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Sex Chromosomes and Sex Determination in Lepidoptera

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Abstract
The speciose insect order Lepidoptera (moths and butterflies) and their closest relatives, Trichoptera (caddis flies), share a female-heterogametic sex chromosome system. Originally a Z/ZZ (female/male) system, it evolved by chromosome rearrangement to a WZ/ZZ (female/male) system in the most species-rich branch of Lepidoptera, a monophyletic group consisting of Ditrysia and Tischeriina, which together comprise more than 98% of all species. Further sporadic rearrangements created multi-sex chromosome systems; sporadic losses of the W changed the system formally back to Z/ZZ in some species. Primary sex determination depends on a Z-counting mechanism in Z/ZZ species, but on a female-determining gene, *Fem,* in the W chromosome of the silkworm. The molecular mechanism is unknown in both cases. The silkworm shares the last step, *dsx,* of the hierarchical sex-determining pathway with *Drosophila* and other insects investigated, but probably not the intermediate steps between the primary signal and *dsx*. The W chromosome is heterochromatic in most species. It contains few genes and is flooded with interspersed repetitive elements. In interphase nuclei of females it is readily discernible as a heterochromatic body which grows with increasing degree of polyploidy in somatic cells. It is used as a marker for the genetic sex in studies of intersexes and *Wolbachia* infections. The sex chromosome system is being exploited in economically important species. Special strains have been devised for mass rearing of male-only broods in the silkworm for higher silk production and in pest species for the release of sterile males in pest management programs.

Sex chromosomes stand out from the rest of the chromosome complement by being structurally different, having a different behaviour in meiosis, sometimes being visible as heterochromatic bodies in interphase nuclei, and by having an associated phenotype, female or male. Since these basic observations are technically not demanding, sex chromosomes have been in the focus of studies from the early days of genetics and cytogenetics, and – with ever refined research tools – up to the present time.

In Lepidoptera, in contrast to most other animal groups, females are the heterogametic sex. They share this property with Trichoptera (caddis flies), their closest relatives, and with snakes and birds. Sex chromosome systems with female heterogamety are referred to as WZ/ZZ and Z/ZZ (alternatively designated WZ and Z0) depending on the presence or absence of a W chromosome.

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It is stimulating to study similarities and differences as well as the consequences associated with this system versus the more common XY systems. Lepidoptera are a large group even among the insects. More than 130,000 Lepidoptera species are known so far (The Global Lepidoptera Names Index, http://www.nhm.ac.uk/entomology/lepindex). But only a few species have attracted more than sporadic interest in genetic research in the past, among them the flour moth *Ephestia kuehniella,* studied mainly for the relationship of single genes and steps in the ommochrome synthesis pathway by A. Kühn and colleagues (1929–1963), *Papilio spp.* and *Danaus spp.* for Batesian mimicry by C.A. Clarke and P.M. Sheppard (1955–1972), the psychid moth *Solenobia triquetrella* and related species for development of sexual intergrades and the evolution of parthenogenesis by J. Seiler and colleagues (1949–1972), and the gypsy moth *Lymantria dispar* for intersexuality and sex determination by R. Goldschmidt and colleagues (1920–1955) [see Robinson, 1971 and references therein]. The economically important silkworm *Bombyx mori* has continuously attracted research for a long time and became an insect model species, second only to *Drosophila melanogaster* in importance [Tazima, 1964; Goldsmith et al., 2005].

After initial efforts in the tens and twenties of the past century, zeal in cytogenetic research dropped – except for counting chromosome numbers – mainly because the mitotic chromosomes were unfavourable for conventional cytogenetics. The conditions have changed dramatically. Vast amounts of sequence data have been acquired including whole-genome shotgun (WGS) sequences of *Bombyx mori* and expressed sequence tag (EST) collections from various species, developmental stages and tissues. A public database devoted to Lepidoptera allows searches in the sequence collections and easy access to the data [http://www.butterflybase.org; Papanicolaou et al., 2005]. Together with the emergence of various fluorescence in situ hybridisation (FISH) techniques and the use of pachytene chromosomes, moth and butterfly chromosomes have become amenable to cytogenetics and gained new interest.

Other reasons for a new interest in Lepidoptera genetics and cytogenetics are the many pest species that belong to this insect group and cause considerable losses in agriculture. Therefore, there is a high demand to develop strategies of pest control against these species, especially species-specific ones that avoid damage to other insect species. Some of the new concepts intend to exploit the sex chromosome system [Marec et al., 2005].

Sex Chromosome Systems

From sex-linked inheritance in the moth *Abraxas grossulariata,* it had been inferred very early in the history of genetics that in contrast to most other groups, females are the heterogametic sex in Lepidoptera [Doncaster and Raynor, 1906; Bateson and Punnet, 1911]. This proved correct when Seiler [1914] identified the sex chromosomes in *Phragmatobia fuliginosa*.

Presently known karyotypes with identified sex chromosome systems are listed in table 1. They are predominantly WZ/ZZ systems, but Z/ZZ, W_1W_2Z/ZZ and WZ_1Z_2/ $Z_1Z_1Z_2Z_2$ systems do occur. Correct distribution of multiple sex chromosomes in meiosis is ensured as they form a single multivalent [e.g. Seiler, 1914; Traut and Marec, 1997; Yoshido et al., 2005a]. Several cases of sex-linked (Z-linked) inheritance [e.g. Kühn, 1939; Glover et al., 1990; Gotter et al., 1999; Suzuki et al., 1999], and a larger data set of species exhibiting sex chromatin (W chromatin) in somatic interphase nuclei [Traut and Marec, 1996] indicate that the table truly represents the majority of butterfly and moth species.

The reason why the sex chromosomes of only a few of the more than 130,000 Lepidoptera species have been identified so far lies in the difficulties that the mitotic chromosomes of Lepidoptera present. They are usually much smaller than vertebrate chromosomes; they are numerous with a modal number of 2n = 60 or 62; they cannot be banded by available methods; and they do not even have localised centromeres (fig. 1a, b, 2a). These difficulties have prevented cytogeneticists for a long time to do more than chromosome counting in this group. In more recent times, the use of pachytene chromosomes and various fluorescence in situ hybridisation (FISH) methods (fig. 1c, d, 2b) opened the field in Lepidoptera for cytogenetic research which is necessary to establish a framework for molecular data.

Meiosis

With respect to crossing-over, Lepidoptera display the reverse situation of the *Drosophila* case: males have crossovers, females do not [Sturtevant, 1915]. Male meiosis follows the conventional cytogenetic scheme with typical chiasma figures, crosses and rings in diplotene and diakinesis [e.g. Maeda, 1939; Traut, 1977; Banno et al., 1996; Traut and Clarke, 1996].

Female meiosis, however, is achiasmatic [Maeda, 1939; Suomalainen, 1965; Traut, 1977; Nokkala, 1987]. This is

Table 1. Lepidopteran karyotypes with identified sex chromosomes

Family	Species	2n chromosome constitution[a]		Reference
		females	males	
Arctiidae	*Lophocampa maculata*	33,Z	34,ZZ	Ennis, 1976
	Phragmatobia fuliginosa[b]	57,W_1W_2Z	56,ZZ	Seiler, 1914; Traut and Marec, 1997
Bombycidae	*Bombyx mori*	56,WZ	56,ZZ	Kawamura and Niino, 1991; Traut et al., 1999
Crambidae	*Ostrinia scapulalis*	62,WZ	62,ZZ	Kageyama and Traut, 2004
Gelechiidae	*Exoteleia dodecella*	23,Z	24,ZZ	Ennis, 1976
	Exoteleia pinifoliella	21,Z	22,ZZ	Ennis, 1976
	Phthorimaea operculella	58,WZ	58,ZZ	Makee and Tafesh, 2006
Lasiocampidae	*Trabala vishnu*	51,WZ_1Z_2	52,$Z_1Z_1Z_2Z_2$	Rishi et al., 1999
Lymantriidae	*Lymantria dispar*	62,WZ	62,ZZ	Traut and Marec, 1997
	Orgyia antiqua	28,WZ	28,ZZ	Traut and Marec, 1997; Yoshido et al., 2005b
	Orgyia thyellina[c]	22,WZ	22,ZZ	Traut and Clarke, 1996
	Orgyia thyellina[c]	23,W_1W_2Z	22,ZZ	Yoshido et al., 2005b
Micropterigidae	*Micropterix calthella*	57,Z	58,ZZ	Traut and Marec, 1997
Noctuidae	*Orthosia gracilis*	27,Z	28,ZZ	Traut and Mosbacher, 1968
Pieridae	*Pieris brassicae*	30,WZ	30,ZZ	Traut and Marec, 1997
Psychidae	*Luffia lapidella*	61,Z	62,ZZ	Narbel-Hofstetter, 1961
	Solenobia triquetrella[b]	61,Z	62,ZZ	Seiler, 1959
	Talaeporia tubulosa	59,Z	60,ZZ	Seiler, 1920
Pyralidae	*Cadra cautella*	60,WZ	60,ZZ	Vítková et al., 2007
	Ectomyelois ceratoniae	62,WZ	62,ZZ	Mediouni et al., 2004
	Ephestia kuehniella	60,WZ	60,ZZ	Traut and Rathjens, 1973
	Galleria mellonella	60,WZ	60,ZZ	Traut et al., 1999
	Witlesia murana	59,W_1W_2Z	58,ZZ	Suomalainen, 1969
	Plodia interpunctella	60,WZ	60,ZZ	Vítková et al., 2007
Saturniidae	*Antheraea yamamai*	62,WZ	62,ZZ	Yoshido et al., 2005b
	Caligula japonica	61,Z	62,ZZ	Yoshido et al., 2006
	Samia cynthia[c]	27,Z	28,ZZ	Yoshido et al., 2005b
	Samia cynthia[c]	26,WZ	26,ZZ	Yoshido et al., 2005b
	Samia cynthia[c]	25,WZ_1Z_2	26,$Z_1Z_1Z_2Z_2$	Yoshido et al., 2005b
	Saturnia pyri	60,WZ	60,ZZ	Traut and Marec, 1997
Sphingidae	*Mimas tiliae*	58,WZ	58,ZZ	Traut and Marec, 1997
Tischeriidae	*Tischeria ekebladella*	46,WZ	46,ZZ	Lukhtanov, 2000
Tortricidae	*Bactra lacteana*	59,W_1W_2Z	58,ZZ	Suomalainen, 1969
	Cydia pomonella	56,WZ	56,ZZ	Fuková et al., 2005
Yponomeutidae	*Yponomeuta cagnagellus*	61,WZ_1Z_2	62,$Z_1Z_1Z_2Z_2$	Nilsson et al., 1988
	Yponomeuta rorellus	61,WZ_1Z_2	62,$Z_1Z_1Z_2Z_2$	Nilsson et al., 1988
	Yponomeuta gigas	61,WZ_1Z_2	62,$Z_1Z_1Z_2Z_2$	Nilsson et al., 1988
	Yponomeuta evonymellus	61,WZ_1Z_2	62,$Z_1Z_1Z_2Z_2$	Nilsson et al., 1988
	Yponomeuta malinellus	61,WZ_1Z_2	62,$Z_1Z_1Z_2Z_2$	Nilsson et al., 1988
	Yponomeuta padellus	61,WZ_1Z_2	62,$Z_1Z_1Z_2Z_2$	Nilsson et al., 1988

[a] Total number, sex chromosomes.
[b] One of several chromosome races described.
[c] Chromosomal races from different localities.

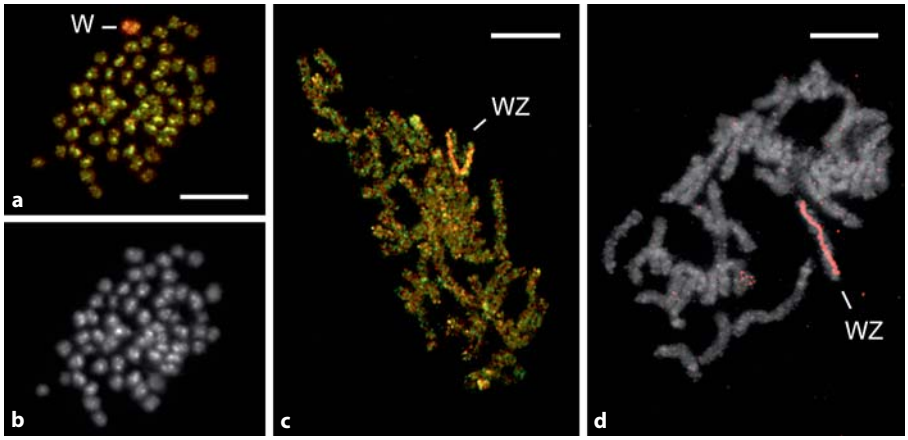

Fig. 1. W chromosome and WZ bivalents of *Ephestia kuehniella*. Mitotic (**a**, **b**) and pachytene (**c**, **d**) complements of females. **a** W chromosome highlighted by CGH, total genomic female DNA (red) competes with total genomic male DNA (green); **b** chromosomes stained with DAPI; **c** W chromosome in the WZ bivalent of a pachytene chromosome complement highlighted by CGH; **d** painted W chromosome in the WZ bivalent, the painting probe was derived from microdissected W chromatin (courtesy of Magda Vitková, České Budéjovice). Bar: 10 μm.

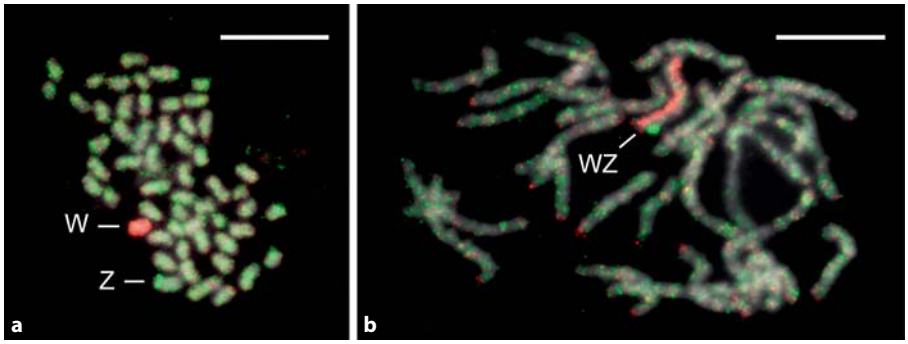

Fig. 2. W and Z chromosomes of *Bombyx mori*. Mitotic (**a**) and pachytene (**b**) complement, the W painted by FISH with a single W-derived BAC (red, BAC 19L6H), the Z labeled with a Z-derived BAC (green, BAC 9A5H), chromosomes stained with DAPI (courtesy of Atsuo Yoshido, Sapporo). Bar: 10 μm.

consistent with not having crossovers. In early prophase I, one can see the conventional sequence of leptotene, zygotene and pachytene but no chiasmata are formed. Hence no diplotene or diakinesis stage can be recognized. A 'modified pachytene' [Rasmussen, 1976] follows instead. The pachytene conformation of paired homologues is conserved while the bivalents become shorter and thicker until metaphase I. At metaphase I, therefore, the homologues are not held together by chiasmata but so-called 'elimination chromatin' can be seen between the paired homologues [Seiler, 1914]. This substance replaces the synaptonemal complex (SC) [Rasmussen, 1977]. It stains dark like chromatin with basic dyes but not with DNA-specific dyes. In fact it is ribonucleoprotein not chromatin [Ris and Kleinfeld, 1952]. The elimination chromatin stays in the metaphase plane when at anaphase I the homologues move to opposite poles [Seiler, 1914; Sorsa and Suomalainen, 1975; Wolf and Joshi, 1996].

Pachytene bivalents consist of long chromosomes. They display a chromomere pattern which allows chromosome mapping in a limited way. Some but not all chromosomes can be identified by the chromomere pattern alone [Traut, 1976; Schulz and Traut, 1979; Traut and Clarke, 1996]. The WZ bivalent of many species is recognized at this stage by the asymmetric pattern of the paired chromosomes. While Z chromosomes have a conventional chromomere-interchromomere pattern, W chromosomes often exhibit a uniformly and densely stained strand, presumed to be heterochromatic (fig. 3a) [Traut and Rathjens, 1973; Traut and Marec, 1997]. In electron microscopy of microspread SC preparations, the heterochromatin presents itself as electron-dense tangled chromatin decorating and marking the W chromosomal lateral element of the WZ bivalent (fig. 3b) [Weith and Traut, 1980; Marec and Traut, 1994]. In a *Bombyx mori* strain with a W to which a large segment of chromosome 2 was translocated, the WZ bivalent was identified as an asymmetric bivalent in pachytene [Kawamura and Niino, 1991]. In many species, the W chromosome can be exposed as a highlighted strand in the WZ bivalent when labeled with comparative genomic hybridisation (CGH) (fig. 1c), genomic in situ hybridisation (GISH) or chromosome painting (fig. 1d), even when it is not recognizable from the chromomere pattern [Traut and Marec, 1997; Traut et al.,

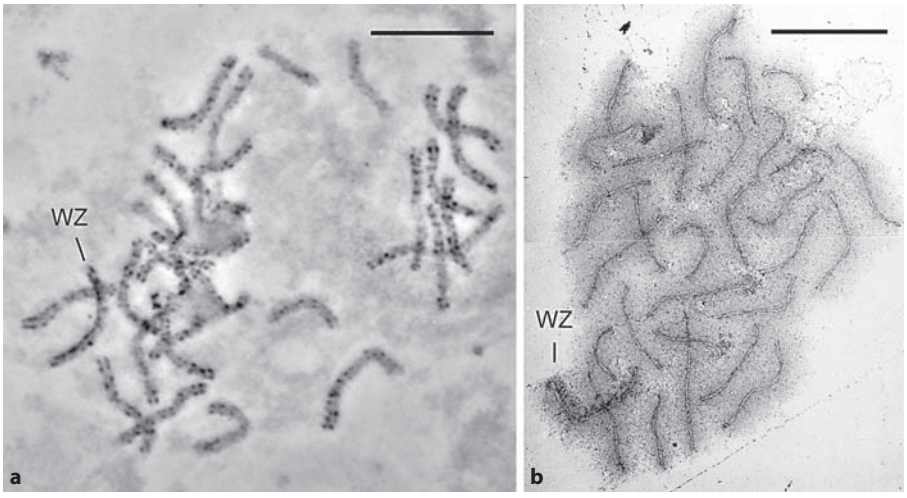

Fig. 3. WZ bivalents of *Ephestia kuehniella*. Pachytene complements of females, orcein-stained and with phase contrast optics (**a**), microspread and with electron microscopy (**b**). Bar: 10 μm. From Traut et al., 1986 with permission of the publisher.

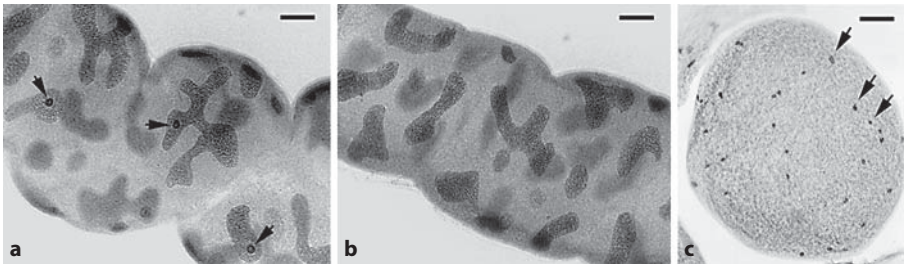

Fig. 4. W chromatin (arrows) in highly polyploid cells of *Ephestia kuehniella*. **a** Malpighian tubule of a female with branched nuclei and one heterochomatin body (arrows) per cell; **b** in male cells there is no heterchromatin body; **c** nurse cell nucleus with multiple heterochromatin bodies (arrows). Bar: 20 μm.

1999; Mediouni et al., 2004; Fuková et al., 2005, 2007; Yoshido et al., 2005a, 2006; Vítková et al., 2007]. Even some single W-derived bacterial artificial chromosomes (BACs), when used as probes in FISH (BAC-FISH), marked the full length of the W chromosome in *B. mori* (fig. 2) [Sahara et al., 2003a]. A breakthrough in chromosome identification was achieved when BAC-FISH cocktails were applied to pachytene chromosome complements. All 28 bivalents of the silkworm karyotype could be identified and associated with the respective linkage groups when 62 selected BACs (0–4 for each bivalent) were hybridized to the pachytene complements of the silkworm [Yoshido et al., 2005b].

Electron microscopy of SC spreads from *E. kuehniella* revealed the pairing process of the WZ bivalent. Pairing of the WZ is delayed with respect to the autosomes. It proceeds in 4 stages: (1) initiation at one end only, (2) partial synapsis of the differently sized W and Z chromosomes, (3) full synapsis achieved by multiple twisting and (4) finally synaptic adjustment to compensate for the length difference [Weith and Traut, 1986; Marec and Traut, 1994].

In all species, in which the WZ was identified in meiosis, full length synapsis between W and Z was observed by light microscopy. Pairing of W and Z in pachytene in spite of extreme sequence divergence poses a conceptual problem. It is generally assumed that homologues recognize each other and finally synapse because of their sequence identity. How do W and Z find each other? One possibility is that W and Z pair because there is no other partner for either of them. Another possibility is the presence of hypothetic recognition sequences – possibly only at one end – which are conserved in the W chromosome in spite of extensive degeneration of most other sequences.

Full-length synapsis of the non-homologous sex chromosomes poses no genetic problem, however. Chromosome integrity is not at risk. The sex chromosomes remain intact since there is no intrachromosomal recombination in female meiosis. Even when two W chromosomes are paired to one Z chromosome in W_1W_2Z/ZZ systems, sex chromosome separation appears to be precise [Seiler, 1914]. Sex chromosome multivalents behave like a single Mendelian factor in meiosis.

Sex Chromatin in Interphase Nuclei

Females but not males of the majority of moth and butterfly species display a heterochromatic body in interphase nuclei [reviewed in Traut and Marec, 1996]. Thus, like mammals, Lepidoptera possess sex chromatin. The source, however, is different in the two taxa. Although present in females of mammals as well as those of Lepidoptera, sex chromatin of Lepidoptera occurs in the heterogametic sex and, therefore, is neither a heterochromatic X or Z chromosome nor associated with dosage compensation. There is ample evidence that the sex chromatin body in Lepidoptera is W chromatin, i.e. derived from the W chromosome. It is absent in species with Z0 sex chromosome systems. W mutants of *Ephestia* display mutant forms of sex chromatin [Rathjens, 1974; Traut et al., 1986; Marec and Traut, 1994]. CGH highlights W chromosomes and sex chromatin alike, and finally, a chromosome painting probe made from microdissected W chromatin paints the W chromosome [Fuková et al., 2007; Vítková et al., 2007].

The general properties of W chromatin are not different from those of most other types of heterochromatin. It is conspicuous in light microscopy because it is considerably denser than the surrounding euchromatin. The higher DNA concentration can be recognised in Feulgen-stained preparations even without DNA cytophotometry. Replication is out of phase with the rest of the chromatin as seen by ^{3}H-thymidine pulse labelling and subsequent autoradiography. Autoradiographic studies of ^{3}H-uridine incorporation show that W chromatin is transcriptionally inactive or at least less active than euchromatin in somatic cells [Traut and Scholz, 1978].

Polyploid cells abound in larvae and adults. In most female tissues, only one W chromatin body per cell is present. Its size increases with increasing degree of polyploidy of the nucleus. The sex chromatin, therefore, is highly conspicuous in these tissues where it forms a single large ball-shaped body (fig. 4a) which is absent in male tissues (fig. 4b). Cytophotometry of the DNA showed proportionate growth of the sex chromatin with that of the nucleus, in a range where measurements were feasible, between 8C and 1000C [Traut and Scholz, 1978]. As the degree of polyploidy attains several thousand C in Malpighian tubules of e.g. *Ephestia kuehniella* [W. Traut, unpublished] and at least 4×10^{5}C in the silk gland of *Bombyx mori* [Gage, 1974], a single sex chromatin body is composed of an enormous number of W chromosomes.

Not all tissues follow these rules. The follicle and nurse cells in *Ephestia* have multiple sex chromatin bodies (fig. 4c) [Guelin, 1968; Mosbacher and Traut, 1968]. Their number increases with the size of the nuclei [Traut and Marec, 1996]. Why the sex chromatin is not sticking together during polyploidisation in these cells, is not clear. But it is tempting to assume that transcriptional activity of the W chromosome in these female-specific tissues is the cause. In contrast to somatic tissues, W chromatin in nurse cells is transcriptionally active during previtellogenesis as shown by EM-autoradiography after ^{3}H-uridine incorporation [Guelin, 1994].

Some Lepidoptera species, e.g. *Orgyia antiqua,* have W chromosomes with a clear euchromatic, autosome-like part besides a heterochromatic segment [Traut and Marec, 1997; Traut et al., 2001; Yoshido et al., 2005a]. They display multiple sex chromatin bodies in all polyploid tissues. The effect can be mimicked by W chromosome mutations in *Ephestia.* When the W chromosome is fused with an autosome, a part of the W chromosome translocated to an autosome, or a part of the Z to the W chromosome, multiple small heterochromatin bodies are displayed even in cells where in the wild-type only a single sex chromatin body is visible [Rathjens, 1974; Traut et al., 1986; Marec and Traut, 1994]. Whether the effect is due to some inherent opposing properties of heterochromatin (stickiness) and euchromatin (disjunction) of the mutant chromosome or simply to the transcriptional activity of the euchromatic part cannot be safely decided.

Composition of the Sex Chromosomes

The first moth genome sequenced was that of the tobacco budworm, *Heliothis virescens,* but the data are proprietary, not publicly available and, therefore, of no use for the scientific community. In contrast, the silkworm *(Bombyx mori)* genome sequenced independently by a Chinese and a Japanese group, is publicly available [Mita et al., 2004; Xia et al., 2004]. In both groups, male genomes were sequenced to avoid the complications expected from the W chromosome. Hence, the two assemblies include contigs of the Z chromosome but not of the W chromosome.

The Z chromosomal sequence contigs in *B. mori* display no obvious general difference in composition between autosomes and Z chromosomes. Conventional genetic maps of the Z have been established not only in the silkworm but also in the European corn borer moth, *Ostrinia nubilalis* [Dopman et al., 2004] and in the butterflies *Heliconius melpomene* [Jiggins et al., 2005] and *Heliconius erato* [Tobler et al., 2005]. Yasukochi et al. [2006]

found that the genes *Tpi* (triose phosphate isomerase) and *apterous* were located in the Z chromosome of *B. mori* and a very distantly related species, the nymphalid butterfly *Heliconius melpomene. Tpi* maps to the Z chromosome also in the tiger swallowtail butterfly *Papilio glaucus* [Scriber et al., 1996] and the crambid moth *Ostrinia nubilalis* [Dopman et al., 2005]. The *period* gene *(per)* maps to the Z chromosome in *B. mori* as well as in *Antheraea pernyi* [Gotter et al., 1999]. Thus there is evidence for conserved synteny in at least a part of the Z chromosome in Lepidoptera.

Z chromosomal genes appear to contribute overproportionately to the speciation process [Sperling, 1994; Naisbit et al., 2002; Dopman et al., 2005]. Pheromone traits, traits for male behavioural response to pheromone, and voltinism traits play an obvious role in reproductive barriers and are often Z-linked, more often than expected under the assumption of random distribution, given the usually high number of linkage groups in Lepidoptera.

Very few genes have been found linked to the W chromosome. The female determining factor, *Fem,* in *B. mori* is the most prominent one [Tazima, 1964]. A giant-egg trait, *Esd,* is associated with the W chromosome but its location has not been confirmed yet [Kawamura, 1988, 1990]. Results obtained from sex chromosome mutants of *Ephestia kuehniella* suggest the presence of a male-killing factor in the W chromosome [Marec et al., 2001]. In *Papilio glaucus,* the dark morph trait is transmitted maternally and, therefore, considered W-linked, but W-linkage is disrupted in some crosses, presumably due to a Z-linked suppressor [Scriber et al., 1996]. However, the lack of association between the maternally transmitted mitochondrial DNA haplotypes and the W-limited phenotype [Andolfatto et al., 2003] make W-linkage questionable. In *Antheraea pernyi,* several copies of the otherwise Z-linked *period* gene have been detected, one of them, *perW,* produces a truncated protein while another one is the source of an antisense RNA [Gotter et al., 1999].

There have been several approaches to analyse DNA composition of the W chromosome. Very general information stems from CGH in which differently labelled genomic DNAs from females and males are competing for binding sites on chromosomes. From these studies, it is obvious that interspersed repetitive sequences abound in the W chromosome of most species. In some species e.g. *Galleria mellonella,* W-specific sequences predominate. In others, e.g. *Bombyx mori,* the W chromosome has accumulated sequences equally present in females and males, presumably autosomal sequences [Traut et al., 1999, 2001; Sahara et al., 2003b; Vítková et al., 2007]. The same information could be gathered from BAC-FISH in *Bombyx.* All autosomal BACs that gave specific signals on the respective autosomes (and Z) hybridised also to scattered sites on the W [Yoshido et al., 2005b]. DNA sequences from the W, isolated as W-specific RAPDs (randomly amplified polymorphic DNA) in *B. mori* turned out to be truncated retrotransposons (reviewed in Abe et al., 2005a]. A direct approach, i.e. cloning of microdissected W sequences, has been performed in the codling moth *Cydia pomonella.* Besides a few non-coding unique W-specific sequences, non-coding and retrotransposon sequences turned up which were also present on autosomes and the Z chromosome [Fuková et al., 2007].

Sex Determination: the Primary Signal

In female-heterogametic systems, the distribution of sex chromosomes to the eggs decides upon the sex chromosome constitution of the zygote and subsequently sexual development of the embryo. ZZ embryos develop into males, WZ embryos (or Z0 embryos in Z/ZZ systems) into females. Since only 1 of the 4 nuclei that result from female meiosis, is becoming the female pronucleus (the egg nucleus), the orientation of the WZ bivalent or the position of the Z univalent, respectively, in the metaphase I spindle determines the sex chromosome constitution of the egg.

In a classical study, Seiler [1920] showed that the Z univalent behaves as a laggard in anaphase I spindles of the psychid moth *Talaeporia tubulosa,* a species with a Z/ZZ sex chromosome mechanism. The position in the spindle, near to the inner or to the outer pole, is influenced by the ambient temperature. At high temperatures (30–37°C), the Z is more often found near the inner pole, thus more Z eggs are produced. At low temperatures (3–8.5°C) it is more often seen moving to the outer pole and, therefore, more nullo-Z eggs are generated. Consequently, the sex ratio among offspring is temperature-dependent.

Although the female-heterogametic system has been conserved in all species so far investigated, the role of sex chromosomes in sex determination has not. WZ systems are not different from XY sex chromosome systems in this respect. Theoretically, the primary sex determining signal in a WZ/ZZ system may be generated by (1) female determining gene(s) on the W chromosome while male development is the default; thus presence of the W causes female development, absence of the W male development; or (2) male promoting gene(s) on the Z acting against female promoting gene(s) located on autosomes

Table 2. Sexual development of polyploids and aneuploids in *Bombyx mori* [data from Kawamura, 1988; Sahara et al., 1997; Tazima, 1964]

Ploidy	Female	Male
2n	WZ AA	ZZ AA Z AA
3n	WZZ AAA WWZ AAA WWZZ AAA	ZZZ AAA ZZ AAA
4n	WZZZ AAAA WWZZ AAAA	ZZZZ AAAA

or a female promoting maternal effect (which may be coded for by a gene on any chromosome including the W). This, in effect, is then a Z-counting mechanism: a single Z chromosome causes female development, 2 Z chromosomes male development. Although we have no precise knowledge of the genes involved in generating the primary sex determining signal, both the 'dominant W' and the 'recessive Z' systems do occur in Lepidoptera.

Thus it cannot be decided a priori whether sex determination in a WZ/ZZ species depends on a 'dominant W' or a Z-counting mechanism. The action of a Z-counting mechanism was nicely shown in the psychid moth *Solenobia triquetrella* when tetraploid females of a parthenogenetic race were crossed with diploid males of a bisexual race. Triploid intersexes with 2 Z chromosomes, WZZAAA, resulted [Seiler, 1949]. All Z/ZZ species necessarily have a Z-counting mechanism. But again, it cannot be decided whether the male-promoting activity of the Z is counteracted by a female-promoting maternal effect or a female-promoting activity encoded by zygotic autosomal genes.

B. mori, on the other hand, represents the 'dominant W' system. The W chromosome carries a female-determining factor, *Fem*. This has been inferred from polyploids and aneuploids (table 2). Whenever a W chromosome is present, a female develops from the embryo. In its absence a male individual develops [Tazima, 1964]. A couple of deletions and translocations of W chromosomal segments in *B. mori* are known that do not include *Fem*. The female-determining function, hence, maps to the remaining segments of the W [Abe et al., 2005b], but the precise location of *Fem* is not yet known nor has *Fem* been isolated to reveal its molecular structure.

Extensive hybridisation experiments between Japanese and European races of the gypsy moth, *Lymantria dispar*, have been performed in the second and third decade of the past century by Richard Goldschmidt (compiled and reviewed in Goldschmidt, 1934]. Basically, European females crossed with Japanese males produced WZ intersexes and ZZ males (F1 hybrid intersexes). The reciprocal crosses, Japanese females crossed with European males, and their daughters backcrossed to European males, produced WZ females and ZZ intersexes (F2R hybrid intersexes). The results were interpreted as showing the effect of strong female- and male-promoting genes in the Japanese but weak female- and male-promoting genes in the European races. Female- and male-promoting activities are well balanced within each race, while inter-race crosses produce the imbalances seen in the development of intersexes. The Z chromosomes carry the male-promoting genes, but whether they act against a zygotic or a maternal female-promoting effect cannot safely be decided.

Sex Determination: the Sex-determining Pathway

In *Drosophila*, the primary sex-determining signal, briefly called the X:A ratio (ratio of X chromosomes to autosome sets), initiates a cascade of gene actions which ultimately regulate the development of sex-specific somatic characters, sex-specific behaviour, and dosage compensation of X-chromosomal genes [for a review see Cline and Meyer, 1996]. The primary signal is in fact an X chromosome-counting device, based on the interaction of a couple of transcription factors. They are encoded by 3 classes of genes: X-linked numerator genes, an autosomal denominator gene, and maternal genes. The primary signal regulates the early expression of *Sxl* which encodes a splice regulator. Sxl regulates its own expression in an auto-feedback cycle and in addition that of *tra*, the next downstream step in the cascade. Tra is again a splice factor which together with Tra2 regulates the splicing of the *dsx* transcript into a female- or male-specific form. Finally, the 2 isoforms Dsx^F and Dsx^M are transcription factors which regulate the expression of genes for somatic sex-specific functions. This well-investigated sex-determining pathway of *Drosophila* had once been considered a paradigm of insect sex determination. But during the last 2 decades, it has become evident that only parts of the cascade are conserved in non-drosophilid insects.

Among Lepidoptera, only *B. mori* has been investigated with respect to sex-determining cascade genes. The orthologue of *Drosophila*'s *Sxl* gene is conserved in *Bombyx,* but the function is not. The *Sxl* transcript is not sex-specifically spliced and, hence, probably not involved in

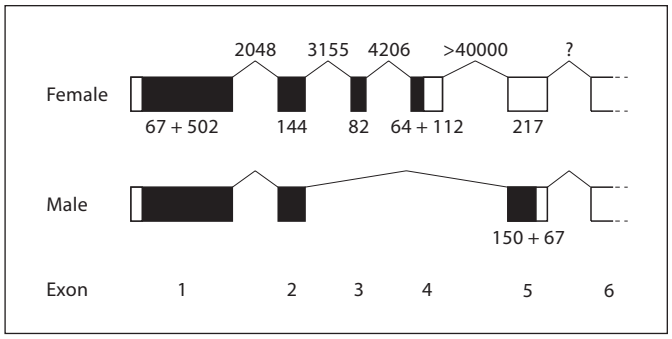

Fig. 5. Sex-specific splicing of the *Bombyx mori doublesex (Bmdsx)* transcript. Exons 3 and 4 are skipped in the male-specific variant. The coding sequence (black boxes) of the female-specific variant ends in exon 4, that of the male-specific variant in exon 5. Sequence data from Acc. No. AB052774 [Ohbayashi et al., 2001; Suzuki et al., 2001].

sex determination [Niimi et al., 2006; Traut et al., 2006]. It is unknown what its function in *B. mori* is. Of the next lower level, *tra-tra2*, only *tra2* has an identified orthologue in *Bombyx* [Niu et al., 2005]. The orthologue of *Drosophila*'s *tra* gene, however, could not be identified yet. This is presumably due to its rapid sequence evolution which had previously been seen even among *Drosophila* species [McAllister and McVean, 2000]. Thus we do not know whether this level of the cascade is functionally conserved. Presumably it is not since the *dsx* transcript of the next lower level of the cascade lacks the characteristic binding motif for Tra-Tra2. Finally, the *dsx* gene at the bottom of the cascade is conserved, structurally as well as functionally. The transcript of *B. mori dsx, Bmdsx*, is sex-specifically spliced into female- and male-specific forms of mRNA (fig. 5). The regulation of splicing, though, differs between *Drosophila* and *Bombyx dsx*. Besides absence of the Tra-Tra2 binding motif, the female-specific 3′ splice site is not weak, and the female form – not the male form – is the default splice variant in *Bombyx,* as shown in in-vitro splicing reactions [Ohbayashi et al., 2001; Suzuki et al., 2001].

Thus at least, the last step of the cascade is conserved in Lepidoptera, as it is in all other insect species investigated so far. The higher levels of the cascade, however, are not shared by all insects. The *Sxl* level is part of the sex-determining cascade only in *Drosophila;* all other species investigated do not use *Sxl* in the sex determining pathway. Particularly the primary sex determination signal is subject to changes. Among flies, epistatic maleness and maternal effect systems exist besides *Drosophila*'s X-counting system. In Lepidoptera, the primary signal has changed even under the cover of a conserved female-heterogametic sex chromosome system. Sex determination is evidently a rapidly evolving system in the upper levels of the cascade and especially so with respect to the primary signal. In the nematode *Caenorhabditis* the primary signal can be altered readily, each of 7 autosomal genes can be made by mutation to adopt the role of the primary sex-determining signal [Hodgkin, 2002].

Dosage Compensation

The double dose of Z chromosomal genes in males and the single dose in females may create a problem if correct gene expression is dosage dependent. Therefore it is interesting to see whether there is a dosage compensation mechanism in Lepidoptera, similar to those in mammals, *Drosophila* or *Caenorhabditis*.

In two *Heliconius* species, *H. melpomene* and *H. elato,* the activity of the Z-linked enzyme 6-phosphogluconate dehydrogenase was twice as high in male as in female tissues [Johnson and Turner, 1979]. Two Z-linked genes, *Bm kettin* and *T15,* were tested on the transcriptional level in *Bombyx mori.* Each of the two genes produced about twice as much transcripts in males as in females [Suzuki et al., 1998, 1999]. Similarly, in another Z-linked gene, *period,* in *Antheraea perny*i, neither the amount of transcript nor that of protein was dosage compensated [Gotter et al., 1999]. Thus, experimental evidence shows that there is no dosage compensation of sex-linked genes in Lepidoptera. The lepidopteran Z appears to contain no genes whose correct functioning is dosage dependent, or else, if it is, the function has to be sex-specific.

Intersexes

The development of sexual intergrades has attracted considerable research in the first half of the past century. Much of the work in this field has been done on two moth species in which intersexes can be easily generated by crossing certain races, the gypsy moth *Lymantria dispar,* and the psychid *Solenobia triquetrella* (see Sex determination: the Primary Signal). The generalizing statements 'Intersexes are mosaics in time' and 'Intersexes are mosaics in space' mark the opposing theories of Richard Goldschmidt and Jakob Seiler, respectively. Goldschmidt based his theory on the investigation of *Lymantria* intersexes where he assumed that intersexes start with either

female or male sexual development and at a 'turning point' switch to sexual development of the opposite sex. Organs or parts of organs that developed before the turning point thus were of one sex while those that developed later were of the opposite sex [Goldschmidt, 1934]. Seiler, instead, based his theory of 'intersexes are mosaics in space' on the investigation of *Solenobia* intersexes, where he found clear indications that there is no time sequence in the patchy distribution of purely female and male areas [Seiler, 1949, 1965].

Mosbacher [1973] reinvestigated *Lymantria* intersexes and found that this was also true for ZZ intersexes of *Lymantria*. The WZ intersexes, however, displayed intermediary sexual characters even in single cell structures like sensilla of antennae and wing scales [Mosbacher, 1975; Mosbacher and Scheffler, 1975]. This is incompatible with either of the two theories. The explanation, why in one case purely female and purely male patches arise and in others even single cells show intermediate characters, is not known. An investigation on the molecular level with a focus on the expression of sex-determining cascade genes in intersexes has not yet been performed.

Wolbachia

Bacteria of the genus *Wolbachia* interfere with the reproductive system of their arthropod hosts in various ways to increase their own transmission. Basically 4 types of interference can be distinguished: (1) cytoplasmic incompatibility, reduced viability of offspring from noninfected mothers and infected fathers, (2) thelytoky, the generation of females from haploid eggs in Hymenoptera, (3) feminisation of genetic males, and (4) killing of males at early developmental stages. Among Lepidoptera, early male killing has been found, e.g. in the butterfly *Hypolimnas bolina* [Dyson et al., 2002] and the crambid moths *Ostrinia furnacalis* and *O. scapulalis* [Kageyama and Traut, 2004; Sakamoto et al., 2007]. Feminisation of genetic males has been reported for the pierid butterfly *Eurema hecabe* [Hiroki et al., 2002]. The parasite obviously interferes with the sex determining pathway in this insect. But even in early male killing interference with the sex determining cascade is involved. Partial curing from *Wolbachia* or incomplete infection resulted in sexual mosaics of purely ZZ individuals [Kageyama and Traut, 2004]. But it has not been investigated yet at which stage of the sex determining pathway of the lepidopteran hosts *Wolbachia* interferes with the normal male-determining sequence. In *Drosophila*, Starr and Cline [2002] showed that *Wolbachia* interacts with *Sex-lethal*, the key regulator in *Drosophila*'s sex determining cascade that is not present in Lepidoptera.

Theoretically, a population that is invaded by *Wolbachia* should die out. Owing to the parasite's strategy, the infection spreads and the male ratio drops until there are not sufficient males to sustain the population. The actual fate, however, can be different. Charlat et al. [2007] investigated two island populations of the butterfly *Hypolimnas bolinae*. Due to male-killing by *Wolbachia*, males were very rare in 2001. Only 10 generations later, in 2005, the populations had recovered, males were common. Both females and males were found infected by the same *Wolbachia* strain as in 2001, but the hosts had acquired tolerance against the parasite.

Evolution of the Lepidopteran Sex Chromosome System

Lepidoptera share female heterogamety with the closely related Trichoptera (caddis flies). Hence, female heterogamety has arisen in the common ancestor of the two orders, which together form the monophyletic group of Amphiesmenoptera. The earliest certain trichopteran and lepidopteran fossiles date from 180–185 and 190 mya, respectively [reviewed in Kristensen and Skalski, 1999; Grimaldi and Engel, 2005]. Female heterogamety in this group, thus, exists for more than 190 myr. All caddis flies investigated so far have a Z/ZZ sex chromosome system and do not display sex chromatin [Klingstedt, 1931; Marec and Novák, 1998; Lukhtanov, 2000]. This is not different from the situation in basic lineages of Lepidoptera. Therefore, it can be assumed that the lepidopteran W chromosome came into being after Lepidoptera had split from the common phylogenetic tree. A larger data set on the distribution of the W chromatin among different families of Lepidoptera indicates that the W chromosome came into being at the common root of Ditrysia and Tischeriina, together containing 98% of the extant Lepidoptera species (fig. 6) [Lukhtanov, 2000; Traut and Marec, 1996]. The earliest evidence for a Ditrysian species is from 97 mya [reviewed in Kristensen and Skalski, 1999]. Thus the female heterogametic system already had a long history before the lepidopteran W arose. As the simplest hypothesis to account for the change from a Z/ZZ to a WZ/ZZ sex chromosome constitution one may assume a fusion between an autosome and the original Z chromosome, giving rise to a neo-W neo-Z sex chromosome constitution that is in fact our present day WZ pair of the majority of

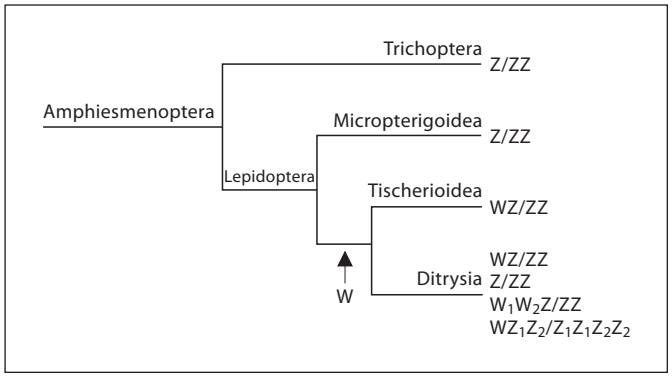

Fig. 6. Phylogeny of the lepidopteran sex chromosome system. Data from table 2, cladogram based on Kristensen and Skalski [1999].

Lepidoptera species [Traut and Marec, 1996]. But other chromosome rearrangements cannot be excluded. Lukhtanov [2000] favours the hypothesis of a B chromosome having adopted the role of a W chromosome.

Chromosome losses and fusion events have evidently contributed to the evolution of the sex chromosomes in more recent times. Sporadic losses of the W chromosome have turned the sex chromosome system formally back to a Z/ZZ system (see table 1), though of course the Z chromosome of the more advanced Lepidoptera may have only partial homology to the ancestral Z, which is common to both, Trichoptera and basic Lepidoptera.

Evidence for sex chromosome fusion has been found by Seiler [1925]. He detected different chromosomal races of the arctiid moth *Phragmatobia fuliginosa*, one with 2n = 58 chromosomes, another one with 2n = 56 chromosomes, and a third race with 2n = 57 in females and 2n = 56 in males. Seiler concluded, the 2n = 58 race possessed a free autosome that had fused with the Z chromosome in the 2n = 57/56 race (W_1W_2/Z females, ZZ males) and with both sex chromosomes in the 2n = 56 race. A re-investigation of the 2n = 57/56 race at pachytene confirmed Seiler's conclusion: the pachytene complement included a trivalent with two W chromosomes, one partly heterochromatic, paired to the single Z chromosome [Traut and Marec, 1997]. A similar event has been deduced in the lymantriid moth *Orgyia thyellina* [Yoshido et al., 2005a]. In *Orgyia antiqua,* traces of past fusion events can be detected. About one third of the W chromosome is heterochromatic and highlighted by CGH and GISH, the remaining two thirds are euchromatic and stain like autosomes [Traut and Marec, 1997; Traut et al., 2001; Yoshido et al., 2005a] and there is evidence of fusion events from meiotic synapsis in the hybrid between *O. antiqua* and *O. thyellina* [Traut and Clarke, 1997]. Fusion events in the saturniid moth *Samia cynthia* have led to Z/ZZ, WZ/ZZ, and $WZ_1Z_2/Z_1Z_1Z_2Z_2$ sex chromosome systems in different subspecies [Yoshido et al., 2005a].

Molecular Evolution of the Sex Chromosomes

It is generally agreed that the evolution of a differential pair of sex chromosomes starts from a pair of homologous chromosomes and requires 2 conditions: (1) possession or acquisition of the sex determining function, i.e. a dominating role in the generation of the primary sex determining signal, and (2) establishment and extension of a non-recombining region. The consequences then are molecular and morphological differentiation between the originally homologous proto-X and proto-Y chromosomes. The evolution is most dramatic in the sex-specific sex chromosome, the Y chromosome in XY/XX systems and the W in WZ/ZZ systems. Decay of genes and accumulation of repetitive sequences like transposons is facilitated in these chromosomes by constant heterozygosity and cannot be repaired or cleaned by recombination. Several mechanisms have been described that act under these conditions and promote the process: Muller's ratchet [Muller, 1964; Charlesworth, 1978], selective sweeps by genetic hitchhiking [Rice, 1987], and background selection [Charlesworth, 1994].

Considering those preconditions, the female-heterogametic sex chromosome system (see previous chapter) as well as suppression of crossover in female meiosis [Suomalainen, 1966], had already been established when Lepidoptera branched off from the common ancestral Trichoptera-Lepidoptera lineage. Both, Trichoptera and basic Lepidoptera, possess the Z/ZZ (Z0) sex chromosome system, hence, no trace is left in these groups of the hypothetical proto-W. But even in the new W chromosome that arose in the Lepidoptera lineage, genetic erosion has advanced rather far, as the few genes known from the W chromosomes and the massive invasion of repetitive elements can tell. Even the sex determining function seen in *Bombyx (Fem)* is certainly absent in species with Z/ZZ sex chromosome systems that have secondarily lost the W chromosome. How much of that W chromosome has been conserved in present-day W chromosomes besides the telomeric sequences is unknown [Yoshido et al., 2005a]. Even among related species, the change in W chromosome sequence composition is considerable, as a comparative FISH study among 4 pyralid

moths has shown [Vítková et al., 2007]. So it may be impossible to trace the W chromosome back to its origin, from its present-day sequence composition.

The proto-Z chromosome, however, may have been conserved throughout Trichoptera and Lepidoptera even though fusion events with autosomes have taken place. As shown above (see chapter: Sequence Composition), there is indeed evidence for conserved synteny of Z chromosomal genes in Lepidoptera. This indicates the stability of the sex chromosome system in Lepidoptera. The sex chromosome pair has not been substituted by another pair of chromosomes in the species considered. But whether the syntenic genes belong to the ancestral Z chromosome or to a later acquisition, e.g. to the presumed addition of an autosome to the Z in connection with the birth of the lepidopteran W chromosome, has yet to be determined.

Applications

Presence or absence of sex chromatin presents an excellent means of recognizing the genetic sex of an embryo or young larva, long before any morphological marker becomes visible. As there are polyploid tissues from a very early stage of development onwards, simple squash preparations are sufficient for this purpose. From older larvae, Malpighian tubules provide an excellent material to determine presence or absence of W chromatin easily. This has been used as a marker for radiation-induced sex chromosome aberrations [Rathjens, 1974; Makee and Tafesh, 2006], in studies on intersexes of *Lymantria dispar* [Mosbacher, 1973] and especially the influence of *Wolbachia* on sex determination and sex-specific lethality of their hosts [Hiroki et al., 2002; Kageyama and Traut, 2004].

In the silkworm *B. mori,* there has been a longstanding interest to develop genetic strains which enable sericulture facilities to discriminate the sex of larvae or even embryos and eliminate females. This is because males produce about 20% more silk than females, and, hence, mass rearing of male progeny has significant economical benefit [Tazima, 1964; Strunnikov, 1987]. The first genetic sexing strains were based on the translocation of autosomal genes for visible traits such as egg colour, larval marking or cocoon colour to the W chromosome [reviewed in Nagaraju, 1996]. This left breeders still with the laborious elimination of females by phenotype. A more sophisticated sexing mechanism was proposed by Strunnikov [1975]. This requires the construction of balanced lethal (BL) strains. Males of such strains are trans-heterozygous for 2 non-allelic, Z-linked recessive lethal mutations, preferably expressed during embryogenesis. Females carry a T(W;Z) translocation, which includes wild-type alleles of both lethal loci, protecting the females from the expression of either lethal allele. Sexing is achieved when BL males are out-crossed to wild-type females (fig. 7). Such crosses produce almost exclusively male progeny, while the female progeny die because they are hemizygous for one of the lethal alleles. Silkworm BL strains, either exactly following Strunnikov's proposal or made according to a modified scheme, were constructed in the former

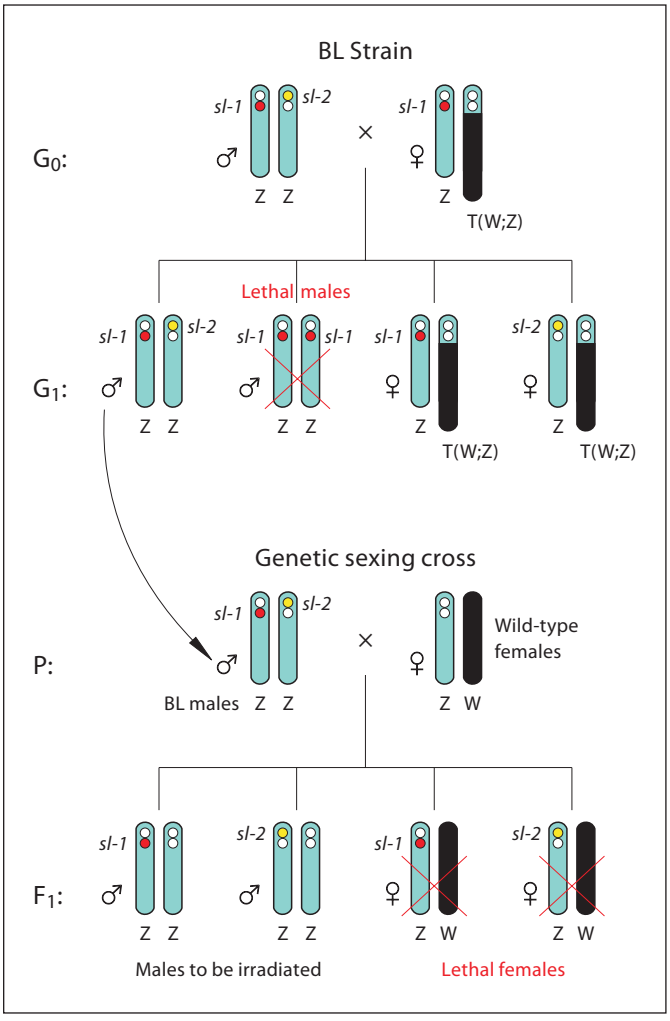

Fig. 7. Scheme of genetic sexing using a balanced lethal (BL) strain suggested by Strunnikov [1975]. A segment of the Z chromosome including wild-type alleles of genes *sl-1* and *sl-2* has been translocated to the W resulting in a T(W;Z) chromosome. The strain is propagated with males heterozygous for lethal alleles (red, yellow) of the 2 genes in trans, and only this type of male offspring is viable. When outcrossed to wild-type females, these males produce male-only progeny [modified from Marec et al., 2005].

Soviet Union [reviewed in Strunnikov, 1987] and in Japan [Ohnuma and Tazima, 1983; Ohnuma, 1988]. Recently, Ohnuma [2005] successfully accomplished the development of an improved BL strain, which carries not only 2 Z-linked lethal mutations and the required T(W;Z) translocation but also a sex-limited larval skin colour gene, introduced by a T(W;A) translocation. The cross-breeding scheme generates a male-only commercial 'strain' named 'Platina Boy' (in fact not a strain but a single generation) with higher yield and better quality of silk than previous attempts [reviewed by Ohnuma, 2006].

There is also an increasing need to develop genetic sexing strains in key lepidopteran pests which are the targets or candidates of the sterile insect technique (SIT) programmes. The SIT relies on the rearing and release of large numbers of genetically sterile insects into a wild population. Current SIT programmes for the management of lepidopteran pests are based on bisexual releases. Male-only releases have never been tested in the field, due to the lack of efficient ways to separate males from females or to produce only males [reviewed in Marec et al., 2005]. The flour moth *E. kuehniella* is currently the only lepidopteran pest in which genetic sexing is possible using Strunnikov's scheme [Marec, 1991]. In the BL-2 strain constructed, laboratory experiments confirmed the potential of BL-2 males to be used for genetic sexing. However, they also revealed several drawbacks, which make the Strunnikov's genetic sexing scheme hardly applicable in mass rearing [Marec et al., 1999]. Recently, a new approach for the development of genetic sexing strains in Lepidoptera has been proposed, which appears feasible under mass rearing conditions. It is based on the construction of transgenic females carrying a dominant conditional lethal gene in the W chromosome [Marec et al., 2005]. A sexing strain of this type could be propagated under permissive conditions, but would eliminate all females while allowing mass-rearing of non-transgenic males under restrictive conditions. The development of a transgenic sexing strain in the codling moth *C. pomonella* is presently underway [Marec et al., 2007].

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