Sex chromosome evolution in moths and butterflies

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Running title: Sex chromosome evolution in Lepidoptera

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Abbreviations
BAC-FISH: FISH using BACs, bacterial artificial chromosomes, as probes
CGH: comparative genomic hybridisation
FISH: fluorescence in situ hybridisation
GISH: genomic in situ hybridisation
MYA: million years ago
Zoo-FISH: FISH using probes from one species on other species
Abstract
Lepidoptera, i.e. moths and butterflies, have a female-heterogametic sex chromosome system, with most females having a WZ constitution while males are ZZ. Besides this predominant WZ/ZZ system, Z/ZZ, WZ1Z2/Z1Z2Z2 and W1W2Z/ZZ systems also occur. Sex is determined by an unknown W-linked gene or genes in Bombyx mori, but by dosage-dependent and equally unknown Z-linked genes in all Z/ZZ species. The female-heterogametic sex chromosome system has been conserved for at least 180 million years in the phylogenetic branch that combines Lepidoptera and Trichoptera. The W chromosome, which is present in most lepidopteran species, was incorporated into the system much later, about 90-100 MYA. Z chromosomes display conserved synteny, much like the Z in birds or the X in mammals. The W, on the other hand, is evolving rapidly. It is crammed with repetitive elements which appear to have a high turnover rate, but poor in or even devoid of protein-coding genes. It has frequently undergone fusion with autosomes or sporadically been lost altogether.

Introduction
Sex chromosomes provide the primary signal in the sex determining pathway and at the same time ensure a balanced sex ratio among offspring. Apart from their function, sex chromosomes are rather often the most conspicuous elements in a chromosome complement in terms of size, form, heterochromatinisation and behaviour in meiosis. This concerns especially the Y chromosomes in XY systems and the W chromosomes in WZ systems, and led to the discovery of sex chromosomes in the early days of cytogenetics. There has been a continuous interest in them since that time.

Sex chromosome systems have evolved independently many times in plants and animals. Some sex chromosome systems appear to be well conserved and have already had a long lifetime, e.g. those of mammals or birds among vertebrates. Others are more shortlived. Quite recently, the common house fly, Musca domestica, replaced its old XY system in many populations worldwide for new ones based on former autosomes (Franco et al. 1982). An extreme case is the fly Megaselia scalaris where replacement of the sex chromosome system by non-homologous new ones can be observed even under laboratory conditions (Traut 2010).

Once a chromosome pair has acquired the sex determining function, it appears to follow a universal evolutionary pathway that leads to crossover suppression and subsequent molecular differentiation, even visible cytogenetic differences between X and Y, or W and Z, and eventually loss of the Y or W, given sufficient evolutionary time.
We present here an ancient female-heterogametic system where we can follow birth and decline of a W chromosome: the lepidopteran sex chromosome system. The insect order Lepidoptera, which contains moths and butterflies, is species-rich, comprises 160,000 described species and an estimated total number of half a million (Kristensen et al. 2007). Among them are species of commercial value and many pest insects. Lepidoptera, hence, offer a rich supply of material to study evolutionary pathways.

**Identification of sex chromosomes in Lepidoptera**

Chromosome numbers are known for more than 1,000 species of Lepidoptera (Robinson 1971), mostly from counting them in the easily accessible male meiotic metaphases. But only in a very limited number of species, the sex chromosome constitution has been uncovered. This is because male meiotic metaphases are inadequate for species with female heterogamety. Mitotic chromosomes in Lepidoptera, on the other hand, are numerous (modal number 2n = 60-62), small and without any landmarks, such as localised centromeres, chromosome bands or obvious heterochromatic segments, and, hence, are rather difficult objects for chromosome study (Fig 1a). Female meiotic pachytene chromosomes proved to be considerably better for that purpose, they are longer and, having an achiasmatic meiosis, the homologues remain synapsed in a pachytene-like stage until metaphase I. The sex chromosome constitution of a number of lepidopteran species was revealed by analysing the pachytene complement. The heterochromatinised W thread in the WZ bivalent was thereby used as the cytogenetical marker for identification of the sex chromosome bivalent (Traut and Rathjens 1973; Traut and Marec 1997).

More recently, various FISH techniques solved the problem of recognising the sex chromosomes. Comparative genomic hybridisation (CGH) was found to be generally applicable for identifying differentiated sex chromosomes, independent of any morphological markers (Fig 1a-c; Traut et al. 1999). The simpler genomic *in situ* hybridisation (GISH) can also highlight sex chromosomes (Fig. 1d). Using GISH, Yoshido et al. (2006) analysed the sex chromosome constitution in 15 species of Lepidoptera. Even single W derived BACs label the W chromosome along most of its length in *B. mori* (BAC-FISH, Sahara et al. 2003b). FISH using probes from microdissected W chromosomes provide another generally applicable method to paint the W chromosome (Fuková et al. 2007; Vitková et al. 2007). The complex sex chromosome multivalents were still a tough problem until Yoshido et al. (2005) introduced telomere-FISH combined with GISH to delineate chromosomes in pachytene sex chromosome multivalents (Fig. 2).
Origin of the lepidopteran sex chromosome system

Lepidoptera and the sister group Trichoptera stand out among insects as the only clade having a female heterogametic sex chromosome system (besides other characters in common). Female heterogamety, therefore, must have arisen in a common ancestor of the two insect orders, more than 180 MYA (the oldest dated fossils are from 180-190 MYA, Kristensen and Skalski 1999; Grimaldi and Engel 2005). All trichopteran species so far investigated (Marec and Novák 1998; Lukhtanov 2000) as well as basal lepidopteran families (Marec et al. 2010) have a Z/ZZ sex chromosome system. The W chromosome, which is considered characteristic for Lepidoptera, only occurs in the more ‘advanced’ families (Fig. 3). In a phylogenetic survey, Traut and Marec (1996) and Lukhtanov (2000) determined the clade in which the W chromosome appeared. The W came into being at the common root of Ditrysia and Tischerioidea, ~97 MYA (the earliest record of a Ditrysian, Kristensen and Skalski 1999). It is not known yet whether the W had originally been an autosome whose homologue was fused to the ancestral Z (Traut and Marec 1996) or a B chromosome which was incorporated in the sex chromosome system by acquiring pairing partnership with the Z chromosome (Lukhtanov 2000).

Variations of the WZ theme

In a recent compilation of known sex chromosome systems in Lepidoptera, the largest fraction, 18 of 38 species, had a WZ/ZZ system (Traut et al. 2007). But they are certainly underrepresented in the compilation. Z/ZZ systems, also called Z0 systems, W1W2Z/ZZ and WZ1Z2/Z1Z2Z2Z2 systems have also been discovered. While the Z/ZZ constitution is the ancestral condition, present in all basal lineages of Lepidoptera, Z/ZZ cases among advanced species are sporadic and considered due to secondary loss of the W chromosome. Multiple sex chromosomes, in contrast, owe their origin to chromosome splitting or to chromosome fusion of the W or the Z with an autosome.

In favourable cases, the origin of a variant sex chromosome constitution can be uncovered and the participating chromosomes identified. In Samia cynthia, a wild silkmoth species, three chromosome races have been discovered in different populations (Yoshido et al. 2005). All three have different sex chromosome systems. The Vietnam population has a Z/ZZ system, the Sapporo population a WZ/ZZ system and the Nagano population a WZ1Z2/Z1Z2Z2Z2 system. With FISH using fosmid and PCR-amplified genomic probes, the original constituents of the sex chromosome system were identified and traced back to homologous
chromosomes of *B. mori* (Fig. 4; Yoshido et al. 2011a; b). In the Z/ZZ race of the Vietnam population, the Z chromosome shows conserved synteny with the *B. mori* Z chromosome while the W was lost in the evolution of the population. The WZ/ZZ system of the Sapporo population is, in fact, a neo-W neo-Z/neo-Z neo-Z system in which a homologue of chromosome 13 from the Vietnam population is fused to the ancestral Z chromosome, thus creating a neo-Z chromosome. The other homologue appears to be fused to a remnant of the ancestral W chromosome, thereby forming a neo-W chromosome. The multiple sex chromosome system in the Nagano population, in turn, appears to be derived from the sex chromosome system found in the Sapporo population whereby the neo-W is seen to be fused with a chromosome 12 of the other populations. This produces a neo-W which pairs with a neo-Z like that from Sapporo, now a Z₁ and a free autosome 12, which is now a Z₂ chromosome.

Lepidopteran chromosomes are holokinetic with kinetochores covering most of the chromosome length (Wolf 1996). Fusions as well as fissions should be facilitated with this type of chromosome. Consistent with this expectation, stable W chromosome fragments and multiple sex chromosome systems have been produced experimentally by γ-ray mutagenesis, in the meal moth *Ephestia kuehniella* (Traut and Rathjens 1973; Rathjens 1974; Traut et al. 1986). Fusion of the W with an autosome was found in four independent cases, giving rise to strains which have now been cultured for more than three decades. The fusion events produced neo-W chromosomes which paired in meiosis with two partners, the original Z chromosome and the former autosome. Formally, these are WZ₁Z₂ /Z₁Z₁Z₂Z₂ sex chromosome systems, and would be called thus if found in the field.

**Sex determination in Lepidoptera**

As noted, in female-heterogametic systems like those of Lepidoptera, the primary signal in the sex-determining pathway is either a female-determining signal encoded in the W chromosome or a dosage-dependent male-determining signal encoded in the Z chromosomes. Both variants occur among lepidopteran species but their molecular basis has not yet been discovered.

In *B. mori*, the W chromosome has a strong female-determining activity. This has been known for a long time from the analysis of polyploids (Tazima 1964; Traut et al. 2007). Whenever an embryo possesses a W, it enters the female pathway, irrespective of the number of Z chromosomes present in its genome. Absence of a W determines the male sex. The feminizing function, *Fem*, has been localised to a small region of the W chromosome by
deletion mapping (Abe et al. 2010). It has not yet, however, been associated with a specific gene or genes.

In Z/ZZ systems, on the other hand, sex is determined by the number of Z chromosomes. Presence of a single Z chromosome in the genome causes female development, whereas presence of two Z chromosomes results in male development. In the chicken, DMRT1 is considered a Z-counting mediator that triggers the sex determining pathway (Smith et al. 2009). In Lepidoptera, however, dosage-dependent male determining genes on the Z chromosome have not been identified.

It is also unknown, whether WZ/ZZ species other than B. mori possess a sex determining mechanism with Fem localised in the W chromosome, or whether sex determination depends on a Z-counting mechanism like in Z/ZZ species.

The subsequent steps of the sex-determining pathway are partially known, mainly from B. mori. Sex-lethal (Sxl), which forms the key switch in Drosophila, is conserved and present in B. mori but Bm-Sxl transcripts are not sex-specifically spliced and, therefore, Bm-Sxl seems not to play a role in the sex-determining pathway (Niimi et al. 2006). In Hymenoptera, Coleoptera and non-drosophilid Diptera, transformer (tra) has taken over the role of a key switch and that of a memory of the sexual state of a cell. But tra has not been found in B. mori or any other lepidopteran species and probably plays no role in regulating the last step in the sex-determining pathway: sex-specific splicing and expression of Bmdsx, the B. mori ortholog of Drosophila’s doublesex (dsx). Instead, the male-specific BmIMP (Bombyx mori homologue of IGF-II mRNA binding protein) together with BmPSI (Bombyx mori homologue of P-element somatic inhibitor) are known to regulate splicing of Bmdsx (Suzuki et al. 2010). Orthologs of dsx with sex-specific splicing are known from all investigated insects and even from a crustacean, Daphnia magna (Kato et al. 2011). The sex-specific Bmdsx transcripts code for two protein isoforms, a female form, BmDSXF, and a male form, BmDSXM. These proteins regulate transcription of sex-specific genes, such as those for yolk proteins. Detailed accounts on sex determination in B. mori have been published recently by Abe et al. (2010) and Suzuki (2010).

Constituents of the W chromosome

Gene and sequence information on the lepidopteran W chromosome are rather scarce. The W chromosome has been exempted from the genome sequencing of B. mori because of the expected problems with the high density of repetitive elements. And classical meiotic recombination mapping cannot be applied to the W as it is present only in females and has no
genetic exchange with its partner, the Z chromosome. However, manipulation of chromosomes, polyploidisation and induced translocations and deletions, allowed researchers to map \textit{Fem} to a limited region of the \textit{B. mori} W chromosome (see above). In \textit{E. kuehniella}, a putative male-killing factor has been assigned to a translocated W segment (Marec et al. 2001).

FISH techniques like CGH and GISH have been applied to a number of species. They give some insight into the molecular composition of the W. The W stands out in these preparations as the most densely labelled chromosome (see Fig 1). Highlighting indicates a high density of repetitive DNA in the W. Using CGH, a conspicuous portion of the repetitives was found to be W-specific in the wax moth, \textit{Galleria mellonella}. The W of \textit{B. mori}, however, is filled with repetitive sequences which are ubiquitous in the genome (Sahara et al. 2003a).

This general property, a W densely populated with repetitive elements, was confirmed by analysis of sequences derived from the W. All DNA fragments hitherto isolated from the W chromosome of \textit{B. mori} contained retrotransposon sequence (Abe et al. 2010). DNA sequences isolated from the W chromosome of the codling moth, \textit{Cydia pomonella}, by microdissection, were multicopy (with three exceptions), but only 3 out of 17 sequence motifs were confirmed retrotransposons (Fuková et al. 2007).

The sequence composition of the W appears to have a rather high turnover rate in evolution. FISH probes generated by microdissection from the W chromosomes of \textit{E. kuehniella}, \textit{Cadra cautella}, \textit{Plodia interpunctella} and \textit{G. mellonella}, all from the family Pyralidae, paint the W of the respective species in chromosome preparations. But when the \textit{E. kuehniella} probe was crosshybridised to W chromosomes of the other species ("Zoo-FISH", Fig 5), the hybridisation signals were rather patchy in \textit{C. cautella} and \textit{P. interpunctella}, and scarce and dot-like or absent in \textit{G. mellonella} (Vítková et al. 2007). So even in related species of the same family, the composition of the W has changed to an extent that it is no longer recognised by hybridisation.

**Constituents of the Z chromosome**

In contrast to the W, the Z chromosome of \textit{B. mori} was included in the whole genome sequencing projects and the assembled sequence made accessible in public databases (The International Silkworm Genome Consortium 2008). The general sequence composition is much like that of autosomes but unlike the composition of its partner W. And again in contrast to the W, the Z chromosome is rather conservative. The Z chromosomal gene content
is conserved among Lepidoptera, much like that of the Z from birds (Nanda et al. 2008) or the X from mammals (Ohno 1973; Rat Genome Sequencing Project Consortium 2004).

Table 1 shows a list of *B. mori* Z chromosomal genes and the presence of their orthologues in Z chromosomes of other lepidopterans. Conserved synteny was detected of genes from position 0.5 Mbp to position 21.8 Mbp of the 22.4 Mbp of the Z chromosomal DNA sequence. Thus conserved synten was found in practically all regions of the *B. mori* Z chromosome, indicating the whole Z chromosome is likely to be conserved. A few exceptions from the synteny rule have been found *Bicyclus anynana* and *Ostrinia nubilalis* (Beldade et al. 2009; Kroemer et al. 2011). The exceptions were corroborated by a search in the *B. mori* sequence database. One gene, *peptidylprolyl isomerase*, from the *B. mori* Z chromosome has an orthologue on *Bicyclus anynana* chromosome 4, but not on the Z. Orthologs of two other *B. mori* Z-linked genes, *ribosomal protein L36* (*RpL36*) and a gene similar to *Aedes aegypti suppressin* (*BGIBMGA002019*) are autosomal in *O. nubilalis*. And one gene (*MOF*) from *B. mori* chromosome 17 has an orthologue on the *B. anynana* Z chromosome. Thus, at least some transpositions or translocations have occurred during evolution of the Z.

Z chromosomes appear to play a major role in the speciation process (Sperling 1994; Dopman et al. 2005). Obvious players in the field of reproductive barriers are genes controlling pheromones, pheromone recognition and voltinism. The Z chromosomes contain many of them, more than expected on the basis of random distribution. A recently discovered example is the tandem array of olfactory receptor genes in the Z chromosome of *O. nubilalis* (Yasukochi et al. 2011). Genes on Z chromosomes are subject to the so-called 'Faster-Z effect' (Mank et al. 2010): they are thought to evolve faster than those on autosomes as they are present in a hemizygous state half of the time, and therefore, more exposed to selection forces.

There is another group of sex-related genes in which the content of the Z differs significantly from that of autosomes in *B. mori*. For unknown reasons, the Z chromosome carries an overproportionate number of genes solely expressed in the testis (Arunkumar et al. 2009, Walters and Hardcastle 2011).

**Dosage compensation**

The dosage of X- and Z-linked genes in one sex is twice that in the other sex. This may pose problems for appropriate expression levels. Species with an XX/XY sex chromosome system, such as mammals, *Caenorhabditis* and *Drosophila*, deal with this problem by using chromosome-wide dosage compensation mechanisms (Chow and Heard 2010). Birds, which are WZ/ZZ species like Lepidoptera, do not possess a chromosome-wide dosage-
compensation, but genes in a male hypermethylated (MHM) region are dosage-compensated while the rest are not (Melamed and Arnold 2007). In Lepidoptera, the transcription levels of several genes from *Bombyx*, *Heliconius* and *Antheraea* have been assayed and shown to have an approximate ratio of 1:2 in the same tissues of females and males, respectively (Johnson and Turner 1979; Gotter et al. 1999; Suzuki et al. 1999). The results were confirmed in most genes of a wider, microarray-based investigation of transcription levels of 579 Z-linked genes in *B. mori*: hence, it appears that Lepidoptera do not have a chromosome-wide dosage-compensation mechanism (Zha et al. 2009). In a new microarray study, Walters and Hardcastle (2011) found a 1:1 ratio of Z-chromosomal transcript levels between females and males relative to autosomal gene expression. From these data, it seems that there is dosage compensation in *B. mori*. The question is not settled though, as the problem of normalising the microarray data appears to be the cause for the different interpretations.

**Exploiting and manipulating lepidopteran sex chromosomes for economic purposes**

Several moth species are economically important, either as useful or harmful insects. The domesticated silkmoth, *B. mori*, and a few wild species are commercially important as producers of silk. Many other moth species are agricultural pests or infest stored goods.

In *B. mori*, males are the preferred sex for commercial applications. Males produce approximately 20% more silk and have a shorter and more homogeneous feeding period. It has been a long-standing wish to discriminate between females and males as early as possible to reduce costs and increase silk production. So-called sexing strains have been developed, therefore, by translocation of autosomal segments carrying genes for visible traits to the W chromosome (Tazima 1964; Nagaraju et al. 1996). The sexes can be distinguished in one of these as early as at the egg stage by colour. Strunnikov (1975) went further and proposed a scheme in which live female offspring do not survive. According to this scheme males with two balanced non-allelic recessive Z-linked lethal factors are crossed to normal females. The resulting progeny consist only of males, since all female progeny are lethal due to the hemizygous lethal allele inherited from the father (Fig. 6). ‘Platina Boy’ is a male-only commercial *B. mori* product in Japan, based on Strunnikov’s proposal (Ohnuma 2006).

Strunnikov’s proposal has also been put into reality in a storage pest, the meal moth *E. kuehniella* (Marec et al. 1999). Trans-heterozygous males carrying two balanced lethals released in sufficient numbers in an infesting population reduced the proportion of females in the succeeding generations.
New schemes are presently introduced into the sterile insect technique (SIT) for pest control, making use of genetically manipulated insects (Alphey et al. 2002), some of them already in the test phase. Manipulating the W chromosome by introduction of a conditional lethal transgene, is a proposal which is being pursued in the codling moth, *C. pomonella*, which infests apples (Marec et al. 2007).

**Outlook**
The introduction of massive parallel sequencing techniques, so-called ‘next gen’ sequencing, has revolutionised research in many fields of genetics. The results are not yet fully realised in the fields discussed here. But in view of the many whole genome sequencing projects presently under way and the intended accessibility of sequence data, it is easy to predict that in the near future all topics of this review will gain massively. Primary sex determining signals, the sex determining cascade, the composition of chromosomes including sex chromosomes, evolution of chromosomes, conserved synteny: all can easily be studied on the sequence level. That will not make cytogenetics obsolete, rather, it will be important for it to persist and improve as a critical framework for organising and extending molecular data.

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**Figure legends**

Fig 1. Identification of W chromosomes by FISH. a-c: Comparative genomic hybridisation (CGH) reveals the W chromosome of the tobacco horn worm, Manduca sexta. Female mitotic metaphase stained with DAPI (a). Female (green) and male (red) whole genomic probes highlight the W chromosome (b, c). d: Genomic in situ hybridisation (GISH) applied to a
female pachytene complement of *Orgyia recens*. Female genomic probe (red) paints the W thread of the WZ bivalent. Bar 10 µm.

Fig 2. A combination of GISH and telomere-FISH delineates individual chromosomes in sex chromosome multivalents. A whole genome female probe (green) detects the ancestral W segment and an insect telomere probe, (TTAGG)_n, (red) the chromosome ends. a: Sex chromosome trivalent in a female pachytene complement of *Orgyia thyellina*. The two W chromosomes, W₁ and W₂, are each composed of an ancestral W segment and a translocated autosomal segment. b: Sex chromosome bivalent in a female pachytene complement of *Orgyia antiqua*. No telomeric signal was detected inside the sex chromosome bivalent. This indicates the presence of a single W with an ancestral W segment and a recently translocated presumably autosomal, segment.

Fig 3. Phylogeny of the sex chromosome system in Lepidoptera and their sister group Trichoptera. The diagram presents superfamilies arranged in a phylogenetic tree, and the female sex chromosome constitution of species investigated (based on Marec et al. 2010 and references therein).

Fig 4. Evolution of variant sex chromosome systems in *Samia cynthia*. Fosmid-FISH detects conserved synteny between chromosomes of *Bombyx mori* and *S. cynthia* and identifies the components of the sex chromosome univalent, bivalent and trivalent of the Vietnam, Sapporo and Nagano populations (for details see Yoshido et al. 2011a; b).

Fig 5. Zoo-FISH showing the decreasing similarity of the DNA content of the W chromosome among the related species *Ephestia kuehniella* (a, b), *Cadra cautella* (c, d), *Plodia interpunctella* (e, f) and *Galleria mellonella* (g, h). The pachytene chromosome preparations were probed with a whole W probe from *E. kuehniella* (red signals). Subsequent hybridisation with species-specific W-chromosome painting probes (green signals) identified the W chromosomes of the respective species (c, e, g) (from Vítková et al. 2007, by courtesy of the authors and with kind permission from Springer Science+Business Media). Bar, 10 µm.

Fig 6. Scheme of genetic sexing proposed by Strunnikov (1975). Males carrying Z-linked recessive trans-heterozygous lethal mutations (*sl*-1 and *sl*-2) are crossed with wild-type
Sahara et al Fig 1
Sahara et al Fig 2
Sahara et al Fig 3
Sahara et al Fig 4
Normal female

Male carrying recessive Z-linked lethal factors

Females

Males

Sahara et al Fig 6
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* included in the table are only those genes that were confirmed as orthologues by a new search in the B. mori genome