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Mechanisms of molecular differentiation of sex chromosomes in Lepidoptera and their evolution

Ph.D. Thesis

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České Budějovice 2017

This thesis should be cited as:

Dalíková M (2017) Mechanisms of molecular differentiation of sex chromosomes in Lepidoptera and their evolution. Ph.D. Thesis. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, CzechRepublic, 118 pp.

Annotation

Sex chromosomes represent a unique part of the genome in many eukaryotic organisms. They differ significantly from autosomes by their evolution, specific features, and meiotic behaviour. Recent advances in the knowledge of sex chromosomes in non-model organisms have been largely enabled by modern cytogenetic methods. The present study explores several topics related to sex chromosomes in Lepidoptera, the largest group of animals with female heterogamety, using methods of molecular cytogenetics, immunocytogenetics, and molecular biology. These topics include physical mapping of chromosomes by BAC-FISH, molecular differentiation and composition of the W chromosome, differences in the evolution of the W and Z chromosome, and meiotic sex chromosome inactivation. The results obtained brought new information not only about the W and Z chromosomes in Lepidoptera, but also about the evolution and specific features of sex chromosomes in general.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 22. 5. 2017

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This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre CAS, supporting doctoral studies in the Molecular and Cell Biology and Genetics study programme.

Financial support

This work was supported by:

The Grant Agency of the AS CR, grant IAA600960925

The Czech Science Foundation, grant 521/08/H042 and 14-22765S

The Grant Agency of the University of South Bohemia, grants 137/2010/P and 067/2012/P

Acknowledgements

First of all, I would like to thank my supervisor František Marec for accepting me in his lab and giving me support and scientific freedom at the same time. I would like to thank to all my former and present colleagues from the lab of molecular cytogenetics; you are the best team ever. My special thanks belong to Anička, Irenka, Jindra, Leo, Magda, Mao, Mirka, and Sander for all the fruitful discussions, both the important and silly ones. My big thanks also belong to Maruška for the care she gives to our moths and butterflies. I would like to thank to Mirek for all the support during last hectic weeks. Last but not least, my never-ending gratitude belongs to my family – my parents and the greatest sister. Without them, none of this would have been possible.

List of papers and author's contribution

The thesis is based on the following papers:

I. Van't Hof A.E., Edmonds N., Dalíková M., Marec F., Saccheri I.J. (2011) Industrial melanism in British peppered moths has a singular and recent mutational origin. *Science* **332**: 958-960. (IF = 34.661)

I hereby declare that Martina Dalíková designed BAC-FISH experiments and performed physical mapping of the chromosome 17 in Biston betularia.

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Ilik J. Saccheri

II. Van't Hof A.E., Nguyen P., Dalíková M., Edmonds N., Marec F., Saccheri I.J. (2013) Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism. *Heredity* **110**: 283-295. (IF = 3.801)

I hereby declare that Martina Dalíková performed BAC-FISH mapping of the Biston betularia W chromosome including data analysis and contributed to the draft of the manuscript.

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Ilik J. Saccheri

III. Dalíková M., Zrzavá M., Hladová I., Nguyen P., Šonský I., Flegrová M., Kubíčková S., Voleníková A., Kawahara A. Y., Peters R. S., Marec F. (2017) New insight into evolution of W chromosome in Lepidoptera. Manuscript submitted to *Journal of Heredity*

I hereby declare that Martina Dalíková took a significant part in the experimental design, performed and analysed all qPCR tests, contributed to CGH and FISH experiments and other procedures, and significantly participated in drafting the manuscript.

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Magda Zrzavá

IV. Dalíková M., Zrzavá M., Kubíčková S., Marec M. (2017) W-enriched satellite sequence in the Indian meal moth, *Plodia interpunctella* (Lepidoptera, Pyralidae). *Chromosome Res.* doi: 10.1007/s10577-017-9558-8 (IF = 2.59)

I hereby declare that Martina Dalíková designed experiments, performed W chromosome-sequence cloning, studied the distribution of PiSAT in the genome, tested the satDNA expression, analysed and interpreted the data obtained, wrote most parts of the first draft of the manuscript, and contributed to its revision.

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Magda Zrzavá

1. Introduction

1.1. Sex chromosomes and their evolution

Sex chromosomes represent a peculiar part of the genome of many eukaryotic organisms with separate sexes. As seen from the results of comparative mapping, they have truly evolved independently in many groups of plants and animals (e.g. Takehana et al. 2008). Despite their independent origin, sex chromosomes share many common features and are a fascinating example of evolutionary convergence. They can be divided into two sex chromosome systems, either with male (XX/XY system and its variations) or female (WZ/ZZ system and its variations) heterogamety (Charlesworth 1996).

According to the generally accepted theory, sex chromosomes originated from a pair of autosomes with two sex determining loci in hermaphroditic species. The first step towards their differentiation is associated with the occurrence of a recessive male sterility mutation in one of those homologues (proto-X) and a dominant female suppressor in the other homologue (proto-Y). This leads to a high selection pressure against recombination between these two loci to prevent the production of infertile individuals. The acquisition of sexually antagonistic genes then leads to the expansion of the region with restricted recombination. As non-recombining parts of the genome are vulnerable to an accumulation of deleterious mutations (Bachtrog 2006) and are often targets for insertion of transposable elements (TEs) (Charlesworth et al. 1994), the newly emerged Y or W chromosomes are predestined to genetic erosion. These chromosomes are usually poor in functional genes, but show accumulation of pseudogenes and various repetitive elements, and are very often heterochromatinized and sometimes can even be lost. In contrast, the X and Z chromosomes retain most of the their ancestral genes and have more autosome-like character, although even these sex chromosomes acquire some unique features (Charlesworth et al. 2005; Kaiser and Bachtrog 2010). Processes like fission, fusion with autosomes, or adoption of B-chromosomes can then lead to the rise of neo-sex chromosomes on the background of already established sex chromosome systems (Carvalho and Clark 2005; Veltsos et al. 2008).

Among many taxa with chromosomal sex determination we can observe sex chromosomes of different ages and therefore at various stages of their evolution. One example of very young sex chromosomes can be papaya, where the male specific non-recombining region forms only 10 % of the Y chromosome (Liu et al. 2004). Nevertheless, even the well-differentiated human sex chromosomes contain evolutionary strata of different ages, suggesting several historical events leading to progressive reduction in recombination with the last one 30-50 million years ago (Lahn and Page 1999; Ross et al. 2005). Additionally, there is even a documented case of an ancient X chromosome turned into an autosome (Vicoso and Bachtrog 2013).

1.2. Chromosomes W and Y

The absence of recombination in sex-limited chromosomes, W and Y, makes them susceptible to accumulation of deleterious mutations. Both positive and negative selection models can explain this degeneration of W and Y chromosomes. In the absence of recombination, selection acting on one locus can interfere with the selection acting on a linked locus. The sex-specific chromosomes have greatly reduced the effective population size (N_e). As the intensity of selection is proportional to N_e, the reduction of N_e in the W and Y chromosomes can increase the rate of fixation of weakly deleterious mutations by background selection (Charlesworth 1994). Moreover, in the absence of recombination and generally low probability of reverse mutations, sex-limited chromosomes with more deleterious mutations can be fixed through process called Muller's ratchet. This involves the stochastic loss of the W or Y chromosome with a lower number of slightly harmful mutations (Charlesworth and Charlesworth 1997). These types of mutations, however, can also be fixed through association with a favourable allele by the so-called genetic hitchhiking (Rice 1987). The relative contribution of these processes to the degeneration of the W and Y sex chromosomes is still questionable (Charlesworth and Charlesworth 2000; Bachtrog 2006).

Pseudogenization (loss of functional genes) is not the only source of degeneration of the W and Y chromosomes. The crucial role in this process is also played by the repetitive genome fraction. Especially in old sex-limited chromosomes the abundance of both tandem repeats and TEs was found. TEs tend to accumulate in parts of the genome with a lower recombination frequency (Charlesworth et al. 1994). The predominance of TEs in the W and Y chromosomes can be explained by their lower elimination from regions with a low genetic activity, as their insertion here is less deleterious than into coding or regulatory regions. Another explanation is based on the reduced frequency of their removal by ectopic recombination (Charlesworth 1991). Changing the copy number of TEs is associated with the early stages of sex chromosome evolution, as their accumulation is visible even in the young Y chromosome of papaya (Na et al. 2014). The accumulated TEs represent the major force driving the evolution of sex-limited chromosomes by chromatin remodelling and heterochromatinization of respective regions including neighbouring genes (Steinemann and Steinemann 2005). Although tandem repeats are also well known for accumulation in heterochromatin (Charlesworth et al. 1994), their role in the sex chromosome evolution is less understood. They usually accumulate in later phases of degeneration (Hobza et al. 2015).

Even old and highly degenerate W and Y chromosomes usually still contain some functional genes, which would be under normal circumstances doomed to accumulate deleterious mutations and lose their function. It was found that some mechanisms have been evolved to help repairing important genes in non-recombining regions. The sequencing of human Y chromosome revealed 8

large palindromic structures containing multiple copies of few genes with extraordinary arm-to-arm nucleotide identity (up to 99.97 %) maintained by gene conversion (Skaletsky et al. 2003). Moreover, orthologues of some of these palindromes were found in Y chromosomes of chimpanzee, bonobo, and gorilla with similar arm-to-arm divergence (Rozen et al. 2003). Multiple copies of a gene homogenized by gene conversion were also found in the W chromosome of some avian species. In New World sparrows and blackbirds (Davis et al. 2010), these copies form a palindrome, whereas in chickens they are tandemly organized (Backström et al. 2005). Although amplification and conversion can prevent accumulation of deleterious mutations in non-recombining chromosomes, there are plenty of genes in the W and Y chromosomes that survive successfully outside palindromes. Thus, the main benefit of these gene multiplications can be an enhancement in adaptive evolution by increasing the potential targets and fixation rates of advantageous mutations (Betrán et al. 2012).

1.3. Chromosomes X and Z

The X and Z sex chromosomes are often characterized as autosome-like although they differ from autosomes in many aspects. On the one hand, these chromosomes spend two-thirds of their time in the homogametic sex, and therefore natural selection has more opportunities to act on mutations beneficial for the homogametic sex. On the other hand, the X and Z chromosomes are present only in one copy in the heterogametic sex, so even recessive mutations beneficial for the heterogametic sex can be quickly fixed as they are immediately available for selection. Thus, the sexually antagonistic selection results in over or under representation of sex-biased genes in X and Z chromosomes (Ellegren and Parsch 2007). This non-random genomic distribution of sex-biased genes was confirmed in most investigated species such as *Drosophila melanogaster* (Parisi et al. 2003; Sturgill et al. 2007) and chicken (Kaiser and Ellegren 2006).

Although the effect of homozygosity on fixation probability is higher in mutations beneficial for the heterogametic sex, any X- and Z- linked favourable recessive mutation is more exposed to positive natural selection. This leads to a higher rate of adaptive evolution in the X and Z chromosomes compared to autosomes. The strength of the so-called “fast-X/Z effect” varies among species. It seems that the influence of the fast-X effect is rather small in *Drosophila* (Connallon 2007; Baines et al. 2008), whereas in mammals the ratio of nonsynonymous to synonymous mutations is significantly higher in the X chromosome compared to autosomes (Torgerson and Singh 2003; Torgerson and Singh 2006). In the case of the fast-Z effect, the Z chromosome in birds and snakes undergoes faster evolution than autosomes (Mank et al. 2007; Vicoso et al. 2013). The extent of fast-X/Z effect is dependent besides other factors also on the relative N_e of the respective sex chromosome and autosomes (Vicoso and Charlesworth 2009; Mank et al. 2010b). Under random

mating the N_e of X and Z is ¾ of the autosomal N_e, however, in the case of populations with only few reproducing males, the male and female heterogamety systems differ significantly. Additionally, mechanisms influenced by N_e vary between the fast-X and fast-Z effects. In species with more reproducing females than males, the N_e of X chromosome is very similar to the N_e of autosomes, whereas the N_e of Z chromosome can drop as low as to half of the autosomal N_e (Ellegren 2009). Due to the small N_e of Z chromosome, other genetic drift may play a role in the fast-Z effect alongside positive natural selection. The genetic drift of slightly deleterious mutations may even be the main force behind the so-called "Faster-Z Evolution" in birds (Mank et al. 2010a).

The decay of genes in sex-limited chromosomes causes imbalance between the heterogametic and homogametic sexes in the gene dose of X- or Z-linked genes. In addition, the difference in the copy number of sex-linked genes can cause variation in protein level, as the heterogametic sex has half the targets for transcriptional machinery, and therefore generates less RNA. As most of the genes do not act in isolation, the differences in gene dose on the X and Z chromosomes could affect large proportion of the genome. Therefore, there should be a clear selection pressure to create a dosage-compensation mechanism that may evolve early after the new sex chromosomes are established, as seen in *Silene latifolia* (Muyle et al. 2012). Although complete dosage compensation was observed in some species, such as *Drosophila* (Larschan et al. 2011), it is not such a prominent feature of sex chromosomes as previously thought. The results for mammals are contradictory, and probably only dosage-sensitive genes are completely compensated (Lin et al. 2012; Pessia et al. 2012). In female heterogamety systems, recent evidence suggests incomplete dosage compensation on the Z chromosome in birds (Itoh et al. 2007; Mank and Ellegren 2009), snakes (Vicoso et al. 2013), and a trematode *Schistosoma mansoni* (Vicoso and Bachtrog 2011). However, even in birds the dosage-sensitive genes are compensated effectively (Zimmer et al. 2016).

1.4. Meiotic sex chromosome inactivation

The differentiation of sex chromosomes leads to loss of homology between them and altered behaviour in meiosis of the heterogametic sex. One of these peculiarities is meiotic sex chromosome inactivation (MSCI) during prophase I. MSCI is connected with variety of chromatin changes, and this process is best understood in mice where the key player is phosphorylated histone H2AX on Ser139 (γH2AX). This histone modification is associated with double strand breaks and meiotic crossing-over. During prophase I there are two waves of accumulation of γH2AX, the first in leptotene is more general, the second in zygotene-pachytene transition concerns only the X and Y chromosomes (Mahadevaiah et al. 2001). The later presence of γH2AX serves as a marker which is quickly followed by other histone modifications associated with transcriptional silencing

and heterochromatinization, such as histone H3 lysines 9 and 27 methylation, histone H4 deacetylation, etc. (Turner 2007). This silencing continues to the end of spermatogenesis (Namekawa et al. 2006).

Although MSCI is best known from male meiosis in eutherian mammals (Namekawa et al. 2007; Turner 2007), it was observed in other species with heterogametic males such as *Caenorhabditis elegans* (Bean et al. 2004), *Drosophila melanogaster* (Hense et al. 2007; Vibranovski 2014), and grasshopper *Eyprepocnemis plorans* (Cabrero et al. 2007). When Schoenmakers et al. (2009) reported the first case of MSCI in an organism with female heterogamety, the chicken, it seemed that this phenomenon could be universal for all differentiated sex chromosomes (Namekawa and Lee 2009). The situation is, however, more complicated as later studies refuted MSCI in chickens (Guioli et al. 2012) and showed the absence of MSCI in platypuses (Daish et al. 2015). Moreover, it seems that even in mammals some miRNA genes are able to escape MSCI (Song et al. 2009; Sosa et al. 2015).

MSCI could evolve not only to avoid potentially damaging meiotic recombination between non-homologous sex chromosomes in the heterogametic sex (McKee and Handel 1993), but also as a result of sexually antagonistic selection (Wu and Xu 2003). As briefly described at the beginning of chapter 1.3., the X and Z chromosomes are subjected to sexually antagonistic selection. Under certain condition they are favourable locations for either male or female advantageous genes. Due to MSCI, male-biased genes with testes specific expression would tend to escape from the X chromosome, e.g. by retroposition (Betrán et al. 2002). The underrepresentation of genes with expression during later spermatogenesis was observed both in mice (Khil et al. 2004) and *Drosophila* (Vibranovski et al. 2009)

1.5. Lepidopteran sex chromosomes

Moths and butterflies (Lepidoptera) represent the largest groups of animals with female heterogamety. Most of the species studied so far have a WZ/ZZ (female/male) chromosomal constitution (Sahara et al. 2012). Besides this predominant system, there is also a plethora of numeric variations, such as W_1W_2Z/ZZ (Naganuma population of *Orgyia thyllina*) or $WZ_1Z_2/Z_1Z_1Z_2Z_2$ (Nagano population of *Samia cynthia*) (Yoshido et al. 2005b). The most complicated sex chromosome system reported so far is for wood white butterflies with up to four W chromosomes in *Leptidea reali* (Šíchová et al. 2015) and up to six different Z chromosomes in *L. amurensis* (Šíchová et al. 2016). However, some lepidopteran species lack the W chromosome. Although the sex chromosome constitution Z0/ZZ was found in species from various families, e.g. in Saturnidae (*Samia cynthia ricini*, Yoshido et al. 2005b), it is typical for basal lepidopteran lineages (Traut et al. 2007). As the Z0/ZZ system is also shared with caddisflies (Marec and Novák 1998), a sister group

of Lepidoptera, the W chromosome absence is considered to be an ancestral trait. According to the generally accepted theory, the W chromosome had evolved in a common ancestor of the family Tischeriidae and the group Ditrysia, the latter comprising about 98 % of the known species of moths and butterflies. It is assumed that the W chromosome originated either from an autosome, whose homologue fused with the Z chromosome, or from a B chromosome (Traut and Marec 1996; Lukhtanov 2000).

The W chromosome is usually formed by constitutive heterochromatin and forms a dense heterochromatic body, the so-called sex chromatin, in interphase nuclei (Traut et al. 2007). The heterochromatin nature of this chromosome suggests that it consists mainly of repetitive DNA, and therefore it was omitted from most sequencing projects. In most species the only information that we have about the W chromosome composition is based mainly on results obtained by methods of fluorescence *in situ* hybridization (FISH) such as comparative genomic hybridization (CGH). These methods confirmed the repetitive nature of the lepidopteran W chromosome as, for example, in the silkworm, *Bombyx mori*, whereby the W-chromosome-derived BAC (Bacterial Artificial Chromosome) clones failed to identify the place of origin and labelled the whole W chromosome (Sahara et al. 2003b). Due to the predominantly repetitive content in the absence of meiotic recombination, the W chromosome evolves quite rapidly (Vítková et al. 2007; Yoshido et al. 2013). According to the CGH results, the W chromosome sequences can be divided into two groups, the W-specific sequences and sequences scattered throughout the genome but accumulated on the W. Individual species may then differ in the proportion of these two groups in their W chromosome (Traut et al. 1999; Sahara et al. 2003a; Fuková et al. 2005; Vítková et al. 2007; Šíchová et al. 2013). Partial sequencing of the W chromosome in *B. mori* and the codling moth, *Cydia pomonella*, revealed an abundance of transposable elements, mainly LTR and non-LTR retrotransposons (Abe et al. 2005; Fuková et al. 2007). The most comprehensive study of the W chromosome composition was performed by Traut et al. (2013). These authors microdissected and sequenced the W chromatin from highly polyploid nuclei of the Mediterranean flour moth, *Ephestia kuehniella*. Unsurprisingly, the most abundant type of sequences were mobile elements. Microsatellites and mitochondrial DNA were present in the flour moth W chromosome as well, although no conventional protein-coding gene was found. This is another typical trait of the lepidopteran W chromosome, as only a few protein coding genes have been found so far (Gotter et al. 1999; Nagaraju et al. 2014) with no overlap between species. Despite its heterochromatic nature, this female specific chromosome is expressed at least in some developmental stages. For example, it is one of sources of piRNAs (small piwi-interacting RNAs) in *B. mori* ovaries (Kawaoka et al. 2008; Kawaoka et al. 2011) and thus, the W chromosome itself seems to be involved in genomic

mechanisms for silencing of transposons. In addition, a piRNA encoded in the W chromosome was recently found to play a central role in sex determination of *B. mori* (Kiuchi et al. 2014).

In contrast to the W chromosome, the Z chromosome is gene rich and rather conservative. The Z chromosome gene content is conserved between *Bombyx mori* (Yoshido et al. 2005a) and many other species (Van't Hof et al. 2008; Yasukochi et al. 2009; Yoshido et al. 2011; Yasukochi et al. 2011; Baxter et al. 2011; Nguyen et al. 2013). Analogically to feminization of X chromosome in *D. melanogaster* (Parisi et al. 2003; Sturgill et al. 2007) and masculinization of the chicken Z chromosome (Kaiser and Ellegren 2006), the Z chromosome of *B. mori* is enriched in genes with expression in testes (Arunkumar et al. 2009). Despite sharing a block of conserved synteny between species from different lepidopteran families, the Z chromosome has a tendency to fuse with autosomes and form the so-called neo-Z chromosome (Nguyen and Carabajal Paladino 2016). The Z chromosome also appears to play important role in speciation (Dopman et al. 2005; Yasukochi et al. 2011; Nguyen et al. 2013). There is, however, no clear evidence for the fast-Z effect in moths and butterflies, as current studies are contradictory, with the presence of this phenomenon in *B. mori* (Sackton et al. 2014) but an absence in two nymphalid species (Rousselle et al. 2016). Although the dosage compensation in Lepidoptera is studied much more extensively than the fast-Z effect, the situation is not much clearer. Older works focused only on the expression of one or few Z-linked genes, and there were significant differences between males and females suggesting a lack of dosage compensation for the respective species (Johnson and Turner 1979; Suzuki et al. 1998; Gotter et al. 1999; Koike et al. 2003). Recent studies are more comprehensive and use RNA-seq or microarrays to study dosage compensation on large-scale data. However, the results are rather species specific without an obvious general conclusion. The complete dosage compensation was observed in *Manduca sexta* (Smith et al. 2014) and *C. pomonella* (Gu et al. 2017). *Heliconius* butterflies exhibit moderate dosage compensation with 5-20 % higher expression in males (Walters et al. 2015). On the other hand, no dosage compensation was observed in *Plodia interpunctella* with females having just over half the male gene expression (Harrison et al. 2012). The most complicated situation is in the silkworm *B. mori* with controversial results supporting either complete dosage compensation (Walters and Hardcastle 2011) or no global compensation and more gene-to-gene regulation (Zha et al. 2009). This inconsistency can be partially caused by the variability of the used tissues or whole bodies in individual studies, as there are tissue-specific differences in the results with especially gonads being less balanced (Zha et al. 2009; Gu et al. 2017). The only common feature among several studies is generally lower expression on the Z chromosome compared with autosomes (Walters and Hardcastle 2011; Smith et al. 2014; Walters et al. 2015; Gu et al. 2017).

Although the W and Z sex chromosomes in Lepidoptera are well differentiated and usually have no obvious regions of homology, they form normal bivalents during meiotic division. The

process of pairing of the W and Z chromosomes was described in *E. kuehniella* (Marec and Traut 1994). In this species, the sex chromosomes are not only lacking homology but the Z chromosome is significantly longer. The bivalent formation is delayed compared to autosomes, and pairing is initiated at the beginning of pachytene by terminal parts of the W and Z chromosome threads which come to close proximity. The next stage is a partially paired bivalent with chromosomes of unequal length. In the next step, the longer Z chromosome thread twists around W chromosome. At the end of this process, the length of both threads is adjusted to each other, and the W and Z chromosomes form a standard bivalent with a fully develop synaptonemal complex.

1.6. Methods of molecular cytogenetics in sex chromosome studies

Since classical cytogenetics is limited to studying highly heteromorphic sex chromosomes, and sequencing projects are usually focused on homogametic sex, molecular cytogenetics plays an irreplaceable role in sex chromosome studies. For these studies, various modifications of fluorescence *in situ* hybridization (FISH) have been developed, such as comparative genomic hybridization (CGH), FISH with chromosome painting probes, and FISH with probes derived from bacterial artificial chromosomes (the so-called BAC-FISH).

CGH was originally established to detect copy number changes between normal and cancer tissues (Kallioniemi et al. 1992). The method was later adapted for the identification and analysis of sex chromosomes by hybridizing differently labelled female and male genomic DNAs, which compete for location on chromosomes (Traut et al. 1999). This method has since been applied in many sex chromosome studies. Because CGH is essentially based on molecular differentiation but not on morphological differences, it was successfully applied for the identification of cryptic sex chromosomes (e.g. Ezaz et al. 2006).

Chromosome painting probes represent a complex mixture of sequences from a single chromosome. First FISHs to visualize a particular chromosome by painting probes was done by Pinkel et al. (1988) and Cremer et al. (1988). The key to success in this method is obtaining DNA from a single chromosome fraction in sufficient quality and quantity. Although painting probes were successfully prepared from a single chromosome (Gribble et al. 2004; Thalhammer et al. 2004), the procedure usually requires multiple copies of a particular chromosome. A very appropriate method to produce such chromosome-specific DNA sample is chromosome sorting by flow cytometry. This approach can purify chromosomes in large numbers, but it is demanding on the amount of input material in the form of a suspension of mitotic cells, and the chromosome of interest needs to be distinguishable by its optical properties (Doležel et al. 2012). Because in many cases it is difficult to meet all requirements of flow cytometry, microdissection is often a better option for the isolation of single chromosomes. Chromosomes were initially dissected manually

with glass needles (Scalenghe et al. 1981) but this method was quickly replaced with laser microdissection (Monajembashi et al. 1986). Laser microdissection was also used for preparation of the first painting probes in Lepidoptera (Fuková et al. 2007; Vítková et al. 2007). However, W chromosome probes were generated from heterochromatin bodies (sex chromatin), easily seen in highly polyploid nuclei of Malpighian tubules. Every such body is composed of several hundred copies of the lepidopteran sex-limited chromosome (Traut and Scholz 1978).

Bacterial artificial chromosomes (BACs) are high-capacity vectors derived from F-plasmid with the ability to carry insert up to 300 kbp long (Shizuya et al. 1992). Due to the size of cloned genomic DNA, BACs can be easily localized on chromosomes by means of FISH with fluorochrome-labelled probes. Since such large DNA fragments contain not only single-copy sequences but also potentially many repeats, BAC-FISH requires the use of unlabelled genomic DNA or C_0t1 DNA as a competitor to suppress hybridization signals produced by sequences shared between the probe and the competitor. BAC-FISH can be used for many applications, such as gene mapping, karyotyping, phylogenetic comparative studies, chromosome rearrangements detection, etc. (Janes et al. 2011). Moreover, individual BAC clones or their combinations can be used for chromosome painting (Lysak et al. 2002; Sahara et al. 2003b). Last but not least, another advantage of BAC libraries is very often the possibility of using them for BAC-FISH in related species (Weise et al. 2005; Yoshido et al. 2007).

2. Outline of research

Lepidoptera represent one of the most diverse groups of animals with many economically important species. Moths and butterflies are also the largest clade with heterogametic females. The lepidopteran species richness is reflected in the variability of their sex chromosomes, from the old well-differentiated W and Z chromosomes, through many numerical variations, to neo-sex chromosomes (Traut et al. 2007; Sahara et al. 2012; Nguyen and Carabajal Paladino 2016). Thus, Lepidoptera are perfect models for studying various aspects of sex chromosomes and their evolution. Despite this, cytogenetic research of moths and butterflies had been limited for a very long time by the peculiarities of their chromosomes, such as small size, absence of defined centromeres, and resistance to common banding techniques (De Prins and Saitoh 2003). Fortunately, modern methods of molecular cytogenetics have proven to be very useful in overcoming these struggles. The main aim of my thesis was to bring new information about mechanisms of molecular differentiation of sex chromosomes in Lepidoptera and to contribute to the understanding of their evolution.

Fluorescence *in situ* hybridization with BAC-derived probes represents one of these trustworthy tools of molecular cytogenetics. The first objective of my thesis was to establish the BAC-FISH method in our laboratory and use its advantages in a joint project with the laboratory of Ilik J. Saccheri (University of Liverpool) on mapping the genome of the peppered moth, *Biston betularia*. This lepidopteran species is well known for its melanic form (*carbonaria*) which rapidly spread in 19th century in Great Britain due to industrial pollution, a phenomenon known as industrial melanism. The genetic basis of this mutation, however, remained unknown. In our study presented in section 3.1, we identified a 200 kb region carrying a gene responsible for the colour change and physically mapped it by BAC-FISH to the autosome, orthologous to the silkworm chromosome 17. Comparison of physical and genetic map of the respective chromosome also revealed regions with uneven recombination rates. Furthermore, we performed a detailed analysis of the whole *B. betularia* karyotype, including the physical mapping of Z-linked genes and the BAC-FISH analysis of the W chromosome (section 3.2). Unurprisingly, this chromosome consisted mainly of heterochromatin and contained sequences of repetitive nature. One end, however, showed remnants of homology with the other sex chromosome, the Z, including part of the *laminin A* gene. A copy of this gene was, most likely, recently transferred from the Z chromosome onto the W by ectopic recombination, because no signs of presudogenization were found in the W copy.

Although the number of species studied for their sex chromosomes has increased significantly in recent years, most studies have focused on advanced ditrysian groups and there is still considerable lack of information about basal lineages. The second objective of my thesis was

to fill this gap and explore the W and Z chromosomes in four selected representatives of basal Ditrysia and Tischeriidae: *Tischeria ekebladella* (Tischeriidae), *Tineola bisselliella* (Tineidae), *Cameraria ohridella* (Gracillariidae), and *Plutella xylostella* (Plutellidae). Using different approaches for each sex chromosome, we have shown that the W chromosome exhibits a rapid and random evolution, while the Z chromosome exhibits conserved synteny of Z-linked genes (section 3.3). Moreover, our results have broadened the spectrum of plausible theories about the origin of W chromosome in Lepidoptera.

Despite the increasing number of sequenced moth and butterfly genomes, the knowledge about the composition of the lepidopteran W chromosome is limited. It seems that retrotransposons are the predominant component of this female specific chromosome (Abe et al. 2005; Fuková et al. 2007; Traut et al. 2013). The third objective of my thesis was to provide an insight into the composition of W chromosome of *Plodia interpunctella*, which surprisingly contained a large W-specific block of heterochromatin (Vítková et al. 2007). In the study presented in section 3.4, we have discovered a new W-enriched satellite sequence, which is expressed throughout all developmental stages and can potentially have both a structural and functional role in *P. interpunctella* genome. Moreover, it is only the fifth satellite DNA known for any lepidopteran species.

The accumulation of repetitive DNA and gene loss on the W chromosome are the most obvious signs of sex chromosome differentiation, which has many consequences that may include alterations in meiotic pairing and inactivation of the heterologous sex chromosomes during meiotic prophase I, the so-called meiotic sex chromosome inactivation (MSCI). This phenomenon was mainly observed in species with heterogametic males (Bean et al. 2004; Cabrero et al. 2007; Namekawa et al. 2007; Turner 2007; Hense et al. 2007), but was recently questioned in the only tested species with female heterogamety, chickens (Guioli et al. 2012). The fourth objective of my thesis was to test a hypothesis on the occurrence of MSCI in the meiosis of lepidopteran females by immunocytogenetic analysis of histone modifications of chromatin in the sex chromosomes of two model species, *Ephestia kuehniella* and *Bombyx mori*. Results presented in section 4.1 suggest the absence of MSCI in moths and butterflies with surprising hyperacetylation of the W chromosome.

3. Original publications

3.1. Industrial melanism in British peppered moths has a singular and recent mutational origin

Van't Hof A.E., Edmonds N., Dalíková M., Marec F., Saccheri I.J.

Science **332**: 958-960 (2011)

Abstract

The rapid spread of a novel black form (known as *carbonaria*) of the peppered moth *Biston betularia* in 19th-century Britain is a textbook example of how an altered environment may produce morphological adaptation through genetic change. However, the underlying genetic basis of the difference between the wild-type (light-colored) and *carbonaria* forms has remained unknown. We have genetically mapped the *carbonaria* morph to a 200-kilobase region orthologous to a segment of silkworm chromosome 17 and show that there is only one core sequence variant associated with the *carbonaria* morph, carrying a signature of recent strong selection. The *carbonaria* region coincides with major wing-patterning loci in other lepidopteran systems, suggesting the existence of basal color-patterning regulators in this region.

3.2. Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism

Van't Hof A.E., Nguyen P., Dalíková M., Edmonds N., Marec F., Saccheri I.J.

Heredity **110**: 283-295 (2013)

Abstract

We have constructed a linkage map for the peppered moth (*Biston betularia*), the classical ecological genetics model of industrial melanism, aimed both at localizing the network of loci controlling melanism and making inferences about chromosome dynamics. The linkage map, which is based primarily on amplified fragment length polymorphisms (AFLPs) and genes, consists of 31 linkage groups (LGs; consistent with the karyotype). Comparison with the evolutionarily distant *Bombyx mori* suggests that the gene content of chromosomes is highly conserved. Gene order is conserved on the autosomes, but noticeably less so on the Z chromosome, as confirmed by physical mapping using bacterial artificial chromosome fluorescence in situ hybridization (BAC-FISH). Synteny mapping identified three pairs of *B. betularia* LGs (11/29, 23/30 and 24/31) as being orthologous to three *B. mori* chromosomes (11, 23 and 24, respectively). A similar finding in an outgroup moth (*Plutella xylostella*) indicates that the *B. mori* karyotype (n=28) is a phylogenetically derived state resulting from three chromosome fusions. As with other Lepidoptera, the *B. betularia* W chromosome consists largely of repetitive sequence, but exceptionally we found a W homolog of a Z-linked gene (*laminin A*), possibly resulting from ectopic recombination between the sex chromosomes. The *B. betularia* linkage map, featuring the network of known melanization genes, serves as a resource for melanism research in Lepidoptera. Moreover, its close resemblance to the ancestral lepidopteran karyotype (n=31) makes it a useful reference point for reconstructing chromosome dynamic events and ancestral genome architectures. Our study highlights the unusual evolutionary stability of lepidopteran autosomes; in contrast, higher rates of intrachromosomal rearrangements support a special role of the Z chromosome in adaptive evolution and speciation.

3.3. New insight into evolution of W chromosome in Lepidoptera

Dalíková M., Zrzavá M., Hladová I., Nguyen P., Šonský I., Flegrová M., Kubíčková S., Voleníková A., Kawahara A. Y., Peters R. S., Marec F

Manuscript submitted to the *Journal of Heredity*

Abstract

Moths and butterflies (Lepidoptera) represent the most diverse group of animals with heterogametic females. Although the vast majority of species has a WZ/ZZ (female/male) sex chromosome system, it is generally accepted that the ancestral system was Z/ZZ and the W chromosome has evolved in a common ancestor of Tischeriidae and Ditrysia. However, the lack of information on sex chromosomes in basal Lepidoptera does not allow further elaboration of this hypothesis. Here we performed a detailed analysis of sex chromosomes in *Tischeria ekebladella* (Tischeriidae) and three species representing lower Ditrysia, *Cameraria ohridella* (Gracillariidae), *Plutella xylostella* (Plutellidae), and *Tineola bisselliella* (Tineidae). Using CGH we show that the first three species have well-differentiated W chromosomes, which vary considerably in their molecular composition, whereas *T. bisselliella* has no W chromosome. Furthermore, our results suggest the presence of neo-sex chromosomes in *C. ohridella*. For Z chromosomes, we selected five genes evenly distributed along the Z chromosome in ditrysian models and tested their Z-linkage using qPCR. The tested genes (*Henna*, *laminin A*, *Paramyosin*, *Tyrosine hydroxylase*, and *6-Phosphogluconate dehydrogenase*) proved to be Z-linked in all species examined. Although conserved synteny of the Z chromosome across Tischeriidae and Ditrysia, along with the W chromosome absence in Psychidae and Tineidae, is compatible with a single origin of the W chromosome in a common ancestor of Tischeriidae and Ditrysia, the independent origin of the W chromosomes in these clades is more parsimonious.

New insight into the evolution of W chromosome in Lepidoptera

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Running title: Evolution of W chromosome in Lepidoptera

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Abstract

Moths and butterflies (Lepidoptera) represent the most diverse group of animals with heterogametic females. Although the vast majority of species has a WZ/ZZ (female/male) sex chromosome system, it is generally accepted that the ancestral system was Z/ZZ and the W chromosome has evolved in a common ancestor of Tischeriidae and Ditrysia. However, the lack of information on sex chromosomes in basal Lepidoptera does not allow further elaboration of this hypothesis. Here we performed a detailed analysis of sex chromosomes in *Tischeria ekebladella* (Tischeriidae) and three species representing lower Ditrysia, *Cameraria ohridella* (Gracillariidae), *Plutella xylostella* (Plutellidae), and *Tineola bisselliella* (Tineidae). Using CGH we show that the first three species have well-differentiated W chromosomes, which vary considerably in their molecular composition, whereas *T. bisselliella* has no W chromosome. Furthermore, our results suggest the presence of neo-sex chromosomes in *C. ohridella*. For Z chromosomes, we selected five genes evenly distributed along the Z chromosome in ditrysian models and tested their Z-linkage using qPCR. The tested genes (*Henna*, *laminin A*, *Paramyosin*, *Tyrosine hydroxylase*, and *6-Phosphogluconate dehydrogenase*) proved to be Z-linked in all species examined. Although conserved synteny of the Z chromosome across Tischeriidae and

Ditrysia, along with the W chromosome absence in Psychidae and Tineidae, is compatible with a single origin of the W chromosome in a common ancestor of Tischeriidae and Ditrysia, the independent origin of the W chromosomes in these clades is more parsimonious.

Key words: comparative genomic hybridization, laser microdissection, quantitative PCR, sex chromosome evolution, synteny mapping.

Introduction

Sex chromosomes represent a special part of the genome which differs from autosomes in its evolutionary dynamics. They evolve *de novo* from a pair of autosomes, when one of the homologs acquires a gene or genes responsible for sex determination. This sets off a sequence of events which results in autosome-like X or Z chromosomes with gene composition changed due to sexual antagonistic selection, degeneration, and ultimate loss of Y or W chromosomes (e.g., Kaiser and Bachtrog 2010). A new sex-limited chromosome may then arise from a B chromosome which starts to pair with the X or Z chromosome. Alternatively, the sex chromosomes may undergo various structural changes such as fusion with another chromosome, which leads to the formation of neo-sex chromosomes and eventually to another round of differentiation (e.g., Carvalho and Clark 2005).

Most insect orders have an XX/XY (female/male) sex chromosome system or its variants (reviewed in Blackmon et al. 2016). There is, however, a single monophyletic group Amphiesmenoptera, which has the alternative sex chromosome system with heterogametic females. It includes two sister groups, namely caddisflies (Trichoptera) and moths and butterflies (Lepidoptera). All caddisflies and basal moths investigated so far have Z0/ZZ (female/male) sex chromosomes (Ennis 1976; Traut and Marec 1996; Marec and Novák 1998; Lukhtanov 2000). At some point in the evolution of Lepidoptera the W chromosome arose; however, the exact phylogenetic position and the mechanism of its origin is not known. As it is evident from the presence of female-specific sex chromatin (see below) and/or direct karyotype investigation, the W chromosome occurs in Tischeriidae and most studied species of the taxon Ditrysia, a mega-diverse group comprising 98% of extant species of moths and butterflies (Powell 1980; sex chromatin and sex chromosome distribution reviewed in Traut and Marec 1996; Lukhtanov 2000; Traut et al. 2007). However, the presence of W chromosome was also reported for a non-ditrysian family Hepialidae in the overlooked study of Kawazoe (1987a).

The W chromosome in the species studied usually represents the largest and often the only segment of heterochromatin in the chromosome complement. This suggests that it consists largely or entirely of repetitive sequences which often accumulate on sex specific chromosomes during their evolution (Kaiser and Bachtrog 2010). This assumption has been supported by detailed studies of the W chromosomes in three ditrysian species, namely *Bombyx mori* (Bombycidae), *Cydia pomonella* (Tortricidae), and *Ephestia kuehniella* (Pyralidae), which showed that the W chromosome is indeed rich in transposable elements (TEs) (Abe et al. 2005; Fuková et al. 2007; Traut et al. 2013). Accordingly, the total number of coding sequences found on the lepidopteran W chromosomes is extremely low with no overlap between species (Kawamura 1988; Gotter et al. 1999; Van't Hof et al. 2012; Kiuchi et al. 2014; Nagaraju et al. 2014; Fujii et al. 2015).

Due to its heterochromatic nature, the W chromosome forms a conspicuous heteropycnotic object in nearly all somatic cells of the most lepidopteran females. This so-called sex chromatin (or W chromatin) body observed in Malpighian tubules has been used as a convenient, yet indirect, evidence for presence of the W chromosome in a particular species (greatest contributions in Traut and Mosbacher 1968; Ennis 1976; Traut and Marec 1996; Lukhtanov 2000). In fact, the vast majority of data on the W chromosome distribution in Lepidoptera were obtained through the analysis of sex chromatin. Interestingly, in ditrysian families thoroughly tested for the W chromosome presence, species without sex chromatin were reported, most likely indicating a secondary loss of the W chromosome (Traut and Marec 1996). This suggests that the W chromosome is dispensable for the genome, which is consistent with its heterochromatic nature and scarcity of genes. This assumption is also in line with recent findings in wild silkmoths, *Samia cynthia* ssp., where the presence of W (or neo-W) chromosome had no influence on sex determination and reproduction of hybrids, demonstrating thus its dispensability (Yoshido et al. 2016; but cf. Kiuchi et al. 2014).

There are several hypotheses on the origin of the W chromosome in Lepidoptera. They state that the W arose from an autosome whose homologue had fused to the Z chromosome in a common ancestor of (i) Heteroneura as supported by decrease in modal chromosome number, or (ii) Euheteroneura (= Ditrysia + Tischeriidae + “Palaephatidae” with the last being probably paraphyletic, see Bazinet et al. 2017), which is less parsimonious as the fusion is not supported by reported modal chromosome numbers and would have to be accompanied by concurrent chromosome fission. Alternatively, (iii) the W chromosome might have originated from a B chromosome in Euheteroneura (Lukhtanov 2000). However, Psychidae, one of the early-diverging lineages of Ditrysia, do not have a W chromosome (Seiler 1919; Seiler 1959; Narbel-Hofstetter 1961), which is a long-known fact never

considered in previous studies (reviewed in Traut et al. 2007; Marec et al. 2010; Sahara et al. 2012). It raises another hypothesis that the W chromosome arose independently in Tischeriidae and in Ditrysia after divergence of Psychidae (Figure 1). In this study, we examined and compared the sex chromosomes in representatives of Tischeriidae and three basal groups of Ditrysia, namely Tineidae, Gracillariidae, and Plutellidae in order to provide additional data, which would shed light on the origin of the W chromosome in Lepidoptera.

Material and Methods

Insects

We used a wild-type laboratory strain of the diamondback moth, *Plutella xylostella* (Plutellidae), obtained from Neil I. Morrison, Oxitec Ltd, Abingdon, UK; for the strain origin see Martins et al. (2012). The larvae were kept on an artificial diet in a room with temperature of 21 ± 1°C, at a 12/12 h (light/dark) regime. The larvae and pupae of the horse-chestnut leaf miner, *Cameraria ohridella* (Gracillariidae), were collected in horse chestnut trees and those of *Tischeria ekebladella* (Tischeriidae) in oak trees from several localities in České Budějovice, CR. The lab strain of the common clothes moth, *Tineola bisselliella* (Tineidae), was established from specimens found in a household in České Budějovice, CR. The larvae were kept on raw sheep wool in a room with temperature of 25 ± 1°C, at a 16/8 h (light/dark) regime.

Chromosomal and Malpighian tubules preparation

Spread chromosome preparations were prepared as described in Mediouni et al. (2004). Meiotic chromosomes were obtained from larval gonads, mitotic preparations were made from larval gonads, brain or wing imaginal discs. The tissue was dissected in a physiological solution for *Ephestia* (Glaser 1917; cited in Lockwood 1961), fixed in Carnoy fixative (6:3:1 ethanol, chloroform, acetic acid) and spread in a drop of 60% acetic acid at 45°C using a hot plate. Optionally, the tissue was hypotonized for 10-15 min in 75 mM KCl prior fixation. Preparations were dehydrated in ethanol series (70%, 80% and 100%, 30 sec each) and stored at -20°C.

Preparations of polyploid interphase nuclei from Malpighian tubules were prepared from the last instar female larvae. The tubules were dissected in physiological solution and fixed on the slide in Carnoy fixative for 1 min. Then they were stained in 2.5% lactic acetic orcein for 3-5 min, mounted in the staining solution and inspected in a light microscope for the presence of sex chromatin (see Traut and Marec 1996).

Comparative genomic hybridization (CGH)

Genomic DNA (gDNA) from *P. xylostella*, *C. ohridella*, and *T. ekebladella* was isolated separately from female and male individuals (larvae, pupae or adults) by standard phenol-chloroform extraction. In *T. bisseliella*, gDNA was extracted from adults by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) and amplified by illustra GenomiPhi HY DNA Amplification Kit (GE Healthcare, Milwaukee, WI, USA). Genomic DNA probes were labeled by Nick Translation Kit (Abbott Molecular, Des Plaines, IL, USA). The 25 µL Nick Translation reaction contained 500 ng of gDNA; 25 µM dATP, dCTP, and dGTP; 9 µM dTTP; 16 µM labeled nucleotides with either Cy3-dUTP (male DNA) or fluorescein-dUTP (female DNA) (both Jena Bioscience, Jena, Germany); 1x nick translation buffer and 5 µl of nick translation enzyme mix. The reaction was incubated at 15°C for 6-7 hrs.

CGH was performed as described in (Traut et al. 1999) with several modifications. Chromosomal preparations were first treated with RNase A (200 ng/µl) (Sigma-Aldrich, St. Louis, MO, USA) in 2x SSC for 1 h at 37°C and denatured in 70% formamide in 2x SSC for 3.5 min at 68°C. The probe mix containing 300 ng of each labeled gDNA probe, 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich) in 10 µl of 50% deionized formamide, 10% dextran sulfate in 2x SSC was denatured for 5 min at 90°C and prehybridized for 90 min at 37°C. Hybridization was carried out for 3 days at 37°C. Then the slides were washed at 62°C for 5 min in 0.1x SSC with 1% Triton X-100 and counterstained with 0.5 µg/ml DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich) in antifade based on DABCO (1,4-diazabicyclo(2.2.2)-octane; Sigma-Aldrich).

W chromosome painting probes

Preparations for laser microdissection of W chromatin bodies were done following the protocol described in Fuková et al. (2007) with slight modifications. Briefly, Malpighian tubules were dissected from the last instar female larvae of *P. xylostella*, *C. ohridella*, and *T. ekebladella*, hypotonized for 15 min in 75 mM KCl and fixed in methanol/acetic acid (3:1) for 15 min. Then the tubules were transferred into a drop of 60% acetic acid on a glass slide coated with a polyethylene naphthalate membrane, spread at 40°C using hot plate and stained with 4% Giemsa (Penta, Czech Republic). Microdissection of W-bodies was performed using a PALM MicroLaser System (Carl Zeiss MicroImaging, Munich, Germany) as described in Kubickova et al. (2002).

DNA amplification and probe labeling was performed according to Drosopoulou et al. (2012). Briefly, 8-12 microdissected sex chromatin bodies per sample were amplified using a GenomePlex Single Cell Whole Genome Amplification Kit (WGA4, Sigma-Aldrich) and then the reaction was

purified by Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The amplified product was labeled using a GenomePlex WGA Reamplification Kit (WGA3, Sigma-Aldrich). The labeling reaction (25 µl total volume) contained 15 ng of amplified DNA, 0.4 mM each dNTP except 0.336 mM dTTP, 40 µM Cy3-dUTP (Jena Bioscience) or Green-dUTP (Abbott Molecular), 1x Amplification mix and 1.7 µl of WGA polymerase.

Telomeric and rDNA probes

Insect telomeric probe $(TTAGG)_n$ was synthetized by means of non-template PCR as described in Sahara et al. (1999) and labeled with Cy3-dUTP. The 18S rRNA gene was amplified by PCR from the codling moth (*Cydia pomonella*) gDNA according to Fuková et al. (2005), cloned using a pGEM-T Easy Vector System (Promega) and labeled with biotin-dUTP (Roche Diagnostics, Mannheim, Germany).
The telomeric and rDNA probes were labeled by using the improved nick translation procedure of Kato et al. (2006) with some modifications. The modified 20 µL reaction contained 1000 ng unlabeled DNA; 0.5 mM dATP, dCTP and dGTP; 0.1 mM dTTP; 20 µM labeled nucleotides; 1x nick translation buffer (50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 0.005% BSA); 10 mM ß-mercaptoethanol; 2.5 x 10^{-4} U DNase I (ThermoFisher) and 1 U DNA polymerase I (ThermoFisher Scientific, Waltham, MA USA). The reaction was incubated at 15°C for 1 hour for both probes and labeling techniques.

Fluorescent *in situ* hybridization with directly labeled probes

Fluorescent *in situ* hybridization (FISH) with directly labeled (i.e. fluorochrome-dUTP-labeled) probes was used for visualization of mitotic chromosome ends (telomeric probe) and for W-chromosome painting. In both cases the FISH procedure was carried out following the protocol of Traut et al. (1999) with some modifications. Chromosomes were denatured at 68°C for 3.5 min in 70% formamide in 2x SSC buffer. For each slide the probe mixture contained 5 µl of labeled W-chromosome and/or 160 ng telomeric probe and 25 µg of sonicated salmon sperm DNA in a total 10 µl volume of 50% formamide, 10% dextran sulfate and 2x SSC. Probes were denatured at 90°C for 5 min. Hybridization was carried out for 3 days at 37°C. Washes and counterstaining were the same as in CGH.

Reprobing

We performed reprobing of FISH slides according to Shibata et al. 2009 in order to investigate the presence of major rDNA clusters in *T. bisselliella* and *T. ekebladella*. Briefly, the slides were

immersed in MiliQ water for 30 min followed by 2x 5 min washes in 2x SSC to remove the cover slip and wash away the mounting medium. Afterwards, to remove the first probe, the slides were incubated for 10 min at 70°C in 50% formamide, 1% Triton X in 0.1x SSC and placed immediately into cold 70% ethanol (prechilled at -20%) for 1 minute and dehydrated through 80% and 100% ethanol (30 s each) at room temperature and air dried. The slides were then immediately used for another round of hybridization.

Fluorescent *in situ* hybridization with biotin-labeled 18S rDNA probe

FISH with biotin-labeled probe was used for 18S rDNA localization in *T. ekebladella* and *T. bisselliella*, essentially following the procedure described in Sahara et al. (1999) with some modifications. Slides were first treated with RNase A (200 ng/µl) (Sigma-Aldrich) in 2x SSC for 1 h followed by two washes in 2x SSC for 5 min, and 30 min incubation in 5x Denhardt's solution, all at 37 °C. Denaturation of chromosomes was done at 68°C in 70% formamide in 2x SSC for 3.5 min. The probe mixture for one slide contained 40 ng of the 18S rDNA probe and 25 µg of sonicated salmon sperm DNA in 10 µl of 50% deionized formamide, 10% dextran sulfate in 2x SSC. Hybridization signals of the biotinylated 18S rDNA probe were visualized and amplified by three-step detection, Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs., West Grove, PA, USA), biotinylated anti-streptavidin (Vector Labs., Burlingame, CA, USA) and Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 µg/ml DAPI in DABCO.

Microscopy and image processing

Preparations were observed in a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) equipped with appropriate fluorescence filter sets and a monochrome CCD camera XM10 (Olympus Europa Holding, Hamburg, Germany). In FISH preparations, black-and-white images were captured separately for each fluorescent dye with cellSens Standard software version 1.9 (Olympus). The images were pseudocolored and merged using Adobe Photoshop CS5.

Isolation of genes for quantitative PCR in *Tischeria ekebladella*

Total RNA was isolated by RNAzol RT (Sigma-Aldrich) from larvae and pupae. First-strand cDNA was synthetized by ImProm-II Reverse Transcription System (Promega) using oligo-dT or gene specific degenerate primers (*Ace2* ACYTTNCCDATRTCYTGNGC; *TH* AYAAYTGYAAYCAYCTBATGAC). Parts of the coding sequences from the genes of interest were amplified by PCR from respective cDNAs using degenerate primers (Supplementary Table 1). The 20 µl reaction contained 2 µl cDNA, 10

µM each primer, 200 µM each dNTP, 1x Ex *Taq* buffer and 1U Ex *Taq* DNA Polymerase (TaKaRa, Otsu, Japan). Amplified fragments were cloned using a pGEM-T Easy Vector System and sequenced using universal M13 primers.

Quantitative PCR (qPCR)

To compare copy number of genes of interest between females and males we used quantitative real-time PCR (qPCR) with gDNA isolated by NucleoSpin Tissue kit (Macherey-Nagel) as template. Relative gene doses of the target genes were determined by comparison with a single-copy autosomal reference gene, *Acetylcholinesterase 2* (*Ace2*). To design primers in *C. ohridella*, *P. xylostella*, and *T. bisselliella* the orthologs of genes of interest were acquired from available genomes (You et al. 2013; Ferguson et al. 2014) and transcriptome (The 1KITE project; NCBI BioProject PRJNA267902; for sequences see Supplementary material). For designing primers in *T. ekebladella* we used sequences obtained in this study (for Gen-Bank accession numbers see Supplementary Table 2). The target and reference genes were analyzed simultaneously in three biological replicas for each sex. For qPCR the reaction mix contained 15-50 ng of gDNA, 1 µM each primer (Supplementary Table 2) and 1x qPCR SYBR Master Mix (Top-Bio, Prague, Czech Republic). The reaction was carried out using the C1000 Thermal cycler CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) and results were analyzed using software Bio-Rad CFX Manager 3.1. To calculate the amplification efficiency (*E*) we used four points of 5 times dilution series. Target to reference gene dose ratio (*R*) was calculated for each biological sample using formula $R = [(1+E_{\text{Reference}})^{\text{CtReference}}]/ [(1+E_{\text{Target}})^{\text{CtTarget}}]$. Two hypothesis were tested statistically by unpaired two-tailed *t* test for unequal variances. The autosomal hypothesis presumes equal *R* value between females and males (1:1). In the Z linkage of target gene hypothesis, the target to reference gene dose ratio would be in males two times higher than in females (*R* values ratio 1:2, females : males).

Results

Chromosome number and sex chromatin status

Chromosome numbers in *Plutella xylostella* (2n=62), *Cameraria ohridella* (2n=60), and *Tischeria ekebladella* (2n=56) are known from previous studies (Kawazoé 1987b; Lukhtanov 2000; Nguyen and Nguyen 2001; De Prins et al. 2002). In this study, we determined the chromosome number in *Tineola bisselliella*, which is 2n=60 in males and 2n=59 in females. For this we used mitotic chromosomes hybridized with telomeric probe in order to visualize the chromosome ends (telomeric

signals not shown). Preparations of polyploid nuclei from Malpighian tubules showed a conspicuous sex chromatin body in females of *P. xylostella*, *C. ohridella*, and *T. ekebladella* (Supplementary Figures 1a, 1e, and 1g). However, the sex chromatin was absent in male nuclei of all four tested species (Supplementary Figures 1b, 1d, 1f, and 1h) as well as in female nuclei of *T. bisselliella* (Supplementary Figure 1c). This suggests that P. *xylostella*, *C. ohridella*, and *T. ekebladella* females have a W chromosome while females of *T. bisselliella* not, which is consistent with a lower number of chromosomes in females by one than in males.

W-chromosome painting and DAPI staining

In *P. xylostella*, *C. ohridella*, and *T. ekebladella*, we prepared W-chromosome painting probes by means of laser microdissection of sex chromatin from female polyploid nuclei of Malpighian tubules. The W-probes were used for FISH on female pachytene chromosomes in order to visualize the W chromosome.

In all three species, the W-painting probe highlighted a single chromosome, the W in the WZ bivalent (Figures 2a-d, 2f, 2h, and 2j). While in *P. xylostella* and *C. ohridella*, the W chromosomes were labeled evenly along their whole length, the probe did not hybridize to one of the W chromosome ends in *T. ekebladella* (Figures 2d).

In *T. ekebladella* and *P. xylostella,* the W chromosome could also be identified by DAPI staining (Figures 2e and 2k) due to its heterochromatin structure. FISH with the W-painting probes thus just confirmed that these chromosomes are indeed the Ws. However, the W chromosome in *C. ohridella* could not be reliably distinguished from the other chromosomes in pachytene nuclei (Figure 2g) as it was highlighted by DAPI only in late pachytene or in highly condensed post-pachytene chromosomes of nurse cells (Figure 2i). In this species, the W-painting probe helped to identify the W chromosome in younger pachytene nuclei. Nevertheless, the highly condensed post-pachytene W chromosome in *C. ohridella* was conspicuous and its heterochromatin was often fragmented into several blocks.

In *T. bisselliella*, we could not prepare the W-chromosome painting probe due to the sex chromatin absence. DAPI staining revealed a thin Z-univalent which was longer than the bivalents in both pachytene (Figure 2l) and highly condensed post-pachytene nuclei of nurse cells (Figure 2m). Since in mitotic metaphases no chromosome was extremely larger than the others (Figure 2n), we suggest that the Z-univalent condenses later than the autosome bivalents. Notably, the Z univalent was often associated with one or two bivalents in a nucleolus of both pachytene and post-pachytene

nurse cells (Figure 2l-m). We performed FISH with 18S rDNA probe in order to test for the presence of rDNA cluster on the Z chromosome. Yet the only rDNA cluster in this species was localized on a pair of autosomes (Supplementary Figure 2).

Molecular differentiation of sex chromosomes by CGH

We used CGH in all four species, but for different reasons. In *P. xylostella*, *C. ohridella*, and *T. ekebladella*, in which we have already confirmed the W chromosome presence by W-painting, we used CGH to roughly characterize the molecular composition of the W chromosome, since the same or different intensities of male and female hybridization signals indicate accumulation of female-specific and ubiquitous sequences, respectively, on the W chromosome (Sahara et al. 2003). In case of *T. bisselliella,* CGH was supposed to provide yet another line of evidence for the absence of the W chromosome. As expected, CGH did not identify any female specific region in *T. bisselliella*, which supports the Z/ZZ sex chromosome system in this species (Figures 3a-d).

In *P. xylostella*, the W chromosome was strongly highlighted by both the female and male probes along its whole length (Figures 3e-h). In addition, both probes showed a similar pattern of hybridization signals suggesting that the W chromosome in this species accumulated autosomal repeats present in both sexes.

Similar to *P. xylostella*, the W chromosome in *C. ohridella* was labeled equally by both gDNA probes. Interestingly, only about two thirds of the W chromosomes were highlighted by these probes (Figures 3i-l). The highlighted part consisted of heterochromatin, while the remaining part was euchromatic. This euchromatic part was labeled by both probes at about the same intensity as autosomes. However, this pattern was not seen in highly condensed W chromosomes in nuclei of nurse cells, where the whole chromosome was labeled strongly by both probes. This different level of hybridization between the two parts of the W chromosome might reflect an existence of two strata, as a result of fusion between W and an autosome.

Unlike *P. xylostella* and *C. ohridella*, the difference between hybridization signals of the female and male gDNA probes in *T. ekebladella* was enormous (Figures 3m-p). While the male probe labeled weakly and nearly evenly all chromosomes including the W, the female probe strongly highlighted the W chromosome. Such hybridization pattern indicates that the W chromosome of *T. ekebladella* consists largely of sequences unique to females. Only a very short segment at one end of the W chromosome was strongly labeled by the male probe as well (Figure 3n, p). Since this terminal segment resembled the autosomal block of heterochromatin probably composed of rDNA loci that

was strongly labeled by both probes, we performed an additional round of FISH with 18S rDNA probe on the CGH slides in order to identify a potential rDNA site on the W chromosome. The 18S rDNA probe confirmed the autosomal location of a single cluster of major rDNA, but did not hybridize to the W chromosome at all. It is thus very likely that the terminal W segment identified by both gDNA probes is composed of repetitive sequences common to both sexes.

qPCR analysis of selected genes

In order to study the synteny of genes between Z chromosomes of basal Ditrysia, Tischeriidae and already investigated lepidopteran lineages, we identified in each species orthologs of five genes distributed along the whole ditrysian Z chromosome as mapped in *Biston betularia* (Supplementary Figure 3), namely *laminin A*, *Tyrosine hydroxylase* (*TH*), *Henna*, *Paramyosin*, *6-Phosphogluconate dehydrogenase* (*6-PGD*) (Van't Hof et al. 2013). A relative gene dose of these orthologs was compared between female and male gDNA by qPCR. The results obtained were statistically tested for two hypotheses, autosomal linkage versus Z- linkage, following the procedure in Nguyen et al. (2013).

In all species studied, the qPCR results (Supplementary Table 3) clearly showed twofold difference between the female and male gene dose in all five genes (female:male ratio 1:2) (Figure 4). In all cases there was a significant difference between the results in females and males, supporting thus the Z-linkage hypothesis. However, the differences were not statistically significant when comparing doubled female values to original male results. Thus, we concluded that a synteny block of genes corresponding to the ditrysian Z chromosome is conserved in all studied species.

Discussion

Species examined in this study differ largely considering their W chromosomes. In two species, namely *P. xylostella* and *T. ekebladella,* we found fully degenerated W chromosomes consisting entirely of heterochromatin. However, according to our CGH results, the W chromosome in *P. xylostella* is composed of repetitive sequences present in both sexes. In contrast, the W chromosome of *T. ekebladella* contains mostly female-specific sequences, while ubiquitous repetitive sequences are largely underrepresented. Such a large difference in molecular composition is not surprising, since the W chromosome evolution through accumulation of repeats has been shown to be a rapid and stochastic process (Abe et al. 2005; Fuková et al. 2007; Traut et al. 2013). Additionally, it has been shown previously that even more closely related species may differ largely in the type of sequences accumulated in this non-recombining chromosome (Vítková et al. 2007).

Similar to *P. xylostella*, the W chromosome of *C. ohridella* also accumulated repetitive sequences common to both sexes. However, these repetitive sequences form about two thirds of the W chromosome which are composed of heterochromatin, while the remaining part is made of euchromatin. The euchromatin part might represent an evolutionary younger stratum which arose by fusion of the ancestral W chromosome with an autosome. Such sex-chromosome-autosome fusions are surprisingly common among moths and butterflies (reviewed in Nguyen and Carabajal Paladino 2016) as they were reported in species from various lepidopteran families such as Tortricidae (Nguyen et al. 2013; Šíchová et al. 2013), Nymphalidae (Smith et al. 2016; Mongue et al. 2017), and Saturniidae (Yoshido et al. 2011). The karyotype of *C. ohridella,* 2n=60, was reported also in its other congeners analyzed so far and represents the modal chromosome number of Gracillariidae (Puplesiene and Noreika 1993; De Prins et al. 2002). Since the ancestral chromosome number is 2n=62 in Ditrysia (Lukhtanov 2000), i.e,. by one chromosome pair more than in Gracillariidae, we hypothesize that there was an old sex-chromosome-autosome fusion which predates the origin of Gracillariidae. Since the autosome fusion with the ancestral W chromosome would result in an immediate cessation of recombination between the neo-W and neo-Z segments due to the absence of crossing-over in all lepidopteran females (e.g., Maeda 1939; Traut 1977; Nokkala 1987), the old fusion is expected to result in a fully heterochromatinized neo-W chromosome. However, the fate of originally autosomal parts of the neo-W chromosomes can vary greatly, and their euchromatic appearance can be maintained in some cases even after old fusion events (Šíchová et al. 2013; Mongue et al. 2017). It should also be noted that the euchromatic nature of a part of the neo-W chromosome does not necessarily reflect the lack of sex chromosome differentiation (Vicoso et al. 2013; Mongue et al. 2017).

Furthermore, our study revealed that *T. bisselliella* (Tineidae) has no W chromosome. The absence of a W chromosome seems to be a shared trait of basal Ditrysia, since all tested species of Psychidae, the most basal ditrysian group studied so far, also lack a W chromosome (Seiler 1919; Seiler 1959; Narbel-Hofstetter 1961). Along with Meessiidae, Tineidae and Psychidae used to constitute the superfamily Tineoidea (reviewed by Mitter et al. 2017) and absence of a W chromosome seems to support their mohophyly. However, recent phylogenetic and phylogenomic studies agree on paraphyly of the Tineoidea (Mutanen et al. 2010; Regier et al. 2015; Bazinet et al. 2017)(see Figure 1).

The W chromosomes in Lepidoptera are currently assumed to have a common origin and arose either via an adoption of a supernumerary B chromosome which started to pair with the Z

chromosome (Figure 5a), or via a fusion of the Z chromosome with an autosome, whose homologue then became the W chromosome (Figure 5b). Both scenarios are possible as found, for instance, in the evolution of Drosophilidae (Carvalho 2002; Bachtrog 2013). Furthermore, a B chromosome became a Y chromosome via meiotic pairing with the X chromosome in *Cacopsylla peregrina* (Psylloidea, Hemiptera) (Nokkala et al. 2003).

However, earlier studies (Lukhtanov 2000; Traut et al. 2007; Marec et al. 2010; Sahara et al. 2012) did not consider the possible significance of the W chromosome absence in Psychidae (Seiler 1919; Seiler 1959; Narbel-Hofstetter 1961). Since the W chromosome is missing also in Tineidae, other scenarios can be brought to play to explain the origin of the W chromosome in Lepidoptera, namely two independent origins of the W in Tischeriidae and Ditrysia excl. Psychidae and Tineidae (and, consequently, also *Palaephatus* and Meessiidae) (Bazinet et al. 2017), with either two Z/autosome fusions (Figure 5c), two B chromosome adoptions (Figure 5d), or their combinations (Figure 5e, f). However, it should be stressed that in Tineidae, information on the absence of the W chromosomes is limited only to a single species and more data is needed to find out whether the loss of the W chromosome applies to all Tineidae.

Whether the W chromosome arose only once or twice independently and whether it originates from a B chromosome or an autosome can be tested by comparison of sex chromosomes between different lepidopteran lineages. The origin of the W chromosome cannot, however, be investigated directly due to the lack of W-linked homologous markers caused by rapid turnover of W chromosome sequences (Bachtrog 2006). An alternative way is to explore synteny of Z-linked genes (Nguyen et al. 2013). Therefore, we have selected five marker genes which evenly cover the Z chromosome in representatives of advanced Ditrysia, namely *Bombyx mori* (Bombycidae) and *Biston betularia* (Geometridae) (Van't Hof et al. 2013), and tested whether they are also Z-linked in the species under study. We showed that all markers are located on the Z chromosome in all examined taxa, which suggests a conserved synteny of Z-linked genes across Tischeriidae and Ditrysia. This largely rules out the hypotheses on the independent W chromosome origin based on the sex-chromosome-autosome fusion in advanced Ditrysia as under both possible scenarios (Figure 5c, f) some of tested markers are expected to be autosomal in representatives of Tischeriidae, Psychidae and Tineidae.

Based solely on the Z chromosome synteny block shared between Tischeriidae and Ditrysia, we cannot discriminate among the other hypotheses. Moreover, lack of the W chromosome in Lepidoptera is largely based on absence of the female specific heterochromatin body, which is an

indirect and not totally reliable proof (Rathjens 1974; Traut et al. 1986; Marec and Traut 1994; Šíchová et al. 2015). Thus, we are unable to score probability of the W chromosome gain nor of its loss. However, these scenarios can be evaluated based on number of evolutionary changes they require. Presuming that the W chromosome is missing in the whole Tineidae, both hypotheses with a single W chromosome origin (Figure 5a, b) would require one acquisition in a common ancestor of Euheteroneura and two independent losses of this chromosome in Psychidae and Tineidae. Moreover, the hypothesis on a single W chromosome origin by chromosome fusion (Figure 5b) would further require this event to be compensated by an autosome fission as the chromosome number of 2n=62 is well supported as the ancestral number in Ditrysia and likely in the whole Heteroneura (Figure 1).

Thus, our data supports the independent origins of the lepidopteran W chromosome as the most parsimonious scenario with two alternatives: (i) two independent B chromosome adoptions in Tischeriidae and Ditrysia (Figure 5 d) or (ii) Z/autosome fusion in Tischeriidae and B chromosome adoption in Ditrysia excl. Psychidae and Tineidae (Figure 5e). Moreover, the latter hypothesis is favored by reduced chromosome numbers observed in Tischeriidae, which suggests overall propensity of their karyotypes to chromosome fusions (Figure 1). The independent origins of W chromosomes during the evolution of Lepidoptera could explain yet another conflict in available data, an anecdotal report of the W chromosome in *Endoclita sinensis*, a representative of Hepialidae (Figure 1, Kawazoé 1987b).

To conclude, our results suggest a conserved synteny of Z-linked genes across Euheteroneura (Tischeriidae and Ditrysia) and, in contrast to previous studies (reviewed in Traut et al. 2007; Marec et al. 2010; Sahara et al. 2012), support independent origin of the W chromosomes in these two groups. The available data further suggests that at least in the Ditrysia excl. Psychidae and Tineidae, the W chromosome arose from a B chromosome which started to pair with the Z chromosome. These results also imply that the abundance of repetitive sequences in the W chromosomes together with the lack of coding sequences do not result from a degeneration process (cf. Bachtrog 2013) and cast doubt on the primary role of W chromosome in sex determination of Lepidoptera (cf. Kiuchi et al. 2014; Yoshido et al. 2016). The present study thus brings a new perspective on understanding the evolution of the lepidopteran sex chromosomes and provides a new framework for their further analysis.

Funding
This research was funded by grant 14-22765S and follow-up grant 17-13713S of the Czech Science Foundation (CSF). PN was supported by CSF grant 14-35819P. SK acknowledges support from The Ministry of Education, Youth, and Sports of the Czech Republic (CEITEC 2020 project LQ1601) and AK from the National Science Foundation grant no. DEB #1557007.

Acknowledgements
We thank Marie Korchová for technical assistance and Jan Zrzavý for critical reading of the manuscript. The 1KITE project is acknowledged for providing access to the transcriptome of *Tineola bisselliella*.

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Figures

Figure 1. Simplified phylogenetic relationships of Lepidoptera. Clades with records of either W chromosome or sex chromatin presence/absence in females are marked with plus and minus, respectively. For Meessiidae and Palaephatidae the sex chromosome status is not known (note that *Palaephatus* might be close to Ditrysia and distant from the rest of „Palaephatidae"; see Bazinet et al. 2016). Clades with studied species, i.e. *Plutella xylostella* (Plutellidae), *Cameraria ohridella* (Gracillariidae), *Tineola bisselliella* (Tineidae), and *Tischeria ekebladella* (Tischeriidae), are in bold. Modal chromosome numbers were based on data in Puplesiene and Noreika 1993; Lukhtanov and Puplesiene 1996; and Lukhtanov 2000. Phylogenetic relationships are according to Regier et al. 2013 and Bazinet et al. 2017.

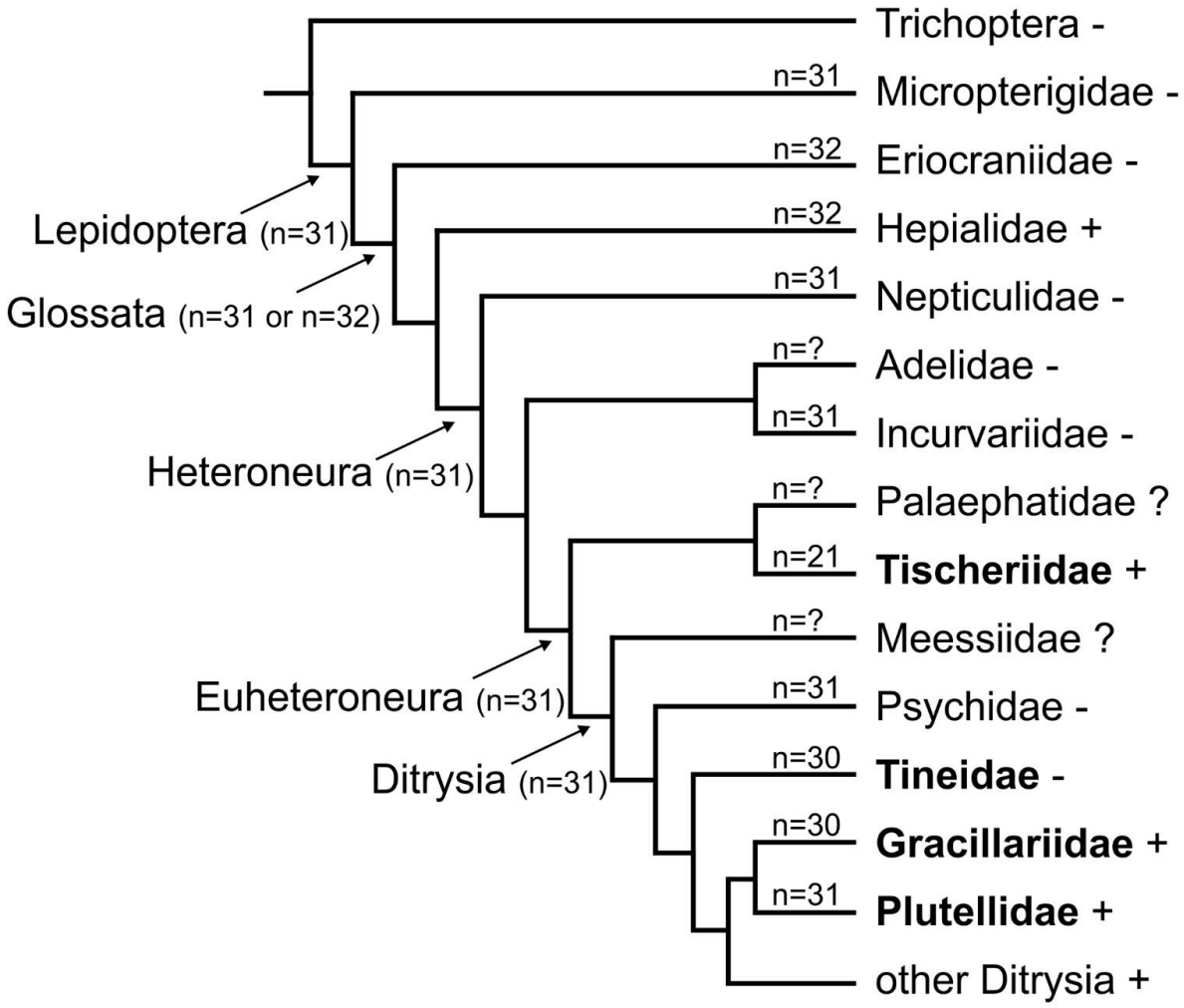


Figure 2. Results of DAPI staining (blue) and FISH with W-painting probes (red) performed on female meiotic chromosomes of *Tischeria ekebladella* (**a**, **d**, and **e**), *Cameraria ohridella* (**b**, **f**-**i**), *Plutella xylostella* (**c**, **j**, and **k**), and *Tineola bisselliella* (**l**-**n**). Whole pachytene nuclei of *T. ekebladella* (**a**), *C. ohridella* (**b**), and *P. xylostella* (**c**) with the W chromosome highlighted by the W-probe. In figures (**d**-**k**) are individual WZ bivalents with and without hybridization signals of the W- probe. In figure (**d**) of *T. ekebladella*, the W-probe hybridized to the whole W chromosome except for one end (arrowhead). In *C. ohridella*, the W chromosome was nearly indistinguishable from other chromosomes during pachytene (**f** and **g**), but became heterochromatinized in highly condensed post-pachytene nuclei of nurse cells (**h** and **i**). In *T. bisselliella*, the W chromosome was absent and the Z univalent was the longest chromosome in pachytene (**l**) as well as in post-pachytene nurse cells (**m**), and was nearly always associated with the nucleolus (N). Since in mitotic metaphase chromosomes (**n**) there is no chromosome conspicuously larger than the others, we suggest that the Z chromosome condensate later than the autosomes.

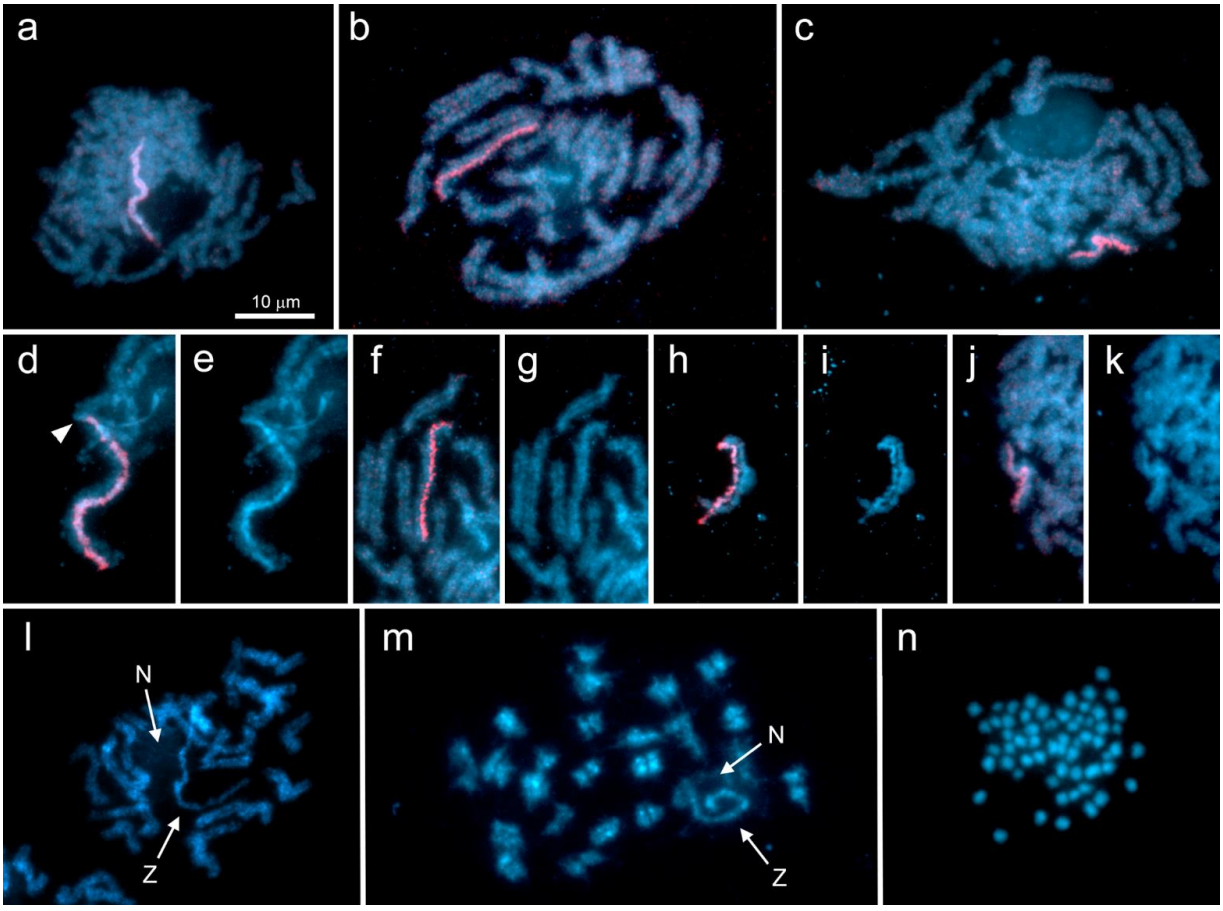


Figure 3. Comparative genomic hybridization on female pachytene bivalents of *Tineola bisselliella* (**a**-**d**), *Plutella xylostella* (**e**-**h**), *Cameraria ohridella* (**i**-**l**), and *Tischeria ekebladella* (**m**-**p**). Figures (**a**, **e**, **i**, and **m**) show DAPI staining (blue) with schematic drawings of the WZ bivalent or the Z univalent in case of *T. bisselliella*. Figures (**b**, **f**, **j**, and **n**) are merged pictures of male (red) and female (green) probes, figures (**c**, **g**, **k**, and **o**) are hybridization signals of female gDNA probes (green), figures (**d**, **h**, **l**, and **p**) are hybridization signals of male gDNA probes (red). Figure (**m**) shows hybridization signals of the 18S rDNA probe (rDNA reprobing on chromosomes after CGH) to demonstrate that there is no rDNA on the W chromosome. Figure (**n**) contains a schematic drawing of the CGH hybridization signals on the W chromosome, where the female probe strongly labeled nearly entire W chromosome except for one end which was highlighted with the male probe (arrowhead). CGH results show that the W chromosome in *T. ekebladella* consists mostly of female specific sequences, while the W chromosome in *C. ohridella* and *P. xylostella* is mainly composed of sequences which are common to both sexes.

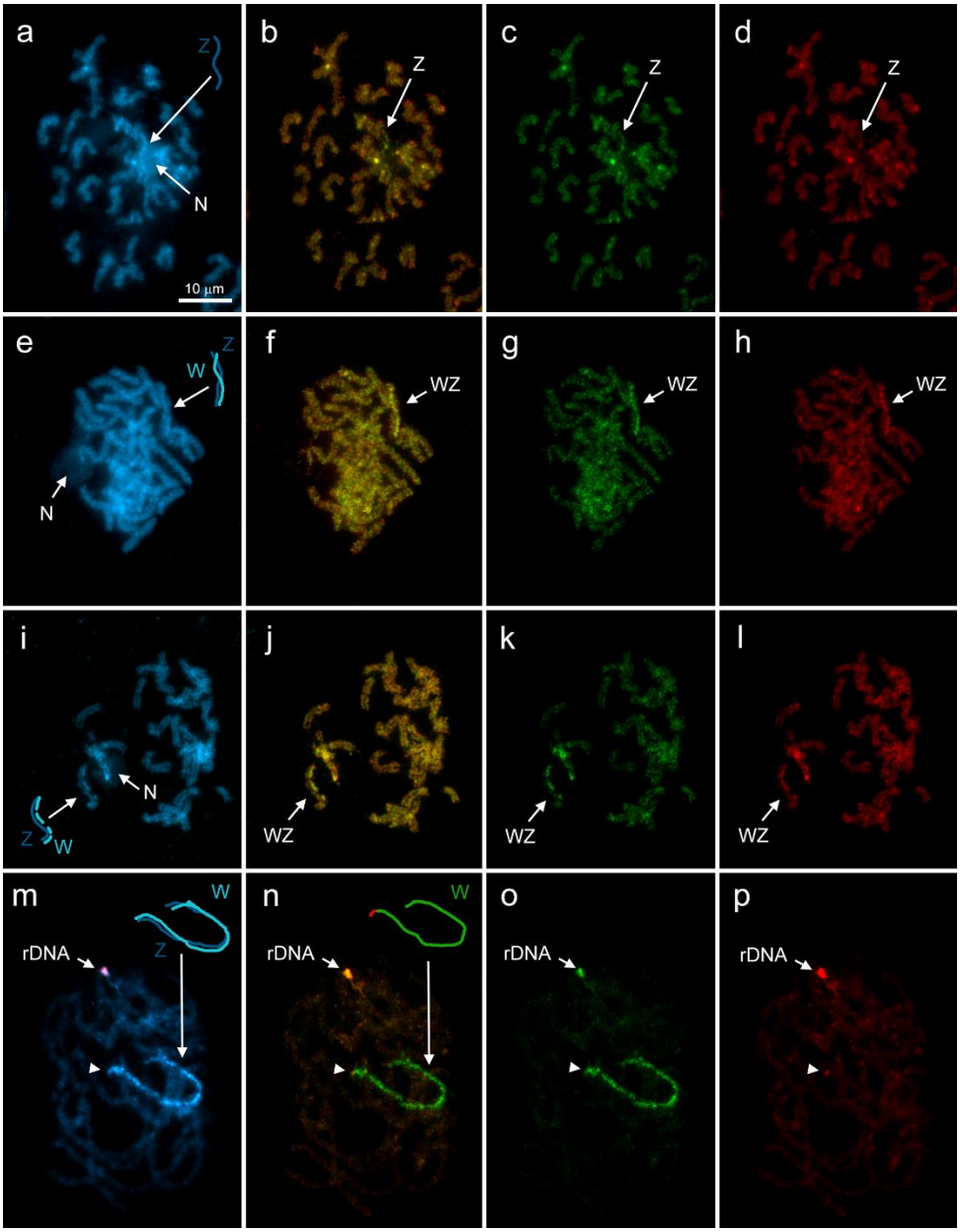


Figure 4. Female-to-male relative gene dose ratio based on qPCR results. Value 0.5 is expected for Z-linked genes, for autosomal genes the expected value would be 1. For summary of qPCR results, see Supplementary Table 3.

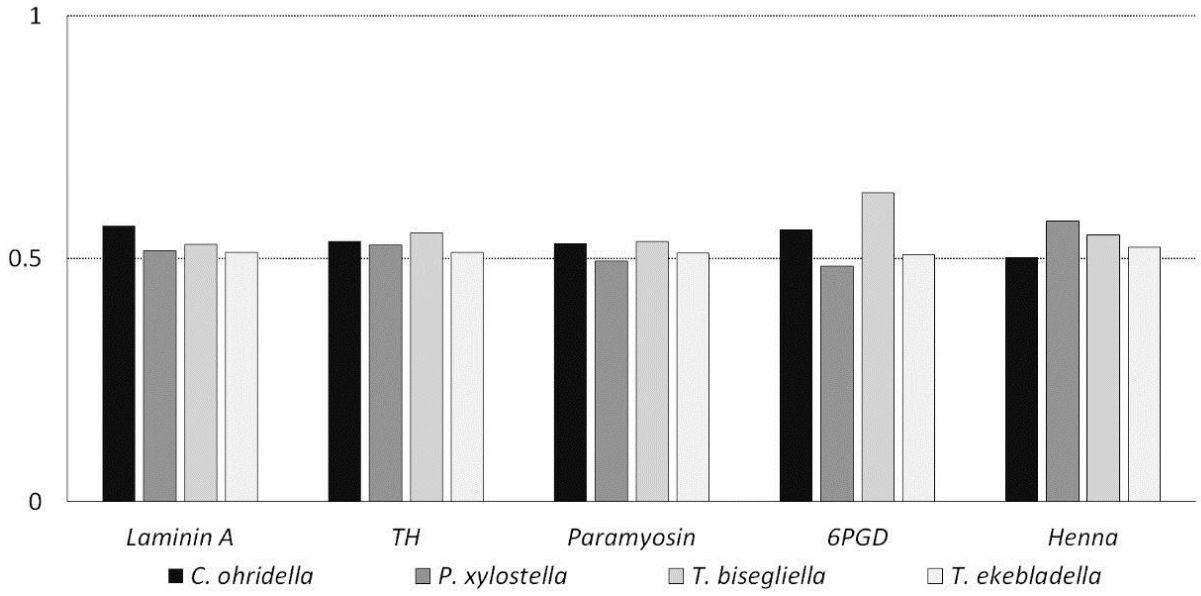
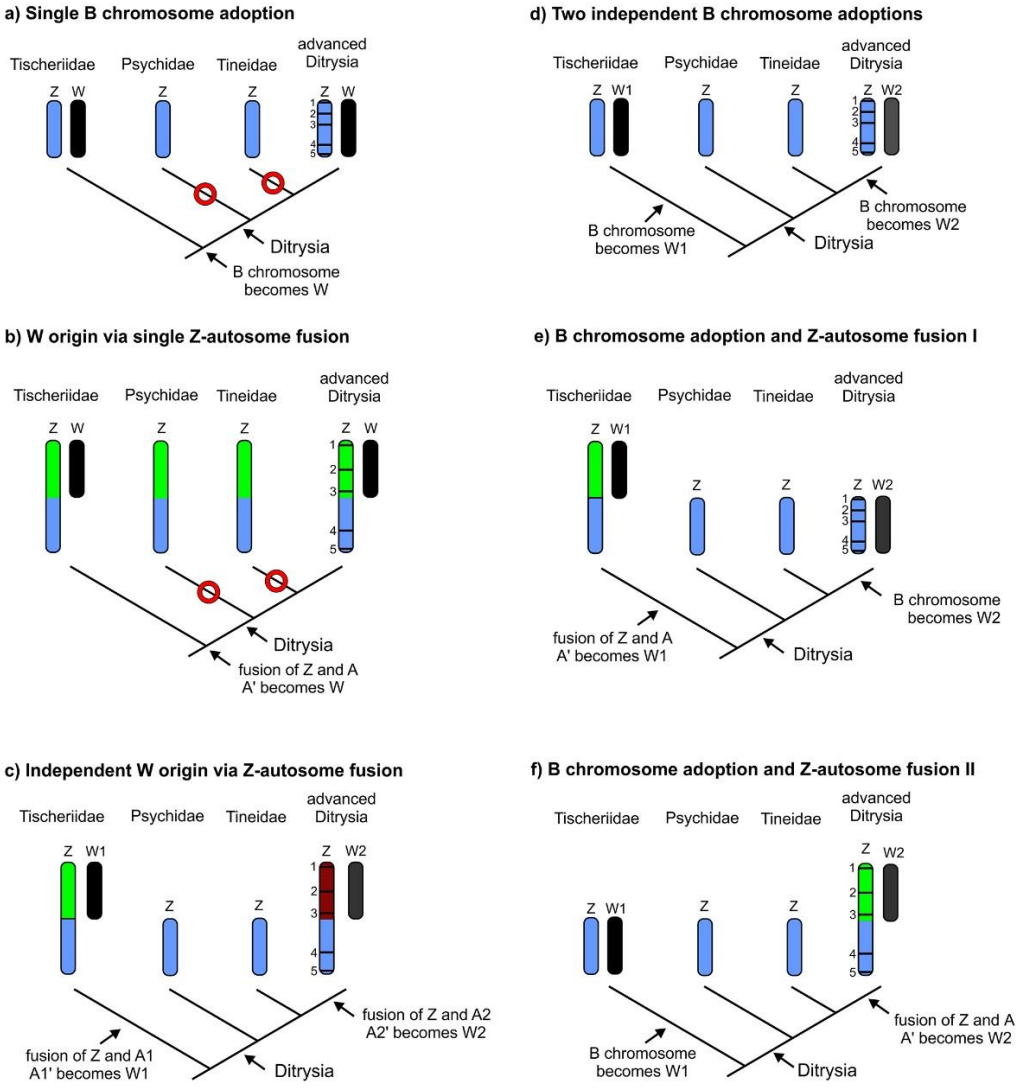
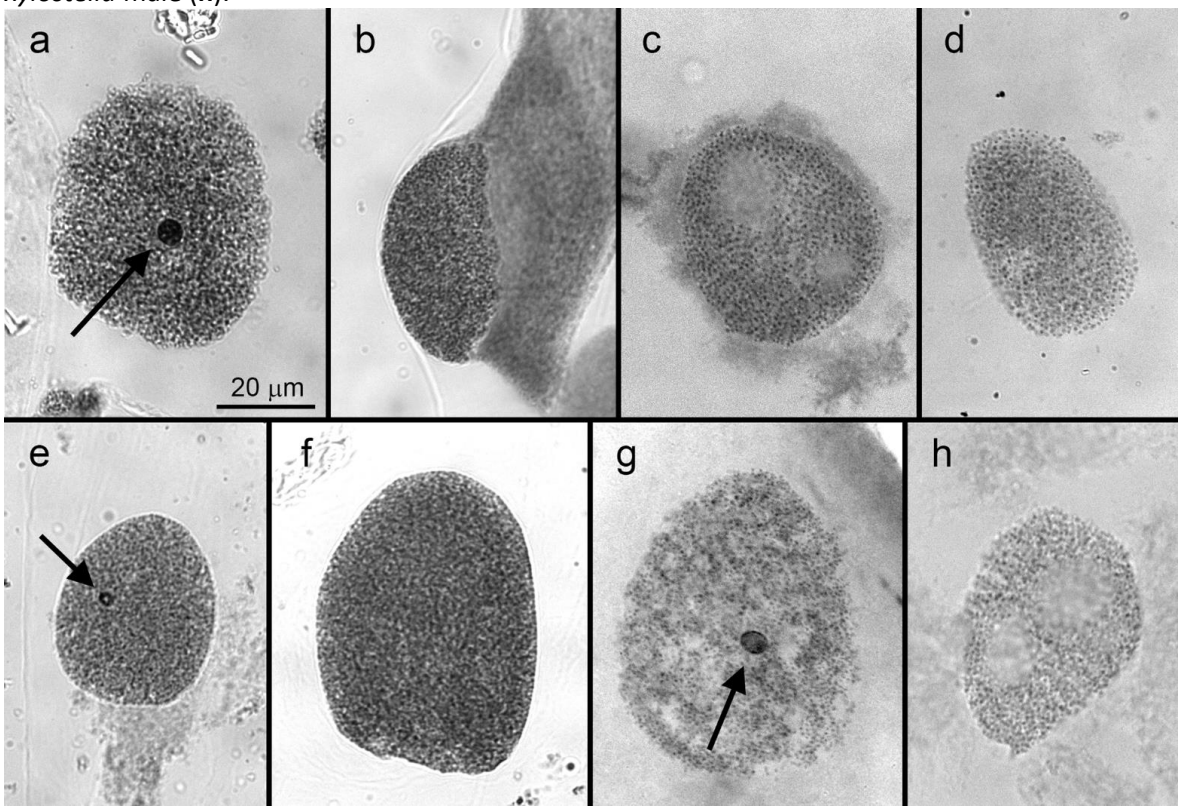


Figure 5. Scenarios of the possible W chromosome origin in Euheteroneura. According to the first scenario (**a**) a B chromosome seized the role of the W chromosome in a common ancestor of Euheteroneura, but was lost secondarily in Tineidae and Psychidae. The second scenario (**b**) describes a single origin of the W chromosome in a common ancestor of Euheteroneura, a group consisting of Tischeriidae, Palaephatiidae (not shown) and Ditrysia. The W chromosome was lost secondarily in Tineidae and Psychidae after branching off from Ditrysia. According to scenarios (**c**) and (**d**) the W chromosome arose twice independently, once in a common ancestor of Tischeriidae and once in a common ancestor of Ditrysia after Psychidae and Tineidae branched off, by chromosomal fusion (**c**) or B chromosome adoption (**d**). In scenarios (**e**) and (**f**) the W chromosome evolve independently by fusion in Tischeriidae and from B chromosome in advanced Ditrysia (**e**) or vice versa (**f**). In scenarios (**c**) and (**f**) part of the marker genes (1-3), which are Z-linked in advanced Ditrysia, should not be present on the Z chromosome in Tineidae and Tischeiidae. In scenarios (**a**), (**b**), (**d**), and (**e**) the Z chromosomes of all three taxa should carry all marker genes. The positions of five Z-linked marker genes are shown as black lines with numbers on the Z chromosome in advanced Ditrysia; markers exclusive for advanced Ditrysia are arbitrarily labeled by numbers 1-3. Scenarios (**a**) and (**b**) are from Lukhtanov (2000); phylogeny is based on Bazinet et al. (2017).

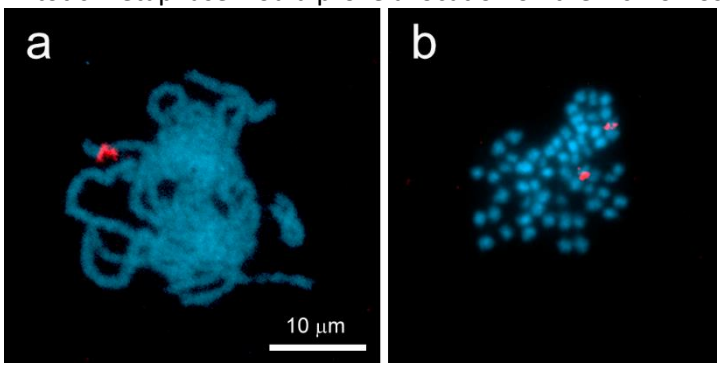


Electronic supplementary material

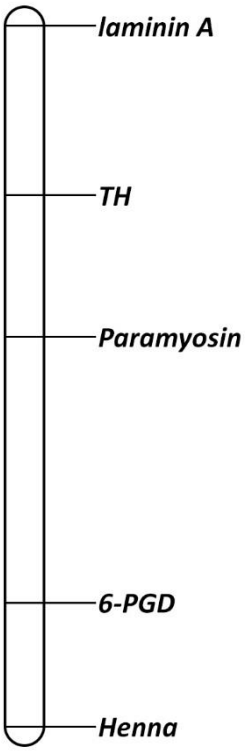
Supplementary Figure 1. Presence or absence of the sex chromatin in polyploid cells of Malpighian tubules. Sex chromatin (arrows) was present in females of *Tischeria ekebladella* (**a**), *Cameraria ohridella* (**e**), and *Plutella xylostella* (**g**), but absent in females of *Tineola bisselliella* (**c**) as well as in males of all four species, i.e. *T. ekebladella* male (**b**), *T. bisselliella* male (**d**), *C. ohridella* male (**f**), *P. xylostella* male (**h**).



Supplementary Figure 2. Location of major rDNA on male and female chromosomes of *Tineola bisselliella* by FISH with the 18S rDNA probe. Chromosomes were counterstained with DAPI (blue). A single cluster of hybridization signals of the probe (red) on male pachytene bivalents (**a**) and two chromosomes with localized hybridization signals in female mitotic metaphase (**b**) clearly show that this species has a single autosomal rDNA cluster. Single chromosome with rDNA signals in female mitotic metaphase would prove a location on the Z chromosome.



Supplementary Figure 3. Scheme of the localization of genes of interest on the Z chromosome of *Biston betularia* based on the physical map published in Van't Hof et al. (2013). For full names of genes, see Supplementary Table 1.



Supplementary Table 1. List of degenerate primers used for obtaining partial gene sequences in *Tischeria ekebladella* and their GenBank accession numbers.

Full gene name	Gene name abbreviation	Forward primer	Reverse primer	GenBank Acc. No.
Acetylcholinesterase 2	*Ace2*	TWTACGGCGGRGGTTACATGAGYGG	ATCCAACTCCASGGTGCATTCAAWG	KY499889
Henna	*Henna*	TAYGARTTYATGGTNGARTGYGARCA	GTYTGNGCRAAYTTDATCATYTT	KY499891
laminin A	*laminin A*	GGNCARGARTTYCAYGTNGC	CAYTTYTTYTGYTCRAANCC	KY499892
Paramyosin	*Paramyosin*	ARAAGGARAARTCSAARTTCCARGC	TGAACTTCYTTRRTDAGYTCRAYRT	KY499893
6-phosphogluconate dehydrogenase	*6-PGD*	GTNATGGGNCARAAYCTNAT	TGNCCNGTCCARTTNGTRTG	KY499890
tyrosine hydroxylase	*TH*	GAYATGAAYCAYCCNGGNTT	CCRAAYTCNACNGTRAACCA	KY499894

Supplementary Table 2. List of primers used for qPCR.

Species	gene	forward	reverse
Tischeria ekebladella	*Ace2*	GACACTATTGTGGCCTCGATG	CCAGGAGTCTCTTCACTTTGC
	Henna	GCTAGCATTTAGAGTGTTTCATAGC	CCAAAAGTTCGTGAACAACATCTG
	6PGD	GAGCGTACGAGATGATTTTGG	GGGACCGGTGAATTCCAAATA
	Paramyosin	GAGAACGTGAGCAAGGAGAAG	TTCAGCTCCTCGATCTTGATG
	TH	ATTCACATTAAGACCAGCCGC	GACGGACGTATTGTGTTGACT
	Laminin A	CTCTTCAGTTCTCCAGGAATGG	GAGAGACAGACATTAGGTGCC
Tineola bisselliella	*Ace2*	GAGGCACCCAAATATTCAAATACAA	ATACGAATATTTTCCAGGATTTGAGG
	Henna	CACACATCTGGTTCTGGTGTATATA	GATTTCTTGGCAGGTTTAGCTTTTA
	6PGD	TAGCATATCTCTCATGACTTGGTAAG	GTGCTGGCCACTTTGTTAAAAT
	Paramyosin	CTTAATGAGCTCGACATTCTCTTG	AGTTGCACATCAAGATTGAGGAG
	TH	TCTGATCTCGATAATTGTAACCACC	GCTCTCTATACTCCTTATCAGCAAA
	Laminin A	TTCGAATGCGGTTCCAATAAATTT	ACTGTTAAGATTGGTAATAAGACACC
Cameraria ohridella	*Ace2*	TACACGGACTGGGAGGAGATAAC	TAGTTTGTAGGGCAGATGAAGAAG
	Henna	CAGTTTTCGCAGGAAATCGGAC	CTTCTCTATGAAGTCATCGGGTG
	6PGD	CCACTTCGTCAAGATGGTCCAC	TCCCTAGGACGTCCTTCATGAG
	Paramyosin	CAGTTCTTCAATCTTGATGTGCAG	TGGAAAATGTGACCAAAGAAAAAGT
	TH	TTTGCTGACAAAGAATACAGAGTGA	GTGTACTCTATTCTCGGGATTTGG
	Laminin A	CAAACGTTATGCTGTTATTGTTGTG	CTAAAGTGCCCTTCTCTGGTTG
Plutella xylostella	*Ace2*	GTACACGGACTGGGAGGAGATC	GGGACACACGAAGAAGTAGTCG
	Henna	CTATTTGCTGATCCCGCCTTCG	GTAGCCAGCTTCTCAATGTAATCAT
	6PGD	GTACGGAGACATGCAGCTCATC	CAGAGGCTAAGAGTTTCTTGTTTTC
	Paramyosin	GAATGTCAGCAAGGAGAAGGTC	GGTTAAGCTCCTCAATCTTGATGT
	TH	CATCCTCAAGACCATCGACAACTA	GATGAGAGCATCGAACTGGATG
	Laminin A	GAGGAGACTGGTCAGTGCAAATG	GTTTGCAGTCAGGGTAGTCGAAG

Supplementary Table 3. Summary of qPCR results. Target gene to reference ratio (*R*) was determined in three biological samples (I-III) in males (M) and females (F) using the reaction efficiencies $E_{Reference}$ and E_{Target}. The mean and its standard error (S.E.) was calculated from these three independent *R* values. Two null hypotheses were tested by unpaired two-tailed *t*-test for unequal variances. In the autosomal hypothesis (A) we tested female-to-male R ratio 1:1, whereas in the Z-linkage hypothesis (Z) the tested female-to-male ratio was 1:2. *P*-value lower than 0.05 means significant difference from tested ratio.

			Target gene to reference ratio (*R*)						*P* value of *t*-test	
Species	Target gene	Sex	sample I	sample II	sample III	$E_{Referemce}$	E_{Target}	mean ± S.E.	A	Z
Plutella xylostella	*Henna*	F	4,889	3,806	3,921	0,745	0,603	4.205±0.343	0,0011	0,1796
		M	7,338	7,456	7,037			7.277±0.125		
	6PGD	F	4,423	2,467	4,207	0,92	0,849	3.699±0.619	0,0037	0,8556
		M	7,720	7,261	7,944			7.641±0.201		
	Paramyosin	F	0,615	0,568	0,471	0,963	0,961	0.551±0.042	0,0013	0,9188
		M	1,040	1,078	1,222			1.114±0.055		
	TH	F	0,064	0,081	0,061	1,012	0,941	0.069±0.006	0,0157	0,7164
		M	0,135	0,104	0,151			0.13±0.014		
	laminin A	F	1,221	0,953	1,037	0,809	0,985	1.07±0.079	0,0041	0,7170
		M	2,109	2,040	2,074			2.075±0.02		
Cameraria ohridella	*Henna*	F	0,260	0,199	0,228	0,946	0,98	0.229±0.018	0,0023	0,9413
		M	0,491	0,401	0,472			0.455±0.027		
	6PGD	F	0,824	0,692	0,778	0,749	0,714	0.765±0.039	0,0003	0,1225
		M	1,424	1,320	1,355			1.366±0.031		
	Paramyosin	F	0,731	0,646	0,642	0,894	0,946	0.673±0.029	0,0002	0,3000
		M	1,242	1,219	1,335			1.265±0.035		
	TH	F	0,609	0,577	0,597	0,983	0,968	0.594±0.009	< 0.0001	0,0535
		M	1,103	1,151	1,074			1.109±0.022		
	laminin A	F	0,970	0,888	1,059	0,869	0,822	0.972±0.049	0,0002	0,0843
		M	1,759	1,698	1,680			1.712±0.024		

Species	Target gene	Sex	Target gene to reference ratio (R)						*P* value of *t*-test	
			sample I	sample II	sample III	$E_{Referemce}$	E_{Target}	mean ± S.E.	A	Z
Tineola bisselliella	*Henna*	F	0,507	0,561	0,486	0,98	0,956	0.518±0.022	0,0054	0,3450
		M	0,987	0,799	1,046			0.944±0.074		
	6PGD	F	0,574	0,566	0,465	0,813	0,83	0.535±0.035	0,0056	0,0527
		M	0,862	0,758	0,908			0.843±0.045		
	Paramyosin	F	0,148	0,152	0,164	0,721	0,83	0.154±0.005	< 0.0001	0,1104
		M	0,293	0,291	0,281			0.288±0.004		
	TH	F	0,436	0,471	0,450	0,84	0,85	0.452±0.01	0,0006	0,0987
		M	0,878	0,755	0,819			0.818±0.036		
	laminin A	F	0,569	0,518	0,596	0,805	0,838	0.561±0.023	0,0003	0,3281
		M	1,118	1,062	0,996			1.059±0.035		
Tischeria ekebladella	*Henna*	F	0,349	0,309	0,371	0,905	0,941	0.343±0.018	0,0003	0,4797
		M	0,634	0,691	0,639			0.655±0.018		
	6PGD	F	1,570	1,336	1,440	0,992	0,88	1.449±0.068	0,0007	0,7904
		M	2,786	2,657	3,090			2.844±0.128		
	Paramyosin	F	2,422	2,273	2,440	0,992	0,844	2.378±0.053	0,0000	0,3835
		M	4,705	4,552	4,674			4.644±0.046		
	TH	F	0,379	0,427	0,493	0,905	0,93	0.433±0.033	0,0004	0,7555
		M	0,850	0,813	0,869			0.844±0.016		
	laminin A	F	2,011	1,934	2,072	0,992	0,856	2.006±0.04	< 0.0001	0,3928
		M	4,050	3,851	3,825			3.909±0.071		

3.4. W-enriched satellite sequence in the Indian meal moth, *Plodia interpunctella* (Lepidoptera, Pyralidae)

Dalíková M., Zrzavá M., Kubíčková S., Marec M.

Chromosome Res (2017) doi: 10.1007/s10577-017-9558-8

Abstract

The W chromosome of most lepidopteran species represents the largest heterochromatin entity in the female genome. Although satellite DNA is a typical component of constitutive heterochromatin, there are only a few known satellite DNAs (satDNAs) located on the W chromosome in moths and butterflies. In this study, we isolated and characterized new satDNA (PiSAT1) from microdissected W chromosomes of the Indian meal moth, *Plodia interpunctella*. Even though the PiSAT1 is mainly localized near the female-specific segment of the W chromosome, short arrays of this satDNA also occur on autosomes and/or the Z chromosome. Probably due to the predominant location in the non-recombining part of the genome, PiSAT1 exhibits a relatively large nucleotide variability in its monomers. However, at least a part of all predicted functional motifs is located in conserved regions. Moreover, we detected polyadenylated transcripts of PiSAT1 in all developmental stages and in both sexes (female and male larvae, pupae and adults). Our results suggest a potential structural and functional role of PiSAT1 in the *P. interpunctella* genome, which is consistent with accumulating evidence for the important role of satDNAs in eukaryotic genomes.

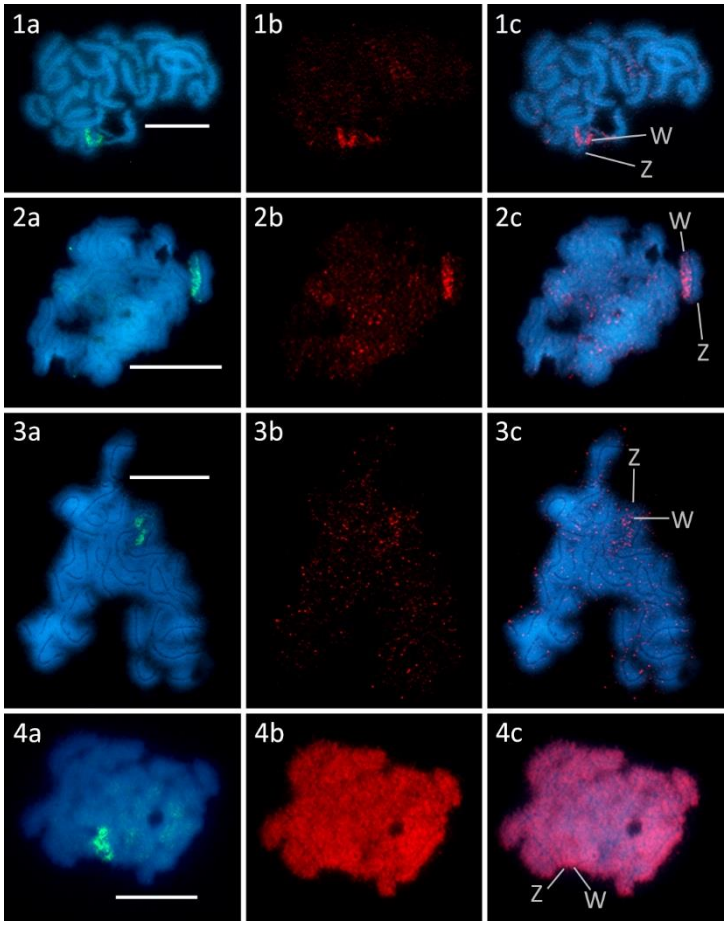
4. Unpublished data

4.1. Meiotic sex chromosome inactivation

Meiotic sex chromosome inactivation (MSCI) is an important phenomenon affecting sex chromosome evolution. Many species have been tested for its presence, but so far MSCI seems to be a widely spread feature of XY sex chromosome systems, whilst absent in chickens, the only tested organism with female heterogamety (Namekawa and Lee 2009; Guioli et al. 2012). Because moths and butterflies represent the largest group of organisms with the WZ/ZZ and derived sex chromosome systems, they are good candidates for further research on MSCI. We studied patterns of several histone modifications frequently associated with MSCI and the presence of an active form of RNA polymerase II by immunolabelling in female pachytene nuclei of two different lepidopteran species, the Mediterranean flour moth (*Ephestia kuehniella*) and the silkworm (*Bombyx mori*). To distinguish the WZ bivalent from autosomes, we used FISH with W chromosome painting probes.

4.1.1. Heterochromatin and transcriptional silencing markers

First we tested the localization of classical heterochromatin markers, histone H3 di- and tri-methylated on lysine K9 (Cowell et al. 2002; Borsatti and Mandrioli 2005; Riddle et al. 2011; Ho et al. 2014). In both species, the dimethylated form of H3K9 showed clear accumulation on the W



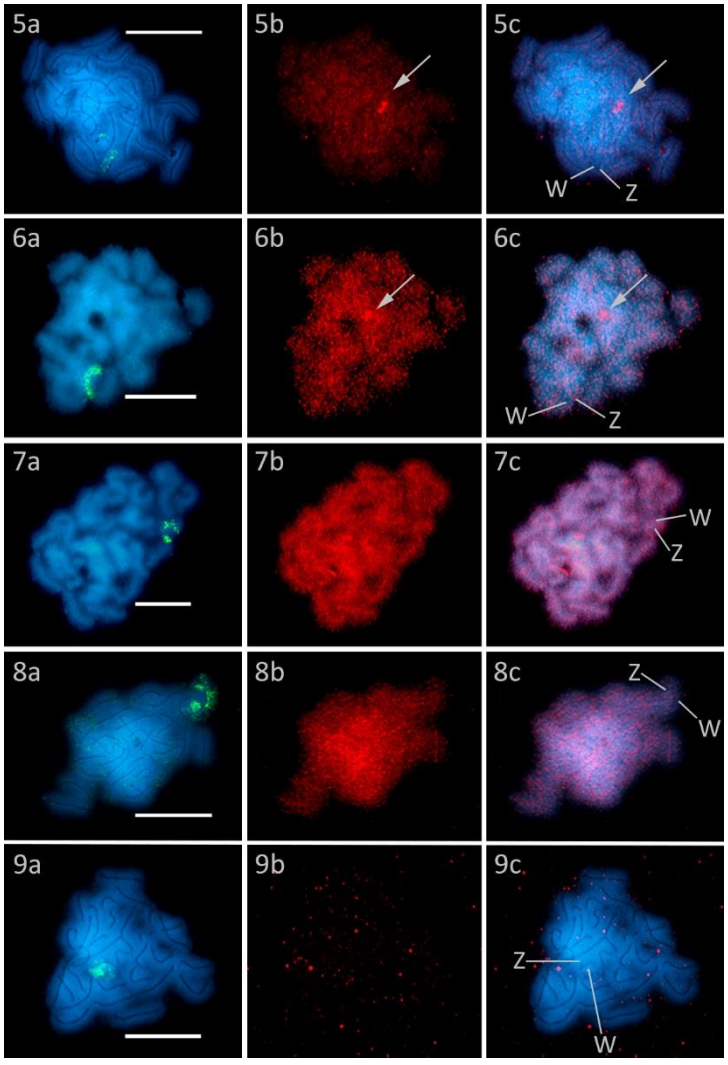
Figures 1-4. Distribution of methylated histone H3 on pachytene oocytes of *Ephestia kuehniella* (**2, 3**) and *Bombyx mori* (**1, 4**). Immunolocalization of histone H3 di-methylated on lysine residue K9 (**1, 2**), histone H3 tri-methylated on lysine residue K9 (**3**), and histone H3 tri-methylated on lysine residue K27 (**4**). (**a**) DAPI stained chromosomes (blue) with the W chromosome detected by FISH with the W-painting probe (green); (**b**) primary antibody distribution detected by secondary antibody conjugated with Cy3 (red); (**c**) DAPI stained chromosomes merged with antibody distribution. Scale bars = 10 µm.

chromosome, while the presence of this modification on both the autosomes and the Z chromosome was very low (Figs. 1-2). The trimethylated version was only slightly enriched on the W chromosome and the difference between the W chromosome and the rest of the genome was less prominent (Fig. 3).

Another repressive mark is trimethylation of lysine K27 histone H3, although this histone is more often associated with facultative heterochromatin (Saksouk et al. 2015) or with heterochromatin during early embryonic development (Vastenhouw and Schier 2012). Surprisingly, this histone modification was highly accumulated on all elements of pachytene nuclei (Fig. 4).

4.1.2. Acetylation of histone H4

Acetylation of lysine residues on histones is generally related to transcriptional activity (Grunstein 1997; Struhl 1998; Smolle and Workman 2013), although some particular variants can have other functions, such as nucleosome positioning, or they can be even found in heterochromatic regions (Turner et al. 1992; Braunstein et al. 1996). Dynamic changes in acetylation are also associated with MSCI (Khalil et al. 2004)

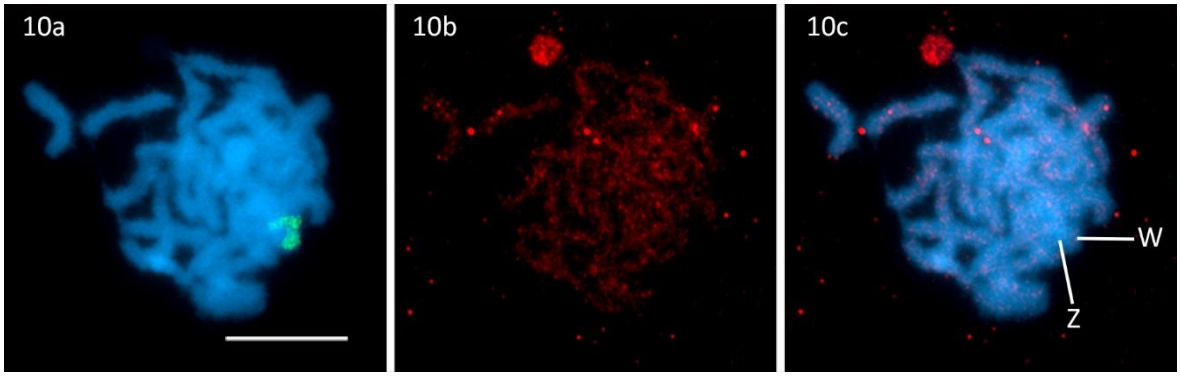


Figures 5-9. Immunolocalization of acetylated and non-acetylated histone H4 on pachytene oocytes of *Ephestia kuehniella* (**5, 8, 9**) and *Bombyx mori* (**6, 7**). Distribution of acetylated histone H4 on lysine residue K5 (**5**), K8 (**6**), K12 (**7**), and K16 (**8**); and of non-acetylated histone H4 (**9**). (**a**) DAPI stained chromosomes (blue) with the W chromosome detected by FISH with the W-painting probe (green); (**b**) primary antibody distribution detected by secondary antibody conjugated with Cy3 (red); (**c**) DAPI stained chromosomes merged with antibody distribution. Arrows indicate autosomal hyperacetylated regions. Scale bars = 10 µm.

We tested the level of acetylation on all possible lysine residues of the N-terminal domain of histone H4 separately. The results in both species were very similar. Moreover, there were no obvious differences between autosomes and sex chromosomes (Figs. 5-8), except small hyperacetylated regions on lysine K5 in *E. kuehniella* (Fig. 5) and on lysine K8 in *B. mori* (Fig. 6). The lowest level of acetylation was observed on K5 (Fig. 5), whereas the highest levels of acetylation were on K12 and K16 (Figs. 7-8). The total hyperacetylation was confirmed by an almost undetectable form of histone H4 without any acetylated residue (Fig. 9).

4.1.3. Detection of RNA polymerase II

To directly evaluate expression on pachytene bivalents, we mapped the distribution of the active C-terminal domain of RNA polymerase II phosphorylated on serine S5 by using a specific antibody. This antibody exhibited very low signals along all chromosomes in pachytene (Fig. 10).



Figs. 10. Immunolocalization of RNA polymerase II C-terminal domain Ser5 phosphorylated in *Bombyx mori*. (**a**) DAPI stained chromosomes (blue) with the W chromosome detected by FISH with W-painting probe (green); (**b**) primary antibody distribution detected by secondary antibody conjugated with Cy3 (red); (**c**) DAPI stained chromosomes merged with antibody distribution. Scale bars = 10 µm.

4.1.4. Conclusions

As expected, in both species the W chromosome is rich in the heterochromatin marker, histone H3 di-methylated on lysine residue K9. However, there is almost no accumulation of the tri-methylated version of this lysine residue. This suggests that the predominant feature of heterochromatin in Lepidoptera is H3K9 dimethylation, similar to that in *Drosophila* (Ebert et al. 2004). Neither of the tested heterochromatin markers are present in a noticeable amount on the Z chromosome.

The overall high level of tri-methylated histone H3 lysine K27 corresponds to the nearly missing RNA polymerase II. This finding indicates a reduced level of transcription in the entire genome during pachytene. Generally low expression in the whole nucleus was previously described in early meiosis of mammals (Page et al. 2012) and birds (Guioli et al. 2012).

Modification	Biological function	W chromosome	Z chromosome	Autosomes
H3K9me2	Heterochromatin[a], X inactivation[b]	high	low	low
H3K9me3	Heterochromatin[a], transcriptional silencing[c]	medium	low	low
H3K27me3	Transcriptional silencing[c], X inactivation[b]	high	high	high
H4K5ac	Transcriptional activation[c]	medium	medium	medium*
H4K8ac	Transcriptional activation[c]	medium-high	medium-high	medium-high^{+}
H4K12ac	Transcriptional activation[c]	high	high	high
H4K16ac	Transcriptional activation[c]	high	high	high

Table 1. Summary of levels of histone modifications on chromosomes from *Ephestia kuehniella* and *Bombyx mori* pachytene oocytes.
An autosomal hyperacetylated region in *E. kuehniella* * and *B. mori*$^{+}$
[a]Cowell et al. (2002) and Ho et al. (2014)
[b]Escamilla-Del-Arenal et al. (2013)
[c]Lawrence et al. (2016)

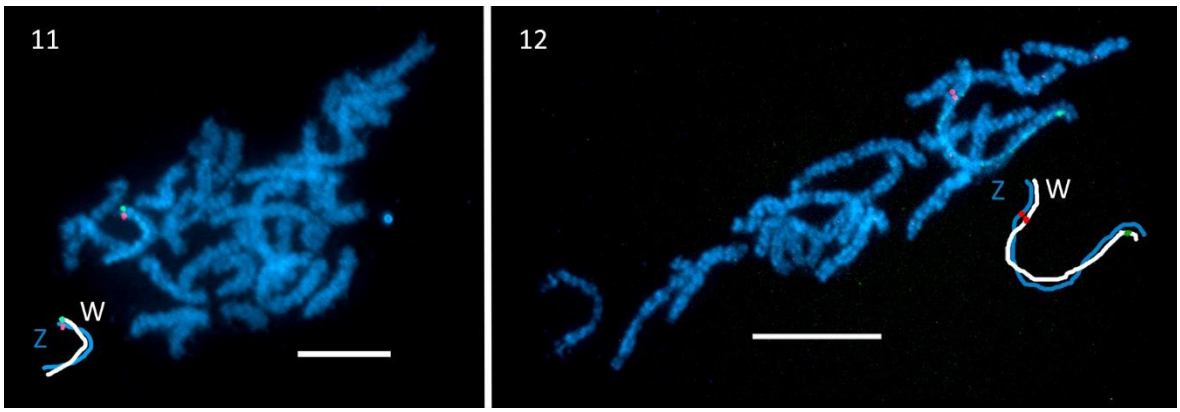
Considering the absence of active transcription, the hyperacetylation of histone H4 including the heterochromatic W chromosome seems to be quite peculiar, especially in the case of acetylation of lysine K16, which is known to be a particularly strong marker of gene activity (Shia et al. 2006). The accumulation of opposing markers (H4 acetylation and H3K27 trimethylation) may be related to the so-called bivalent histone modifications. This phenomenon is well known from mammalian embryonic stem cells, where promoters of developmental genes have often both activating and repressing modifications (Voigt et al. 2013; Matsumura et al. 2015; Harikumar and Meshorer 2015). In addition, the high level of histone acetylation on the W chromosome may indicate the transcriptional activity of this heterochromatic sex chromosome during meiosis, as the ovary-specific transcription of the W chromosome was found in both species, *E. kuehniella* (Traut 1977) and *B. mori* (Kawaoka et al. 2011).

The absence of heterochromatin markers in the Z chromosome together with its overall autosome-like histone modifications (Table 1) in both species examined suggests the absence of MSCI in Lepidoptera. However, further research is needed to make a final conclusion. The optimal way to collect more data would be to study the histone modifications and gene expression from different chromosomes in various stages of meiosis and compare them with the pachytene stage. There would, however, be a problem in determining leptotene/zygotene cells as the early prophase I needs to be identified by some protein markers, often by γH2AX. This histone modification is associated with the ongoing crossing-over (Mahadevaiah et al. 2001). As lepidopteran females have

achiasmatic meiosis, and therefore this marker is not applicable in moths and butterflies. Moreover, the detection of transcripts from different chromosomes by RNA-FISH only in pachytene is useless as according to our results, there is almost no active RNA polymerase II attached to chromosomes during this stage of meiosis.

4.2. Meiotic sex chromosome pairing

The gradual loss of homology between sex chromosomes during their evolution leads to changes in meiotic pairing. Very often, the sex chromosomes pair only through the region with residual homology, the so-called pseudoautosomal region (PAR), while other parts of the chromosomes remained unsynapsed, such as in placental mammals (Checchi and Engebrecht 2011). Birds are, however, an exception to this scenario, as their sex chromosomes form completely synapsed bivalents despite the small PAR (Pigozzi and Solari 1997; Smeds et al. 2014). When sex chromosomes lack any PAR, they can either stay completely unsynapsed, e.g. in marsupials (Page et al. 2005), or pair through some shared sequence, such as intergenic spacers of the nucleolus organizer in *Drosophila melanogaster* (McKee et al. 1992). Although the W and Z chromosome in Lepidoptera obviously lack homology between each other, they form a fully synapsed WZ bivalent as in birds (Marec and Traut 1994). There is, however, little information available regarding the orientation of sex chromosomes in the bivalent. This feature was examined only in a mutant line of *Ephestia kuehniella* with aberrant sex chromosomes, so the results may not be valid for wild type individuals (Marec et al. 2001).



Figures 11-12. BAC-FISH in pachytene oocyte complements of *Biston betularia* (**11**) and *Cydia pomonella* (**12**) with red labelled (Cy3) Z-BAC probes and green labelled (FITC) W-BAC probes. Chromosomes are counterstained with DAPI. The location of hybridization signals is also shown on schematic drawings of WZ bivalents. Scale bars = 10 μm

We decided to take advantage of the newly established method in our laboratory, BAC-FISH, and study the relative position of sex chromosomes in the WZ pachytene bivalent in *Biston betularia* and *Cydia pomonella*. First we tested several BAC clones, derived from the respective W

chromosome, to choose the appropriate markers with acentric localization and discrete signal. In both species, we used a BAC clone carrying the sex-linked gene *kettin* to mark the Z chromosome. Orientation of the W chromosome was detected by a BAC clone (1) with a female copy of the *laminine A* gene in *Biston betularia* (Van't Hof et al. 2013) and (2) with female enriched repeat W27 (Fuková et al. 2007) in *Cydia pomonella*. In both species, the mutual orientation of the sex chromosome markers remained the same in all examined nuclei (Figs. 11-12). This finding may suggest the existence of some shared sequences between the W and Z chromosomes, which helps to guide the specific pairing during meiosis. Since the initiation of sex chromosome pairing is mediated by one chromosomal end in Lepidoptera (Marec and Traut 1994), a suitable candidate for such a sequence could be one of subtelomeric repeats, such as the TRAS mobile element (Kubo et al. 2001). The aberrant WZ pairing in the ASF mutant lines of *Ephestia kuehniella* (Marec et al. 2001) could be then caused by the absence of some chromosomal ends.

5. Synthesis and perspectives

5.1. BAC-FISH as a tool for sex chromosome study

Not long after their discovery (Shizuya et al. 1992), the new high capacity cloning vectors, bacterial artificial chromosomes (BACs), were used as probes in fluorescence *in situ* hybridization, the so-called BAC-FISH (Cai et al. 1995; Hanson et al. 1995; Jiang et al. 1995). Since then this method has gained important place in many cytogenetic studies both in plants and animals including Lepidoptera (e.g. Yamamoto et al. 2008). In this thesis I used BAC-FISH to construct a physical map of an autosome in *Biston betularia* (Van't Hof et al. 2011), localize BAC clones selected according to a gene shared by both sex chromosomes in this species (Van't Hof et al. 2013) and study the mutual orientation of sex chromosomes in the WZ pachytene bivalent in *B. betularia* and *Cydia pomonella* (section 4.2).

The previous studies used W-derived BAC clones as chromosome markers (Sahara et al. 2003b) or to examine interspecific divergence of W chromosome sequences (Yoshido et al. 2007). Here we have shown that even BAC clones originating from highly repetitive chromosomes can be used for positional mapping and can reveal subtle details of the W chromosome structure. In this respect, the hybridization signals of BACs with *laminin A* were particularly interesting (Van't Hof et al. 2013). Out of four BAC clones containing this gene in *B. betularia*, two carried the Z-copy of *laminin* A and two the W-copy. While one of the Z-derived clones hybridized only to the place of its origin, the second clone labelled the end of each sex chromosome. This hybridization pattern implies the presence of common sequences at the end of W and Z chromosomes outside the *laminin A* gene. Since the most likely origin of the W-copy of this gene is through ectopic recombination, the other shared sequence could either be also transferred from the Z chromosome or, on the other hand, be the source of homology enabling ectopic recombination in this region. The W chromosome derived BACs differed in their hybridization pattern as well. While one of them labelled the discrete part at the end of the W chromosome, the other produced scattered signals along the entire chromosome length with stronger signals at the corresponding end. These results demonstrate the repetitive nature of the W chromosome of *B. betularia* with variable copy number of different repeats. However, the results may also indicate that some sequences are dispersed over most of the W chromosome, while others are accumulated in one spot.

To conclude, the BAC-FISH has a broad use in sex chromosome studies in moths and butterflies. It can be used not only for gene mapping and labelling of chromosomes or their parts, but it can also help to better understand the highly repetitive content of the heterochromatic W chromosome.

5.2. W chromosome content and its expression

Since the W chromosome in Lepidoptera is mainly formed by repetitive DNA, the amount of known sequences from this chromosome is quite limited. Several existing studies agree that the dominant type of repeats are LTR and non-LTR retrotransposons (Abe et al. 2005; Fuková et al. 2007; Traut et al. 2013). This is consistent with the general composition of lepidopteran genomes. In *Bombyx mori*, mobile elements form 35 % of the entire genome, and solely retrotransposons occupy 28 % of silkworm genome (Osanai-Futahashi et al. 2008).

The W chromosome is usually the only or the largest block of constitutive heterochromatin in karyotypes of moths and butterflies (Traut et al. 2007). As this type of chromatin is usually composed of satellite DNA (Charlesworth et al. 1994), it is quite surprising that from four previously known satDNAs in Lepidoptera, only two are enriched (Lu et al. 1994; Mandrioli et al. 2003) and one is even depleted (Věchtová et al. 2016)on this sex-specific chromosome, especially considering the variety of long tandem repeats on the W and Y chromosomes in other organisms (Evans et al. 1974; Bonaccorsi and Lohe 1991; Solari and Dresser 1995; Krzywinski et al. 2005; Hobza et al. 2006). In the present study, we described and characterized a new satellite DNA, PiSAT1 in *Plodia interpunctella* (Dalíková et al. 2017). The PiSAT1 represents an important addition to the small collection of lepidopteran long tandem repeats not only for its W-enriched genomic distribution but also for its transcription throughout the development.

The number of protein coding genes on the W chromosome is very low. To date, only a handful of gene sequences are known. Except for several zinc finger transcriptional factors (Nagaraju et al. 2014) other genes represent copies of Z-linked sequences, such as *period* in *Antherea pernyi* (Gotter et al. 1999) and *laminin A* in *Biston betularia* (Van't Hof et al. 2013). Moreover, the circadian clock *period* gene is found in the *A. pernyi* W chromosome in multiple incomplete copies, which are the source of female specific antisense RNA and even truncated protein. However, neither of these product is essential for the core circadian clock mechanisms.

These genes shared between the W and Z chromosome most probably do not represent remnants of previous homology but later transfers. Given that lepidopteran females have achiasmatic meiosis and lack meiotic homologous recombination (Traut et al. 2007), possible mechanisms of duplication Z-linked gene onto the W chromosome are ectopic recombination and retroposition. Both options are likely because the W chromosome contains many repeats that can be interspersed throughout the genome and thus can provide partial homologies with other chromosomes to allow recombination. At the same time, retropositions into gene poor heterochromatic areas cannot interfere either with coding or regulatory regions and thus they are under low to negligible selection pressure.

Despite its heterochromatic nature, transcriptional activity of the W chromosome in ovaries has been known for a long time (Traut 1977), although the sources of this activity have been found quite recently. The W chromosome of *B. mori* encodes numerous female-enriched small piwi-interacting RNAs (piRNAs) (Kawaoka et al. 2008; Kawaoka et al. 2011), which are responsible for silencing transposition in the germline. Moreover, a large portion of piRNAs is also localized in the putative sex determining region. Silkworm females of the W-chromosome-mutated strain KG display many masculine features including expression of testes-specific genes in the ovaries. The modified transcription profiles of ovaries of KG females also exhibit downregulation of female-specific piRNAs (Hara et al. 2012). As shown later one of these piRNAs from the W chromosome is in fact the primary determiner of the sex determination in *B. mori* (Kiuchi et al. 2014). The so-called *Fem* piRNA inhibits mRNA of the Z-linked gene *Masculinizer* (*Masc*) which is required for the male-specific splicing of the *doublesex* gene transcripts. The integral role of the W chromosome in sex determination is not, however, conserved among Lepidoptera (Yoshido et al. 2016).

Additionally, the transcriptional activity of the W chromosome was also shown in other tissues, e.g. W copies of *period* in the brain of *A. pernyi*. In *P. interpunctella*, we found expression of the W enriched PiSAT1 satellite DNA in all developmental stages. Although our current results (Dalíková et al. 2017) do not allow us to exclude PiSAT1 transcription from autosome and/or Z chromosome copies only, this sequence may represent another potentially functional non-coding RNA originating from the lepidopteran W chromosome. As shown in *Drosophila melanogaster*, the Y chromosome can have a massive effect on the transcription of plethora genes both from the autosomes and the X chromosome (Lemos et al. 2008). In general, transcribed repeats from the W chromosome, possibly including the newly discovered PiSAT1, may affect the expression of various genes. Both tandem repeats (Volpe et al. 2002) and mobile elements (Sijen and Plasterk 2003) have been proven to cause their own heterochromatinization via RNA interference pathway. Since copies of some repeats can be scattered throughout the genome, their epigenetic silencing could potentially affect the transcription of adjacent genes due to the position-effect variegation, a phenomenon well known in *Drosophila* (Schotta et al. 2003).

Although the W chromosome was considered to be an almost transcriptionally inert element with limited functions, recent data show its importance in multiple biological functions associated with transcription of non-coding RNAs. As evidence of the significance of such RNAs has accumulated recently (Kaikkonen et al. 2011), this topic represents an interesting field for future research on the W chromosome in Lepidoptera.

5.3. Meiotic sex chromosome inactivation

Meiotic sex chromosome inactivation (MSCI) represents one of the adaptive responses to sex chromosome differentiation and loss of shared sequences. This phenomenon is, however, far from ubiquitous. In this study, we present Lepidoptera as yet another group without MSCI (section 4.1), although our data needs further support. From the currently investigated organisms, MSCI was confirmed in therian mammals (Namekawa et al. 2007; Turner 2007; de Vries et al. 2012) and invertebrates with heterogametic males (Bean et al. 2004; Cabrero et al. 2007; Hense et al. 2007; Vibranovski 2014). This phenomenon is, however, absent in birds (Guioli et al. 2012), monotremes (Daish et al. 2015), and moths and butterflies (section 4.1). It is clear that the evolution of MSCI cannot be attributed to one or the other sex chromosome system, neither has it had a single evolutionary origin. If a rule could be found to describe this, according the available data, the presence or absence of MSCI is mostly dependent on the sex chromosome pairing in given group of organisms. In therian mammals, the sex chromosomes remain largely unsynapsed with changes in the cohesin component of the synaptonemal complex compared to autosomes (Page et al. 2005; Page et al. 2006). Whereas both the avian and lepidopteran WZ bivalents are well-paired and fully synapsed (Marec and Traut 1994; Pigozzi and Solari 1997), the sex chromosomes in platypuses pair only with PARs but form a dense element with accumulated SMC3 cohesin component (Daish et al. 2015). Thus, it seems that MSCI is mainly a specialized version of meiotic silencing of unsynapsed chromosomes (Turner et al. 2004) and evolved to overcome the pachytene checkpoint, which would normally arrest meiosis in cells with incomplete synapses (Roeder and Bailis 2000).

The MSCI results in longer postmeiotic inactivation (Namekawa et al. 2006), which has a dramatic impact on the X and Z sex chromosome, respectively. These chromosomes should be theoretically the ideal place for sex biased genes. Due to their hemizygous state in the heterogametic sex, even recessive mutations are immediately visible for selection, so those mutations favourable for the heterogametic sex should be fixed quickly. However, such genes with testes expression were found to be depleted from the X chromosome in *Drosophila* (Betrán et al. 2002; Vibranovski et al. 2009) and other species with MSCI (Khil et al. 2004), while the absence of MSCI in Lepidoptera should prevent such gene exodus from the Z chromosome. However, two recent studies have brought contradictory conclusions to this topic (Toups et al. 2011; Wang et al. 2012), with the latter, more comprehensive study, suggesting a higher rate of retroposition of ovary specific genes from Z chromosome.

5.4. New insights into the evolution of sex chromosome in Lepidoptera

Because most of the data on sex chromosomes come from a handful of lepidopteran families and mainly from more advanced lineages, we decided to study the W and Z chromosomes

in basal Ditrysia and Tischeriidae. Our data confirmed the strikingly conserved synteny of Z-linked genes (section 3.3, van't Hof et al. 2013; Sahara et al. 2012; Nguyen et al. 2013; Sahara et al. 2013). However, sex-specific chromosomes differed considerably from each other. Of the four tested species, *Tischeria ekebladella* (Tischeriidae) showed accumulated W-specific repeats, *Plutella xylostella* (Plutellidae) contained more interspersed repetitive DNA in the W chromosome, results in *Cameraria ohridella* (Gracillariidae) indicated the presence of neo-sex chromosomes, and results in *Tineolla biseglielle* (Tineidae) showed the complete absence of the W chromosome. Due to the high variability of tested W chromosomes and the overall rapid evolution (Yoshido et al. 2007; Vítková et al. 2007; Yoshido et al. 2013), the origin of the lepidopteran sex-specific chromosome cannot be tracked directly. However, based on the Z chromosome synteny, the presence or absence of the W chromosome, chromosome number, and new phylogeny of basal Lepidoptera(Regier et al. 2013; Bazinet et al. 2017), we could evaluate a number of necessary evolutionary events in individual scenarios of the W chromosome origin (section 3.3 Fig. 5). Although both currently favoured hypotheses about a single origin of the W (Lukhtanov 2000; Traut et al. 2007) are definitely possible, we proposed new scenario with two independent origins of the W chromosome via an autosome fusion and B chromosome acquisition, which is more parsimonious. Unfortunately, we have not been able to use any weighed method to distinguish between individual hypotheses. It is currently impossible to evaluate the probability of loss of W chromosome nor the formation of neo-sex chromosomes as both events happened numerous times in different lepidopteran families (Traut et al. 2007; Nguyen and Carabajal Paladino 2016).

Sex chromosomes have fascinated researchers since their discovery as they represent part of the genome with many intriguing characteristics. Long-term research on their evolution and differentiation, focused on limited amount of model organisms, has given rise to many hypotheses and has revealed a number of general phenomena. Modern methods of molecular biology allow us to expand the spectrum of studied groups of plants and animals. Recent research on sex chromosomes in non-model species, including this thesis, sheds new light on the forces behind the sex chromosome evolution and demonstrates the importance of broadening the spectrum of species of interest.

6. References

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7. Curriculum vitae

Martina Dalíková

Birth Date: 6.7.1984
Birth Place: Havlíčkův Brod, Czech Republic
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Education

2006 Bachelor's degree with honours in Biology, Faculty of Biological Sciences, University of South Bohemia. Thesis: Histone H4 acetylation on the W chromosome of Mediterranean flour moth *Ephestia kuehniella*.

2009 Master's degree with honours in Experimental Biology, Faculty of Science, University of South Bohemia. Thesis: BAC clones as a tool for the study of codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) W chromosome.

Since 2009 PhD studies, Faculty of Science, University of South Bohemia, Thesis: Mechanisms of molecular differentiation of sex chromosomes in Lepidoptera and their evolution.

Research interests

Sex chromosome evolution, epigenetics, karyotype evolution, comparative genomics

Internships

2008 Bacterial artificial chromosome (BAC) library of *Cydia pomonella* screening (two months stay), Dr. Lisa G. Neven lab, USDA ARS, Wapato, Washington, USA

2010 Synaptonemal complex chromosomal preparations and immunodetection (one month stay), Dr. María I. Pigozzi lab, Faculty of Medicine, University of Buenos Aires, Argentina

Teaching and Consulted Thesis

- Genetics (KGN 245) and Cytogenetics (KGN 604) practical courses
- Lectures and practical courses for high-school students and teachers
- Voleníková A. (2012) The occurrence of an aberrant heterochromatin in the Mediterranean flour moth *Ephestia kuehniella* (Lepidoptera). Bc. Thesis, in Czech, 43 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Grants

Grant SGA2008/019 "The difference in the level of histone H3 methylation on W chromosome of *Ephestia kuehniella* between somatic and reproductive cells" of the Student Grant Agency of Faculty of Science, University of South Bohemia in České Budějovice

Grant 067/2012/P "The meiotic sex chromosome inactivation in Lepidoptera" of the Grant Agency of University of South Bohemia in České Budějovice

Selected Conferences

Dalíková M., Marec F. (2014) Whole genome silencing but no MSCI in meiosis of lepidopteran females In: 9th International Workshop on the Molecular Biology and Genetics of the Lepidoptera, 17-23 August 2014, Kolympari, Greece, talk.

Dalíková M., Marec F. (2013) Meiotic sex chromosome inactivation in Lepidoptera: new data. In: 19th International Chromosome Conference, 2-6 September 2013, Bologna, Italy, poster.

Dalíková M., Pigozzi M.I., Toscani M.A., Marec F. (2011) Is the lepidopteran Z chromosome meiotically inactivated? In: 18th International Chromosome Conference, 29 August - 2 September 2011, Manchester, UK, poster.

Dalíková M., Vítková M., Neven L.G., Nguyen P., Šíchová J., Fuková I., Marec F. (2009) BAC clones as a tool for the study of the codling moth sex chromosomes. In: The 8th International Workshop on the Molecular Biology and Genetics of the Lepidoptera, 23-29 August 2009, Kolympari, Greece, poster.

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Publications

Dalíková M., Zrzavá M., Kubíčková S., Marec M. (2017) W-enriched satellite sequence in the Indian meal moth, *Plodia interpunctella* (Lepidoptera, Pyralidae). *Chromosome Res* doi: 10.1007/s10577-017-9558-8

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Věchtová P., **Dalíková M.**, Sýkorová M., Žurovcová M., Füssy Z., Zrzavá M. (2016) CpSAT-1, a transcribed satellite sequence from the codling moth, *Cydia pomonella*. *Genetica* **144**: 385-395.

Nguyen P., Sýkorová M., Šíchová J., Kůta V., **Dalíková M.**, Frydrychová R., Neven L.G., Sahara K., Marec F. (2013) Neo-sex chromosomes and adaptive potential in tortricid pests. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 6931-6936.

Šíchová J., Nguyen P., **Dalíková M.**, Marec F. (2013) Chromosomal evolution in tortricid moths: conserved karyotypes with diverged features. *PLoS ONE* **8**: e64520.

Van't Hof A.E., Nguyen P., **Dalíková M.**, Edmonds N., Marec F., Saccheri I.J. (2013) Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism. *Heredity* **110**: 283-295.

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Fuková I., Neven L.G., Bárcenas N.M., Gund N.A., **Dalíková M.**, Marec F. (2009) Rapid assessment of the sex of codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) eggs and larvae. *Journal of Applied Entomology* **133**: 249-261.

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