body was clearly visible in highly polyploid nuclei (Frizzi 1948; Ito 1977). The W-body was also found by Traut and Mosbacher (1968) in *Ephestia kuehniella*. In the meiotic WZ bivalent of *E. kuehniella*, the W chromosome was easily identified as a deeply stained thread (Traut and Rathjens 1973), however, no such thread was apparent in the silkworm (Traut 1976).

Kawazoe (1987) observed one pair of different chromosomes among 28 pseudopair-like parallel arrangements of homologous element in parthenogenetic female individuals. He claimed that the pair represents the W and Z sex chromosomes. Kawamura and Niino (1991) identified the silkworm WZ bivalent for the first time in a radiation-induced mutant strain, the sex-limited yellow cocoon (*Sy*) (Kimura et al. 1971). Because the quality of silk and its yield are different in females and males, discrimination of sex is important. The removal of juvenile female larvae, easily distinguishable by appearance, has been one of ideas to improve the silk production. The *Sy* strain allowed easy discrimination of sex by yellow larval hecomatin body or W-body was clearly visible in highly polyploid nuclei (Frizzi 1948; Ito 1977). The W-body was also found by Traut and Mosbacher (1968) in *Ephestia kuehniella*. In the meiotic WZ bivalent of *E. kuehniella*, the W chromosome was easily identified as a deeply stained thread (Traut and Rathjens 1973), however, no such thread was apparent in the silkworm (Traut 1976).

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**Sex chromosomes**

Comparative genomic hybridization (CGH) using female and male genomic probes made the W chromosome identification in *B. mori* feasible (Traut et al. 1999). Hybridization signals of the probes highlighted a single chromosome, the W chromosome in female mitotic complements and a thread of a single bivalent, the WZ in pachytene oocytes. In addition, strong hybridization signals were observed in a segment of another pair of chromosomes in both sexes, designated by these authors as the NOR-chromosomes. However, we later showed that the chromosomes were not the NOR-chromosomes but a pair of chromosome 24 (Yoshido et al. 2005a). Although the identification of silkworm sex chromosomes by CGH was quite convincing, direct evidence was obtained using BAC-FISH, i.e. FISH with bacterial artificial chromosome (BAC) probes. Sahara et al. (2003) selected W-derived BAC clones as probes and showed that hybridization signals of the probes identified the same W chromosome as CGH. Using the W-derived BAC probes Sahara et al. (2003) further identified both the W chromosome and the WZ bivalent in a mutant silkworm strain, ZWII. Although classical genetic analyses revealed that two fragments of chromosome 2 and a Z fragment are translocated onto the W in ZWII, conventional staining only showed asymmetric WZ bivalents, and the W-BAC probes hybridized only to the W compartment of the translocated chromosome in the asymmetric bivalents. In addition, the W-BAC probes only painted more than half of the mitotic W chromosomes in the ZWII strain but not the whole W chromosome as in the normal strain (Sahara et al. 2003). Later, we confirmed that the component with no hybridization signals of the W-BAC probes is composed of the chromosome 2 fragments (Figure 1).

Since there were no doubts that female genomic DNA probes in CGH experiments painted the W chromosome of *B. mori* and two other lepidopteran species (Traut et al. 1999), genomic in situ hybridization (GISH) technique was thought to be also applicable to identify lepidopteran W chromosomes (Medioni et al. 2004; Fuková et al. 2005; Yoshido et al. 2005b; 2006). For example, Yoshido et al. (2006) confirmed the usefulness of GISH in 9 lepidopteran species. GISH clearly identified WZ bivalents in pachytene oocytes of 8 species, while in one species showed the absence of the W chromosome in female meiotic complements, indicating that the species have a Z0/ZZ sex chromosome system.

GISH in combination with telomeric probes has been successfully used to analyze multiple sex chromosome systems in Lepidoptera. In most insects including Lepidoptera, the telomeres are composed of pentanucleotide repeats, (TTAGGG)n (Okazaki et al. 1993; Sahara et al. 1999), which is thought to evolve from the ancestral (TTAGGG)n repeats (Vitková et al. 2005). Because telomere-FISH can detect chromosomal ends, Yoshido et al. (2005b) combined GISH probe (female genomic DNA) with (TTAGGG)n probe. This technique enabled them to decipher unusual sex chromosome systems, which involved sex-chromosome-autosome fusions in lymantrid moths, *Orgyia* sp., and in wild silkmoths, *Samia cynthia* sp. Recently, the combined technique proved very useful in the analysis of complex sex-chromosome multivalents in *Leptidea* wood white butterflies (Šichová et al. 2015). In *L. amurensis*, this technique revealed a curious multiple sex chromosome constitution with one of the highest numbers of sex chromosomes, 9 in females (W;W;W;Z;Z;Z;Z;Z;Z;Z) and 12 in males (Z;Z;Z;Z;Z;Z;Z;Z;Z;Z;Z;Z) (Šichová et al. 2016).

**Application of FISH techniques for the identification of**

**Bombyx mori karyotype**

Kawazoe (1987) developed a very sophisticated but too dif-
Sahara et al. made mitotic preparations from very early embryos by an air-dry method and selected longer prometaphase complements to arrange pairs of chromosomes for karyotyping. For *B. mori*, he additionally used parthenogenetically developed female embryos which were induced by hot water treatment at 48°C for 15 min as originally developed by Astaurov (1940) (cited in a review of Klymenko 2001). Most probably it has been the best technique to prepare mitotic karyotype in *B. mori* so far. However, no further evidence was shown whether the karyotype in the silkworm was accurate or not.

Traut (1976) assembled the *B. mori* karyotype by means of pachytene mapping. For lepidopteran karyotyping, pachytene nuclei have two advantages: reduced number of elements to half and much longer bivalents compared to mitotic chromosomes. However, in *B. mori* only 6 bivalents but not the WZ bivalent could be discriminated by chromomere patterns, length and/or the NOR presence. Finally, the precise karyotype of *B. mori* has been assembled by means of BAC-FISH (Yoshido et al. 2005a). Since a dense RAPD linkage map (Yasukochi 1998) has been constructed, Yoshido et al. (2005a) selected clones from a *B. mori* BAC library (Wu et al. 1999) which were assigned to molecular linkage groups. Then the *B. mori* karyotype was established using two color-labelled 62 BAC-DNA probes (Figure 2).

**Comparative genomics in Lepidoptera**

The BAC-FISH mapping together with genome data of *B. mori* (Mita et al. 2004; Xia et al. 2004) further opened the gate for comparative study on lepidopteran chromosomes. Fine genome comparisons were feasible if genome sequences were assigned to each chromosome of other lepidopteran species such as *Heliconius melpomene* and *Melitaea cinxia* (The Heliconius genome consortium 2012; Ahola et al. 2014). Molecular linkage analysis (e.g. Van’t Hof et al. 2013) or BAC-FISH mapping (Yasukochi et al. 2009; 2016; Yoshido et al. 2011; Sahara et al. 2013) of single-copy orthologs of *B. mori* genes also provided whole genome comparisons without large-scale genome sequencing (Figure 3). Comparative genomics in Lepidoptera so far surprisingly revealed the chromosome collinearity. The conserved gene order is inconsistent with an idea that holocentric chromosomes can survive even if fissions occur, which...
Figure 3. The *Manduca sexta* karyotype. Sixty-three BAC probes in the first attempt and 4 additional reprobed BACs in the second attempt were used to identify individual chromosomes. (a) A spermatocyte pachytene complement showing 28 bivalents identified by BAC probes. (b) The bivalents from this nucleus arranged according to corresponding *Bombyx mori* chromosome numbers (black italic numbers). (c) Identification of the sex chromosome bivalent WZ in a pachytene oocyte by genomic *in situ* hybridization (GISH) (green signals) combined with BAC-FISH of *M. sexta* Z probes (red and cyan). Z, sex chromosome bivalent (ZZ). Bar = 10 µm. After Figure 1 in Yasukochi et al. (2009).
accelerates chromosomal rearrangements. Although newly synthesized telomeres were revealed in radiation-induced \textit{L.\textit{usulana elegans}} holocentric chromosomes (Jankowska et al. 2015), chromosome 3 fragment in mottled \textit{B.\textit{mori}} strain seemed to carry telomeric repeat in only one end (Fujiwara et al. 2000). This scientific piece of puzzle is not yet well arranged.

**Future chromosome studies in \textit{Bombyx mori}**

Both spontaneously and artificially induced mutants with aberrant chromosomes have been isolated and maintained in \textit{B.\textit{mori}}. For instance, the possibility of reciprocal translocation was reported for a strain, r521 (Sakaida B. mori). Future chromosome studies in \textit{Bombyx mori} will carry telomeric repeat in only one end (Fujiwara et al. 2000). The \textit{B.\textit{mori}} chromosome 3 fragment in mottled strain seemed to harbor a homeotic mutation named Double star (\textit{E \textit{Ds}}). Synthesized telomeres were revealed in radiation-induced accelerates chromosomal rearrangements. Although newly synthesized telomeres were revealed in radiation-induced \textit{L.\textit{usulana elegans}} holocentric chromosomes (Jankowska et al. 2015), chromosome 3 fragment in mottled \textit{B.\textit{mori}} strain seemed to carry telomeric repeat in only one end (Fujiwara et al. 2000). This scientific piece of puzzle is not yet well arranged.

Radiation-induced mutants with translocated chromosomes were well documented in classical genetics (see Tazima 1964). However, most of them are not yet cytogenetically examined. For examples, the \textit{W}-translocated strain (TWPB) and its derivatives were studied by Tanaka et al. (2000). The \textit{B.\textit{mori}} chromosome 3 fragment in mottled strain seemed to harbor a homeotic mutation named Double star (\textit{E \textit{Ds}}). Synthesized telomeres were revealed in radiation-induced accelerates chromosomal rearrangements. Although newly synthesized telomeres were revealed in radiation-induced \textit{L.\textit{usulana elegans}} holocentric chromosomes (Jankowska et al. 2015), chromosome 3 fragment in mottled \textit{B.\textit{mori}} strain seemed to carry telomeric repeat in only one end (Fujiwara et al. 2000). This scientific piece of puzzle is not yet well arranged.

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