University of South Bohemia in České Budějovice

Faculty of Science

Cytogenetic analysis of sex chromosomes in representatives of the tribe Heliconiini

Bachelor thesis

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Annotation

The present thesis focuses on sex chromosomes in *Dryas iulia* and *Eueides isabella* from the tribe Heliconiini. It highlights the importance of verification of in silico results with cytogenetics.

Declaration

I hereby declare that I am the author of this Bachelor thesis and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 13 .4. 2023

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Magdaléna Vališová

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I would like to thank my supervisor Petr Nguyen who gave me such an amazing opportunity to work on this very insightful and revolutionary project. He shared his extensive knowledge with me, was very patient and kindly answered all my questions. He accepted me to his lab, where I immediately felt at home, and I got to work with so many kind and loving people.

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1. Introduction

1.1. Sex chromosomes

Sex chromosomes have evolved independently in different taxa. Some of them are well conserved and old (for example mammals and birds), others appear to be more short-lived. For example, *Musca domestica* replaced its XY chromosomes with new ones derived from autosomes (Meisel et al., 2020). Sex chromosomes are a rapidly evolving part of the genome, and they are also one of the most prominent parts of the chromosome complement regarding their degree of heterochromatinization and their behavior during meiosis. This is true especially for the Y chromosome (in XY systems) and W chromosome (in WZ systems) (Sahara et al., 2012). They differ from autosomes, even though most sex chromosomes originate from autosomes. During evolution the pair of sex chromosomes accumulated genetic changes, which distinguished them from autosomes and from one another (Traut et al., 2019). The main role of sex chromosomes lies in sex determination – they are the source of first sex-determining signal, and they secure an equal sex ratio among offspring (Sahara et al., 2012).

The widely accepted hypothesis about evolution of sex chromosomes says, that sex chromosomes developed from a pair of autosomes, which gained the sex-determining function (Bull, 1983, Charlesworth, 1991, Traut, 1994). For the sex chromosome differentiation is also crucial a region which is very closely linked to the sex determining sequence. The differentiation of a chromosome restricted to a heterogametic sex is a result of restricted recombination (Charlerworth, 1991, Bergero & Charlesworth, 2009). The process of sex chromosome differentiation includes crossover suppression which often spreads among the whole length of the chromosome. Crossover suppression is a result of sexually antagonistic genes, which occur on the chromosomes (Rice, 1987). As a result, sex chromosomes evolve separately in the segments, where crossover is not happening and accumulate differences in molecular composition and structure. In heterogametic species the degrading chromosome (Y or W) can lose some of its genes without negatively affecting the fitness of the individual (Muller, 1918). The loss of genes cannot be reversed via crossover. The sex chromosome often loses most of the informational content and tends to degenerate except for sex-specific functions like sex determination or fertility (Traut, 1994). Among insect degree of differentiation between the two sex chromosomes differs greatly in different groups (Kaiser & Bachtrog, 2010). In the order Lepidoptera, the W chromosome in often smaller than Z chromosome and forms heterochromatic bodies in interphase nuclei (Traut et al., 2008).

The sex chromosome systems occur in diploid organisms such as animals and flowering plants. The first is the sex chromosome system XX/X Y with male heterogamety, which means that males carry two different alleles X and Y, and females are homogametic carrying two X chromosomes. This system can be observed in mammals and flies. The other chromosomal system is WZ/ZZ. In this case, the females are heterogametic with W and Z chromosomes and the males are homogametic with constitution ZZ. This system can be found for example in birds and butterflies (Yoshido et al., 2005). Systems with heterogametic males are more common in nature (Traut, 1996) and female heterogametic systems are understudied.

1.2. Lepidopteran cytogenetics

The chromosome number is known in more than 1000 lepidopteran species (Robinson, 1971). Most lepidopteran species have maintained conserved karyotype during evolution. The haploid number of chromosomes is in the range of n = 29 to n = 31 (Robinson, 1971). The most common chromosome number n = 31 is considered to be ancestral. It probably evolved more than 125 million years ago in common ancestor of group Ditrysia which includes 98 % of the representatives of order Lepidoptera we know today (Ahola et al., 2014).

Order Lepidoptera with their sister clade Trichoptera contain species with holokinetic chromosomes. Holokinetic chromosomes do not have structurally defined centromere and their kinetochore form along the whole length of a chromosome (Wolf, 1996, Neumann et al., 2012).

However, absence of localized centromere complicates identification of individual chromosomes. Moreover, lepidopteran chromosomes are typically numerous and small with uniform shape (Kreklová, 2017). That is why pachytene chromosomes are preferably used in cytogenetic analyses as they are much longer and show a specific chromomere pattern. Yet even chromomere pattern does not allow identification of all pachytene bivalents. For instance, only XYZ bivalents were identified in *Bombyx mori* by Traut (1976). The field of lepidopteran cytogenetics was revolutionized by molecular-cytogenetic methods such as fluorescence in situ hybridization and its variations (for example comparative genomic hybridization – CGH or genomic in situ hybridization – GISH), which enabled fluorescence tagging of specific part of genome and identification of individual chromosomes (Goldsmith MR, 2010). Thanks to these methods, we are now able to identify and observe W chromosomes in many lepidopteran species under fluorescent microscope.

1.3. W chromosome in Lepidoptera

Lepidoptera are the most species rich group with heterogametic females (Dalíková et al., 2017). Some of the species in this order have chromosomal system WZ/ZZ (♀/♂), while others are missing the W sex chromosome and have chromosomal system Z0/ZZ (♀/♂). This can be either an ancestral state in early diverging lineages or result of secondary loss of the W sex chromosome (Hejníčková et al., 2019, Traut et al., 2008). W chromosome has been found in 98 % of the species in the order Lepidoptera. Further variation in sex chromosome system in some species can be caused by sex chromosome fission or their fusion with an autosome (Traut et al., 2008). Lepidopteran Z and W chromosomes can be similar in their size but differ greatly in their structure. Z chromosome has similar features to autosomes and is rich in genes. Many studies confirmed highly conserved synteny of Z-linked genes across the order Lepidoptera (Beldade et al., 2009, Van'T Hof et al., 2013, Fraïsse et al., 2017, Dalíková et al., 2017, Hejníčková et al., 2019). W chromosome, however, is poor in genes and accumulates many repetitive and sequences (Kaiser & Bachtrog, 2010).

There are two hypotheses on the origin of the lepidopteran W chromosome (Lukhtanov, 2000). The first suggests that the W chromosome originated from fusion of the ancestral Z chromosome with an autosome (Figure 1). This would mean that the W chromosome is in fact a neo-W of autosomal origin (Traut et al., 2008). The second hypothesis says that W chromosome evolved from a supernumerary chromosome (Figure 2), the so-called B chromosome, in Tischeriidae and Ditrysia (Dalíková et al., 2017). The B chromosomes are not present in all species, but only in some populations or even only in some individuals (Camacho et al., 2000). The commonly accepted theory is that B chromosomes evolved from regular A chromosomes (Jones & Rees, 1982) are a byproduct of standard karyotype evolution. They could evolve from centric fragments which are a result of chromosomal fusion or from pericentromeric segment of fragmented chromosome. They do not follow the Mendelian inheritance as they do not segregate regularly in mitosis and meiosis (Camacho et al., 2000).

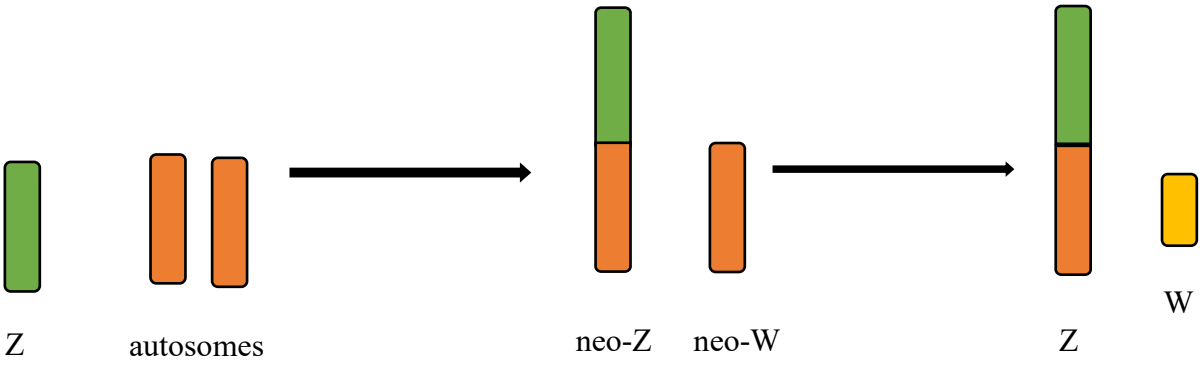

Fig. 1: Evolution of W chromosome from fusion between Z chromosome and autosome, creating neo-W chromosome.

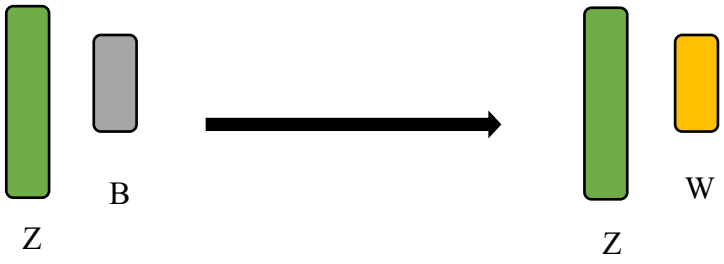

Fig. 2: Evolution of W chromosome from B chromosome.

1.4. Genomics of Lepidoptera

In the order Lepidoptera, complete physical maps based on genes were only constructed for a few species. First, it was the silkworm (*Bombyx mori*) (Yasukochi et al., 2006), another species was the tobacco hornworm (*Manduca sexta*) (Yasukochi et al., 2009). The chromosome map is also known for two butterflies *Heliconius Melpomene* (Jiggins et al., 2005, Pringle et al., 2007) and *Bicyclus anynana* (Beldade et al., 2009). Gene mapping for these species was performed either via genetic linkage analysis, which is a very time-consuming method or via locating the genes physically using the method fluorescence in situ hybridization with bacterial artificial chromosomes (BAC FISH) which requires a BAC library.

The first sequenced lepidopteran genome was the genome of the silkworm *Bombyx mori*, which is an important model in insect genetics (The International Silkworm Genome Consortium, 2008). First drafts were published even sooner in 2004 by Chinese and Japanese researchers which independently started whole genome sequencing of male *B, mori* (Mita et

al., 2004, Xia et al., 2004). Unfortunately, these data were not sufficient for building the whole genome of the insect due to their low sequencing coverage and lack of fosmid or BAC libraries. Four years later, the whole genome was put together. It was discovered that the genome of *B. mori* contains 14 623 genes, which is only slightly more than number of genes in *Drosophila melanogaster*, the main insect model in biomedical science sequenced earlier in March 2000 (Adams et al., 2000). 3 223 of the *B. mori* genes were species-specific (The International Silkworm Genome Consortium, 2008).

The big revolution in genome sequencing came with next generation sequencing (NGS) technologies. The goal of NGS is to make sequencing faster and cheaper. It can be achieved by processing a big amount of DNA fragments in parallel on one sequencing platform. With the advances in sequencing technologies, the Earth BioGenome Project set a goal to sequence all known eukaryotic species on our planet, which has been adopted by many national initiatives. For instance, The Darwin Tree of Life Project aims at assembling sequences from as many specimens as possible in the islands of Britain and Ireland and releasing these sequences to public. To this day (12.4.2023), 168 chromosomally complete assemblies for order Lepidoptera were published (Darwin Tree of Life Project Consortium, 2022, https://portal.darwintreeoflife.org/).

The first genomes sequenced using NGS was the one of the Monarch butterflies *Danaus plexippus* (Zhan et al., 2011) and *Heliconius melpomene* (The Heliconius Genome Consortium, 2012).

1.5. Sex chromosome sequencing

Despite the fact that X and Y (or Z and W) chromosomes both have their origin in a pair of autosomes, they differ from each other significantly. It is because the Y and W chromosomes are partly or fully eliminated from the process of recombination during meiosis and they become very different from recombining autosomes and X or Z sex chromosomes (Traut et al., 2013). The Y and W chromosomes have lost most of their protein-coding genes, kept only few especially the ones with sex-specific functions and acquired some new genes from autosomes (Alföldi, 2008, Carvalho et al., 2001, Skaletsky et al., 2003). It is very difficult to sequence Y and W chromosomes, due to the number of repetitive sequences they contain. The sequence is hard to assemble, and it requires special assembly programs, accurate maps and a wide range of libraries. These are the reasons why Y and W chromosomes have been usually excluded from whole genomic sequencing and the structure of most of them remains unknown

(Traut et al., 2013). On the other hand, the sequencing technologies are rapidly evolving and sequencing whole sex chromosomes has become feasible (Tomaszkiewicz et al., 2017, Carey et al., 2022).

In Lepidoptera, some genome projects reported sequences of W chromosomes albeit not correct. For example, report of the *Cydia pomonella* genome by Wan et al. (2019) showed that the W chromosome sequence is much smaller than expected based on previous cytogenetic analyses (Fuková et al., 2005). Yet another example of dubious W chromosome sequence was reported in *Kallima inachus* (Yang et al., 2020). The report indicated that the W chromosome is rich in genes, which is in stark contrast with our current knowledge on molecular composition of W chromosomes in Lepidoptera (see above).

In 2021 the genome sequence of *Dryas iulia* was published along with its putative W chromosome, which should be very small with the length of only 2,2 Mb. The research was done again only in silico with the use of bioinformatic methods. The whole genome of *Dryas iulia* was assembled into 31 chromosomes and the remaining sequences ("chromosome 32") were declared as the chromosome W. The evidence for the sequences being chromosome W was absence of similarity to the other 31 chromosomes, but it was not confirmed via molecular or cytogenetic methods. Lewis et al. (2021) supported their findings by referring to a small W chromosome of the postman butterfly *Heliconius melpomene* (Jiggins et al., 2005). However, the referred article focuses only on genetic map which cannot indicate a size of the non-recombining W chromosomes. Lewis et al. (2021) also used the W sequence in support of the hypothesis of W chromosome originating from a B chromosome. The assumption for the W chromosome evolving from B chromosome is that the structure of W chromosome is very repetitive, contains domesticated repetitive elements, and the sequence of the chromosome has no shared features with Z chromosome or autosomes (Lewis et al., 2021). The authors also compared W chromosome in *Dryas iulia* with other lepidopteran female genomes and found no matches. Therefore, they concluded that W chromosome evolved independently multiple times throughout the order Lepidoptera.

2. Aims

It is vital to confirm results obtained in silico with molecular methods (Traut et al., 2017). This crucial step was omitted by Lewis et al. (2021) when publishing the genome sequence of *Dryas iulia* including its chromosome W. The aim of this thesis was to verify the presence of W chromosome, its size and differentiation in representatives of the tribe Heliconiini *Dryas iulia* and *Eueides isabella*. Partial aims were (i) to verify species identity of available material (by barcoding) and (ii) detect the W chromosome by means of genomic in situ hybridization.

3. Material and methods

3.1. Insects

The pupae of *Dryas iulia* and *Eueides isabella* were bought at Stratford Butterfly Farm, UK and shipped via mail. The pupae as well as adult butterflies enclosed in our laboratory under ambient conditions were used for chromosome preparations and for verifying the species by barcode.

3.2. Chromosome preparations

In each species meiotic chromosomes were obtained from ovaries using methods following the protocol by Mediouni et al. (2004). The ovaries were dissected in physiological solution for *Ephestia kuehniella* (0,9% NaCl, 0,042% KCl, 0,025% $CaCl_2$, 0,02% $NaHCO_3$; Glaser 1917 cited in Lockwood 1961) under a stereo microscope. They were fixed in freshly prepared Carnoy´s fixative (ethanol:chloroform:acetic acid, 6:3:1) for 15 minutes. The ovaries were transferred using wolfram needle onto SuperFrost slide cleaned in acidic ethanol (1% HCl in ethanol). They were macerated in a drop of 60% acetic acid and the drop of resulting cell suspension was spread with the wolfram needle on a hot plate at 45 °C until it nearly evaporated. The slides were then dehydrated in an ethanol series, air dried, and stored at -20 °C until further use.

The pupal and adult carcasses were frozen in liquid nitrogen and stored at -80 °C until further use for DNA extraction.

3.3. DNA extraction

Genomic DNA (gDNA) used for probe construction and barcode was extracted from male and female pupae and adult carcasses using cetyltrimethylammonium bromide (CTAB) method following the protocol by Winnepenninckx et al. (1993). Briefly, the tissue was homogenized and mixed with an extraction buffer (0,1 g tissue/2,5 ml buffer) containing 2% CTAB, 100 mM Tris-HCl (pH 8), 40 mM EDTA, 1,4 M NaCl, 0,2% β-mercaptoethanol a 0,1 mg/ml proteinase K and incubated for 15 to 17 hours at 37 °C. The same amount of stabilized chloroform was then added to the lysed tissue, thoroughly mixed, and centrifuged at 9000 rpm for 15 minutes at 4 °C. The water phase with genomic DNA was then transported to a clean Eppendorf tube. RNA was eliminated by using RNase with a concentration of 50 mg/ml extraction buffer. It was incubated for 30 minutes at 37 °C. A third of the volume of isopropanol was added to precipitate DNA which was sedimented by centrifuging the sample

for 15 minutes at 4 °C at 9000 rpm. The supernatant was then discarded, and DNA was washed with 70% ethanol and air dried for 2 minutes for the residual ethanol to evaporate. DNA was dissolved in 50 µl of water and stored at -80 °C until further use.

The concentration of DNA was measured by Qubit 4 with broad range DNA kit (Thermo Fisher Scientific) and the purity was checked via electrophoresis and Nanodrop one (Thermo Fisher Scientific).

3.4. Barcode

The species identity of studied butterflies was verified using barcoding according to Hebert et al. (2004) with slight modifications. Partial mitochondrial COI sequence was amplified using universal lepidopteran primers: forward primer Lep-F1 5′-ATTCAACCAATCATAAAGATAT-3′ and reverse primer Lep-R1 5′-TAAACTTCTGGATGTCCAAAAA-3′. The reaction mix of total volume 25 µl contained: 10 ng template DNA, 0,5 µM primer Lep-F1, 0,5 µM primer Lep-R1, 0,2 mM dNTP, 1x *ExTaq* buffer and 2 U *ExTaq* Polymerase (TaKaRa, Otsu, Japan). The thermocycling profile was one cycle of 1 minute at 94 °C, six cycles of 1 minute at 94 °C, 90 seconds at 45 °C, and 75 seconds at 72 °C, followed by 36 cycles of 1 minute at 94 °C, 90 seconds at 51 °C, and 75 seconds at 72 °C, with a final step of 5 minutes at 72 °C. The PCR product was purified using Wizard SV Gel & PCR Clean Up System (Promega, Madison, WI, USA) and the concentration was measured by Qubit 4 with broad range DNA kit (Thermo Fisher Scientific). Each PCR product was sequenced from both ends by SEQme, s.r.r. (Dobříš, Czech Republic) using the barcoding primers. The sequences were processed in Geneious Prime program (version 2022.1.1). Low quality bases were trimmed, the reverse complement was created for reverse sequence, forward and reverse sequences were aligned based on complementarity. The resulting consensus sequence was compared against NCBI nt/nr collection and boldsystems.org using megablast.

3.5. Probe construction

Probes for genomic in situ hybridization (GISH) were constructed from female gDNA using the nick-translation method using Cy3- dUTP (Jenna Bioscience, Jena, Germany). The 40 µl labeling reaction mix contained 2 µg DNA, 1x nick-translation buffer (5 mM Tris-HCl, 0,5 mM MgCl2, 0,0005 % BSA; pH 7,5), 10 mM β-mercaptoethanol, 50 µM dATP, 50 µM dCTP, 50 µM dGTP, 10 µM dTTP, 20 µM Cy3-dUTP, 40 U DNA polymerase I (Thermo Fisher Scientific, Waltham, MA, USA) and 0,01 U DNase I. This mix was incubated for 3,5

hours at 15 °C. The reaction was stopped with 10x loading buffer u (50% glycerol, 250 mM EDTA, 5,9 mM bromphenol blue). The probes were stored at -20 °C until further use.

3.6. Genomic in situ hybridization

To detect female specific sequences on the W chromosome, genomic in situ hybridization was performed following the protocol by Mediouni et al. (2004). The Cy3-labeled female whole genome DNA was used as a probe. A hybridization mix contained 250 ng of the probe, 3 μg of male DNA fragmented by heat at 99 °C for 20 minutes, 25 μg of salmon sperm DNA (Sigma-Aldrich). To this solution was then added 1/10 of final volume 3 M NaAc and 2,5x of final volume cold 100% ethanol. The hybridization mix was washed with 70% ethanol and dissolved in 50% deionized formamide, 10% dextran sulphate and 2x SSC.

The slides were treated with RNase A solution (100 μg/ml) for 1 hour at 37 °C. The chromosomes were denatured using denaturation mix (50 % deionized formamide, 2xSSC, H_2O) on a hot plate at 68 °C for 3,5 minutes.

Finally, 10 μl of hybridization mix were applied to the slides and covered with 24 x 32 slip. The edges of the cover slip were sealed using Rubber cement glue (Marabuwerke GmbH, Tamm, SRN). The preparations were incubated in a wet chamber at 37 °C for three days.

After the incubation, the Rubber cement glue with cover glass was removed and the slides were washed in 1% Triton x-100 in 0.1x SSC at 62 °C for 5 minutes and in 1% Kodak PhotoFlo in H_2O for 2 minutes. Chromosomal preparations were then mounted in DAPI in DABCO with the final concentration of 0,5 μg/ml. The slides were covered with 24 x 40 mm cover slip, its edges were sealed with nail polish and the slides were stored at -4 °C.

3.7. Fluorescence in situ hybridization with telomeric probes

Fluorescence in situ hybridization was used for detection of telomeric sequences at the ends of chromosomes. It followed the protocols of Mediouni et al. (2004) and Sahara et al. (1999) with some modifications. The same slides used in genomic in situ hybridization were reprobed by telomeric probe.

Briefly, for hybridization solution were mixed 150 ng telo-CY3 oligo probe (kindly provided by Dr. Magda Zrzavá) and 25 μg salmon sperm. The final concentration of telomeric probe in hybridization mix was 150 ng/slide.

For reprobing, the nail polish and cover slip were removed from the slide with forceps, and the slides were washed in 2x SSC for 5 minutes at room temperature. To postfix preparation, the slides were washed in freshly prepared 4% paraformaldehyde in 2x SSC for 10 minutes and then in 2x SSC for 5 minutes. The slides were incubated in 50% formamide 1% Triton X in 0,1% SSC at 70 °C for 10 minutes. The slides were then placed into 70% cold ethanol and dehydrated through ethanol series (80% and 100%).

The hybridization mix was applied on the slides, and they were incubated, washed and stored as is mentioned in genomic in situ hybridization (see above).

3.8. Microscopy and image processing

Chromosomal preparations were observed in Leica DM6 microscope (Leica, Germany) equipped with standard fluorescent filter set. Images of chromosomes were recorded by DFC9000 camera. All the images were captured separately for each fluorescent dye with Leica LAS X software, version 3.7.3.23245. The images were pseudo colored (light blue for DAPI, red for Cy-3 and yellow for telomeric probe) and the layers were put together in Adobe Photoshop, version 20.0.2.

4. Results

To verify the species identity of the examined material, I sequenced the fragment of the cytochrome oxidase I gene (COI). The identity of both species *Dryas iulia* and *Eueides isabella* was confirmed as the consensus sequence matched records in nt/nr database of NCBI and BOLT databases (boltsystems.org). The sequence identity of the closest hit was for *Dryas iulia* 99,8 % (GenBank accession number MZ229728) and for *Eueides isabella* 100 % (GenBank accession number GU659584).

The W chromosome was detected using genomic in situ hybridization (GISH). The method confirmed the presence of W chromosome in both species *Dryas iulia* and *Eueides isabella*. In *Dryas iulia* was also used fluorescent in situ hybridization with telomeric probes (FISH) for the W chromosome length determination. Both methods GISH and FISH are subtypes of fluorescent in situ hybridization. This method uses probe DNA which is labelled with fluorescent dye. The fluorescent probe is denatured and hybridized on chromosomes, which have been denaturized as well. This enables us to detect particular parts of the genome, individual chromosomes and even desired parts of the chromosomes.

4.1. *Dryas iulia*

Genomic in situ hybridization showed scattered signal on the *D. iulia* W chromosome. The chromosome was well visible under fluorescent microscope. It was tagged along its whole length, but the signal was concentrated into several blocks. The signal was weaker in other parts of the chromosome – especially on one of the chromosome ends (Figure 1, a-c).

The W chromosome paired regularly with the Z chromosome and was approximately the same size. Providing the *D. iulia* genome size of 440 Mb and number of chromosomes n=31 (Lewis et al. 2021), the average length of a bivalent is thus about 14 Mb. It is reasonable to assume that the W chromosome is bigger than average as the Z chromosome is evolutionary conserved and represent one of the largest chromosomes across Lepidoptera (Dalíková et al., 2017). Results of telomeric FISH do not suggest presence of smaller W chromosome fragments (Fig. 2).

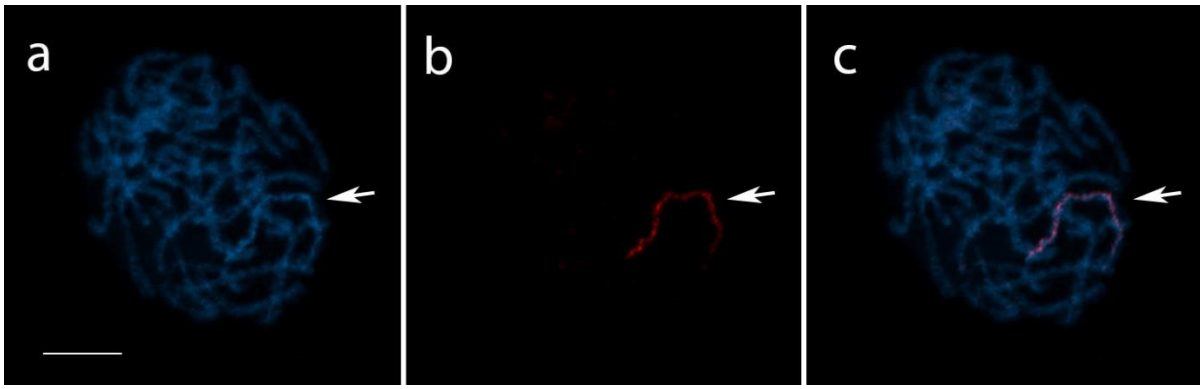

Fig. 1: Genomic in situ hybridization on *Dryas iulia* female pachytene nucleus (a-c). a - Chromosomes stained with DAPI (blue), the arrow marks the WZ bivalent. b - Chromosome W tagged with Cy-3 labelled female genomic probe (red). The signal is scattered and concentrated into several blocks and significantly weaker on one of the ends of the chromosome. c - Overlay of DAPI in DABCO and fluorescent coloring. Scale = 10 μm.

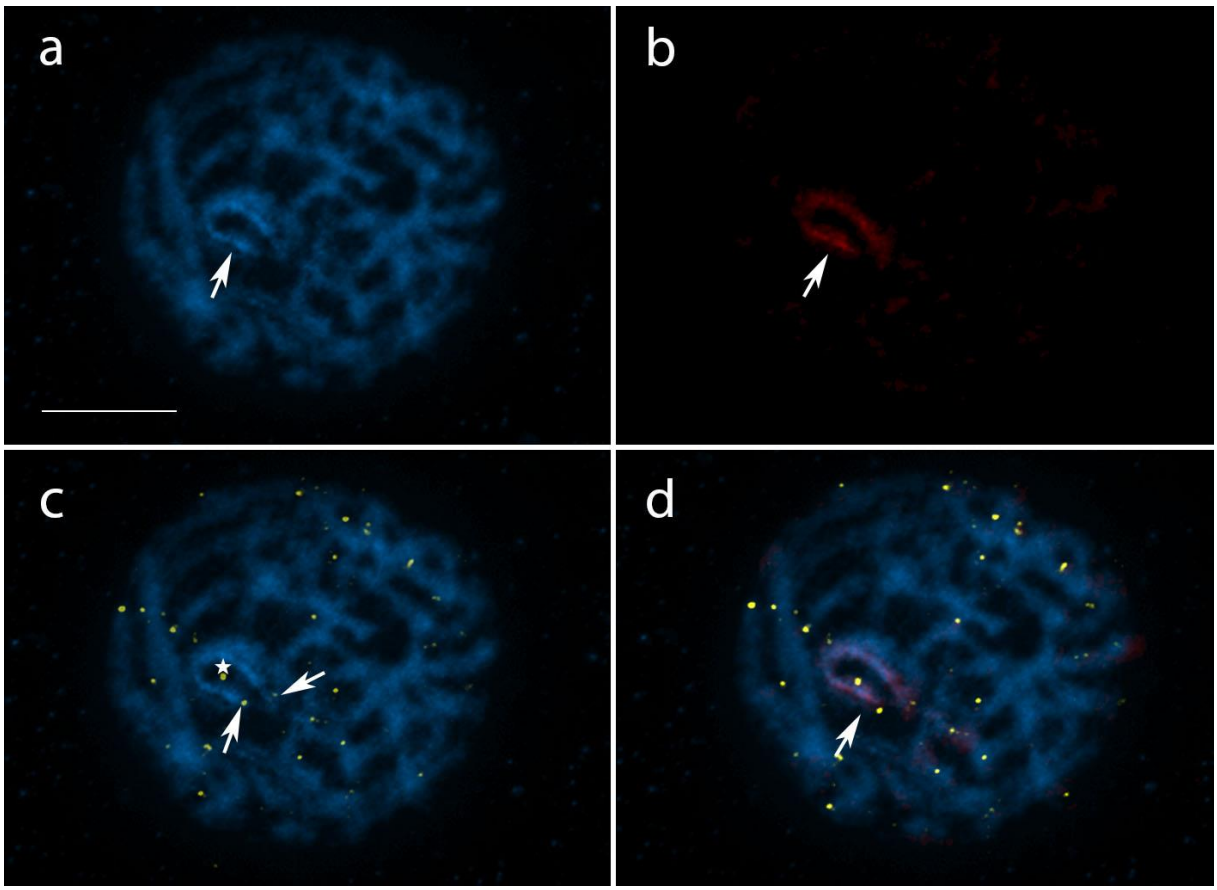

Fig. 2: Fluorescence and genomic in situ hybridization with telomeric probes on *Dryas iulia* pachytene nucleus (a-d). a - Chromosomes stained with DAPI (blue), the arrow marks the ZW bivalent. b - The W chromosome tagged with Cy-3 labelled female genomic probe (red). c - Telomeric sequences at the ends of chromosomes detected with oligo probe $(TTAGG)_n$, the

arrows mark the beginning and the end of W chromosome. The five-pointed star marks a telomeric signal which is probably an artefact. It is not an interstitial telomeric signal on the WZ bivalent since it only appeared in one nuclei. d - Overlay of DAPI in DABCO and fluorescent tagging of W chromosome telomeric sequences. The arrow marks the W chromosome. Scale = 10 μm.

4.2. *Eueides isabella*

The number of chromosomes in *Eueides isabella* was determined on mitotic nuclei and it equals n = 31. From the mitosis is also evident, that the W chromosome was DAPI positive and was not significantly smaller than the autosomes (Fig. 3, a-c). Yet, genomic in situ hybridization revealed different morphology of the W chromosome as it appeared to be smaller in the majority of nuclei. The chromosome was typically dot shaped (Figure 3, d-f) or rod-like (Figure 3, g-i), which were observed in the majority of nuclei. The chromosome W in these polymorphisms probably pairs with itself and the Z chromosome is folded around it. The least frequent morphology of W chromosome is full length (Figure 3, j-l). Within one individual could be seen all three morphologies. The signal on the W chromosome is very dense and it is tagged along its whole length. In figure 3, j-l is the signal on W chromosome interrupted.

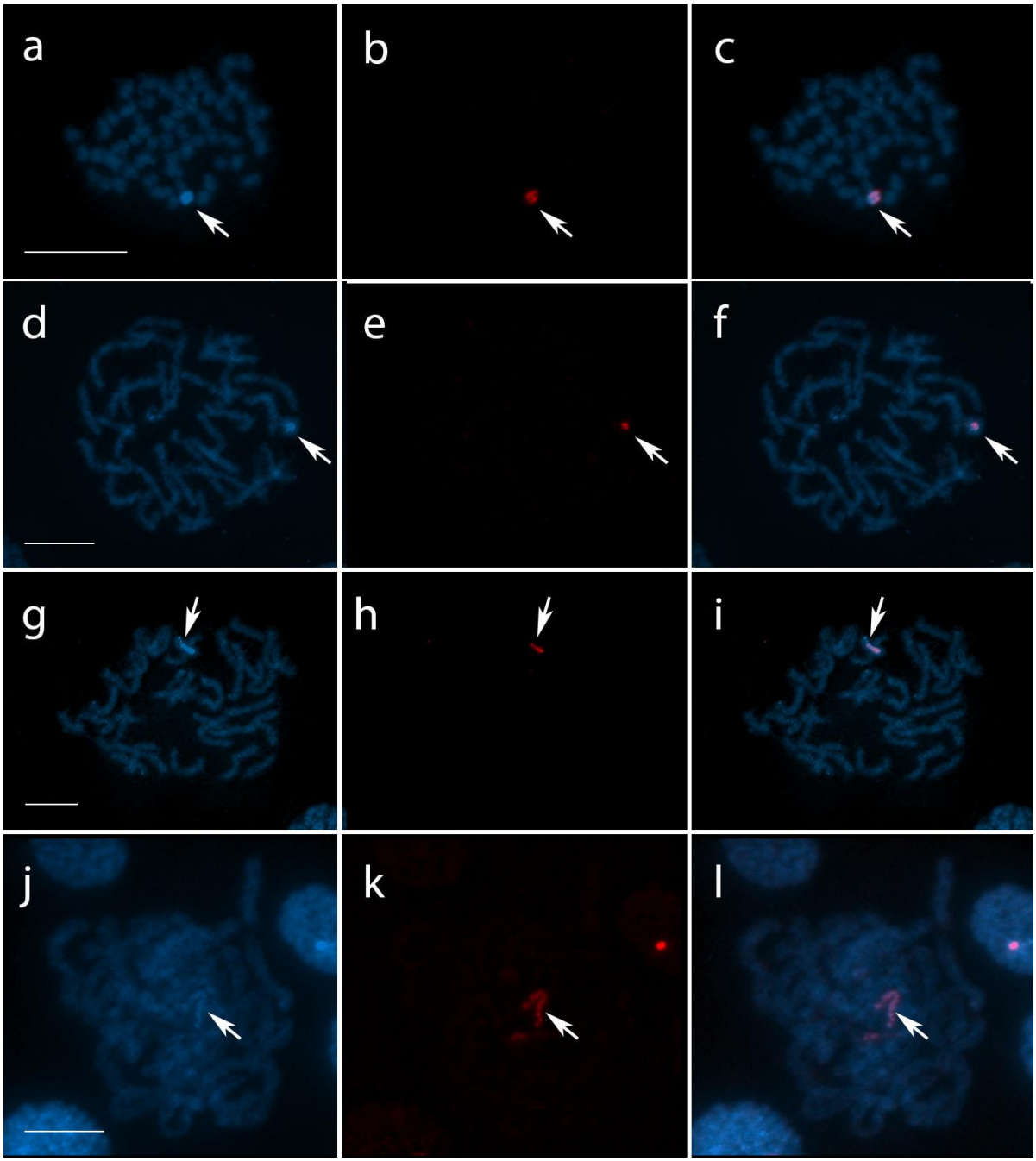

Fig. 3: Genomic in situ hybridization in *Eueides isabella* on female mitotic (a-c) and pachytene (d-l) complements. a - Chromosomes in mitosis consisting of 2n = 62. The arrow marks chromosome W, which is comparable in size to the other chromosomes and is highly DAPI positive. The chromosomes were stained with DAPI (blue). b - Chromosome W detected with Cy-3 labelled female genomic probe (red). c - Overlay of DAPI stained chromosomes and GISH signals. d - Pachytene chromosomes stained with DAPI, the arrow marks a DAPI positive dot-like W chromosome. e – The chromosome W highlighted by female genomic probe labelled by Cy3. f - Overlay of DAPI and GISH signals. g – Pachytene chromosomes stained by DAPI, the arrow marks a rod-like W chromosome. h – The W chromosome

highlighted by female Cy-3 labelled genomic probe. i - Overlay of DAPI and GISH channels. j - Chromosomes in pachytene stained by DAPI, the arrow marks W chromosome of regular length. k - The chromosome W highlighted by female genomic probe. l - Overlay of DAPI and GISH signal. Scale = 10 μm.

5. Discussion

In the present thesis I used genomic in situ hybridization (GISH) in the two representatives of the tribe Heliconiini, namely *Dryas iulia* and *Eueides isabella*. The aim was to determine the presence, size and differentiation of W chromosome in both species. The results confirmed the presence of W chromosome in *D. iulia* as well as in *E. isabella*. The length of the chromosome in *D. iulia* is inconsistent with the predictions of Lewis et al. (2021). The W chromosome in *E. isabella* displayed strange behavior concerning the morphology and chromosome pairing.

5.1. W chromosome in *Dryas iulia*

GISH showed that the W chromosome in *D. iulia* is not as small as reported by Lewis et al. (2021). It is not smaller than the autosomes and based on its standard pairing with the Z chromosome the sequence should be at least 14 Mb long. Whereas the published sequence comprises only 2,2 Mb. Fluorescent in situ hybridization with telomeric probe was further performed to test for presence of multiple sex chromosome system, suggesting possible fragmentation of the W chromosome. The results did not suggest an interstitial telomeric signal, so the W chromosome does not seem to be fragmented. Lewis et al. (2021) did not confirm their in silico results. However, it is crucial to complement and verify the genomics by any other means (Traut et al., 2017). Currently there are many methods which are able to detect chromosomes. One of them is fluorescence in situ hybridization of oligonucleotide probes, the so-called oligopaint (Rosin et al., 2021), which allows differential painting of chromosome regions. Similarly, the *Cydia pomonella* genome project published incomplete sequence of W chromosome (Wan et al., 2019). The authors claimed that the sequence of W chromosome is 3,3 Mb long. However, thanks to previous cytogenetic analyses (Fuková et al., 2005), it was clear that it should be much longer. Lewis et al. (2021) support their findings with the *Heliconius melpomene* linkage map (Jiggins et al., 2005). However, the map provides no information on the size of the non-recombining W chromosome. Based on my findings, Lewis et al. (2021) can be considered an artefact. Therefore, there is no evidence supporting the multiple origin of the W chromosome from B chromosome throughout the order Lepidoptera. Absence of any similarity between the W and the other chromosomes can be explained by lack of recombination and a rapid turnover of its repetitive content (Vítková et al., 2007).

5.2. Sex chromosomes in meiosis

The W chromosome in *Eueides isabella* showed interesting behavior as its morphology varied even in one individual from dot- or rod-like to its regular pairing with the Z chromosome. Chromosome pairing is one of crucial steps in meiosis in which diploid genome is reduced by half and haploid gametes are formed. The main steps of meiosis are replication of genome, chromosome pairing, synapsis stabilizing chromosomal alignment via formation of synaptonemal complex, and chromosome recombination (Kleckner, 1996, Roeder, 1997, Zickler & Kleckner, 1999, Page & Hawley, 2003). The synaptonemal complex plays also a role in segregation of homologous chromosomes as crossovers form connections (chiasmata) within the complex. Meiotic division results in cellular differentiation and in the end in differentiated gametes (Checchi & Engebrecht, 2011). Errors in meiotic process can lead to sterility of the individuals or inviable or defective offspring. Since sex chromosomes are heterologous, their nondisjunction rate is significantly higher than in autosomes (Shi et al., 2001).

The chromosome pairing and recombination are processes which usually depend on the chromosome homology. However, X and Y chromosomes are homologous only in certain parts called the pseudo-autosomal regions and their recombination and synapsis occur only in these regions (Handel, 2004). Therefore, multiple strategies in different species evolved ensuring that heteromorphic sex chromosomes segregate equally (Checchi & Engebrecht, 2011). One of the strategies is that pairing of sex chromosomes takes place later in meiotic prophase than the pairing of autosomes (Schoenmakers et al., 2009). Delay in synapsis and incomplete pairing of heterologous chromosomes W and Z is typical also for Lepidoptera (Marec & Traut, 1994, Wang et al., 1993). Moreover, an adjustment in synapsis occured as the non-homologous chromosomes W and Z paired for example in a flour moth *Ephestia kuehniella* (Weith & Traut, 1986, Marec, 1996). .

There are some common features among sex chromosomes of species with nonhomologous chromosomes which usually do not pair standardly. They all must go through process called meiotic sex chromosome inactivation. This process occurs during meiotic prophase I and its main principle is expanding heterochromatic domain and silencing heteromorphic sex chromosome via transcription (Turner, 2007). Meiotic sex chromosome inactivation is a result of sexual antagonism in which the expression of one gene can be beneficial for one sex and harmful for the other. Its main role is to prevent a rise of selfish genetic elements (Wu & Yujun Xu, 2003, Meiklejohn & Tao, 2010). It is also supposed to prevent recombination between

two non-homologous chromosomes (McKee & Handel, 1993, Inagaki et al., 2010). All the chromosomes must pair to avoid meiotic checkpoints. It was hypothesized that meiotic sex chromosome inactivation can play part in avoiding the checkpoints, but this theory is yet to be proven (Vibranovski et al., 2009).

Based on the GISH results, we can assume intrachromosomal interactions among various regions of the *E. isabella* W chromosome, which seems to pair even with itself. The Z chromosome wraps around the tightly packed the W chromosome as a specific form of synaptic adjustment (Marec, 1996) which may be due to delayed pairing of sex chromosomes in meiosis and lack of homology between the sex chromosomes as mentioned above.

The chromosome pairing is a process usually dependent on the homology of chromosomes. In *B. mori* pairing is initiated by telomeric regions (Holm 1985). Rasmussen (1986) suggested that there are so called recognition sites at the chromosome ends, which can initiate pairing and stimulate the formation of synaptonemal. Van't hof et al. (2013) reported that terminally located Z-linked gene, *laminin A*, was translocated to the W chromosome by ectopic recombination, possibly creating a recognition site in *Biston betularia*. Indeed, physical mapping revealed that W and Z sex chromosomes paired non-randomly in this species. We can assume that since the Z and W chromosomes are nonhomologous that they are looking for similar sequences to pair with, which can result in W chromosome pairing with itself, providing its homogenous and highly repetitive sequence composition. *E. isabella* is not the only species with the irregular pairing of W and Z chromosomes. In monarch butterfly the W chromosome folds in meiosis and appears to be pairing with itself and the Z chromosome wraps around it in a horseshoe manner (Mongue et al., 2017). Similar pairing was observed in a blue butterfly *Polyommatus escheri* (Kreklová, 2020). There are two possible explanations for this phenomenon. First, the W chromosome can pair with itself due to the highly homologous regions, possibly forming a synaptonemal complex within the U-shaped W chromosome. Indeed, the monarch W consists of highly identical transposable elements organized in tandem repeats (Dalíková, Walters, personal communication). Moreover, Rasmussen (1987) reported that individual chromosomes are able to pair with more than one chromosome in *B. mori* polyploid meiocytes. This hypothesis could be tested with the use of immunostaining of proteins of synaptonemal complex (Xiang et al., 2023). The second hypothesis is that due to the presence of tandem repeats in a W chromosome, the chromosome form heterochromatin, and can fold into heterochromatic body similar to sex chromatin comprising multiple W copies observed in polyploid interphase nuclei of Lepidoptera (Traut

& Marec, 1996). Unfortunately, there is no detailed information on epigenetic silencing of W chromosome in Lepidoptera and further research is needed.

6. Conclusion

The aim of this thesis was to confirm the presence of W chromosome in species *Dryas iulia* and *Eueides isabella* and to determine its size and level of differentiation. The species identity was confirmed via barcoding. The W chromosome was detected by genomic in situ hybridization using fluorescently labelled female probes. This method showed that the length of W chromosome in *Dryas iulia* does not correspond to the results of Lewis et al., (2021). The W chromosome in *Eueides isabella* displayed three morphological states and unusual pairing with Z chromosome. The mechanism of chromosome pairing in this species is yet to be discovered and it can be a topic for further research.

7. References

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD et al. (2000) The Genome Sequence of Drosophila melanogaster. *Science* **287**: 2185–2195.

Ahola V, Lehtonen R, Somervuo P, Salmela L, Koskinen P et al. (2014) The Glanville fritillary genome retains an ancient karyotype and reveals selective chromosomal fusions in Lepidoptera. *Nature Communications* **5**(1).

Alföldi, J. E. (2008). Sequence of the Mouse Y Chromosome.

Beldade P, Saenko SV, Pul N, Long AD (2009) A gene-based linkage map for Bicyclus anynana butterflies allows for a comprehensive analysis of synteny with the lepidopteran reference genome. *PLoS Genetics* **5**(2): 1000366.

Bergero R, Charlesworth D (2009) The evolution of restricted recombination in sex chromosomes. *Trends in Ecology and Evolution* **24**(2): 94-102.

Bull, J. J. (1983). Evolution of sex determining mechanisms.

Camacho JPM, Sharbel TF, Beukeboom LW (2000) B-chromosome evolution. Philosophical Transactions of the Royal Society B: *Biological Sciences* **355**(1394): 163-178.

Carey SB, Lovell JT, Jenkins J, Leebens-Mack J, Schmutz J et al (2022) Representing sex chromosomes in genome assemblies. *Cell Genomics* **2**(5): 100132.

Charlesworth B (1991) The Evoluton of Sex Chromosomes. *Science* **251**(4997): 1030-3.

Carvalho AB, Dobo BA, Vibranovski MD, Clark AG (2001) Identification of five new genes on the Y chromosome of Drosophila melanogaster. *Genetics* **98**(23): 13225-1330.

Checchi PM, Engebrecht J (2011) Heteromorphic sex chromosomes: Navigating meiosis without a homologous partner. *Molecular Reproduction and Development* **78**(9): 623-632.

Cole F, Keeney S, Jasin M (2010) Evolutionary conservation of meiotic DSB proteins: more than just Spo11. *Genes & Development* **24**(12): 1201-1207.

Dalíková M, Zrzavá M, Hladová I, Nguyen P, Šonský I et al. (2017) New insights into the evolution of the W chromosome in Lepidoptera. *Heredity* **108**(7): 709-719.

Darwin Tree of Life Project Consortium (2022) Sequence locally, think globally: The Darwin Tree of Life Project. *Perspective* **119**(4): e2115642118.

Fraïsse C, Picard MAL, Vicoso B (2017) The deep conservation of the Lepidoptera Z chromosome suggests a non-canonical origin of the W. *Nature Communications* **8**(1): 1486.

Fuková I, Traut W, Vítková M, Nguyen P, Kubíčková S (2005) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. Chromosoma **116**(2): 135–145.

Glaser RW (1917) The growth of insect blood cells in vitro. *Psyche* **24**: 1-7.

Goldsmith MR, Marec F (2010) Molecular Biology and Genetics of the Lepidoptera. CRC Press, Boca Raton, FL, USA, 368 pp.

Handel M (2004) The XY body: a specialized meiotic chromatin domain. *Experimental Cell Research* **296**(1): 57–63.

Hejníčková M, Koutecký P, Potocký P, Provazníková I, Voleníková A et al. (2019) Absence of W chromosome in psychidae moths and implications for the theory of sex chromosome evolution in lepidoptera. *Genes* **10**(12).

Herbert PND, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. *PNAS* **101**(41): 14812-14817.

Holm PB (1985) Ultrastructural characterization of meiosis. *Biol. Skif. (Copenhagen)* **25**: 39-90.

Inagaki A, Schoenmakers S, Baarends WM (2010) DNA double strand break repair, chromosome synapsis and transcriptional silencing in meiosis. *Epigenetics* **5**(4): 255–266.

Jiggins CD, Mavarez J, Beltrán M, McMillan WO, Johnston JS et al. (2005) A genetic linkage map of the mimetic butterfly Heliconius melpomene. *Genetics* **171**(2): 557–570.

Jones RN, Rees H (1982) B Chromosomes. Academic Press, London, UK, 266 pp.

Kaiser VB, Bachtrog D (2010) Evolution of sex chromosomes in insects. Annual Review of *Genetics* **44**: 91-112.

Kleckner N (1996) Meiosis: how could it work? *Proceedings of the National Academy of Sciences* **93**(16): 8167–8174.

Kreklová M (2017) Holokinetický drive a jeho role v karyotypové evoluci motýlů [Holokinetic drive and his role in karyotypic evolution of Lepidoptera. Bc. Thesis, in Czech] - p. 42, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Kreklová M (2020) Analýza pohlavních chromozomů modrásků (Lycaenidae) [Analysis of sex chromosomes of blue butterflies (Lycaenidae). Mgr. Thesis, in Czech] – 58 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Lewis J J, Cicconardi F, Martin SH, Reed RD, Danko CG et al. (2021) The Dryas iulia Genome Supports Multiple Gains of a W Chromosome from a B Chromosome in Butterflies. *Genome Biology and Evolution* **13**(7): evab128.

Lockwood APM (1961) "Ringer" solutions andsome notes on the physiological basis of their ionic composition. *Comparative Biochemistry and Physiology* **2**: 241-289.

Lukhtanov VA (2000) Sex chromatin and sex chromosome systems in nonditrysian Lepidoptera (Insecta). *Journal of Zoological Systematics and Evolutionary Research* **38**(2): 73–79.

Marec F (1996) Synaptonemal complexes in insects. *International Journal of Insect Morphology and Embryology* **25**(3): 205–233.

Marec F, Traut W (1994) Sex chromosome pairing and sex chromatin bodies in W–Z translocation strains of Ephestia kuehniella (Lepidoptera). *Genome* **37**(3): 426–435.

McKee BD, Handel MA (1993) Sex chromosomes, recombination, and chromatin conformation. *Chromosoma* **102**(2): 71–80.

Mediouni J, Fuková I, Frydrychová R, Marec F, Fuková I et al. (2004) Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, Ectomyelois ceratoniae (Lepidoptera: Pyralidae). *Caryologia* **57**(2): 184–194.

Meiklejohn CD, Tao Y (2010) Genetic conflict and sex chromosome evolution. *Trends in Ecology & Evolution* **25**(4): 215–223.

Meisel RP, Olafson PU, Adhikari K, Guerrero FD, Konganti K et al. (2020) Sex Chromosome Evolution in Muscid Flies. *G3* **10**(4): 1341–1352.

Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T et al. (2004) The genome sequence of silkworm, Bombyx mori. DNA Research: *An International Journal for Rapid Publication of Reports on Genes and Genomes* **11**(1): 27–35.

Muller HJ (1918) Genetic variability, twin hybrids and constant hybrids, in a café of balanced lethal factors. *Genetics* **3**(5): 422–499.

Neumann P, Navrátilová A, Schroeder-Reiter E, Koblížková A, Steinbauerová V et al. (2012) Stretching the rules: Monocentric chromosomes with multiple centromere domains. *PLoS Genetics* **8**(6): e110277.

Page SL, Hawley RS (2003) Chromosome Choreography: The Meiotic Ballet. *Science* **301**(5634): 785–789.

Pringle EG, Baxter SW, Webster CL, Papanicolaou A, Lee SF et al. (2007) Synteny and Chromosome Evolution in the Lepidoptera: Evidence from Mapping in Heliconius melpomene. *Genetics* **177**(1): 417–426.

Rasmussen SW (1986) Initiation of synapsis and interlocking of chromosomes during zygotene in Bombyx spermatocytes. *Carlsberg Research Communications* **51**: 401-432.

Rasmussen SW (1987) Chromosome pairing in autotetraploid Bombyx males. Inhibition of multivalent correction by crossing over. *Carlsberg Research Communications* **52**(3): 211–242.

Rice WR (1987) The Accumulation of Sexually Antagonistic Genes as a Selective Agent Promoting the Evolution of Reduced Recombination between Primitive Sex Chromosomes. *Evolution* **41**(4): 911.

Robinson R (1971) Lepidoptera Genetics. Pergamon Press, Oxford, 698 pp.

Roeder GS (1997) Meiotic chromosomes: it takes two to tango. *Genes & Development* **11**(20): 2600–2621.

Rosin LF, Gil J, Drinnenberg IA, Lei EP. (2021). Oligopaint DNA FISH as a tool for investigating meiotic chromosome dynamics in the silkworm, Bombyx mori. *BioRxiv* (preprint).

Sahara K, Marec F, Traut W (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. *Chromosome Research* **7**: 449-460.

Sahara K, Yoshido A, Traut W (2012) Sex chromosome evolution in moths and butterflies. *Chromosome Research* **20**(1): 83–94.

Schoenmakers S, Wassenaar E, Hoogerbrugge JW, Laven JSE, Grootegoed JA et al. (2009) Female Meiotic Sex Chromosome Inactivation in Chicken. *PLoS Genetics* **5**(5): e1000466.

Shi Q, Spriggs E, Field LL, Ko E, Barclay L, Martin RH (2001) Single sperm typing demonstrates that reduced recombination is associated with the production of aneuploid 24, XY human sperm. *American Journal of Medical Genetics* **99**(1): 34–38.

Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L et al. (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* **423**(6942): 825–837.

The Heliconius Genome Consortium (2012) Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature* **487**(7405): 94–98.

The International Silkworm Genome Consortium (2008) The genome of a lepidopteran model insect, the silkworm Bombyx mori. *Insect Biochemistry and Molecular Biology* **38**(12): 1036–1045.

Tomaszkiewicz M, Medvedev P, Makova KD (2017) Y and W Chromosome Assemblies: Approaches and Discoveries. *Trends in Genetics* **33**(4): 566-282.

Traut W (1976) Pachytene mapping in the female silkworm, Bombyx mori L. *Chromosoma* **58**: 275-284.

Traut W (1994) Sex Determination in the Fly Megaselia scalaris, a Model System for Primary Steps of Sex Chromosome Evolution. *Genetics* **136**(3): 1097-104.

Traut W, Marec F (1996) Sex chromatin in Lepidoptera. *The quarterly review of biology* **71**(2): 239-256.

Traut W, Ahola V, Smith DAS, Gordon IJ, Constant RH (2017) Karyotypes versus Genomes: The Nymphalid Butterflies Melitaea cinxia, Danaus plexipuss and D. chrysippus. *Cytogenetic and Genome Research* **153**(1): 46–53.

Traut W, Sahara K, Marec F (2008) Sex chromosomes and sex determination in Lepidoptera. *Sexual Development* **1**(6): 332-346.

Traut W, Schubert V, Daliková M, Marec F, Sahara K (2019) Activity and inactivity of moth sex chromosomes in somatic and meiotic cells. *Chromosoma* **128**(4): 533–545.

Traut W, Vogel H, Glöckner G, Hartmann E, Heckel DG (2013) High-throughput sequencing of a single chromosome: A moth W chromosome. *Chromosome Research* **21**(5): 491–505.

Turner JMA (2007) Meiotic sex chromosome inactivation. *Development* **134**(10): 1823–1831.

Van'T Hof AE, Nguyen P, Dalíková M, Edmonds N, Marec F et al. (2013) Linkage map of the peppered moth, Biston betularia (Lepidoptera, Geometridae): A model of industrial melanism. *Heredity* **110**(3): 283–295.

Vibranovski MD, Lopes HF, Karr TL, Long M (2009) Stage-Specific Expression Profiling of Drosophila Spermatogenesis Suggests that Meiotic Sex Chromosome Inactivation Drives Genomic Relocation of Testis-Expressed Genes. *PLoS Genetics* **5**(11): e1000731.

Vítková M, Fuková I, Kubíčková S, Marec F (2007) Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera). *Chromosome Research* **15**(7): 917–930.

Wan F, Yin C, Tang R, Chen M, Wu Q et al. (2019 A chromosome-level genome assembly of Cydia pomonella provides insights into chemical ecology and insecticide resistance. *Nature Communications* **10**(1): 4237.

Wang YX, Marec F, Traut W (1993) The synaptonemal complex complement of the wax moth, Galleria mellonella. *Hereditas* **118**: 113-19.

Weith A, Traut W (1986) Synaptic adjustment, non-homologous pairing, and non-pairing of homologous segments in sex chromosome mutants of Ephestia kuehniella (Insecta, Lepidoptera). *Chromosoma* **94**(2): 125–131.

Winnepenninck B, Backeljau, De Wachter R (1993) Extraction of high molecular weight DNA from molluscs. *Trends in Genetics* **9**(12): 407.

Wolf KW (1996) The structure of condensed chromosomes in mitosis and meiosis of insects. *International Journal of Insect Morphology and Embryology* **25**: 37-62

Wu CI, Yujun XuE (2003) Sexual antagonism and X inactivation – the SAXI hypothesis. *Trends in Genetics* **19**(5): 243–247.

Xia Q, Zhou Z, Lu C, Cheng D, Dai Fet al. (2004) A Draft Sequence for the Genome of the Domesticated Silkworm (Bombyx mori). *Science* **306**(5703): 1937–1940.

Xiang Y, Tsuchiya D, Guo F, Gardner J, McCroskey S et al. (2023) A molecular cell biology toolkit for the study of meiosis in the silkworm Bombyx mori. *G3* jkad058.

Yang J, Wan W, Xie M, Mao J, Dong Z et al. (2020) Chromosome-level reference genome assembly and gene editing of the dead-leaf butterfly Kallima inachus. Molecular Ecology *Resources* **20**(4): 1080–1092.

Yasukochi Y, Ashakumary LA, Baba K, Yoshido A, Sahara K (2006) A Second-Generation Integrated Map of the Silkworm Reveals Synteny and Conserved Gene Order Between Lepidopteran Insects. *Genetics* **173**(3): 1319–1328.

Yasukochi Y, Tanaka-Okuyama M, Shibata F, Yoshido A, Marec F et al. (2009) Extensive Conserved Synteny of Genes between the Karyotypes of Manduca sexta and Bombyx mori Revealed by BAC-FISH Mapping. *PLoS ONE* **4**(10): e7465.

Yoshido A, Marec F, & Sahara K (2005) Resolution of sex chromosome constitution by genomic in situ hybridization and fluorescence in situ hybridization with (TTAGG)n telomeric probe in some species of Lepidoptera. *Chromosoma* **114**(3): 193–202.

Zhan S, Merlin C, Boore JL, Reppert SM (2011) The Monarch Butterfly Genome Yields Insights into Long-Distance Migration. *Cell* **147**(5): 1171–1185.

Zickler D, Kleckner N (1999) Meiotic Chromosomes: Integrating Structure and Function. *Annual Review of Genetics* **33**(1): 603–754.