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W chromosomes in Lepidoptera: evolution, diversity and molecular features

Ph.D. Thesis

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Annotation

Sex chromosome evolution is a fascinating and very dynamic process, which is best to be studied on diverse groups of organisms. Moths and butterflies (Lepidoptera) are known for their species diversity and female heterogamety, which makes them an ideal research model. Most species of Lepidoptera have a WZ/ZZ (female/male) system, although some species lack the W chromosome. The first part of this thesis discusses possible mechanisms and points of its origin in terms of phylogeny. Specifically, it focuses on the lack of W chromosome in the group of bagworms (Psychidae), supporting recent theory about the independent origin of W chromosomes in Lepidoptera. The second part of the thesis provides valuable information about the W chromosome variability and molecular content within the group of loopers (Geometridae). Finally, the third part describes the accumulation of retrotransposons on the W chromosome in *Peribatodes rhomboidaria* and emphasizes their importance in the process of sex chromosome differentiation.

Declaration

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

České Budějovice, 25.11. 2022 .....

Martina Hejníčková

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Presented publications and author's contribution

1) **Hejníčková M**, Koutecký P, Potocký P, Provazníková I, Voleníková A, Dalíková M, Visser S, Marec F, Zrzavá M (2019) Absence of W Chromosome in Psychidae Moths and Implications for the Theory of Sex Chromosome Evolution in Lepidoptera. *Genes* 10: 1016 (IF = 4.47).

I hereby declare that Martina Hejníčková carried out most of the experimental procedures including material collection, chromosome preparations, CGH and flow cytometry experiments and data analysis, and wrote the first draft of the manuscript. MH contributed 80%.

2) **Hejníčková M**, Dalíková M, Potocký P, Tammaru T, Trehubenko M, Kubíčková S, Marec F, Zrzavá M (2021) Undifferentiated, Rearranged, Lost: High Variability of Sex Chromosomes in Geometridae (Lepidoptera) Identified by Sex Chromatin. Cells 10: 2230 (IF = 7.66).

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3) **Hejníčková M**, Dalíková M, Zrzavá M, Marec F, Lorite P, Montiel EE (2022) Exploring the W chromosome: accumulation of retrotransposons contributes to sex chromosome differentiation in the willow beauty *Peribatodes rhomboidaria* (Lepidoptera: Geometridae). Preprint on ResearchSquare; submitted to Scientific Reports (IF = 4.99).

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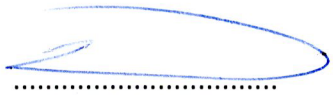
Co-author agreement

Magda Zrzavá, the supervisor of this thesis and a corresponding author in presented papers fully acknowledges Martina Hejníčková as the first author and her contributions as stated above.



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1. Introduction

1.1. Sex determination

Sexual reproduction is an effective way to mix genetic material and produce variable progeny, thus enhancing its chance of survival and fitness in eukaryotic organisms. Sex determination systems may have evolved from mating types found in fungi or yeast, whose amount ranges from two up to 23 328 mating types found in *Schizophyllum commune* (Kothe 1999).

The sex of a developing individual can be determined in two basic ways: either by an environmental factor (ESD) or genetically (GSD). There is a plethora of unique sex-determination pathways with different molecular mechanisms. In the case of ESD, the main sex-determination factor affecting embryonic development may be population structure or temperature. Temperature-dependent ESD has been documented for example in crocodiles, where higher temperatures lead to male-only offspring. An opposite pattern can be found e.g. in most turtles, where higher temperatures produce female-only offspring. In these cases, the range of temperatures where both sexes can hatch is usually quite narrow. Alternatively, a specific range of temperatures may lead to the development of one sex, while both cooler or warmer conditions will produce the other one (Lang & Andrews 1994; González et al. 2019).

Another type of ESD mechanism is based on social interactions. For instance, sex in *Bonellia* worms depends on whether or not the larva encounters another female. The slipper snail (*Crepidula fornicata*) forms a pile composed of multiple individuals, whose sex depends on their position in the pile (Coe 1936). Another remarkable case of social sex determination can be found in fish; however, these occur in the form of sequential hermaphroditism (Gemmell et al. 2019). For instance, teleost fish display extreme phenotypic plasticity and may change sex during their lifetime as a response to changes in their social structure, as seen e.g. in the bluehead wrasse (*Thalassoma bifasciatum)* in which the largest female of a social group may turn into a male when necessary (Godwin 2009). In general, sex reversal is relatively common in fish and amphibians, which may be facilitated by a high frequency of homomorphic sex chromosomes. Although the sex of an individual may be genetically predisposed, certain environmental factors can trigger its change in order to produce the optimal sex ratios

(Baroiller & D'Cotta 2016; Flament 2016). For instance, in snow skink lizard sex determination mechanism depends on the weather conditions affected by the altitude (Pen et al. 2010).

The borderline between environmental and genetic sex determination lies in haplodiploid system. In this case, the sex of the offspring depends on fertilization, since fertilized eggs produce diploid females while unfertilized eggs produce haploid males, as seen e.g. in honeybees and other Hymenoptera (Leather & Hardie 2018).

Nevertheless, genetic sex determination is the most widespread system in eukaryotic organisms. It is typical for example in mammals and birds but can be also found across various other groups of vertebrates, as well as insects and some flowering plants. GSD is mediated by the presence of sex chromosomes, which carry genes responsible for sexual development and differ to varying degrees between sexes. There are two basic sex chromosome systems: male heterogamety (XX/XY), typical for mammals and most insects, and female heterogamety (WZ/ZZ), typical for moths and butterflies, caddisflies, birds, and snakes. These systems may occur in many variants, ranging from the absence of the heteromorphic chromosome (XX/X0 or Z0/ZZ) to the presence of multiple sex chromosomes/neo-sex chromosomes (Bachtrog et al. 2014).

In some taxa (e.g. fish, amphibians, reptiles, or insects) both types of heterogamety may exist. Usually, each species carries only one version, although turnovers are relatively common. Occasionally, these systems may coexist in different populations of the same species as documented in the Japanese frog *Rana rugosa* (Miura et al. 1998).

1.2. Sex chromosome evolution

Sex chromosomes have evolved repeatedly and independently across multiple taxa. Yet, they represent the most fascinating example of convergent evolution by displaying identical features of composition, function, and degeneration.

The universal model of sex chromosome evolution assumes the acquisition of a sex-determining factor by one of the homologous autosomes, which triggers a spiral of events slowly leading to its genetic erosion (Charlesworth 1991). Since it is evolutionarily advantageous to preserve a successful combination of sexually antagonistic mutations,

recombination is halted as they start to accumulate near the sex-determining factor. However, recombination is also one of the fundamental mechanisms of chromosomal rejuvenation. Thus, this new sex-specific chromosome (Y or W) gradually fills up with pseudogenes, tandem repeats, and mobile elements, accelerating its differentiation and progressing heterochromatinization. Such chromosome may become dispensable, and eventually, in case the sex-determining factor is translocated or replaced, it may be completely eradicated (as seen e.g. in mole voles of the genus *Ellobius;* Kolomiets et al. 1991). Nevertheless, it seems that some species evolved a coping mechanism to overcome the degeneration process since in several species the evolutionary old sex chromosomes are still homomorphic (Vicoso et al. 2013; Han et al. 2022).

Furthermore, evolutionary mechanisms might be even more complicated. Sex chromosomes may fuse with other chromosomes, giving rise to neo-sex chromosomes which compose of several evolutionary strata (e.g. Yoshido et al. 2005b; Vlašánek et al. 2017) or they may undergo fissions which generate multiple sex chromosomes (e.g. Yoshido et al. 2020). Alternatively, new sex chromosomes may arise via adoption of a B chromosome - a supernumerary chromosome with a non-mendelian inheritance pattern, usually containing a large portion of repetitive DNA. The adoption mechanisms may involve the gain of a sex determining factor (Clark & Kocher 2019) or simply pairing with an existing Z or X univalent (Nokkala et al. 2003). Taken together, sex chromosomes may come in various shapes, sizes, and numbers and differ greatly across taxa regarding their content.

1.3. Sex chromosome evolution in Lepidoptera

Moths and butterflies (Lepidoptera) represent the ideal research model for sex chromosome evolution studies since they are rich in species and genetic diversity, which allows observing the sex chromosome differentiation process in various stages. Additionally, the female heterogamety system is quite exceptional in insects, since it is by default present only in Lepidoptera and their sister group caddisflies (Trichoptera).

Naturally, there are multiple variants of this system across taxa. Basal Lepidoptera and Trichoptera share the ancestral sex chromosome constitution Z0/ZZ (Sahara et al. 2012). The presence of the W chromosome is more typical for advanced moths and butterflies from the

phylogenetic group Ditrysia, which comprise around 98% of all known species (Kristensen et al. 2007**,** Figure 1). However, there are some exceptions on both sides: for example, in a basal non-dytrisian group Hepialidae W chromosome was discovered (Kawazoé 1987; Voleníková 2015). On the other hand, W chromosome absence was recorded in the Japanese giant silkworm (*Caligula japonica)* which belongs to Saturniidae, Ditrysia where the W chromosome is expected (Yoshido et al. 2006).

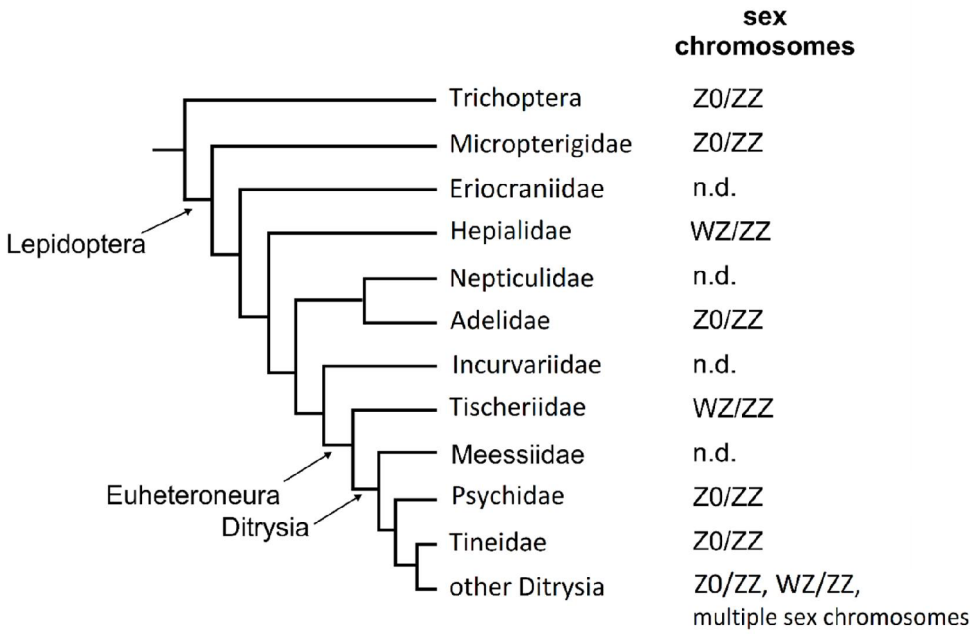

Figure 1: Simplified phylogeny of Lepidoptera with various sex chromosome systems. Based on Hejníčková et al. (2019) and references within. n.d. = not determined.

Historically, widely accepted theories about the origin of the W chromosome in Lepidoptera assumed that it evolved in the common ancestor of Ditrysia and Tischeriidae (a small group of leaf-miners where the W chromosome is present; Lukhtanov 2000). However, this hypothesis was partially based on indirect evidence and did not reflect information about sex chromosomes in bagworms (Psychidae), one of the most basal groups of Ditrysia.

Based on previous research (Seiler 1919; Seiler 1922; Seiler 1960; Narbel-Hofstetter 1961), which inspected various species of Psychidae, it seems that there are odd chromosome numbers in females. These findings suggest the absence of the W chromosome, which was recently confirmed in yet another group of basal Ditrysia - Tineidae, namely in the common clothes moth *Tineola bisselliella* (Dalíková et al. 2017a).

These observations cast doubt on the original hypothesis and contribute to a growing body of evidence for the non-canonical origin of W chromosomes in different groups of Lepidoptera. As proposed by Dalíková et al. (2017a), the W chromosome arose independently in Tischeriidae either via B chromosome adoption or via Z chromosome-autosome fusion, and in Ditrysia via B chromosome adoption, after the divergence of Psychidae and some other groups. Non-canonical origin was also suggested by Fraïsse et al. (2017), supporting the hypothesis of two separate secondary W chromosome acquisitions in Tischeriidae and Ditrysia.

1.4. Sex chromosome features in Lepidoptera

The Z chromosome resembles autosomes by its size, structure, and content. It plays an important role in speciation since it contains genes that control pheromone production and recognition (Dopman et al. 2005; Yasukochi et al. 2011). Despite being evolutionary much older than the W chromosome, it displays well-preserved synteny across various taxa, as shown in widespread comparative studies (Beldade et al. 2009; Yasukochi et al. 2009; Van't Hof et al. 2013; Dalíková et al. 2017a). Thus, the Z chromosomes are homologous and probably have a common origin in Lepidoptera, as well as Z chromosomes in birds (Nanda et al. 2008) and X chromosomes in mammals (Mácha et al. 2012).

In contrast, the W chromosomes differ significantly even between closely related species (Vítková et al. 2007; Zrzavá et al. 2018; Cabral-de-Mello et al. 2021), and their mutation rate may be accelerated because of the absence of meiotic recombination in females (Bauer 1933; Wolf 1994). They are typically filled with ubiquitous transposable elements and satellites (Abe et al. 2005; Sahara et al. 2012; Traut et al. 2013).

Furthermore, W-enriched and even W-specific sequences were recorded e.g. in the Indianmeal moth (*Plodia interpunctella,* Dalíková et al. 2017b) and the codling moth (*Cydia pomonella,* Fuková et al. 2007). Such sequences contribute significantly to the process of sex chromosome differentiation, which may lead to sexual dimorphism or speciation (reviewed in Snell and Turner 2018).

Functional genes are very scarce on the W chromosome; one of the very few W-linked genes is the *Fem* factor, which is a small piRNA coding region discovered in silkworm (*Bombyx mori)* and it serves as a primary signal for the development of a female embryo (Kiuchi et al. 2014). Briefly, this piRNA targets and silences the *Masculinizer* gene located on the Z chromosome, which causes female-specific splicing of the *doublesex* gene. Recently, a similar factor was identified also on the W chromosome of the Mediterranean flour moth (*Ephestia kuehniella*, Visser 2021) and the diamondback moth (*Plutella xylostella*, Harvey-Samuel et al. 2022).

However, the actual importance of the W chromosome for the sex determination process is not clear. On one hand, in *B. mori* it is essential for female development, regardless of the number of Z chromosomes (Traut et al. 2008). On the other hand, in the wild silkmoths (*Samia cynthia* ssp.) the W chromosome plays no role in sex determination (Yoshido et al. 2016). In such cases, sexual development may depend on the number of Z chromosomes and/or the Z: autosome ratio.

1.5. Sex chromatin

W chromosomes typically display partial or total heterochromatinization due to their significant level of differentiation and repetitive DNA content. High heterochromatin load usually induces the formation of a conspicuous sex chromatin body in female somatic polyploid cells, which consists of hundreds to thousands of copies of the W chromosome. As noted by Traut and Marec (1996), who compiled data from 238 species of Lepidoptera, the occurrence of sex chromatin body is indeed common. More specifically, it was recorded in 81% of examined females, which all belonged to Ditrysia. The authors pointed out that sex chromatin assay is a fast and cost-efficient diagnostic method for sexing embryos and young larvae. Further, they suggested that the sex chromatin body might be an indicator of sex chromosome aberrations in mutagenesis screens.

This was already observed in the irradiated lines of *E. kuehniella* or *C. pomonella*, where the sex chromatin body fragmentation or disappearance forecasted structural changes involving sex chromosomes (Rathjens 1974; Traut et al. 1986; Makee & Tafesh 2007; Marec & Traut 1994). Similarly, this phenomenon was recorded in species where sex chromosome rearrangements occurred naturally, such as the vapourer moth (*Orgyia antiqua,* Traut and Clarke 1997), or the clouded Apollo (*Parnassius mnemosyne,* Vlašánek et al. 2017).

These observations suggest that in the case of fusions or translocations involving the W chromosome, it might be the newly acquired, transcriptionally active part of the chromosome that disrupts sex chromatin formation. Suspected ongoing transcription of W-localized genes in follicles and nurse cells during previtellogenesis probably caused sex chromatin to disintegrate into multiple scattered bodies in *E. kuehniella* (Guelin 1994). In general, however, changes in sex chromatin occurrence are quite exceptional, since most of the inspected Ditrysia display normal sex chromatin bodies.

1.6. Sex chromatin and W chromosome in Geometridae

Geometridae is one of the largest groups of Ditrysia with approximately 23 000 described species (Sihvonen et al. 2011). Their phylogenetic position predetermines presence of the W chromosome and the sex chromatin body, respectively. However, relatively high frequencies of sex chromatin deviations were recorded in this group: out of 23 examined species, 7 of them showed abnormalities despite being phylogenetically distant (Traut & Mosbacher 1968; Ennis 1976). Additionally, total chromosome numbers in some species of Geometridae are different from the ancestral karyotype n=31 (Robinson 1971), which is otherwise relatively stable within Lepidoptera. These features of Geometridae indicate dynamic karyotype evolution possibly involving sex chromosome rearrangements, which have the potential to disrupt formation of a sex chromatin body. However, until now we were not able to link these traits together because of a lack of data from this group. From the cytogenetical point of view, so far the WZ bivalents were only detected in the magpie moths *Abraxas sylvata* and *Abraxas grossulariata,* which differ greatly in the W chromosome content despite being congeners (Zrzavá et al. 2018), and in the peppered moth *Biston betularia,* which is probably the only

species of Geometridae with a detailed karyotype description including a linkage map (Van't Hof et al. 2013).

1.7. Tools for sex chromosome studies

Chromosome numbers in Lepidoptera were traditionally obtained from preparations of squashed and stained gonads (Robinson 1971; Ennis 1976; Lukhtanov 2000). This method provided valuable insight into the lepidopteran karyotypes, although it did not allow to study sex chromosomes in detail. Another classical cytogenetic approach is the sex chromatin assay, which enables to inspect the presence and features of the sex chromatin body in female polyploid somatic interphase cells of the Malpighian tubules. The preparation is fixed with Carnoy's fixative and stained with lactic acetic orcein (LAO), which is a purple substance that binds to chromatin. Sex chromatin assay helped to uncover sex chromosome systems in many species of Lepidoptera (Traut & Marec 1996), sex chromosome rearrangements and aberrations (Rathjens 1974; Traut et al. 1986; Makee & Tafesh 2007; Marec & Traut 1994) and helped with sexing of young larvae (Fuková et al. 2009).

Regarding the methods of molecular cytogenetics, fluorescence *in situ* hybridization (FISH) plays a fundamental role. It is used for the physical localization of the examined sequence on the chromosomal preparation, and its principle lies in the simultaneous denaturation of the labeled probe(s) and the chromosomal DNA, and their subsequent hybridization based on sequential homology. Once the probe binds to the chromosomal DNA, the signal detection may be performed either directly (i.e. the probe itself contains nucleotides conjugated with fluorochromes), or with the use of a signal amplification agent. Signal amplification is mainly used for mapping shorter single-copy genes which may be difficult to detect, and often it is based on affinity interactions between fluorochrome-conjugated molecules. In Lepidoptera, FISH has been used to study various cytogenetic markers which allow to uncover the karyotype dynamics and distinguish between individual chromosomes (Provazníková et al. 2021), including the sex chromosomes (Šíchová et al. 2013; Carabajal Paladino et al. 2014).

While the essence of FISH remains the same, there are multiple variations to serve specific purposes. For instance, BAC-FISH employs bacterial artificial chromosomes (BACs) as probes. BAC libraries constructed for the selected species were successfully applied to study

karyotypes and chromosomal rearrangements (Yoshido et al. 2005a; Nguyen et al. 2013; Van't Hof et al. 2013). Further, an actual chromosomal painting probe can be acquired for the W chromosome in species with normal sex chromatin constitution. W-chromosome DNA is obtained by laser microdissection of the sex chromatin body and used as a probe to study and compare W chromosome features across species (Fuková et al. 2007; Vítková et al. 2007; Zrzavá et al. 2018).

Other variations of FISH utilize whole genomic DNAs as hybridization probes. One of them is comparative genomic hybridization (CGH) which employs two separately labeled genomes (e.g. male and female). The probes compete for homologous sequences during hybridization, which eventually allows for a simple, yet efficient comparison of genomic differences between sexes since the potential overrepresentation of a certain sequence will be highlighted by the respective probe, while the rest will hybridize equally. Therefore, CGH enables to identify sex chromosomes, study their differentiation level, and estimate their sequence composition (Traut et al. 1999; Sahara et al. 2003).

Another common tool for cytogenetic sex chromosome studies is genomic *in situ* hybridization (GISH). Unlike CGH, GISH only employs one type of labeled genomic probe (e.g. the female gDNA), which competes for binding sites with a surplus of unlabeled competitor DNA (e.g. the male gDNA). Thus, the differences between the sexes are still highlighted by the probe. In addition, since GISH only requires one type of fluorochrome, it is possible to combine this approach with the classical version of FISH. For instance, GISH was successfully applied with the telomeric probe, which allowed to distinguish individual chromosomes in sex chromosome multivalents (Yoshido et al. 2005b).

Regarding the methods of molecular biology, sex chromosomes are usually studied at the gene level. One of the commonly used methods is quantitative real-time PCR (qPCR), which is a variation to a classical polymerase chain reaction that allows performing quantitative analysis of the desired product by comparing it to a single-copy autosomal reference gene (e.g. acetylcholinesterase 2). Thus, it enables to estimate copy numbers of sex-linked genes between individuals. For instance, it was used to demonstrate Z chromosome homology across Lepidoptera (Dalíková et al. 2017a), to uncover neo-sex chromosomes in the family Tortricidae (Nguyen et al. 2013) and superfamily Gelechioidea (Carabajal Paladino et al. 2019), or to localize *Masculinizer* gene on the Z chromosome of *E. kuehniella* (Visser et al. 2021).

The current era offers an endless variety of genomic and bioinformatic tools to study the sex chromosomes as whole entities. Unfortunately, a universal procedure to study the molecular composition of sex chromosomes of Lepidoptera has not been developed yet, and attempts to do so are rather scarce. Perhaps it is due to the repetitive nature of the W chromosome connected with inevitable assembly difficulties, and also due to the very dynamic environment in these fields. Although shotgun sequencing of BAC clones combined with PCR-based RAPD markers (Abe et al. 2005) and whole genome shotgun sequencing comparative assemblies (Abe et al. 2010) were successfully applied in the beginning, they are now considered outdated. In contrast, sequencing of microdissected sex chromatin seems to be an effective approach to studying W chromosomes (Fuková et al. 2007; Traut et al. 2013; Yoshido et al. 2016), although it has its limitations since not every species has suitable sex chromatin features. Further, a combination of contemporary genomic and bioinformatic approaches allowed e.g. *in silico* comparative analysis of Z chromosomes in several species (Fraïsse et al. 2017), as well as elaborate assemblies of Z chromosomes (Massardo et al. 2020; Seixas et al. 2021), and recently even the W chromosome (Lewis et al. 2021). It is expected that the recent expansion of advanced long-read sequencing techniques such as Nanopore might help to overcome current W-chromosome assembly problems and provide more information about its content and function.

Modern approaches are also applied to investigate differences in expression levels of sex-linked genes. Transcriptomic data are processed through optimized pipelines to identify potential genes of interest, which may be further investigated. For instance, such studies shed light on dosage compensation mechanisms in several species (e.g. Harrison et al. 2012; Walters et al. 2015; Gu et al. 2017).

Finally, one of the unexplored research pathways is the repeat content examination. This field seems promising in Lepidoptera; however, it is not clear to what extent the repetitive DNA present on the W chromosome is W-specific, and this feature varies between species. Individual satellite sequences, as well as transposable elements, were identified on the W chromosome using various techniques (Abe et al. 2005; Fuková et al. 2007; Věchtová et al. 2016; Dalíková et al. 2017b). Nevertheless, a revolutionary tool RepeatExplorer (Novák et al. 2013) now enables to extract and compare the whole repeat content from genomic data. Recently it was applied to Lepidoptera for the first time and it allowed the characterization of

three W-chromosome-located satellites (Cabral-de-Mello et al. 2021). Therefore, plenty of options to study W-linked repetitive DNA remain to be explored.

2. Outline of research

2.1. History of sex chromosome research in Lepidoptera

Research on sex chromosomes in moths and butterflies started more than a century ago. Female heterogamety was first described in the magpie moth, *Abraxas grossulariata*, and has been triggering our curiosity ever since (Doncaster 1907). Another important milestone was achieved by the identification of a female-specific heterochromatin body in the eastern spruce budworm (*Choristoneura fumiferana;* Smith 1945). Following studies have shown that the presence of the so-called sex-chromatin body in female somatic cells is a common trait in a majority of species of Lepidoptera (Traut & Mosbacher 1968; Ennis 1976).

Basic karyotype research was complicated by the fact that the chromosomes of Lepidoptera are small, holokinetic, and lack distinct morphological features. In addition, traditional cytogenetic methods (such as G-banding) were not applicable. Significant progress was accomplished by the use of the meiotic pachytene stage (Traut 1976; Traut & Scholz 1978), which enabled the identification of sex chromosomes, and together with the microspreading technique for electronic microscopy allowed to study synaptonemal complexes and meiotic pairing (Traut et al. 1986; Wang et al. 1993; Marec & Traut 1994). Another breakthrough was brought by molecular cytogenetic methods such as FISH and its variations. The most important one was the application of CGH (Traut et al. 1999), which enabled to distinguish and study various features of sex chromosomes and estimate their molecular composition. Simultaneously, detailed karyotype studies started to uncover amazing variability in sex chromosome composition, structure, and size, as well as the occurrence of neo-sex chromosomes and multivalents (e.g. Yoshido et al. 2005b; Yoshido et al. 2006; Nguyen et al. 2013; Šíchová et al. 2013).

Previous cytogenetic research indicated that the molecular content of the W chromosome is gene-poor and mainly consists of repetitive sequences. Unfortunately, this caused many sequencing projects to use male DNA only, in order to avoid problems with genome assembly. Significant progress was achieved by sequencing W chromosome-derived BAC clones (Abe et al. 2005), and W-chromosome DNA obtained from microdissected sex chromatin bodies (Fuková et al. 2007; Traut et al. 2013; Yoshido et al. 2016). Such experiments confirmed the

prevalence of repetitive DNA on the W chromosome; other enabled the identification of W-linked and even W-specific satellites (Věchtová et al. 2016; Dalíková et al. 2017b). Simultaneously, the first W-linked gene *Fem* factor was described in *B. mori*, being the very first piRNA molecule ever discovered to have a sex-determining function (Kiuchi et al. 2014).

2.2. Current state of art & presented papers

Nowadays, the main lines of research focus on the origin and evolution of the W chromosome, its molecular composition, and its role in sex determination processes. The work presented in this dissertation thesis addresses these topics and hopefully brings new insights into the field.

In the first paper (Hejníčková et al. 2019), we provide thorough evidence regarding the absence of the W chromosome in three species of Psychidae, which was previously suspected based on odd chromosome numbers in females (Seiler 1919; Seiler 1922; Seiler 1960; Narbel-Hofstetter 1961). Psychidae played a crucial role in the W chromosome origin theories due to their phylogenetic position at the very base of Ditrysia. Previously, the emergence of the lepidopteran W chromosome was dated to the common ancestor of Ditrysia and Tischeriidae, meaning that it should also be present in Psychidae. However, cytogenetic approaches combined with comparative genome hybridization applied on chromosomal preparations of both sexes confirmed that Psychidae lack the W chromosome. Further, this observation was supported by the results of flow cytometry, which showed that female genome size is significantly reduced compared to males. Based on these results, we conclude that Z0/ZZ sex chromosome system is prevalent in Psychidae. This finding further supports the non-canonical W chromosome evolution theory, which presumes independent origins of the W chromosomes in Tischeriidae and in advanced Ditrysia.

The formulation of the original W chromosome evolution theory was partially influenced by the occurrence of sex chromatin in various lepidopteran species. Thus, we decided to investigate this feature in the second paper (Hejníčková et al. 2021). For this purpose, we selected the family Geometridae, which are known to have frequent sex chromatin and chromosome number abnormalities. In the beginning, we conducted an extensive sex chromatin screening in 50 species of Geometridae and subsequently selected 8 species for a

thorough analysis of karyotypes and sex chromosomes. The selected species represented all types of sex chromatin ranging from atypical to normal bodies, including its multiple occurrences. Our results uncovered astonishing variability in sex chromosome number, constitution, size, and content, and suggested a link between atypical sex chromatin and sex chromosome abnormalities. However, it also showed that sex chromatin is not an unambiguous marker of the W presence and should be used with caution. According to our knowledge, this is the most comprehensive study of sex chromosomes in a single family of Lepidoptera, which helped us to understand sex chromosome diversity and differentiation in general.

Interestingly, even the two control species with regular sex chromatin from the previous study displayed very different W chromosome features. The first one is the common heath, *Ematurga atomaria,* which has a small, heavily degenerated W chromosome filled mostly with common repetitive sequences. On the contrary, the W chromosome of the willow beauty, *Peribatodes rhomboidaria*, is the same size as the Z chromosome and contains a large portion of female-enriched sequences (according to our published CGH results). We decided to compare the repeat content of these two W chromosomes and eventually characterize some W-enriched sequences. For this purpose, we generated NGS data from three females and three males of each species and searched for differences in repeat content between sexes, using the RepeatExplorer tool. According to our expectation, we found no W-enriched sequences in *E. atomaria*, as described in the bachelor thesis of Marie Kubínová (2022). Nevertheless, in the third manuscript (Hejníčková et al. 2022; preprint) we presented the comparative analysis in *P. rhomboidaria,* which yielded 10 putative W-enriched sequences (mostly retrotransposons). We characterized the two most abundant of them, which were amplified and successfully mapped back on the W chromosome by FISH. The results confirmed their W chromosome enrichment and emphasized the role of retrotransposons in the sex chromosome differentiation process. In addition, we validated a novel approach to repeat content comparison and contributed to our knowledge of W chromosome composition in Lepidoptera.

3. Publications

3.1. Absence of W Chromosome in Psychidae Moths and Implications for the Theory of Sex Chromosome Evolution in Lepidoptera

Martina Hejníčková [1,2], Petr Koutecký [2], Pavel Potocký [1], Irena Provazníková [1,2], Anna Voleníková [1,2], Martina Dalíková [1,2], Sander Visser [1,2], František Marec [1], Magda Zrzavá [1,2]*

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Genes 10, 1016 (2019).

Abstract

Moths and butterflies (Lepidoptera) are the largest group with heterogametic females. Although the ancestral sex chromosome system is probably Z0/ZZ, most lepidopteran species have the W chromosome. When and how the W chromosome arose remains elusive. Existing hypotheses place the W origin either at the common ancestor of Ditrysia and Tischeriidae, or prefer independent origins of W chromosomes in these two groups. Due to their phylogenetic position at the base of Ditrysia, bagworms (Psychidae) play an important role in investigating the W chromosome origin. Therefore, we examined the W chromosome status in three Psychidae species, namely *Proutia betulina*, *Taleporia tubulosa*, and *Diplodoma laichartingella*, using both classical and molecular cytogenetic methods such as sex chromatin assay, comparative genomic hybridization (CGH), and male vs. female genome size comparison by flow cytometry. In females of all three species, no sex chromatin was found, no female-specific chromosome regions were revealed by CGH, and a Z-chromosome univalent was observed in pachytene oocytes. In addition, the genome size of females was significantly smaller than males. Overall, our study provides strong evidence for the absence of the W chromosome in Psychidae, thus supporting the hypothesis of two independent W chromosome origins in Tischeriidae and in advanced Ditrysia.

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Absence of W Chromosome in Psychidae Moths and Implications for the Theory of Sex Chromosome Evolution in Lepidoptera

Martina Hejníčková [1,2], Petr Koutecký [2], Pavel Potocký [1], Irena Provazníková [1,2,†], Anna Voleníková [1,2], Martina Dalíková [1,2], Sander Visser [1,2], František Marec [1] and Magda Zrzavá [1,2,*]

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Abstract: Moths and butterflies (Lepidoptera) are the largest group with heterogametic females. Although the ancestral sex chromosome system is probably Z0/ZZ, most lepidopteran species have the W chromosome. When and how the W chromosome arose remains elusive. Existing hypotheses place the W origin either at the common ancestor of Ditrysia and Tischeriidae, or prefer independent origins of W chromosomes in these two groups. Due to their phylogenetic position at the base of Ditrysia, bagworms (Psychidae) play an important role in investigating the W chromosome origin. Therefore, we examined the W chromosome status in three Psychidae species, namely *Proutia betulina*, *Taleporia tubulosa*, and *Diplodoma laichartingella*, using both classical and molecular cytogenetic methods such as sex chromatin assay, comparative genomic hybridization (CGH), and male vs. female genome size comparison by flow cytometry. In females of all three species, no sex chromatin was found, no female-specific chromosome regions were revealed by CGH, and a Z-chromosome univalent was observed in pachytene oocytes. In addition, the genome size of females was significantly smaller than males. Overall, our study provides strong evidence for the absence of the W chromosome in Psychidae, thus supporting the hypothesis of two independent W chromosome origins in Tischeriidae and in advanced Ditrysia.

Keywords: Psychidae; Lepidoptera; sex chromosome; evolution; W chromosome; Z chromosome; genome size; sex chromatin; comparative genomic hybridization; flow cytometry

1. Introduction

Moths and butterflies (Lepidoptera) are among the most species-rich groups of insects and represent the largest animal taxon with heterogametic females. Most lepidopteran species have a WZ/ZZ (♀/♂) sex chromosome constitution, but some species lack the W chromosome and have a Z0/ZZ (♀/♂) constitution, either as an ancestral sex chromosome system or as a result of a secondary loss of the W chromosome [1]. Generally, the lepidopteran Z and W chromosomes, though often similar in size, differ greatly in their composition. The Z chromosome resembles autosomes and contains many genes. Multiple studies have shown a highly conserved synteny of Z-linked genes between species across the phylogenetic tree of Lepidoptera [2–5]. In contrast, the W chromosome is largely composed of repetitive sequences and is partially or completely degenerated, possibly as a result of the absence of recombination in lepidopteran females [6]. Genetic erosion and accumulation of repetitive sequences

lead to heterochromatinization of the W chromosome, multiple copies of which form a conspicuous spherical body called sex chromatin in somatic polyploid nuclei [1]. The presence of this sex chromatin body has been used as a simple, though not entirely reliable, assay to determine the presence of the W chromosome in particular species [7–10].

Female heterogamety has most probably evolved in the common ancestor of caddisflies (Trichoptera) and Lepidoptera. Since neither caddisflies nor basal moths have a W chromosome, it is believed that Z0/ZZ is the ancestral sex chromosome constitution for these sister clades [9,11]. Apparently, the W chromosome is present in the majority of lepidopteran species studied; although it should be noted that the amount of available cytogenetic data is still scarce in some groups [1]. Nevertheless, the origin of the W chromosome remains elusive. There are several hypotheses about when and how the W chromosome arose [5,11,12]. The generally accepted hypothesis of Lukhtanov [12] assumed that the W chromosome evolved in a common ancestor of Tischeriidae (Tischeriina in the original paper), a small group of leaf-mining moths, and Ditrysia, an evolutionary younger, multidiverse group comprising 98% of lepidopteran species. This assumption was mainly based on indirect evidence (such as sex chromatin absence) from taxa which had branched off earlier. However, neither this hypothesis nor the following reviews [1,6,13] considered the available data in bagworms (Psychidae), one of the most basal families of Ditrysia. In several species of bagworms, odd chromosome numbers were reported [14–18], possibly suggesting the absence of the W chromosome.

Recently, we investigated sex chromosomes in representatives of other basal ditrysian families, namely Gracillariidae, Plutellidae, and Tineidae, and found that the common clothes moth, *Tineola bisselliella* from the last family, lacks the W chromosome [5]. This finding, combined with odd chromosome numbers in yet more basal bagworms, casted doubts on the single W chromosome origin and led to the postulation of an alternative hypothesis of two independent origins of the W chromosome: one in Tischeriidae and one within Ditrysia after Psychidae and Tineidae branched off [5]. The odd chromosome number in females, however, does not necessarily exclude the W chromosome presence. For example, a W chromosome-autosome fusion can lead to the neo-WZ_1Z_2 chromosome system, which also results in an odd chromosome number in females and even number in males (see [6,19–21]). Nevertheless, the potential absence of the W chromosome in Psychidae is currently based solely on unequal chromosome numbers between females and males.

Considering the importance of basal families for understanding the origin of the W chromosome in Lepidoptera, we performed a detailed analysis of karyotypes and sex chromosomes in representatives of Psychidae to clarify the presence or absence of the W chromosome in this group. Implications of our findings for hypotheses on the evolution of sex chromosomes in basal moths are discussed.

2. Materials and Methods

We investigated three species of bagworms, namely *Proutia betulina* (Psychinae), *Taleporia tubulosa* (Taleporinae), and *Diplodoma laichartingella* (Naryciinae). Male and female larvae of Psychidae were collected in deciduous woods surrounding České Budějovice, Czechia. Larvae of the penultimate instar were found mostly on beech trunks in the period between March and June 2015–2019 and determined according to common morphological features (e.g., shape of the bag). All specimens were kept in a plastic box with moistened vegetation at a stable temperature of 4 °C and dissected within one week; residual tissues were immediately frozen in liquid nitrogen and stored at −20 °C until further use. As a control species for sex chromatin assay and flow cytometry for *P. betulina*, we used a laboratory wild-type strain WT-C of the Mediterranean flour moth, *Ephestia kuehniella* (Pyralidae), kept at the Institute of Entomology BC CAS, České Budějovice (see [22]). As a control species for the flow cytometry of *T. tubulosa*, we used a *Drosophila melanogaster* strain with white eye mutation [23] to avoid potential difficulties with the eye pigments.

2.1. Chromosome and Polyploid Nuclei Preparations

Meiotic chromosomes were obtained from larval gonads, mitotic chromosomes from larval gonads and brains, and in male larvae also from wing imaginal discs. Dissections were performed in physiological solution according to Glaser [24] with modifications described in Dalíková et al. [25]. Brains, wing imaginal discs, and male gonads were hypotonized in 75 mM KCl for 8 min and fixed in Carnoy fixative (6:3:1 ethanol, chloroform, acetic acid) for 15 min. Female gonads were fixed immediately after dissection in order to preserve the heterochromatin pattern of the W chromosome, if present. Fixed tissues were macerated and spread in a drop of 60% acetic acid on the slide at 45 °C using a hot plate. Preparations were dehydrated in an ethanol series (70%, 80% and 100%, 30 s each), air-dried, and stored at −20 °C until further use. Preparations used for simple staining with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) were mounted in antifade based on DABCO (1,4-diazabicyclo(2.2.2)-octane; Sigma-Aldrich) containing 0.5 µg/mL DAPI and sealed with nail polish.

For the sex chromatin assay, polyploid interphase nuclei were prepared from Malpighian tubules of the last instar larvae as described in Mediouni et al. [26]. Tubules were dissected in physiological solution, briefly fixed on a slide with Carnoy fixative, and stained with 1.25% lactic acetic orcein for 3–5 min. Each preparation was then covered with a cover slip and redundant dye was drained using filter paper.

2.2. DNA Extraction

Genomic DNA (gDNA) was extracted from larvae stored at −20 °C by NucleoSpin DNA Insect kit (Macherey-Nagel, Düren, Germany) with the following modifications: the tissue was initially crushed by pestles in 1.5 mL microcentrifuge tubes with 100 µL of Elution buffer BE to maximize DNA yield and then transferred to a NucleoSpin Bead Tube type D (provided by the producer). The next steps were performed according to the manufacturer's instructions. Final concentrations of the extracted DNA were measured on a Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

2.3. Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) was performed according to Traut et al. [27] with modifications described in Dalíková et al. [5]. Female and male gDNAs were fluorescently labelled using an improved nick translation protocol based on Kato et al. [28]. The 20 µL labelling reaction contained 1000 ng of gDNA; 0.05 mM each dATP, dCTP, and dGTP; 0.01 mM dTTP; 0.02 mM labelled nucleotides with either Cy3-dUTP (male gDNA) or fluorescein-dUTP (female gDNA) (both Jena Bioscience, Jena, Germany), nick translation buffer (50 mM TrisHCl, pH 7.5, 5 mM $MgCl_2$, 0.005% BSA), 10 mM β-mercaptoethanol, 20 U DNA Polymerase I (ThermoFisher Scientific, Waltham, MA, USA) and 0.005 U DNase I (ThermoFisher Scientific). The reaction was incubated at 15 °C for 2.5 h. For hybridization mixture per slide, 250 ng of each labelled probe and 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich) were mixed together, precipitated, and re-dissolved in 50% deionized formamide, 10% dextran sulphate, and 2× SSC. The mixture was denaturated at 90 °C for 5 min and prehybridized at 37 °C for 1.5 h. After prehybridization, the mixture was applied on a female meiotic preparation, which had been previously treated with RNAse A (200 ng/µL; Sigma–Aldrich) in 2× SSC for 1 h at 37 °C, washed 2× 5 min in 2× SSC, and denaturated at 68 °C in 70% formamide solution in 2× SSC for 3.5 min. The slides were incubated with the hybridization mixture for 3 days at 37 °C, washed in 0.1× SSC with 1% Triton X-100 at 62 °C, and counterstained with 0.5 µg/mL DAPI in antifade based on DABCO.

2.4. Microscopy and Image Processing

Chromosome and polyploid nuclei preparations were examined using 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). Black-and-white images were captured with CellSens Standard software version 1.9 (Olympus). Preparations of polyploid nuclei were investigated using light microscopy. For CGH preparations, black-and-white images were captured separately for each fluorescent dye, and the images were pseudocolored and merged using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

2.5. Flow Cytometry

Flow cytometry was used to estimate the genome size, and to uncover potential differences between males and females which might correspond to the absence of the W chromosome. Two bagworm species—*P. betulina* and *T. tubulosa*—were examined. *D. laichartingella* was not examined due to lack of material. For the genome size measurement, larval brain tissue was frozen in liquid nitrogen and stored at −20 °C until next use. As the internal standard, we used fresh *E. kuehniella* adult males (1/4 of head per sample) for *P. betulina*, and fresh *D. melanogaster* adult males (1/4 of head per sample) for *T. tubulosa*; it was not possible to use a single standard for both species studied due to the overlap of peaks. The entire head of the measured bagworm individuals as well as part of the head of the standard were chopped using a sharp razor blade in 500 µL of nuclei isolation buffer (0.1 M Tris-HCl pH 7.5, 2 mM $MgCl_2$, 1% Triton X-100) [29]. The suspension was filtered through a 42-µm nylon mesh, the volume was adjusted with the buffer to 1 mL, and propidium iodide (PI) and RNase IIa were added, both at final concentration of 50 µg/mL. The samples were stained at least for 20 min and analyzed with a Partec CyFlow SL flow cytometer (Partec, Münster, Germany; now Sysmex) equipped with a 100 mW 532 nm (green) solid-state laser. Fluorescence intensity and SSC (side-scattered light) parameter of 10,000–30,000 particles (depending on number of peaks and amount of debris) were recorded. Data were analyzed using FlowJo 10 software (TreeStar, Inc., Ashland, OR, USA). Due to the relatively large amount of fluorescent debris, gating based on a combination of SSC and PI fluorescence signals was applied to the samples before evaluating histograms of PI fluorescence. Mean, coefficient of variation (CV), and number of nuclei were recorded for 2C peaks of both the sample and the standard.

The genome size was calculated from the ratio of the mean fluorescence of the sample and the internal standard, *E. kuehniella* (2C = 0.90 pg; [29]) or *D. melanogaster* (2C = 0.36 pg; [30]). Basic statistics (mean, standard error, standard deviation, and variation range) of the genome size were calculated for each species and sex. Genome sizes of males and females of each species were compared using the two-sample *t*-test.

3. Results

3.1. Sex Chromatin and Chromosome Number

All species studied were tested for the presence of sex chromatin in the polyploid nuclei from Malpighian tubules in both males and females. Sex chromatin was absent in all specimens examined (Figure 1).

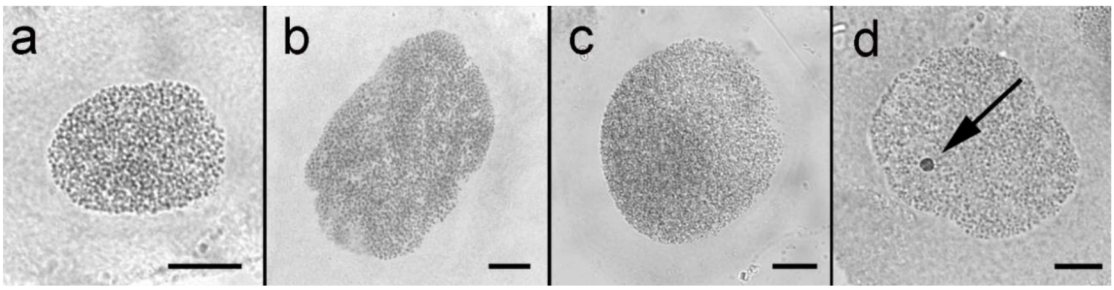

Figure 1. Sex chromatin assay in polyploid nuclei from Malpighian tubules of bagworm (Psychidae) female larvae. No sex chromatin was found in any species of Psychidae, namely *Proutia betulina* (**a**), *Taleporia tubulosa* (**b**), and *Diplodoma laichartingella* (**c**). In contrast, all female nuclei of the control species, *Ephestia kuehniella* (Pyralidae) showed a conspicuous sex chromatin body (arrow; (**d**)). Bar = 10 µm.

Chromosome preparations stained with DAPI were examined using a fluorescence microscope. We determined de novo the chromosome number in *P. betulina*, which is $2n = 61$ in females and $2n = 62$ in males (Figure 2a,b). Chromosome numbers of *T. tubulosa* ($2n = 59/60$ in female/male) were published by Seiler [14]. Unfortunately, we failed to determine the chromosome number in *D. laichartingella* due to lack of material.

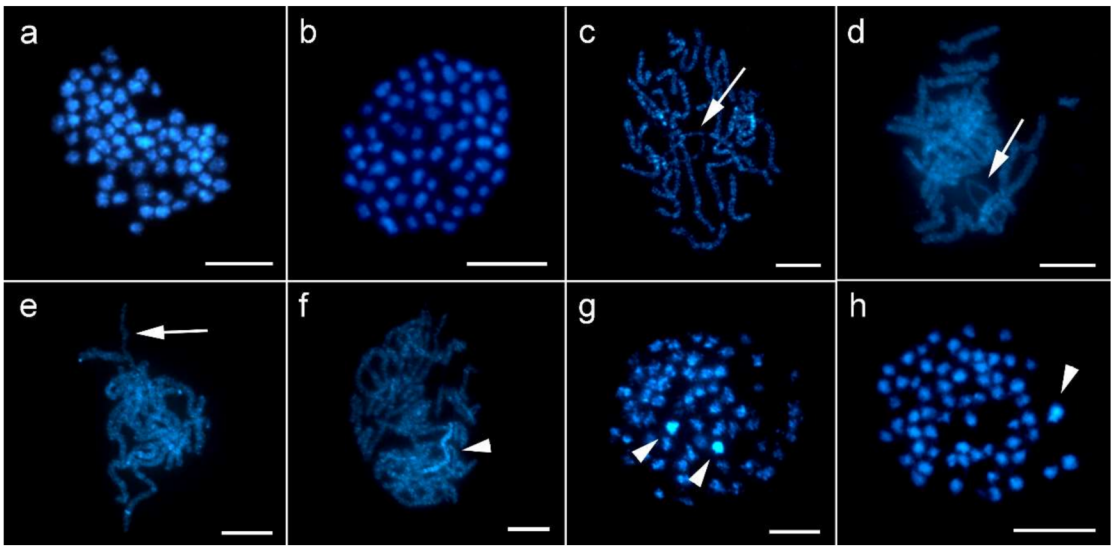

Figure 2. DAPI (4',6-diamidino-2-phenylindole)-stained mitotic and meiotic chromosomes of *P. betulina*, *T. tubulosa*, and *D. laichartingella*. Different numbers of chromosomes in mitotic metaphases of *P. betulina* females with $2n = 61$ (**a**) and males with $2n = 62$ (**b**), and the presence of a Z-chromosome univalent (arrows) in female pachytenes of *P. betulina* (**c**), *T. tubulosa* (**d**), and *D. laichartingella* (**e**) indicate the absence of the W chromosome in these species. In *P. betulina*, a strongly heterochromatinized bivalent was observed in male pachytenes (**f**); mitotic metaphases with less condensed chromosomes showed two DAPI-highlighted chromosomes in males (**g**), and only one DAPI-highlighted chromosome in females (**h**). These heterochromatin DAPI-positive elements (arrowheads) probably represent a Z-chromosome bivalent (**f**) and Z chromosomes (**g**,**h**). Bar = 10 µm.

Interestingly, in *P. betulina* two large, strongly heterochromatinized chromosomes were observed in male preparations, both as a bivalent in the pachytene stage (Figure 2f) and as two individual chromosomes in most mitotic metaphases (Figure 2g). In female preparations, however, only one such chromosome was found in most mitotic metaphases (Figure 2h). Based on the difference between male and female preparations, we presume that this conspicuous chromosome is the Z chromosome.

Importantly, a single unpaired chromosome was repeatedly observed in female pachytenes of *P. betulina*, *T. tubulosa*, and *D. laichartingella* (Figure 2c–e). Such an element was not observed in male pachytenes. This finding suggests that the unpaired element is the Z-chromosome univalent.

3.2. Comparative Genomic Hybridization (CGH)

To verify the absence of the W chromosome in Psychidae, we performed CGH on female meiotic preparations of *P. betulina*, *T. tubulosa*, and *D. laichartingella*. Both the female and male genomic probes hybridized evenly to all chromosomes and no chromosome was highlighted by the probes, thus supporting the absence of the W chromosome in the karyotype of these species (Figure 3a–l).

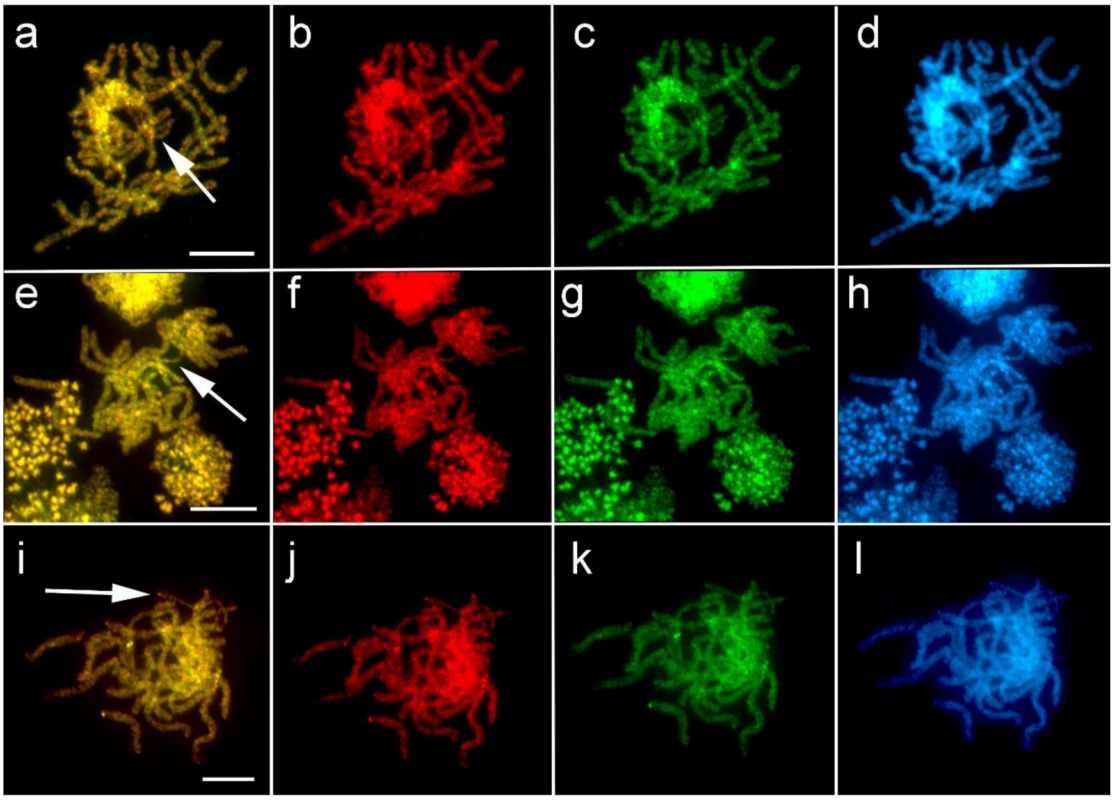

Figure 3. Comparative genomic hybridization (CGH) on female pachytene bivalents in *P. betulina* (**a**–**d**), *T. tubulosa* (**e**–**h**), and *D. laichartingella* (**i**–**l**). Panels **a**, **e**, **i** show merged pictures of both probes. Hybridization signals of male probes (red) are shown in panels **b**, **f** and **j**; female probes (green) in **c**, **g** and **k**. DAPI counterstaining (blue) is shown in panels **d**, **h** and **l**. Z-chromosome univalents are marked with arrows. CGH did not identify any female-specific/enriched element in pachytene complements, confirming the absence of the W chromosome. Bar = 10 μm.

3.3. Flow Cytometry

To confirm the cytogenetic data, we used flow cytometry to measure the genome sizes of both sexes in *P. betulina* and *T. tubulosa*. Flow cytometric analysis of Psychidae is a challenge due to the small amount of brain tissue in the species studied and the need to use frozen material due to the short season of occurrence. However, after optimizing the amount of the internal standard and exhausting the entire volume of the sample, we were able to obtain at least three measurements of sufficient quality (clear peaks with a mean CV = 3.7% and 4.7% for the sample and the standard, respectively, and enough particles) for both sexes of both taxa. The genome size of *P. betulina* females was 2C = 2.32 ± 0.03 pg (mean ± standard error; N = 3; Figure 4a), whereas in males it was 2.45 ± 0.02 pg (N = 7; Figure 4b); on average, male genomes are bigger than female genomes by 5.6% and this difference is statistically significant (t = 2.876, df = 8, p = 0.021). The genome size of *T. tubulosa* females was 2C = 0.75 ± 0.01 pg (N = 6; Figure 4c), while the genome size of *T. tubulosa* males was 2C = 0.78 ± 0.01 pg (N = 6; Figure 4d). On average, *T. tubulosa* male genomes are bigger than female genomes by 4% and this difference is also statistically significant (t = 2.362, df = 10, p = 0.040).

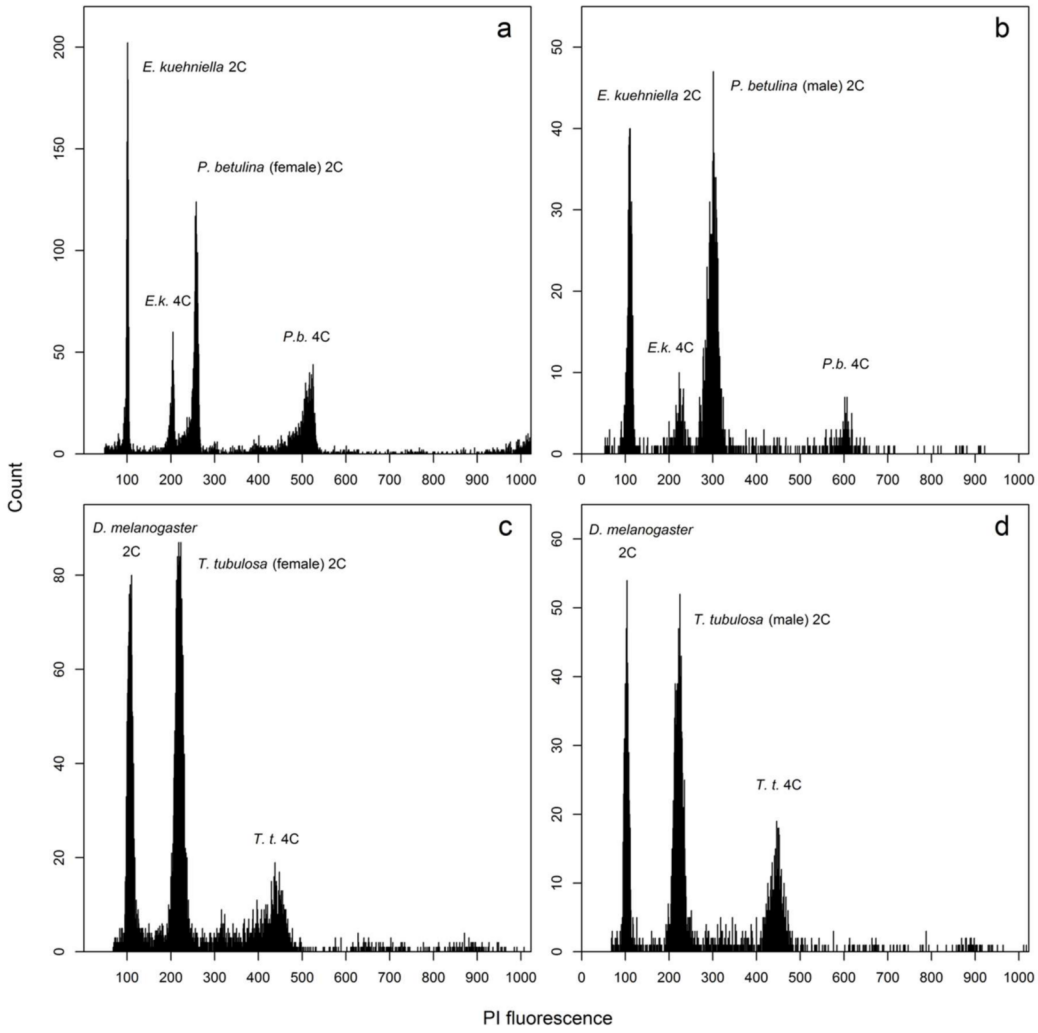

Figure 4. Examples of flow cytometric fluorescence histograms of Psychidae species *P. betulina* (**a**)—female, (**b**) male and *T. tubulosa* (**c**)—female, (**d**)—male). In all cases, 2C and 4C peaks are clearly visible. Peaks of the internal standards *E. kuehniella* and *Drosophila melanogaster* are denoted in a similar way. PI = propidium iodide.

4. Discussion

Psychidae, placed at the base of Ditrysia, have a crucial phylogenetic position for understanding the W chromosome emergence in Lepidoptera. Several cytogenetic papers on Psychidae were published by J. Seiler [14–16,31,32], but the methods available at the time did not allow to obtain unambiguous information about the constitution of sex chromosomes. In our study, we provide clear evidence of the W chromosome absence and a Z0/ZZ (female/male) sex chromosome system in three representatives of two major bagworm clades using a combination of classical and molecular cytogenetics and genome size measurements.

The sex chromatin assay was negative in all bagworm specimens examined, indicating the absence of the W chromosome. This method was used as indirect evidence of the W chromosome presence/absence in many lepidopteran species, which was consequently used as a basis for hypotheses about the W chromosome origin (e.g., [8,9,12,33]). However, there are some limitations, since certain chromosomal rearrangements involving the W chromosome are known to disrupt sex chromatin formation. This has been demonstrated, for example, in mutant strains of *E. kuehniella*, with radiation-induced sex chromosome rearrangements. Part of the Z chromosome was translocated on the W chromosome causing deformation and fragmentation of the sex chromatin body, while a fusion of

the W chromosome with an autosome resulted in complete disintegration and disappearance of sex chromatin [22,34]. A similar sex chromatin disruption was observed in natural populations of three species of *Leptidea* wood white butterflies, in which multiple sex chromosomes originated by complex rearrangements with autosomes [35]. Recently, the absence of sex chromatin was found in females of the clouded Apollo, *Parnassius mnemosyne* (Papilionidae), although examination of sex chromosomes revealed that the W chromosome is present in this species [10]. Therefore, though the presence of sex chromatin in polyploid nuclei often corresponds to the presence of the W chromosome, its absence cannot be used as definitive proof for the absence of the W chromosome in Lepidoptera.

Due to the limitations of sex chromatin assay, it was necessary to perform a more detailed chromosome analysis of the Psychidae. Firstly, we de novo determined chromosome numbers in *P. betulina* ($2n$ = 61 in females and 62 in males); the chromosome numbers in *T. tubulosa* ($2n$ = 59 in females and 60 in males), were already published by Seiler [14]. This resembles data on different chromosome numbers between males and females in other species, namely *Psyche* (syn. *Fumea*) *casta* and *Luffia lapidella*, and odd chromosome number in parthenogenetic females of *Luffia fernauchtella* (all Psychinae; see Table S1 for an overview of sex chromosomes in Psychidae).

Interestingly, a Psychidae species *Apterona helix* (Oiketicinae) was reported to have equal numbers of chromosomes in females and males (reviewed in Robinson [36]). However, the original publication of Narbel [18] mentioned two possible karyotype variants that differed from each other by one chromosome. Given that the chromosome numbers in females are lower by one than in males in other Psychidae species, we believe that this could be the case for this species as well, and that the results from Narbel [18] were possibly misinterpreted by Robinson [36]. However, the karyotypes of *A. helix* should be re-analyzed to confirm their true sex chromosome constitution.

In another species of Psychidae, *Dahlica* (syn. *Solenobia*) *triquetrella*, different sex chromosome systems (Z0 and WZ) were found in local populations [16,37]. However, this "W" chromosome was described as nondisjuctional and sporadically appearing in both sexes, even in multiple copies (i.e., Z0 and WZ females and ZZ, WZZ, and WWZZ males were observed, all phenotypically normal). As initially pointed out by Robinson [36], these characteristics are more typical for B chromosomes rather than a sex chromosome. In addition, crossing experiments between diploid and tetraploid populations suggest that it is the ratio between the number of autosomal chromosome sets and Z chromosomes which determines the sex of an individual in *D. triquetrella*, not the presence of the "W" chromosome [32]. Taken together, it is not clear whether the "W" chromosome in this species is a true sex chromosome, but if so, it would certainly represent an evolutionary novelty that is not common to other Psychidae.

As mentioned above, the difference in chromosome number of males and females does not solely prove the absence of the W chromosome. However, the application of CGH did not reveal any potential female-specific or female-enriched sequences that would make the W visible, thus providing further evidence of the W chromosome absence. Consistent with this finding, the female pachytene nuclei showed a univalent not seen in male pachytenes, which was interpreted as a single Z chromosome. Taken together, we conclude that the odd chromosome number in females of studied species is the result of W chromosome absence.

Finally, we compared the genome size of males and females in *P. betulina* and *T. tubulosa*. Our results showed that the female genomes are significantly smaller than the male genomes in both species, specifically by 5.6% in *P. betulina* and by 4% in *T. tubulosa*, which supports the absence of the W chromosome in females and corresponds well with chromosome counts. The differences between male (ZZ) and female (Z0) genomes should therefore correlate with the size of the Z chromosomes, which seem to be remarkably larger than most autosomes in these species. For example, the Z chromosomes in *P. betulina* were the most conspicuous chromosomes in the karyotype. Apart from being the largest chromosomes, they were often strongly stained by DAPI, which suggests a high abundance of AT-rich repetitive sequences and partial heterochromatinization. This feature was especially noticeable in mitotic metaphases with less condensed chromosomes. In contrast, no heterochromatinization was

observed in chromosomal preparations of *T. tubulosa*. Our data on the large Z chromosomes correspond to previous observations from multiple taxa that the sex chromosomes in Lepidoptera are often the largest chromosomes of the karyotype [1,21,38–40].

In terms of phylogeny, Psychidae form two major clades, the Arrhenophanine lineage, which includes, besides others, Naryciinae (e.g., *D. laichartingella*) and Taleporinae (e.g., *T. tubulosa*), and the Psychinae lineage, which includes Psychinae (e.g., *P. betulina*) and Oiketicinae ([41]; but see [42]). In both clades, species with odd chromosome numbers in females predominate, probably having a Z0/ZZ sex chromosome system (Table S1). Even though an exception was found in Arrhenophaninae, where some populations of *D. triquetrella* presumably have a supernumerary chromosome, available data suggests that the Z0/ZZ sex chromosome system is ancestral in Psychidae.

The W chromosome absence in Psychidae is important for understanding the origin of the W chromosome in Lepidoptera. It was generally believed that the W chromosome arose in the common ancestor of Euheteroneura (Tischeriidae + Ditrysia; [6,12]; see simplified phylogenetic tree of basal Lepidoptera with known sex chromosome system in Figure 5). According to this hypothesis, the presence of the W chromosome in the earliest diverging lineages of Ditrysia, i.e., Meessiidae, Psychidae, and Tineidae, was expected. However, there are no data on sex chromosomes in Meessiidae, and the W chromosome absence was recently reported in a representative of Tineidae, *T. bisselliella*, in Dalíková et al. [5], where all possible scenarios of W chromosome origin were described in detail. Briefly, based on the conserved Z chromosome synteny across Euheteroneura and the reduced chromosome number in Tischeriidae, the authors favoured (i) Z chromosome-autosome fusion in Tischeriidae and (ii) B chromosome acquisition in advanced Ditrysia (after Psychidae and Tineidae branched off) as two different ways of W chromosome birth. Both options, however, operated with the presumed absence of the W chromosome in Psychidae, which has so far been based only on odd chromosome numbers in females. Our data confirm absence of the W chromosome in Psychidae, thereby supporting the proposed hypothesis by Dalíková et al. [5].

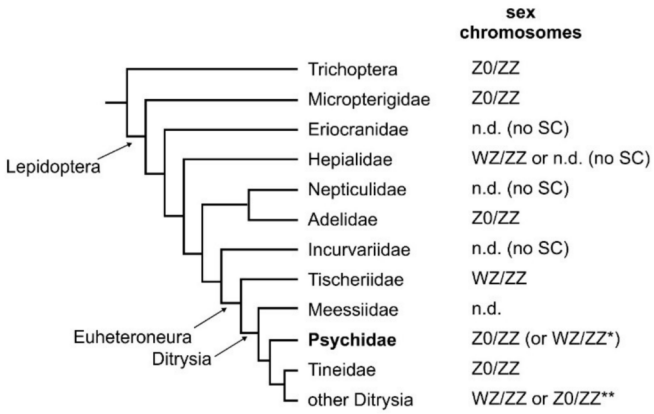

Figure 5. Simplified phylogenetic tree of basal Lepidoptera with known sex chromosome constitution. Records on sex chromosome status originate either from this work or from references listed in the text. In three families, only the sex chromatin (SC) assay data is available, but the actual sex chromosome status was not determined (n.d.). There are no cytogenetic data from the most basal family of Ditrysia, Meessiidae. In two species of Psychidae, namely *Dahlica triquetrella* with a supernumerary chromosome in some populations and *Apterona helix* with alleged chromosome number differences in males and females (but see the main text), the ambiguity is indicated (*). Based on the internal Psychidae phylogeny, we consider both traits to be evolutionary novelties and suggest that the ancestral state is Z0/ZZ. In other Ditrysia, the vast majority of species has a WZ/ZZ system or derived variants with multiple W and/or Z chromosomes, but some species have lost the W secondarily(**). Phylogenetic relationships are according to previous studies [43,44].

To conclude, females of the studied species of Psychidae lack sex chromatin, have a lower chromosome number and a smaller genome size than males (not determined in *D. laichartingella*), and have a univalent in pachytene nuclei, which is absent in males. The data presented in this study thus clearly show that these species lack the W chromosome. Taken together with previous data, species with a Z0/ZZ sex chromosome system predominate in both clades of Psychidae. The putative W chromosomes, if any, have occurred infrequently and more recently in the evolution of Psychidae, suggesting an independent adoption rather than an original trait. In addition, all these findings support the hypothesis of independent origins of the W chromosomes in Euheteroneura; i.e., in Tischeriidae and in advanced Ditrysia.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/10/12/1016/s1, Table S1: Available information on chromosome numbers and sex chromosomes in Psychidae [45–48].

Author Contributions: Conceptualization M.Z. and M.H.; methodology M.Z. and M.D.; formal analysis P.K.; investigation M.H.; resources M.H., P.P., I.P., S.V., and A.V.; writing—original draft preparation M.H.; writing—review and editing M.Z., M.D., A.V., I.P., S.V., P.K., and F.M.; visualization M.H.; supervision M.Z., M.D., and P.K.; project administration M.H. and M.Z.; funding acquisition F.M. and P.K.

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Table S1. Available information on chromosome numbers and sex chromosomes in Psychidae.

Species	**Subfamily**	**Reproduction mode**	**2n (♀/♂)**	**Sex chromosomes**	**Available data**	**References**
Taleporia tubulosa	Taleporiinae	sexual	59/60	Z0/ZZ	- Z univalent in female pachytene - absence of sex chromatin - female genome smaller than male genome	[14]; this study
Dahlica triquetrella syn. *Solenobia triquetrella*	Naryciinae	sexual or parthenogenetic	61/62 or 62/62	females Z0 and WZ, males ZZ, WZZ, WWZZ	- individuals with and without W do not differ in phenotype - W suggested to be a B chromosome in Robinson [39]	[18,36,37,45]
Diplodoma laichartingella	Naryciinae	sexual	n.d.	Z0/ZZ	- Z univalent in female pachytene - absence of sex chromatin	this study
Psyche casta syn. *Fumea casta*	Psychinae	sexual	63/64	probably Z0/ZZ	- 31 elements in female metaphase I (probably 30 bivalents, one univalent) - daughter plates have either 30 or 31 chromosomes	[14]
Luffia ferchaultella	Psychinae	parthenogenetic	61 (♀ only)	probably Z0	- 30 bivalents and a single chromosome, probably Z, in female metaphase I - Z called X in the paper	[17,36,46]
Luffia lapidella	Psychinae	sexual	61/62	probably Z0/ZZ	- odd chromosome number in females	[47]
Proutia betulina	Psychinae	sexual	61/62	Z0/ZZ	- Z univalent in female pachytene - absence of sex chromatin - female genome smaller than male genome	this study
Apterona helix	Oiketicinae	sexual or parthenogenetic	61-62	probably Z0/ZZ	- chromosome counts in original publication range from 61-62 - probably misinterpreted in Robinson [39]	[18,36,48]

3.2. Degenerated, Undifferentiated, Rearranged, Lost: High Variability of Sex Chromosomes in Geometridae (Lepidoptera) Identified by Sex Chromatin

Martina Hejníčková [1,2], Martina Dalíková [1,2], Pavel Potocký [2], Toomas Tammaru [3], Marharyta Trehubenko [1,2], Svatava Kubíčková [4], František Marec [2], Magda Zrzavá [1,2]*

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Abstract

Sex chromatin is a conspicuous body that occurs in polyploid nuclei of most lepidopteran females and consists of numerous copies of the W sex chromosome. It is also a cytogenetic tool used to rapidly assess the W chromosome presence in Lepidoptera. However, certain chromosomal features could disrupt the formation of sex chromatin and lead to the false conclusion that the W chromosome is absent in the respective species. Here we tested the sex chromatin presence in 50 species of Geometridae. In eight selected species with either missing, atypical, or normal sex chromatin patterns, we performed a detailed karyotype analysis by means of comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). The results showed a high diversity of W chromosomes and clarified the reasons for atypical sex chromatin, including the absence or poor differentiation of W, rearrangements leading to the neo-W emergence, possible association with the nucleolus, and the existence of multiple W chromosomes. In two species, we detected intraspecific variability in the sex chromatin status and sex chromosome constitution. We show that the sex chromatin is not a sufficient marker of the W chromosome presence, but it may be an excellent tool to pinpoint species with atypical sex chromosomes.

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Degenerated, Undifferentiated, Rearranged, Lost: High Variability of Sex Chromosomes in Geometridae (Lepidoptera) Identified by Sex Chromatin

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Abstract: Sex chromatin is a conspicuous body that occurs in polyploid nuclei of most lepidopteran females and consists of numerous copies of the W sex chromosome. It is also a cytogenetic tool used to rapidly assess the W chromosome presence in Lepidoptera. However, certain chromosomal features could disrupt the formation of sex chromatin and lead to the false conclusion that the W chromosome is absent in the respective species. Here we tested the sex chromatin presence in 50 species of Geometridae. In eight selected species with either missing, atypical, or normal sex chromatin patterns, we performed a detailed karyotype analysis by means of comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). The results showed a high diversity of W chromosomes and clarified the reasons for atypical sex chromatin, including the absence or poor differentiation of W, rearrangements leading to the neo-W emergence, possible association with the nucleolus, and the existence of multiple W chromosomes. In two species, we detected intraspecific variability in the sex chromatin status and sex chromosome constitution. We show that the sex chromatin is not a sufficient marker of the W chromosome presence, but it may be an excellent tool to pinpoint species with atypical sex chromosomes.

Keywords: sex chromosome evolution; W chromosome; neo-sex chromosomes; sex chromatin; Lepidoptera; Geometridae; comparative genomic hybridization; intraspecific chromosomal variability

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1. Introduction

Sex chromosomes represent a rapidly evolving part of the genome. It is generally accepted that they originate from a pair of autosomes when one of the homologs has acquired a sex-determining factor [1]. This triggers a sequence of events conditioned by the cessation of recombination, leading to the degeneration of a sex-specific sex chromosome, i.e., Y or W [2]. Typical features of the Y and W chromosomes are gene deficiency, the presence of pseudogenes, and the abundance of repetitive sequences such as mobile elements and tandem repeats. Eventually, the sex-specific chromosome may lose its sex-determining function and disappear, resulting in the X/XX or Z/ZZ sex chromosome system (reviewed, e.g., in the work of [3,4]). Alternatively, new heteromorphic sex chromosomes may arise from a B chromosome, which acquires a sex-determining locus or simply begins to pair with the X or Z chromosome in the heterogametic sex [5,6].

The evolution of sex chromosomes, however, may not be so direct. Apart from the canonical XX/XY or WZ/ZZ systems, neo-sex chromosomes may arise by fusion between the ancestral sex chromosomes and autosomes. These neo-sex chromosomes

then consist of multiple evolutionary strata, which may be clearly visible on the neo-Y or neo-W chromosome of some species due to the heterochromatin part of the ancestral degenerate sex chromosome and the euchromatin part of the attached autosome (e.g., the work of [7–9]). In addition, multiple sex chromosome systems may arise by fissions of ancestral sex chromosomes or by fusions and translocations between sex chromosomes and autosomes (e.g., the work of [10,11]).

The evolution of sex chromosomes thus proceeds in cycles: from non-degenerate, autosome-like sex chromosomes, through various stages of ongoing degeneration of the Y and W chromosomes, to their ultimate loss, while taking detours through neo-sex chromosomes in the meantime. Therefore, differentiated sex chromosomes in different species can be achieved at various stages of the process, and their number, appearance, and molecular content can vary enormously, even among related species [12–14]).

Moths and butterflies (Lepidoptera) are the most species-rich group with female heterogamety. Diverse sex chromosome systems have been described in this group, including primary absence or secondary loss of the W chromosome, neo-sex chromosomes, and multiple sex chromosomes, allowing to study the sex chromosomes at various stages of their evolution. Lepidoptera, therefore, represent an ideal model for the study of sex chromosome evolution. The basal clades of the order Lepidoptera and the sister order Trichoptera (caddisflies) lack the W chromosome, the absence of which is thus considered an ancestral state [15,16]. The exact mechanism and time of the W chromosome origin are not yet clear [17], but it is typically present in Ditrysia, a megadiverse group comprising 98% known lepidopteran species. Accordingly, the most common sex chromosome constitution in this group is WZ/ZZ (♀/♂) [15].

The lepidopteran Z chromosome resembles autosomes in content and structure [18]. It is rich in genes and exhibits a highly conserved synteny block of "ancestral" Z-linked genes across Lepidoptera [17,19–21]. In contrast, most of the W chromosomes investigated so far consist mainly of repetitive sequences and probably contain very few functional genes [22,23]. A high load of repeats leads to the conversion of the W chromosome to heterochromatin, which is often the largest or only heterochromatin block in the karyotype. As a consequence, the W chromosome of most species forms a round condensed body in female interphase polyploid nuclei, the sex chromatin, which contains up to several thousand W copies [22].

In a comprehensive study monitoring the occurrence of sex chromatin in 238 species, Traut and Marec [24] concluded that sex chromatin is a common trait of females in Ditrysia. They also emphasized the usefulness of sex chromatin as a diagnostic marker of sex in young developmental stages and a marker for the identification of W chromosome aberrations. Since then, sex chromatin has been used in a number of other studies as an indicator of the W chromosome presence and its condition (e.g., the work of [7,17,25–27]). However, the use of sex chromatin as a marker should be considered with caution, as there are certain cytogenetic factors that might influence its occurrence and appearance. For example, the fusion of a W chromosome with a Z or an autosome or translocation from another chromosome onto a W chromosome may result in fragmentation, an aberrant shape, or even disappearance of the sex chromatin body. This has been observed in irradiated strains of the Mediterranean flour moth (*Ephestia kuehniella*) [18,28,29] or the codling moth (*Cydia pomonella*) [30], but also in species in which the fusion of W with another chromosome occurred naturally, such as the vapourer moth (*Orgyia antiqua*), or the clouded Apollo (*Parnassius mnemosyne*) [9,31].

Sex chromatin, if present, represents an excellent source of the W chromosome DNA that can be used either to prepare a W-painting probe for W chromosome identification by fluorescence in situ hybridization (FISH) [13,17,32,33] or for sequencing [23]. Another technique used to study W chromosomes is comparative genomic hybridization (CGH), in which differently labeled male and female genomic probes compete on complementary loci on chromosomes of heterogametic sex [34,35]. CGH thus not only identifies the W chromosome but also provides information about its sequence composition. This

method has, however, its limitations as it fails to detect molecularly undifferentiated sex chromosomes [36].

In this study, we investigated the W chromosomes in geometrid moths. Geometrids are one of the most diverse groups of Ditrysia, with over 23,000 species [37]. Due to their advanced phylogenetic position, the presence of sex chromatin and W chromosome is expected. However, available studies show an unusual occurrence of sex chromatin in this group. Of the 23 species tested so far, the sex chromatin pattern was atypical in females in 7 of them, despite the fact that the species were phylogenetically distant ([38,39]; reviewed in the work of [24]). Furthermore, geometrids differ noticeably in chromosome numbers, suggesting dynamic karyotype evolution by fusions and fissions that could include sex chromosomes [40,41]. Yet, very little has been done regarding cytogenetic analysis of geometrid sex chromosomes [13,20]. Therefore, we conducted an extensive survey of the sex chromatin status in geometrids, which included 50 species. Based on the results, we selected eight species with atypical and normal sex chromatin patterns and performed a detailed cytogenetic analysis to identify the cause of sex chromatin malformation or absence. We also addressed questions on the use of sex chromatin as a W chromosome marker and as a data source for theories on the W chromosome origin (e.g., the work of [15,17,25]).

2. Materials and Methods

2.1. Specimens

The list of 50 species of Geometridae screened for the presence of sex chromatin in adulthood is included in Table S1. Based on the results, 8 species were chosen for thorough cytogenetic investigation: *Aethalura punctulata*, *Chiasmia clathrata*, *Epirrhoe alternata*, *Hylaea fasciaria*, *Hypomecis atomaria*, *Operophtera brumata*, *Peribatodes rhomboidaria*, and *Pseudopanthera macularia*. Except for *E. alternata* and *O. brumata* (Larentiinae), all species belong to the subfamily Ennominae [42]. Adult moths were collected in Czechia and Estonia in the years 2018–2020, either using UV light traps at night or captured by entomological nets in daylight; brachypterous females were collected from tree trunks at night. All specimens were identified according to their morphology. Mated females were kept individually in moisturized plastic containers with host plants, where they laid eggs. The hatched larvae were reared on their host plants until the last instar or pupa, dissected, and the residual tissue was immediately frozen in liquid nitrogen and stored at −20 °C. From each selected species, we examined several offspring of both sexes from different mothers and from different localities (for more details, see Table S2).

2.2. Chromosomal Preparations and Sex Chromatin Assay

Chromosomal preparations were obtained from penultimate instar larvae from gonads (meiotic chromosomes) and from wing imaginal disc or brains (mitotic chromosomes) as described in the work of [27]. Polyploid interphase nuclei for sex chromatin assay were obtained from Malpighian tubules of both larvae and adult specimens and stained with lactic acetic orcein as described in the work of [43].

2.3. DNA Isolation

Genomic DNA (gDNA) was isolated by CTAB (hexadecyltrimethylammonium bromide; Sigma-Aldrich, St. Louis, MO, USA) according to the protocol of [44] with the following modifications. Insect tissues were crushed in 800 µL of extraction buffer prepared according to the protocol and incubated in a heat block at 60 °C overnight. Afterward, an equal amount of pure chloroform was added, and the sample was centrifuged, the upper aqueous phase was transferred into a new tube, and the chloroform extraction step was repeated. The solution was then treated with 5 µL of RNase A (10 mg/mL; Sigma-Aldrich) and incubated for 30 min at 37 °C. To precipitate DNA, 2/3 of the final volume of isopropyl alcohol (Sigma-Aldrich) was added, and the mixture was incubated for at least 2 h at room temperature. Finally, the solution was centrifuged for 15 min at 14,000 g, the super-

natant was removed, and the pellet was washed in 70% ethanol, air-dried, and dissolved in PCR-grade water. Final concentrations of the extracted gDNA were measured on a Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA), and DNA purity was assessed by 260/280 ratio on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Comparative Genomic Hybridization (CGH)

Genomic DNAs were fluorescently labeled by the improved nick translation procedure of [45] with some modifications [27]. Male gDNAs were labeled with Cy3-dUTP, female gDNAs with either fluorescein-dUTP or ATTO 488-dUTP (all Jena Bioscience, Jena, Germany). Nick translation reactions were incubated at 15 °C and stopped after 3.5 h either by 10% of the reaction volume loading dye buffer (25 mM EDTA pH 8, 0.6 mM bromophenol blue, and 5% glycerol) or by 10 min inactivation at 70 °C. Labeled probes were checked on a standard 1.5% agarose gel in TAE buffer.

CGH was carried out according to the protocol of [34] with modifications described in the work of [17]. Briefly, the hybridization mixture per slide consisted of 250–500 ng of each labeled gDNA probe (exactly the same amount of gDNA of each sex per slide) and 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich) as a non-specific competitor. The mixture was precipitated and dissolved in 50% deionized formamide, 10% dextran sulfate, and 2 × SSC. After 5 min incubation at 90 °C, it was cooled down on ice and prehybridized for 1.5 h at 37 °C. Finally, the mixture was applied on a slide, which had already been incubated with RNase A (200 ng/µL, Sigma-Aldrich) in 2 × SSC for 1 h at 37 °C, twice washed in 2 × SCC for 5 min, and denatured with 70% formamide in 2 × SSC at 68 °C for 3.5 min, then cooled down in 70% cold ethanol (1 min) and dehydrated in 80% and 100% ethanol at room temperature, 30 s each. Slides were incubated with the hybridization mixture at 37 °C for 3 nights. They were then washed for 5 min at 62 °C in 0.1 × SSC with 1% Triton X-100, stained with 0.5 µg/mL DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich), and mounted in DABCO antifade (1,4-diazabicyclo(2.2.2)-octane; Sigma-Aldrich). In each species studied, several specimens of both sexes were examined by CGH.

2.5. Genomic In Situ Hybridization (GISH) with 18S rDNA Probe

GISH was performed according to the CGH protocol (see above), only without the prehybridization step. The hybridization mixture consisted of 250–500 ng of a female gDNA probe labeled by nick translation (see above), 3 µg of unlabeled male gDNA (fragmented by boiling at 99 °C for 20 min), 25 µg of sonicated salmon sperm DNA, and 300 ng of labeled 18S rDNA probe. This probe was prepared by PCR from the codling moth, *Cydia pomonella*, following the protocol described in the work of [46] and labeled with Cy3-dUTP by nick translation with 1.5 h incubation.

2.6. Reprobing and Fluorescence In Situ Hybridization (FISH) with Telomeric Probe

In species with supposed sex chromosome multivalents, we performed FISH with a telomeric probe on slides previously used for CGH to visualize chromosome ends. The insect telomeric probe $(TTAGG)_n$ [47] was generated by non-template PCR [48] as described in the work of [49] and labeled with biotin-14-dUTP (Invitrogen) by nick translation (see above) with 50 min incubation. Genomic probes were removed from slides as described in the work of [50]. The telomeric probe was then hybridized on slides followed by multiple signal amplification steps, using streptavidin-Cy3 conjugated antibodies (Jackson ImmunoRes. Labs., Inc., West Grove, PA, USA) and biotinylated antistreptavidin antibodies (Vector Labs., Inc., Burlingame, CA, USA) according to the protocol described in the work of [49]. Finally, the slides were counterstained with 0.5 µg/mL DAPI in DABCO antifade.

2.7. FISH with W-Painting Probe

In *C. clathrata* and *O. brumata*, we microdissected sex chromatin and used it as a painting probe to visualize the W chromosome. Microdissection was performed essentially

as described in the work of [32], and probe amplification and labeling were performed as described in the work of [17]. In *C. clathrata*, the W-painting probe labeled with Cy3-dUTP was hybridized along with the telomeric probe labeled with ATTO 488-dUTP as described in the work of [17] with the following modifications: the amount of telomeric probe was 300 ng per slide, and post-hybridization washes were 2 × 5 min in 2 × SSC, 2 × 5 min in 1 × SSC, 1 min in 1 × PBS and 1 min in 1% Kodak Photo-Flo in miliQ water, all at 25 °C. These low stringency washes were used due to the low intensity of hybridization signal of the W-painting probe, observed after the high stringency wash in 0.1 × SSC with 1% Triton at 62 °C for 5 min, conditions that we otherwise use for FISH with fluorochrome-labeled probes. In *O. brumata*, the W-painting probe labeled with Cy3-dUTP was used without the telomeric probe, and the high stringency post-hybridization wash was applied.

2.8. Microscopy and Image Processing

Chromosomal preparations were examined under a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany). For fluorescently stained chromosomes, we used narrow fluorescence filter sets and a monochrome CCD camera XM10 (Olympus Europa Holding, Hamburg, Germany). Polyploid nuclei preparations stained with lactic acetic orcein were examined by light microscopy using 40x and 63x objectives depending on the size of the nuclei. Black-and-white images were captured by cellSens Standard software version 1.9 (Olympus). For slides after CGH, GISH, or FISH, pictures were captured separately for each fluorescent dye, then pseudocoloured and merged using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

3. Results

We inspected 50 species from five subfamilies of Geometridae for the presence of a sex chromatin body in polyploid nuclei of the Malpighian tubule cells. Including data from the literature, the female sex chromatin assay in 69 species revealed several variants: normal sex chromatin (i.e., the usual conspicuous body) was present in 48 species, while atypical sex chromatin (i.e., missing, scattered, miniature, multiple or variable) was found in 21 species (Figure 1; Table S1). Males of all inspected species lacked sex chromatin.

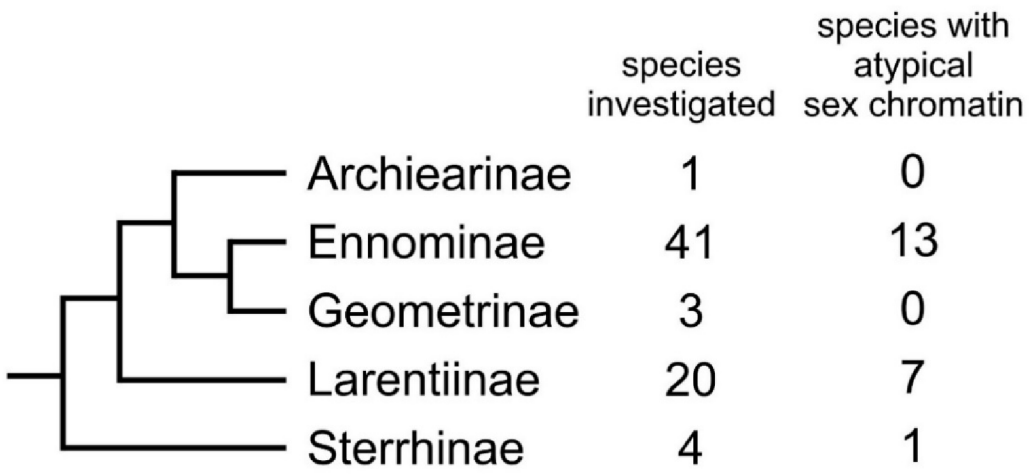

Figure 1. Species inspected for the sex chromatin presence. Data on 69 species of Geometridae belonging to five subfamilies are included. An atypical sex chromatin pattern was found in almost one-third of the species (see Table S1 for details). Phylogeny based on the work of [42].

Based on the sex chromatin assay, we selected six species with atypical sex chromatin as well as two species with normal sex chromatin as controls for a thorough cytogenetic investigation (see Table 1 for a summary of results).

3.1. *Aethalura Punctulata (Ennominae)*

Sex chromatin was completely missing in *A. punctulata* (Figure 2a,b). Subsequently, the absence of the W chromosome was confirmed by chromosome counts, in which the chromosome number of $2n = 61/62$ (♀/♂) was lower by one in females (Figure 2c,d). CGH on female pachytene chromosomes showed no differentiated regions in the genome, and a single Z univalent was observed (Figure 2e–h). No differentiation nor the Z univalent was found in males (Figure 2i–l).

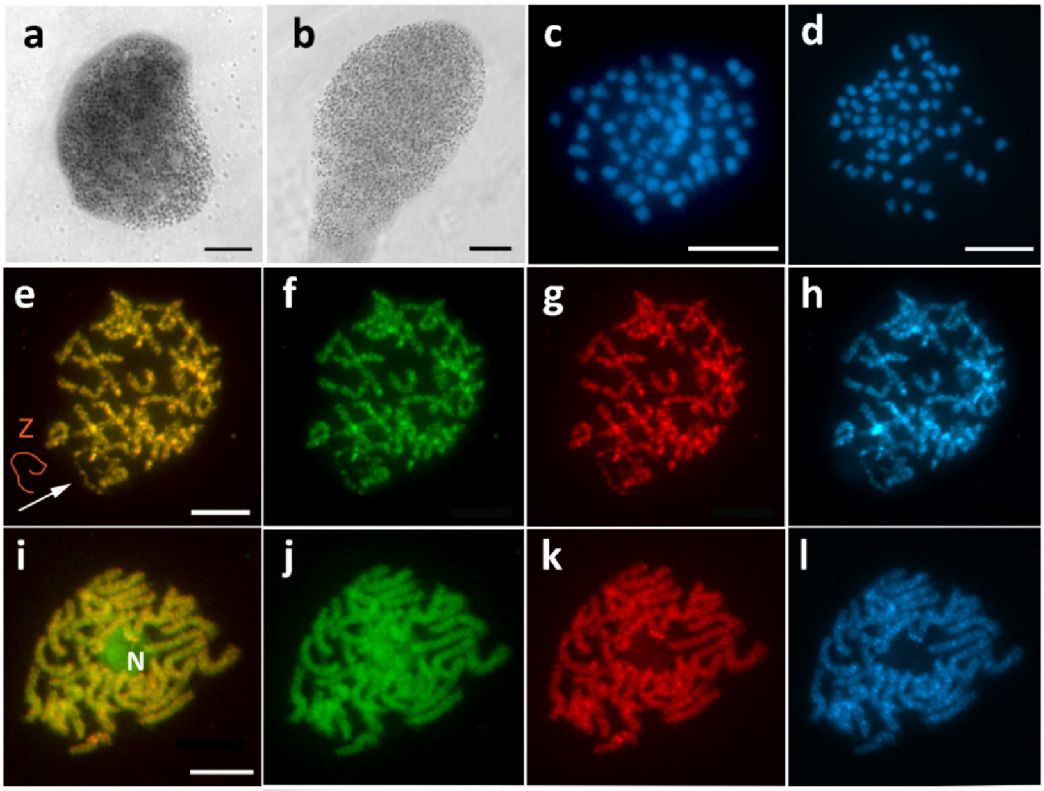

Figure 2. Missing W chromosome in *Aethalura punctulata***.** Sex chromatin was absent in female (**a**) and male (**b**) polyploid nuclei stained with orcein. Mitotic metaphase chromosomes stained with DAPI show $2n = 61$ in females (**c**) and $2n = 62$ in males (**d**). Comparative genomic hybridization (CGH) on female pachytene chromosomes (**e**–**h**) did not reveal any differentiated chromosome. The Z univalent is indicated by an arrow and shown in the scheme (**e**). CGH on male pachytene chromosomes showed similar results, but no univalent was found (**i**–**l**); note nucleolus (N). Panels (**e**,**i**)—merged pictures of both probes; (**f**,**j**)—female genomic probe (green); (**g**,**k**)—male genomic probe (red); (**h**,**l**)—DAPI staining (light blue). Bar = 10 µm.

3.2. *Chiasmia Clathrata (Ennominae)*

In *C. clathrata*, we detected variability in the sex chromatin appearance. Female offspring in some broods showed normal sex chromatin (Figure 3a), while in other broods, they showed miniature sex chromatin bodies (Figure 3b). No sex chromatin was found in males (Figure 3c). The following cytogenetic analysis using CGH distinguished two types of sex chromosome systems: in broods with normal sex chromatin, we found the classical WZ system (Figure 3d–g), while in broods with miniature or scattered sex chromatin we found a WZ_1Z_2 system (Figure 3h–k). In both cases, the W chromosomes were not composed of conspicuous heterochromatin and were identified by both genomic probes, with the female probe signals being slightly stronger. They were mostly indiscernible by DAPI staining, although when observed in the pachytene nuclei of nurse cells, they were occasionally highlighted with DAPI, probably due to higher chromosome condensation. The existence of two sex chromosome systems was confirmed by FISH with the telomeric probe combined

with the W-painting probe, which was prepared from microdissected sex chromatin bodies of WZ females. In females with normal sex chromatin, the W-painting probe labeled the W chromosome along its entire length and the telomeric probe hybridized to the ends of the WZ bivalent (Figure 3p,q). However, in females from two broods with fragmented sex chromatin, only part of the W chromosome was labeled with the W-painting probe, and telomeric signals were detected at both ends of this large W chromosome as well as at both ends of its two pairing partners, Z_1 and Z_2 (Figure 3r,s). These results strongly suggest that the ancestral W chromosome underwent fusion with an autosome, forming a neo-W chromosome. The homologous autosome thus became the Z_2 sex chromosome. No chromosome was differentiated by CGH in male pachytene complements (Figure 3l–o). The exact chromosome number could not be identified due to an insufficient number of suitable-quality mitotic metaphases.

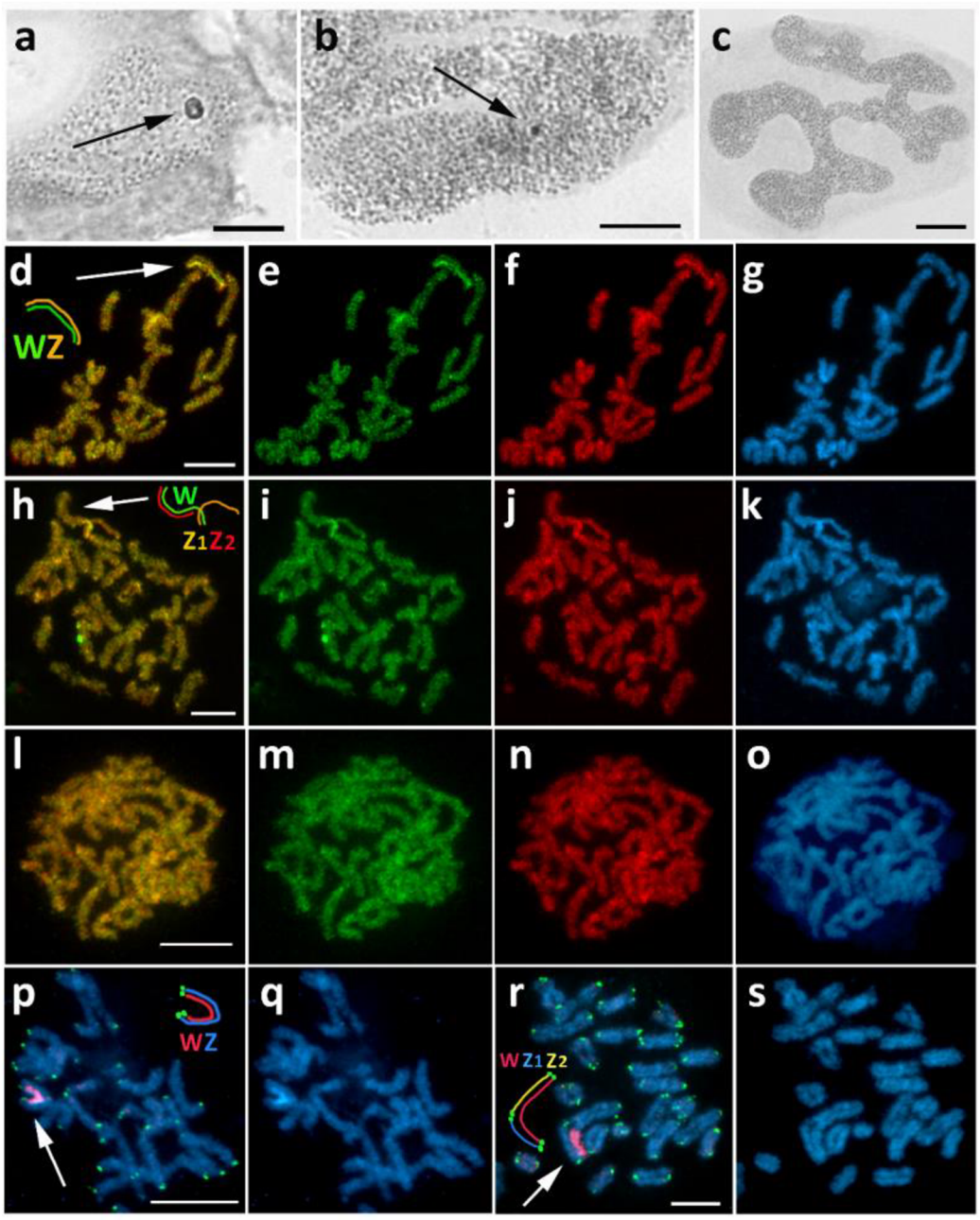

Figure 3. Sex chromosome systems in *Chiasmia clathrata*. (**a**–**c**) Polyploid nuclei stained with orcein

showing a variable sex chromatin pattern in females from different broods, either normal (**a**) or miniature body (**b**), whereas it is absent in males (**c**). (**d–o**) Comparative genomic hybridization (CGH) on pachytene chromosomes revealed a WZ sex chromosome bivalent ((**d**), arrow and scheme) in females with normal sex chromatin (**d–g**) and a WZ1Z2 trivalent (**h**, arrow and scheme) in females with scattered sex chromatin (**h–k**); no chromosome was differentiated by CGH in males (**l–o**). Panels (**d**,**h**,**l**)—merged pictures of both probes; (**e**,**i**,**m**)—female genomic probe (green); (**f**,**j**,**n**)—male genomic probe (red); (**g**,**k**,**o**)—DAPI staining (light blue). (**p–s**) Fluorescence in situ hybridization (FISH) with W-painting probe (red) and $(TTAGG)_n$ telomeric probe (green) on pachytene chromosomes of females with the WZ bivalent (**p**,**q**) and females with the WZ_1Z_2 trivalent (**r**,**s**). Panels (**p**,**r**)—merged pictures of both probes; (**q**,**s**)—DAPI staining (light blue). In the WZ bivalent, the W-painting probe labeled the entire W chromosome (**p**, arrow and scheme). In the WZ_1Z_2 trivalent, less than half of the W chromosome was labeled with the W-painting probe, and telomeric signals confirmed the presence of two Z chromosomes (**r**, arrow and scheme). Bar = 10 µm.

3.3. *Epirrhoe Alternata (Larentiinae)*

Females of *E. alternata* showed scattered sex chromatin with a variable appearance, often forming either one or two miniature heterochromatic bodies, occasionally even smaller dot-like bodies (Figure 4a). No sex chromatin was found in males (Figure 4b). In all females examined, CGH analysis uncovered a sex chromosome multivalent consisting of differentiated W chromosomes, highlighted by both genomic probes with the female signal being slightly stronger (Figure 4j–m), which was not seen in males (Figure 4n–q). Since it was necessary to distinguish between the individual chromosomes of the multivalent, the CGH slides were reprobed by FISH with a telomeric probe (Figure 4e–i). In addition to standard telomeric signals at the ends of the multivalent, we detected two additional pairs of hybridization signals (Figure 4i). One of them also colocalized with DAPI-positive heterochromatin blocks, which are located terminally on the respective W chromosomes (Figure 4h,i). Taken together, these results suggest the presence of a sex chromosome quadrivalent consisting of three separate W chromosomes paired with a single Z chromosome (Figure 4e). Therefore, the sex chromosome constitution in this species is $W_1W_2W_3Z/ZZ$ (♀/♂). Consistent with this finding, the diploid chromosome number was determined to be $2n = 64$ in females and $2n = 62$ in males (Figure 4c,d).

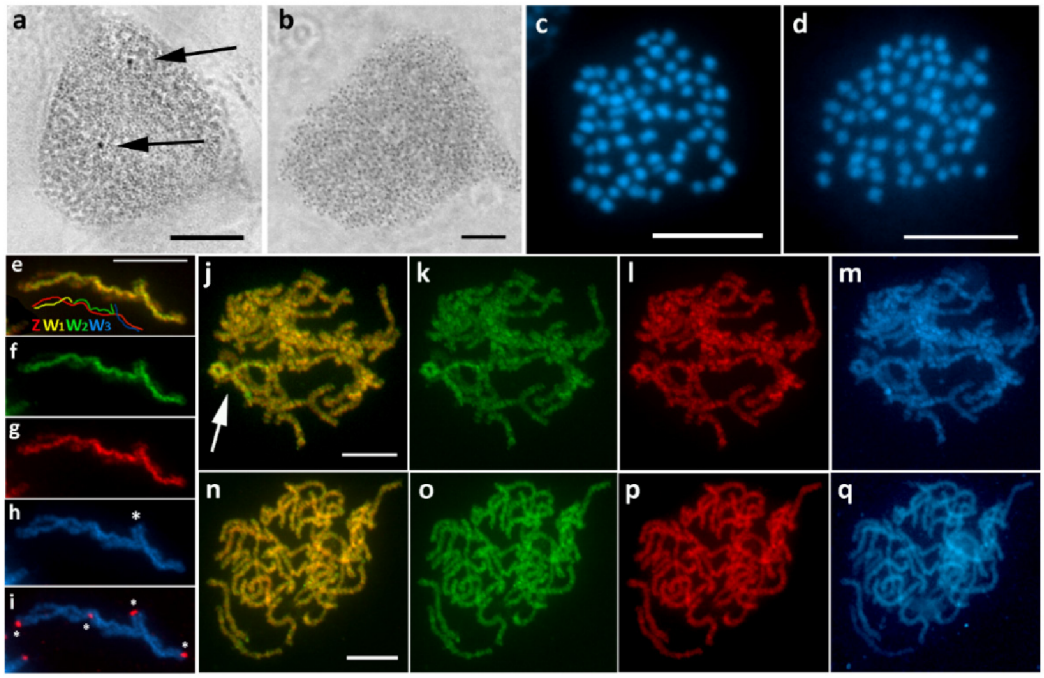

Figure 4. Sex chromosome multivalent in *Epirrhoe alternata*. (**a**,**b**) Polyploid nuclei stained with

orcein showing miniature dot-like sex chromatin bodies in females ((**a**), arrows) but not in males (**b**). (**c**,**d**) Mitotic metaphase chromosomes stained with DAPI showing $2n = 64$ in females (**c**) and $2n = 62$ in males (**d**). (**e**–**h**) A sex chromosome quadrivalent, $W_1W_2W_3Z$, revealed by CGH ((**e**), scheme) and confirmed by reprobing using FISH with telomeric probe ((**i**), asterisks); note a pair of telomeric signals colocalized with two DAPI-positive heterochromatin blocks at the ends of W_2 and W_3 ((**h**), asterisk). (**j**–**m**) CGH on female pachytene chromosomes showing the sex chromosome multivalent highlighted with both genomic probes, with female signal being slightly stronger ((**j**), arrow). (**n**–**q**) CGH on male pachytene chromosomes without any differentiated regions. Panels (**e**,**j**,**n**)—merged pictures of both genomic probes; (**f**,**k**,**o**)—female genomic probe (green); (**g**,**l**,**p**)—male genomic probe (red); (**h**,**m**,**q**)—DAPI staining (light blue); (**i**)—merged picture of DAPI staining and $(TTAGG)_n$ telomeric probe (red). Bar = 10 μm.

3.4. Hylaea Fasciaria (Ennominae)

Sex chromatin in *H. fasciaria* females was rather unusual. Apart from a single spherical conspicuous body (Figure 5a), we also observed two or more bodies within one polyploid nucleus, often differing in size and/or shape (Figure 5d,e); occasionally, we found a single oval-shaped or otherwise deformed body (Figure 5b,c). No sex chromatin was found in males (Figure 5f). These findings were partially elucidated by the CGH results, as the W chromosome was preferentially labeled with the female genomic probe and composed of two parts: a strongly heterochromatic part and a euchromatic-like part (Figure 5g–k). Interestingly, the latter part of the WZ bivalent was associated with the nucleolus (Figure 5j,k). Application of the 18S rDNA probe combined with GISH detected many scattered, disorganized signals throughout the nucleolus region (Figure 5l–n). We assume that this is due to loose chromatin loops, suggesting continuous high transcriptional activity in the nucleolar organizer region (NOR). In males, no differentiated chromosomes were identified (Figure 5o–r). Due to the lack of material, it was not possible to determine the number of chromosomes in this species.

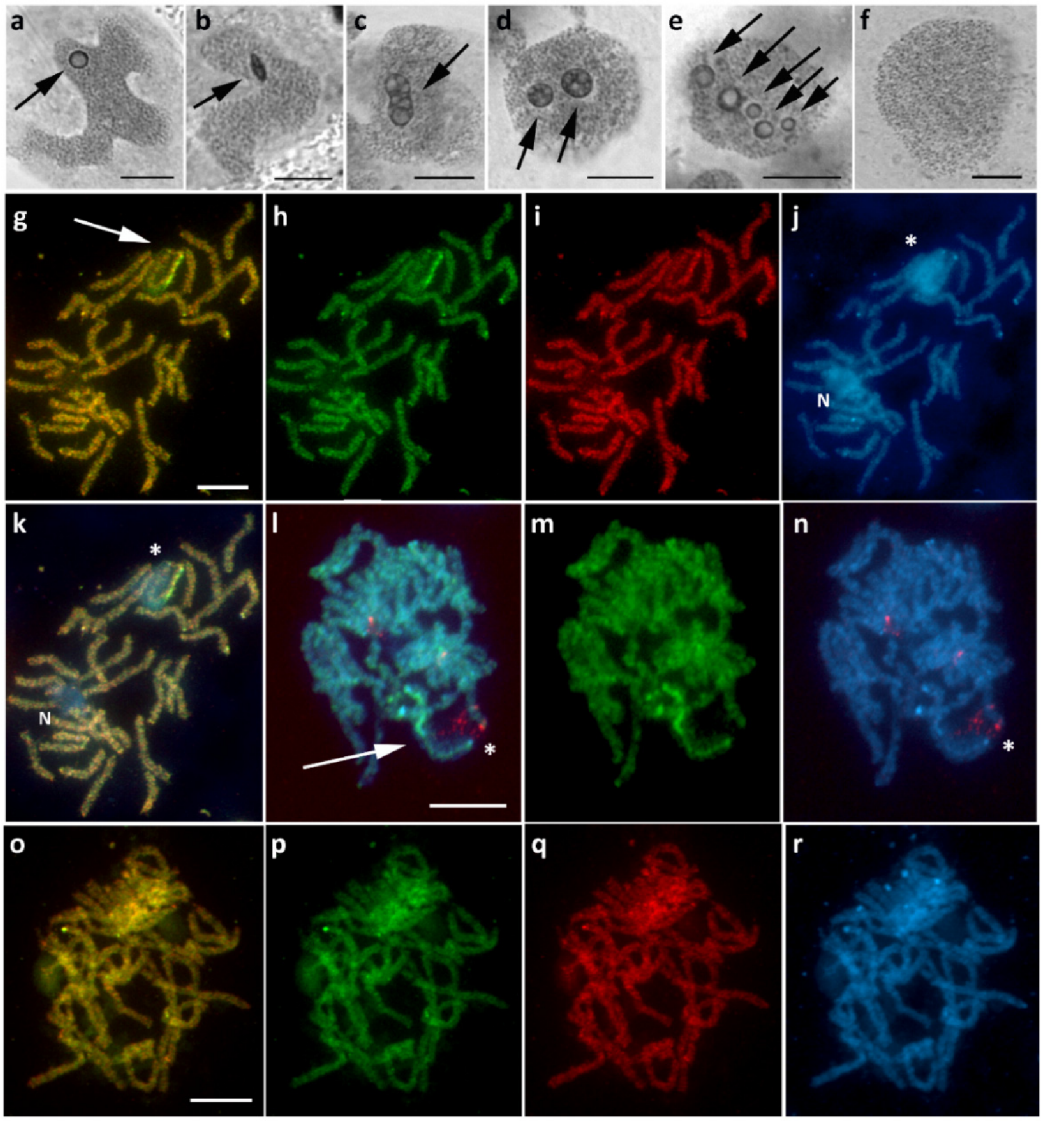

Figure 5. Multiple sex chromatin bodies and nucleolus-associated W chromosome in *Hylaea fasciaria*. (**a**–**f**) Polyploid nuclei stained with orcein showing variable forms of sex chromatin in females ((**a**–**e**), arrows), from a regular single body or deformed body to multiple bodies, while it was absent in males (**f**). (**g**–**k**) CGH on female pachytene chromosomes identified a WZ bivalent ((**g**), arrow), in which the strongly heterochromatinized part of the W chromosome was preferentially labeled with the female genomic probe (**g**–**i**), and the other part of the WZ bivalent was associated with the nucleolus, well visible after DAPI staining ((**j**,**k**); asterisk); note the second nucleolus (N) in the same pachytene complement (**j**,**k**). (**l**–**n**) GISH combined with FISH with 18S rDNA probe on female pachytene chromosomes identified the WZ bivalent ((**l**), arrow) according to the W chromosome labeled by the female genomic probe (**l**,**m**) and the associated nucleolus by scattered signals of the 18S rDNA probe (**l**,**n**; red signals, asterisk). (**o**–**r**) CGH on male pachytene chromosomes without any differentiated region. Panels (**g**,**o**)—merged pictures of both genomic probes; (**h**,**m**,**p**)—female genomic probe (green); (**i**,**q**)—male genomic probe (red); (**j**,**r**)—DAPI staining (light blue); (**k**)—merged picture of both genomic probes and DAPI staining; (**l**)—merged picture of the female genomic probe, 18S rDNA probe, and DAPI staining; (**n**)—merged picture of 18S rDNA probe and DAPI staining. Bar = 10 μm.

3.5. Hypomecis Atomaria (Ennominae)

Hypomecis atomaria (previously *Ematurga atomaria*, see the work of [51] for the nomenclatural change) was used as our first control species. In highly polyploid nuclei, sex

chromatin formed a typical, roundish and conspicuous body in females, but not in males (Figure 6a,b). The diploid number of chromosomes was the same in both sexes, $2n = 62$ (Figure 6c,d), suggesting a WZ/ZZ sex chromosome system. Using CGH, we identified a curious WZ bivalent in female pachytene complements (Figure 6e–h), with a remarkably small, fully heterochromatic W chromosome that was often completely surrounded by the Z chromosome (Figure 6i). Since both genomic probes hybridized evenly to the W chromosome (Figure 6f,g), we assume that it is predominantly composed of repetitive DNA sequences common to both sexes. In males, no chromosome was differentiated by CGH (Figure 6j–m).

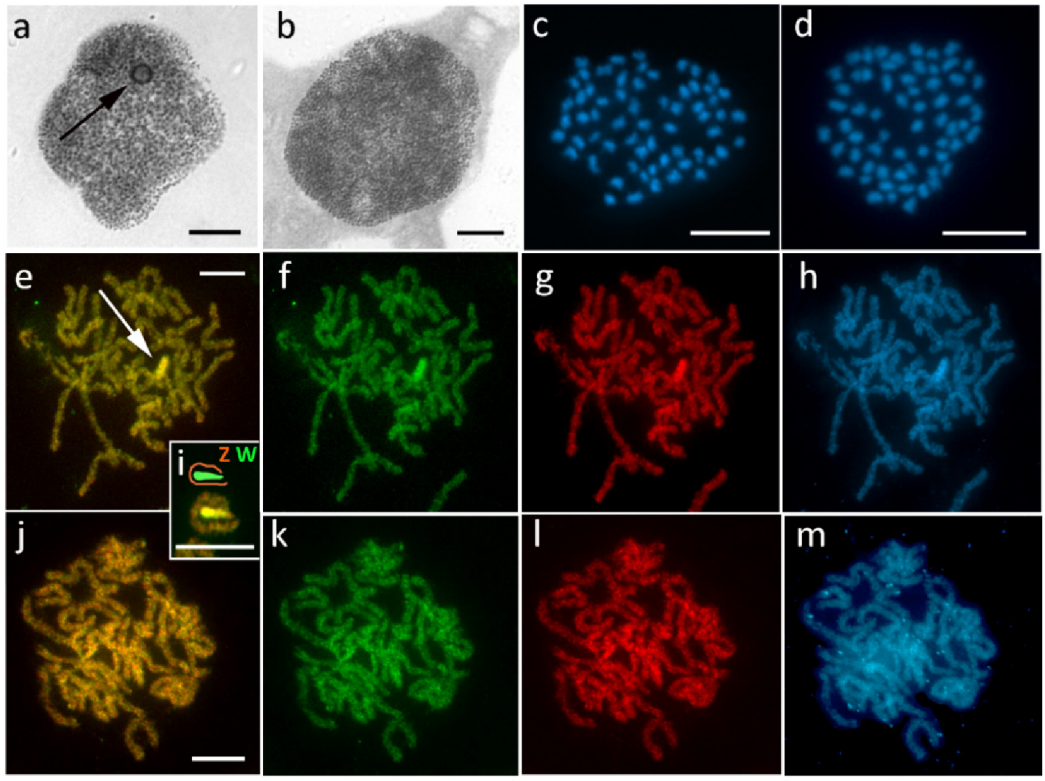

Figure 6. Highly differentiated WZ sex chromosomes in *Hypomecis atomaria*. (**a**,**b**) Polyploid nuclei stained with orcein showing a voluminous sex chromatin body in females ((**a**), arrow) and missing sex chromatin in males (**b**). (**c**,**d**) Mitotic metaphase chromosomes stained with DAPI showing $2n = 62$ in both females (**c**) and males (**d**). (**e**–**m**) Comparative genomic hybridization (CGH) on pachytene chromosomes identified in females (**e**–**i**) a highly differentiated W chromosome ((**e**), arrow), composed of prominent DAPI-positive heterochromatin (**h**). In the WZ bivalent, the W chromosome formed a short, thick rod surrounded by a long Z chromosome ((**i**), scheme). In males, no chromosome was differentiated by CGH (**j**–**m**). Panels (**e**,**i**,**j**)—merged pictures of both probes; (**f**,**k**)—female genomic probe (green); (**g**,**l**)—male genomic probe (red); (**h**,**m**)—DAPI staining (light blue). Bar = 10 µm.

3.6. Operophtera Brumata (Larentiinae)

In *O. brumata*, a well-visible sex chromatin body (eventually two bodies) foreshadowed a highly differentiated W chromosome in females (Figure 7a,b), while no sex chromatin was found in males (Figure 7c). The diploid number of chromosomes was significantly reduced and differed between the sexes, with $2n = 30$ in females and $2n = 28$ in males; the individual chromosomes also varied greatly in size (Figure 7d,e), some of them being much larger than usual lepidopteran chromosomes. These two features combined indicate multiple fusions that formed the karyotype of this species. In pachytene oocytes, CGH strongly highlighted a relatively small chromosomal segment, the anticipated W chromosome (Figure 7f–m), which was not seen in pachytene spermatocytes (Figure 7n–q). Slightly preferential labeling with the female genomic probe was observed, although the male

genomic probe also hybridized to the W chromosome (Figure 7g,h,k,l). This finding suggests the presence of female-specific sequences in combination with an abundance of common repetitive sequences on this chromosome. In most pachytene figures, the W chromosome was condensed into a compact roundish body (Figure 7f–i,r,t). Occasionally we could also observe it in a stretched form (Figure 7j–m,s). The peculiar shape of the heterochromatin part of W suggested that there could be multiple W chromosomes pairing with the Z chromosome. Thus, we performed reprobing by FISH with a telomeric probe to detect the ends of potential multiple sex chromosomes. Based on all of the results, we concluded that this highly degenerate heterochromatic chromosome is probably the ancestral W chromosome, W_1 (Figure 7t). It was also strongly labeled with the painting probe prepared by microdissection of sex chromatin, thus confirming that sex chromatin consists only of W_1 chromosome copies (Figure 7r,s). Given the different number of chromosomes between the sexes and the location of telomeres, we concluded that there are two other undifferentiated sex chromosomes, W_2 and W_3, pairing with a long Z chromosome (Figure 7t'). Since the chromosome number in *O. brumata* is much lower than the ancestral lepidopteran number ($2n = 62$), and only one of the W chromosomes is heterochromatin-rich, we assume that the Z chromosome underwent fusions with two autosomes, forming the neo-Z chromosome. The homologs of the fused autosomes then became the euchromatin-rich W_2 and W_3.

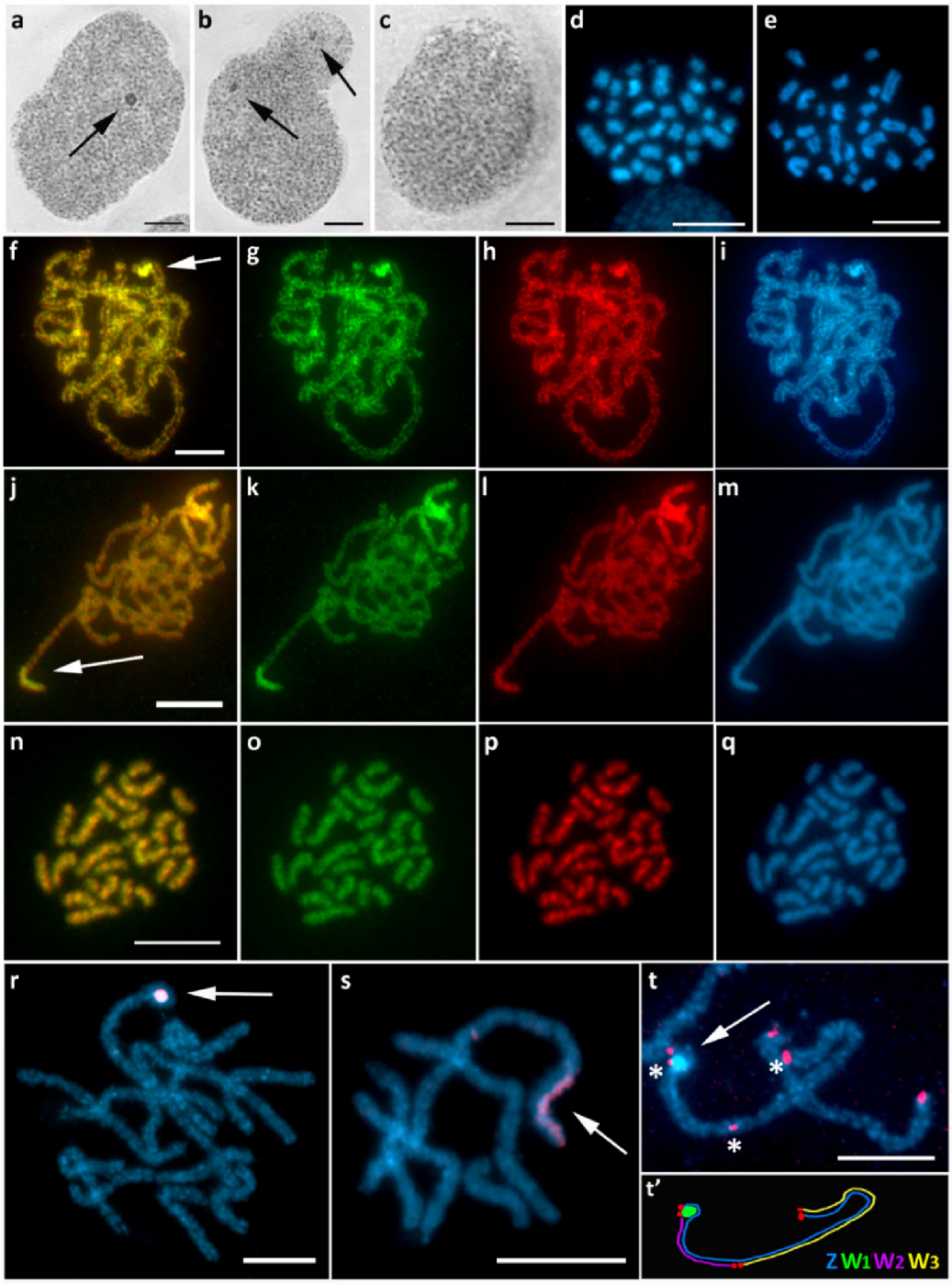

Figure 7. Sex chromosome multivalent and reduced chromosome number in *Operophtera brumata*. (**a**–**c**) Polyploid nuclei stained with orcein showing a single conspicuous sex chromatin body ((**a**), arrow) and occasionally two bodies (**b**, arrows) in females, but no sex chromatin in males (**c**). (**d**,**e**) Mitotic chromosomes stained with DAPI showing $2n = 30$ in females (**d**) and $2n = 28$ in males (**e**). (**f**–**m**) CGH on female pachytene chromosomes identified a terminal W chromosome segment, either forming a heterochromatin body (**f**–**i**, arrow) or a short region paired with the Z chromosome ((**j**–**m**), arrow), highly differentiated with the female genomic probe, but also highlighted with the male genomic probe. (**n**–**q**) No differentiated regions were found after CGH on male mitotic chromosomes. (**r**,**s**) FISH with the W_1-painting probe showing two forms of the W_1 chromosome, condensed ((**r**), arrow) and stretched ((**s**), arrow). (**t**,**t'**) FISH with the $(TTAGG)_n$ telomeric probe revealed a sex chromosome quadrivalent consisting of a fully differentiated W_1 chromosome ((**t**), arrow) and two

undifferentiated sex chromosomes, W_2 and W_3, separated by telomeric signals ((**t**), asterisks), as shown in the scheme (**t'**). Panels (**f**,**j**,**n**)—merged pictures of both genomic probes; (**g**,**k**,**o**)—female genomic probe (green); (**h**,**l**,**p**)—male genomic probe (red); (**i**,**m**,**q**)—DAPI staining (light blue); (**r**,**s**)—merged picture of W_1-painting probe (red) and DAPI; (**t**)—merged picture of $(TTAGG)_n$ telomeric probe (red) and DAPI. Bar = 10 μm.

3.7. Peribatodes Rhomboidaria (Ennominae)

Peribatodes rhomboidaria was used as our second control species due to the typical, conspicuous sex chromatin body found in females but not in males (Figure 8a,b) and also due to the ancestral number of chromosomes in both sexes, $2n = 62$ (Figure 8c,d). Accordingly, CGH revealed a highly differentiated, DAPI-positive W chromosome in females, preferentially labeled with the female genomic probe along almost the entire length of the chromosome, except for one terminal region that resembled the Z chromosome and autosomes (Figure 8e–i). No differentiated chromosome was found in males (Figure 8j–m).

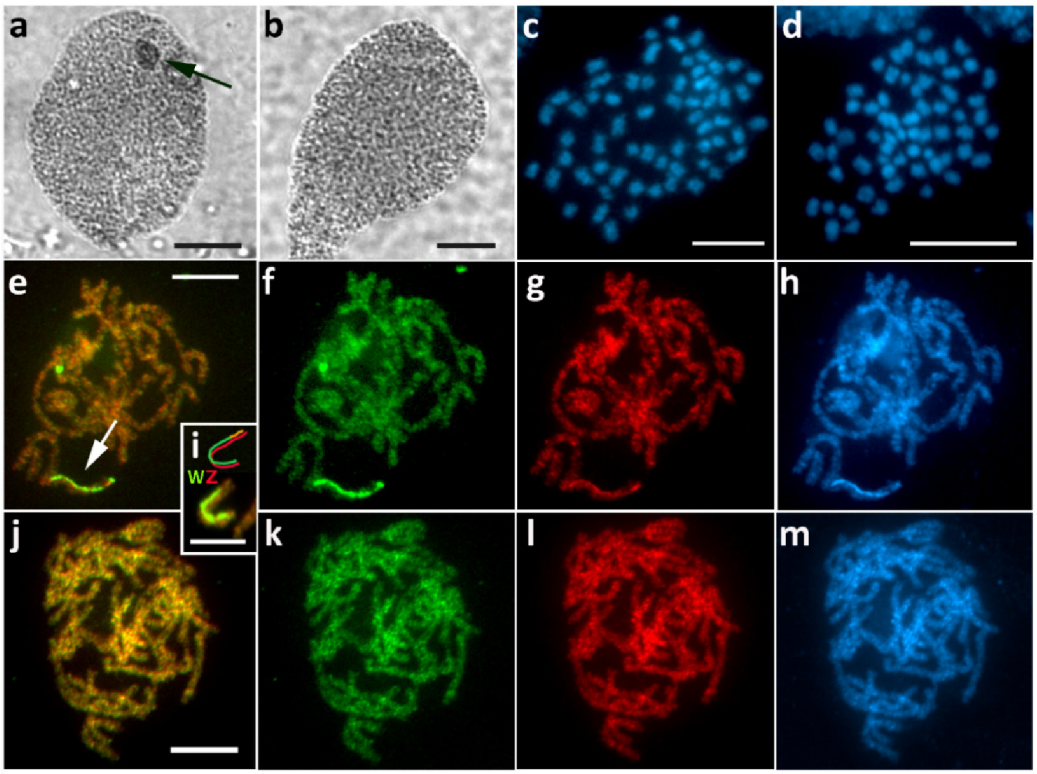

Figure 8. WZ sex chromosomes of *Peribatodes rhomboidaria* with W enriched in female-specific sequences**.** (**a**,**b**) Polyploid nuclei stained with orcein showing conspicuous sex chromatin in females (**a**) but not in males (**b**). (**c**,**d**) Mitotic metaphase chromosomes stained with DAPI showing $2n = 62$ in both females (**c**) and males (**d**). (**e**–**i**) CGH on female pachytene chromosomes identified a WZ bivalent with a well-differentiated, DAPI-positive W chromosome, strongly labeled by the female genomic probe except for the terminal region (**e**, arrow; **i** and scheme). (**j**–**m**) CGH on male pachytene chromosomes without any differentiated region. Panels (**e**,**i**,**j**)—merged pictures of both genomic probes; (**f**,**k**)—female genomic probe (green); (**g**,**l**)—male genomic probe (red); (**h**,**m**)—DAPI staining (light blue). Bar = 10 μm.

3.8. Pseudopanthera Macularia (Ennominae)

Intraspecific variability was observed in *P. macularia*. In the first brood, sex chromatin was absent in female progeny (Figure 9a), and CGH did not reveal any differentiated chromosome (Figure 9f–i). Such findings would indicate the absence of a W chromosome; however, since the chromosome number was the same $2n = 62$ in both sexes (Figure 9d,e) and no Z univalent was found in females, we assume a WZ sex chromosome system with an

undifferentiated W chromosome. In the other two examined broods, we found a relatively small sex chromatin body in females (Figure 9b), and CGH identified a WZ bivalent with a W chromosome preferentially labeled with the female genomic probe (Figure 9j–m). We did not detect any sex chromatin (Figure 9c) or any differentiated chromosome by CGH in all examined males (Figure 9n–q).

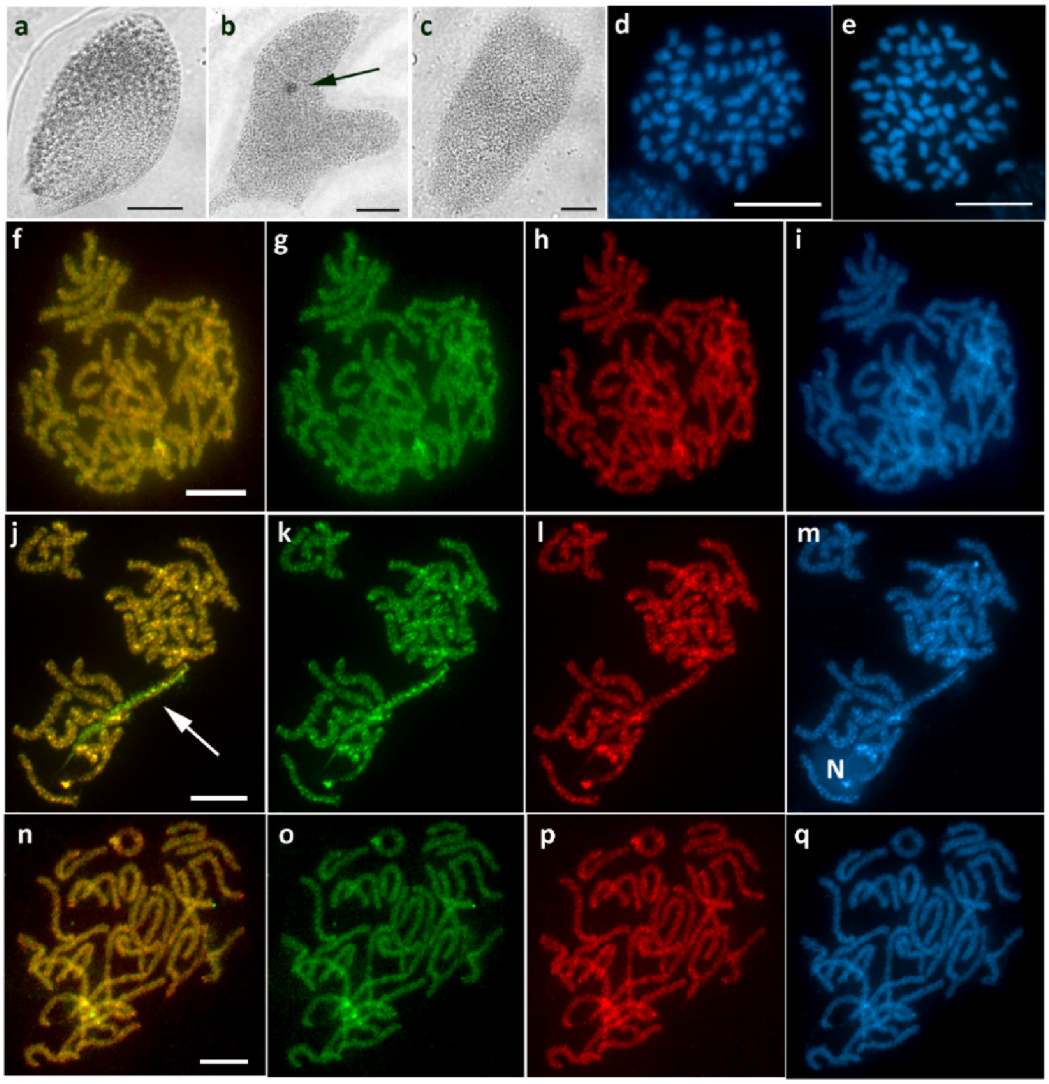

Figure 9. WZ sex chromosomes with variable W in *Pseudopanthera macularia*. (**a**–**c**) Orcein-stained polyploid nuclei showing the absence of sex chromatin in females from the first brood (**a**), a small sex chromatin body in females from another brood (**b**), and no sex chromatin in males (**c**). (**d**,**e**) Mitotic metaphase chromosomes stained with DAPI showing $2n = 62$ in both females (**d**) and males (**e**). (**f**–**m**) CGH on female pachytene chromosomes failed to identify the WZ bivalent, suggesting the presence of an undifferentiated W chromosome in the first brood (**f**–**i**), whereas, in two other broods, the W chromosome was strongly labeled with the female genomic probe ((**j**–**m**), arrow); note nucleolus (N). (**n**–**q**) CGH on male pachytene chromosomes without any differentiated chromosome. Panels (**f**,**j**,**n**)—merged pictures of both probes; (**g**,**k**,**o**)—female genomic probe (green); (**h**,**l**,**p**)—male genomic probe (red); (**i**,**m**,**q**)—DAPI staining (light blue). Bar = 10 μm.

To summarize, we detected various forms of W chromosomes in females of all species studied, except *A. punctulata*, in which a Z univalent was found. As expected, we did not find any W chromosomes in males. We also determined chromosome numbers when possible. Most species showed a diploid number of $2n = 62$, which corresponds to the ancestral number of chromosomes in Lepidoptera [20,25]. However, in *A. punctulata*,

E. alternata, and *O. brumata*, there were differences between sexes, indicating either the W chromosome absence or the existence of multiple sex chromosomes. Moreover, the chromosome number in *O. brumata* was significantly reduced. The results obtained are summarized in Table 1.

Table 1. Overview of results.

Species	2n ♀/♂	SC ♀	W Composition + Features	Sex Chromatin
A. punctulata	61/62	Z0	absent (Z univalent recorded)	absent
C. clathrata	n.d.	WZ	female enriched/common repetitive (slightly DAPI+)	normal
	n.d.	WZ_1Z_2	neo-W with 2 parts: female enriched/common repetitive (slightly DAPI+) and undifferentiated	scattered
E. alternata	64/62	$W_1W_2W_3Z$	common repeats/female enriched, DAPI+ blocks on W_2, W_3	scattered
H. fasciaria	n.d.	WZ	2 parts: female enriched (DAPI+) and undifferentiated (nucleolus-associated)	normal/multiple
H. atomaria	62/62	WZ	common repeats, DAPI+, small size	normal
O. brumata	30/28	$W_1W_2W_3Z$	W_1 female enriched/common repeats, DAPI+, W_2 and W_3 undifferentiated; neo-Z	normal/double
P. rhomboidaria	62/62	WZ	female enriched, DAPI+	normal
P. macularia	62/62	WZ	female enriched	normal
			undifferentiated	absent

2n ♀/♂, diploid chromosome number in females/males. SC ♀, sex chromosome constitution in females. W composition, prevalence of certain types of sequences determined by CGH (female enriched versus common repetitive sequences). DAPI+, DAPI-positive heterochromatin. n.d., not determined.

4. Discussion

In this work, we performed an extensive sex chromatin survey in 50 species of the family Geometridae. While females of most species displayed a single, normal-looking sex chromatin body, various exceptions were found, including miniature or scattered bodies, multiple bodies, or the complete absence of sex chromatin. Subsequent cytogenetic analysis of eight selected species, representing different types of sex chromatin, revealed a wide spectrum of W chromosome variants (including its absence), ranging from non-differentiated to fully degenerate W chromosomes, differing in number, size, and molecular composition. In addition, two cases of intraspecific W chromosome polymorphisms were recorded.

Our results, combined with the available literature, suggest a link between the sex chromatin presence and appearance and the constitution of sex chromosomes, specifically the W chromosome(s). In particular, the large conspicuous sex chromatin body (i.e., normal sex chromatin pattern) correlates with a high level of differentiation and consequent heterochromatinization of the W chromosome, which also often makes it easily recognizable after DAPI staining, such as in *E. kuehniella* [29], *C. pomonella* [46], *P. rhomboidaria,* and *H. atomaria* (this study). The correlation between the heterochromatin-rich W chromosome and the normal sex chromatin body occurs regardless of the actual W chromosome size. For example, the large W in *P. rhomboidaria* (this study) and small degenerated Ws in *H. atomaria* (this study) and in *Bicyclus anynana* [52] display similar sex chromatin bodies.

Hybridization of the W-painting probe in *O. brumata* further supports the opinion that especially the heterochromatin-rich W chromosome forms a typical sex chromatin body. In females of this species, we found a $W_1W_2W_3$neo-Z sex chromosome constitution. We presume that W_1 is most probably the ancestral highly degenerate heterochromatin-rich W chromosome, while W_2 and W_3 are of autosomal origin and consist of euchromatin. The W-painting probe prepared from microdissected sex chromatin in this species hybridized exclusively to the degenerated W_1 chromosome. Therefore, the other two W chromosomes, which consist of euchromatin and resemble autosomes, do not form sex chromatin, indicating that it is the heterochromatin nature, not the presence of the W chromosome, which is vital for the sex chromatin formation.

Deviations from the typical appearance of sex chromatin can have several causes. For instance, we observed a variable occurrence of miniature or small sex chromatin bodies in *E. alternata*, although sex chromosome analysis showed the same results in all broods studied, i.e., a $W_1W_2W_3Z$ system in females. Although DAPI staining failed to clearly

identify the W chromosomes, CGH reliably detected all three of them. The fact that the ancestral chromosome number of $2n = 62$ is preserved in males of this species, while it is increased by 2 in females ($2n = 64$), suggests the origin of 3 Ws by fission of the ancestral W chromosome. However, a more complex origin involving fusions of sex chromosomes with autosomes and subsequent fissions, as demonstrated in *Leptidea* butterflies [11], cannot be ruled out without further research.

The sex chromatin status can also be influenced by an intraspecific polymorphism in the W chromosome composition. In Lepidoptera, intraspecific sex chromosome polymorphism has so far been found in *Samia cynthia* ssp. and *Orgyia thyellina* [8], *Danaus chrysippus* ssp. [53], and *C. clathrata* and *P. macularia* (this study). In the latter species, we were able to detect the W chromosome by CGH in females with regular sex chromatin. However, in females with a miniature or disintegrated sex chromatin, we failed to differentiate the W chromosome by CGH and thus to identify a WZ bivalent. Hence, the WZ/ZZ sex chromosome system was only deduced due to the same number of chromosomes in both sexes, $2n = 62$. Because each W chromosome is inherited independently only in the female lineage and without meiotic recombination, we suggest that the differentiated and undifferentiated W chromosomes may have diverged by the acquisition of different types of sequences.

Variability of the W chromosome in *P. macularia* with consequent variability in sex chromatin presence shows that it is the particular sequence composition of the W in respective species, populations, or females that will or will not lead to the formation of normal sex chromatin. Impaired heterochromatinization might have various causes, including the lack of "booster" sequences (e.g., LINE elements) promoting the spread of a silencing factor [54]. Considering the high sex chromosome variability within Lepidoptera, individual sex chromosomes may or may not foster the silencing signals sufficiently. Additionally, the mere existence of heterochromatin on the sex chromosome does not automatically lead to the formation of the sex chromatin body. Thus, higher-order heterochromatin organization is required, such as changes in the phosphorylation of heterochromatin proteins, as seen in protozoan *Tetrahymena* [55]. In Lepidoptera, however, such molecular mechanism remains elusive.

Sex chromosome ability to establish and maintain heterochromatin on any level may be further impaired by translocation of a euchromatic (e.g., autosomal) segment. In *C. clathrata*, we observed two distinct forms of sex chromatin: (i) normal sex chromatin corresponding to the WZ system and (ii) miniature or scattered sex chromatin in females with the neo-WZ_1Z_2 system. In both cases, the ancestral W chromosome or its part in the neo-W chromosome was differentiated using CGH or FISH with the W-painting probe. In neo-WZ_1Z_2 females, the formation of normal sex chromatin was probably disrupted by the undifferentiated, autosome-derived part of the neo-W chromosome. Loss of the sex chromatin was already observed in natural species with a neo-W chromosome, such as the clouded Apollo, *Parnassius mnemosyne* [9], as well as in structural mutants of the W chromosome in *E. kuehniella* [18,29]. These authors suggested that the disruption of sex chromatin is caused by the tendency of transcriptionally active autosomal chromatin to disperse. The sex chromatin presence and appearance also depend on the size of translocated fragments, as shown in the T(W;Z) mutant lines of *E. kuehniella* [18], and on the specific sequence content. On the other hand, the formation of neo-W chromosomes does not always lead to the absence of sex chromatin. For example, the monarch butterfly, *Danaus plexippus*, has a neo-W chromosome with two clearly differentiated parts (old heterochromatic and new euchromatic) but still shows a single-sex chromatin body in female polyploid cells [56].

The euchromatic part of the W chromosome is probably also responsible for the formation of multiple sex chromatin bodies observed in *H. fasciaria* females (this study). However, unlike *C. clathrata*, these sex chromatin bodies were conspicuous and only had different sizes and shapes. In this species, we encountered an intriguing phenomenon. The WZ bivalent appeared to be immersed into the nucleolus, so the presence of a large cluster of genes for ribosomal RNA was expected. Yet, FISH with the 18S rDNA probe failed to

localize such a cluster directly on the W and/or Z chromosomes. Instead, we detected scattered signals nearby the bivalent, possibly in loosen chromatin loops. Nevertheless, we suggest that the unusual appearance and number of sex chromatin bodies may be a side effect of the W chromosome association with the nucleolus and its potential link to high transcriptional activity in this region. Moreover, since the other part of the W in *H. fasciaria* is fully differentiated and heterochromatic, it is also possible that this chromosome is, in fact, a neo-W that arose by fusion between the ancestral W and an NOR-bearing autosome. Further research is needed to verify this option.

Finally, the absence of sex chromatin may in some cases truly indicate the absence of the W chromosome, as shown in *A. punctulata* (this study) or, e.g., in the common clothes moth, *Tineola bisselliella* [17], and three species of bagworms (Psychidae) [27]. The loss of the W chromosome in some species points to the dispensability of this chromosome and indicates the presence of yet unknown molecular mechanism of sex determination [57], different from the lepidopteran model species, *B. mori*, in which the W chromosome carries a sex-determining factor [58]. On the contrary, in the sex chromatin absence in one of *P. macularia* broods was probably caused by the low level of differentiation of the W chromosome (see above). This finding suggests that the actual number of lepidopteran species lacking the W chromosome, estimated by the sex chromatin absence (see the work of [24]), is, in fact, lower. As follows from this study, a detailed analysis of karyotype and sex chromosomes using methods of molecular cytogenetics is crucial to determine the presence or absence of the W chromosome. Moreover, multiple females of each species, preferably from more than one population, should be examined for possible intraspecific polymorphisms or anomalies. Such a thorough cytogenetic investigation may also lead to the discovery of derived sex chromosome systems, thus contributing to the understanding of the evolution of sex chromosomes in Lepidoptera (e.g., the work of [12,56,59–61]).

5. Conclusions

Our results and literature data suggest that there are two main factors influencing the sex chromatin formation: (i) the actual level of differentiation or degeneration of the W chromosome and (ii) the presence of a euchromatin segment on the W chromosome with potential transcriptional activity, usually acquired by structural rearrangements with autosomes or the Z chromosome. Our work revealed the astonishing variability of sex chromosomes in Geometridae species, emphasized the need to inspect sex chromosomes in more populations because of possible intraspecific polymorphisms, and showed that the sex chromatin assay is an easy tool to pinpoint species with atypical sex chromosomes. These species can then be studied at more detailed genomic and transcriptomic levels to unveil the molecular mechanisms of speciation, adaptation, sex determination, and sex chromatin formation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10092230/s1, Table S1: Overview of sex chromatin occurrence in Geometridae, Table S2: List of species selected for detailed cytogenetic study.

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Subfamily	Species	SC* female	SC* male	Reference
Ennominae	*Abraxas grossulariata*	yes	no	[13, 38]
Ennominae	*Abraxas sylvata*	yes	no	[13]
Ennominae	***Aethalura punctulata***	no	no	This study
Ennominae	*Agriopis leucophaearia*	no	no	This study
Ennominae	*Agriopis marginaria*	yes	no	This study
Ennominae	*Alcis repandata*	yes	n.d.	This study
Ennominae	*Alsophila aescularia*	no	no	This study
Ennominae	*Angerona prunaria*	yes	no	This study
Larentiinae	*Aplocera plagiata*	yes	n.d.	This study
Archiearinae	*Archiearis notha*	yes	no	This study
Ennominae	*Biston betularia*	yes	no	[20]
Ennominae	*Bupalus piniaria*	yes	no	[38], this study
Ennominae	*Cabera exanthemata*	yes	no	This study
Ennominae	*Cabera pusaria*	yes	n.d.	This study
Ennominae	*Campaea margaritaria*	yes	no	This study
Ennominae	*Campaea perlata*	no	no	[39]
Larentiinae	*Camptogramma bilineata*	yes	n.d.	This study
Ennominae	*Caripeta angustiorata*	yes	no	[39]
Ennominae	*Caripeta divisata*	yes	no	[39]
Ennominae	***Chiasmia clathrata***	variable	no	This study
Larentiinae	*Chloroclysta siterata*	yes	n.d.	This study
Ennominae	*Colotois pennaria*	yes	n.d.	This study
Sterrhinae	*Cyclophora pendularia*	yes	n.d.	This study
Sterrhinae	*Cyclophora punctaria*	yes	no	[38]
Ennominae	*Ectropis crepuscularia*	yes	no	This study
Ennominae	*Ennomos erosaria*	yes	no	This study
Larentiinae	*Entephria caesiata*	no	n.d.	This study
Larentiinae	***Epirrhoe alternata***	variable	no	This study
Larentiinae	*Epirrhoe tristata*	yes	no	[38], this study
Ennominae	*Erannis defoliaria*	yes	n.d.	This study
Ennominae	*Erannis tiliaria*	yes	no	[39]
Larentiinae	*Eulithis populata*	yes	n.d.	This study
Larentiinae	*Eupithecia tantillaria*	yes	no	This study
Geometrinae	*Geometra papilionaria*	yes	no	This study
Geometrinae	*Hemithea aestivaria*	yes	no	This study
Larentiinae	*Hydria prunivorata*	no	no	[39]
Ennominae	***Hylaea fasciaria***	variable	no	This study
Ennominae	***Hypomecis atomaria***	yes	no	This study
Ennominae	*Hypomecis punctinalis*	yes	no	This study
Ennominae	*Hypomecis roboraria*	yes	no	This study
Sterrhinae	*Idaea luteolaria*	no	no	[38]
Geometrinae	*Jodis putata*	yes	no	This study
Ennominae	*Lambdina f. fiscellaria*	no	no	[39]
Ennominae	*Macaria alternata*	no	no	This study
Ennominae	*Macaria liturata*	yes	n.d.	This study
Ennominae	*Macaria notata*	no	no	This study
Ennominae	*Nepytia canosaria*	yes	no	[39]

Ennominae	*Opistograptis luteolata*	no	no	[38], this study
Larentiinae	*Odezia atrata*	variable	no	This study
Larentiinae	*Operophtera bruceata*	yes	no	[39]
Larentiinae	***Operophtera brumata***	yes	no	This study
Ennominae	*Paleacrita vernata*	yes	no	[39]
Ennominae	*Parectropis similaria*	no	n.d.	This study
Ennominae	***Peribatodes rhomboidaria***	yes	no	This study
Ennominae	*Phigalia titea*	yes	no	[39]
Ennominae	*Plagodis alcoolaria*	no	no	[39]
Ennominae	*Plagodis dolabraria*	yes	n.d.	This study
Ennominae	*Protoboarmia porcelaria indicataria*	yes	no	[39]
Ennominae	***Pseudopanthera macularia***	variable	no	This study
Sterrhinae	*Scopula floslactata*	yes	no	[38]
Larentiinae	*Scotopteryx chenopodiata*	no	no	This study
Ennominae	*Siona lineata*	yes	no	This study
Larentiinae	*Thera britannica*	yes	n.d.	This study
Larentiinae	*Thera juniperata*	yes	no	[39]
Larentiinae	*Thera variata*	no	no	This study
Larentiinae	*Xantorhoe ferrugata*	yes	n.d.	This study
Larentiinae	*Xantorhoe fluctuata*	yes	n.d.	This study
Larentiinae	*Xantorhoe montanata*	variable	no	[38], this study
Larentiinae	*Xantorhoe quadrifasciata*	yes	n.d.	This study

* **SC** = Sex chromatin in polyploid nuclei of Malpighian tubules
yes = normal sex chromatin present
no = sex chromatin absent
variable = variable occurence/atypical appearance
n.d. = not determined

Species	Sex chromatin F/M *	Cytogenetics F/M **	Broods analysed ***	Localities of collection ****
Aethalura punctulata	8/6	5/6	2	České Budějovice CZ
Chiasmia clathrata	28/15	11/2	4	České Budějovice + Kuří (Benešov nad Černou) CZ
Epirrhoe alternata	25/9	19/5	5	České Budějovice + Kuří (Benešov nad Černou) + Vyšenské kopce (Český Krumlov) CZ + Karilatsi (Põlva) EE
Hylaea fasciaria	7/3	4/2	1	Karilatsi (Põlva) EE
Hypomecis atomaria	12/5	7/6	3	České Budějovice + Vyšenské kopce (Český Krumlov) CZ
Operophtera brumata	11/3	9/6	4	České Budějovice CZ
Peribatodes rhomboidaria	7/8	7/4	3	České Budějovice + Javorník (Hodonín) CZ
Pseudopanthera macularia	5/4	4/5	3	Brněnec (Svitavy) + Sulíkov (Blansko) + Chvalnov-Lísky (Kroměříž) CZ

* Total number of individuals (including adults) inspected for sex chromatin: females/males

** Total number of individuals in analysed/karyotyped by molecular cytogenetics (CGH, GISH, FISH, DAPI staining): females/males

*** Number of broods (i.e. offsprings of a single female) inspected by molecular cytogenetics

**** Localities of collection: CZ = Czech Republic; EE = Estonia

3.3. Exploring the W chromosome: accumulation of retrotransposons contributes to sex chromosome differentiation in the willow beauty *Peribatodes rhomboidaria* (Lepidoptera: Geometridae)

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Abstract

The evolution and molecular differentiation of heteromorphic sex chromosomes involves the accumulation of different repetitive DNA sequences due to restricted recombination. This also applies to moths and butterflies (Lepidoptera), which mostly have the WZ sex chromosome system. The femalespecific W chromosome is typically rich in heterochromatin, harbours few or no genes and carries a large number of repetitive sequences. Molecular cytogenetic approaches such as comparative genomic hybridization (CGH) or fluorescence in situ hybridization (FISH) have shown that the W chromosome has evolved rapidly and varies widely among species. However, our knowledge of the composition of the W chromosome is still very limited. Here we present the analysis of repeats on the W chromosome in the willow beauty, *Peribatodes rhomboidaria* (Geometridae). RepeatExplorer comparative analysis of male and female genomes identified 10 putative W chromosome-enriched repeats, most of them being LTR or LINE mobile elements. We analysed two repeats with the largest difference between sexes: PRW LINE-like and PRW Bel-Pao. The results of FISH mapping and bioinformatic analysis confirm their W chromosome enrichment, thus supporting the hypothesis that mobile elements are the driving force of W chromosome differentiation in Lepidoptera.

Exploring the W chromosome: accumulation of retrotransposons contributes to sex chromosome differentiation in the willow beauty Peribatodes rhomboidaria (Lepidoptera: Geometridae)

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Abstract

The evolution and molecular differentiation of heteromorphic sex chromosomes involves the accumulation of different repetitive DNA sequences due to restricted recombination. This also applies to moths and butterflies (Lepidoptera), which mostly have the WZ sex chromosome system. The female-specific W chromosome is typically rich in heterochromatin, harbours few or no genes and carries a large number of repetitive sequences. Molecular cytogenetic approaches such as comparative genomic hybridization (CGH) or fluorescence in situ hybridization (FISH) have shown that the W chromosome has evolved rapidly and varies widely among species. However, our knowledge of the composition of the W chromosome is still very limited. Here we present the analysis of repeats on the W chromosome in the willow beauty, Peribatodes rhomboidaria (Geometridae). RepeatExplorer comparative analysis of male and female genomes identified 10 putative W chromosome-enriched repeats, most of them being LTR or LINE mobile elements. We analysed two repeats with the largest difference between sexes: PRW LINE-like and PRW Bel-Pao. The results of FISH mapping and bioinformatic analysis confirm their W chromosome enrichment, thus supporting the hypothesis that mobile elements are the driving force of W chromosome differentiation in Lepidoptera.

Introduction

Sex chromosomes have evolved independently in multiple taxa. In most cases, their evolution seems to follow similar trajectories with predictable outcomes, although various exceptions and derivations are relatively common. Nevertheless, the classical model of sex chromosome evolution assumes that the sex-determining factor is acquired by one of the homologous chromosomes, resulting in a new pair of sex chromosomes[1]. The new heteromorphic chromosome (usually Y or W, depending on the type of heterogamety) eventually begins to attract sexually antagonistic genes, and these evolutionarily advantageous mutations can be further fixed, e.g. by inversions. However, such progressive differentiation simultaneously restricts recombination in this region. This is usually followed by gradual degenerative changes such as the accumulation of repetitive sequences and pseudogenization, often leading to heterochromatinization and subsequently even to the dispensability of this particular chromosome. If the sex-determining factor is translocated or substituted, such a sex chromosome may eventually be lost forever and the entire sex chromosome life cycle may be restarted[2,3].

The processes of sex chromosome evolution are best studied in diverse groups of organisms that likely share an ancestral karyotype while exhibiting various abnormalities at different evolutionary stages. One of these is the insect order Lepidoptera (moths and butterflies), the largest group with female heterogamety. Until recently, however, very little was known about the actual molecular composition of their W chromosomes, which were regularly excluded from NGS projects due to their repetitive nature causing assembly problems. So far, only a few genes have been found on the W chromosome, such as the sex-determining female factor generating *Fem* piRNA[4]. In contrast, the lepidopteran W chromosomes are typically filled with various types of ubiquitous transposable elements (TE)[5–7] and may also contain

satellite DNA[8]. Occasionally, some of these sequences are enriched on the W chromosome, such as piSAT1 in *Plodia interpunctella*[9], or even W-specific (i.e. present only on the W), such as CpW2 and CpW5 in *Cydia pomonella*[10].

Apart from Lepidoptera, the accumulation of transposable elements and other repetitive DNA by the heterogametic sex chromosomes has been observed in plants[11], fungi[12], humans[13], other mammals[14], birds[15], *Drosophila* flies[16], beetles[17] and many others[18]. Such events usually lead to the formation of heterochromatin, which may attract even more TEs, as some of them preferentially insert into silenced regions[19]. This could be the result of opposing selection forces balancing the deleterious effects of integration on the host cell on the one hand, and the propagation of the specific transposable element in the genome on the other. Some degree of preference for integration sites can be observed in almost all TE types[20]. Their population dynamics within the genome are described by the deleterious insertion model, which assumes that transposons are eliminated from gene-rich regions by selection pressure, or by the ectopic recombination model, which assumes a higher abundance of transposons in regions with low recombination rates where they cannot be removed by ectopic recombination[21]. These models offer an explanation for the accumulation of transposable elements on heterogametic sex chromosomes (i.e. Y and W).

Hence, transposable elements may play an important role in the evolution and differentiation of sex chromosomes. In fact, it has already been shown that TEs can influence whole genomes, both in destructive and constructive ways. Destructive forces primarily include insertions into genes or their promoters that impede their function, which may eventually lead to lower fitness or the development of various diseases[22]. On the other hand, some of our vital proteins originated via domestication of ancient transposable elements, such as RAG proteins that carry out recombination in immunoglobulin genes[23], or telomerase which maintains chromosome ends[24]. In addition, they may contribute to genome size expansion or speciation, as e.g. LINE-1 retrotransposons may reduce gene expression, provide new exons for protein-coding genes[25], or serve as “booster stations” for the spread of silencing Xist RNA during X chromosome inactivation in mammals[26]. To sum up, transposable elements can shape genomes in various ways and influence sex chromosome differentiation, potentially leading to speciation or sexual dimorphism[27].

Previous cytogenetic studies in Lepidoptera have shown striking differences between W chromosome composition and size even between closely related species, suggesting rapid evolution[28–30]. One of the most informative cytogenetic tools is comparative genomic hybridization (CGH), which enables to estimate the differentiation level and the ratio of common to female-enriched sequences on the W chromosome. In our recent study[28], CGH revealed a high degree of sex chromosome differentiation in many species of Geometridae (Lepidoptera), including the willow beauty (*Peribatodes rhomboidaria)*. This species has a standard lepidopteran karyotype (n = 31, WZ/ZZ), and its normal-sized W chromosome is strongly DAPI positive and contains a large amount of heterochromatin. Furthermore, it shows a remarkable hybridization pattern after CGH, as it is strongly marked by the female genomic

probe (Fig. S1), suggesting the presence of female-enriched and/or female-specific sequences. In order to classify them, we performed low coverage NGS sequencing of three individuals of each sex and compared their repeat content using the RepeatExplorer pipeline[31]. The results describe the repeatome of *P. rhomboidaria* in general and reveal two W-enriched sequences, contributing to our knowledge of W chromosome evolution in Lepidoptera. Finally, this study validates a novel approach for analysing differences in repeat content that can be applied to other species.

Materials And Methods

Insects

Adult specimens of *P. rhomboidaria* were collected in the field from June to September 2019 – 2020 in the vicinity of České Budějovice, Czech Republic, using light traps and entomological nets. Females were kept in plastic containers with host plants (mostly leaves of *Achillea millefolium*) until they laid eggs. After hatching, the larvae were reared to the penultimate instar stage, dissected for chromosome preparations and the remaining tissues were frozen in liquid nitrogen and stored at − 20°C.

Chromosome preparations

Meiotic chromosomes were obtained from gonads of penultimate instar larvae and spread chromosome preparations were made as previously described[32], dehydrated in an ethanol series (70%, 80% and 100%, 30 s each), air-dried and stored at − 20°C until further use.

DNA isolation and sequencing

Genomic DNA (gDNA) was extracted from one half of the larva using CTAB (hexadecyltrimethylammonium bromide; Sigma-Aldrich, St. Louis, MO, USA) according to the published protocol[33] with modifications previously described[28]. The concentration of isolated DNA was measured using Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA), and its quality was assessed by the absorbance ratio at 260/280 nm using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For each of the three broods studied (i.e. offspring of the same mother), we selected a sample of optimal quality from one male and one female (6 samples in total). Paired-end sequencing of 150 bp long reads from a library with 450 bp inserts was performed by Novogene (HK) Co., Ltd. (Hong Kong, China) using the Illumina HiSeq 4000 platform.

NGS data processing and repeatome analysis

The obtained Illumina raw reads were first processed by Trimmomatic version 0.32[34], the Illumina sequencing adapters were trimmed and finally all reads were cropped to a final length of 140 bp. The

quality of the reads before and after processing was checked using FastQC version 0.10.1[35]. The remaining paired sequences were converted to FASTA format using FastQtoFasta software from the FASTX-Toolkit version 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit). The following procedures were performed using the RepeatExplorer2 Utilities Kit version 0.3.8–451 (https://repeatexplorer-elixir.cerit-sc.cz). Sequences were interlaced and a random sample of 100 000 reads was generated for each individual (corresponding to approximately 0.02× genome coverage) and tagged with a specific read name prefix. Alternatively, read sampling was done using the seqtk tool (https://github.com/lh3/seqtk). Samples were concatenated into a single fasta file, which was processed by RepeatExplorer[31] using the default parameters for comparative analysis. To identify W-enriched repeats, the number of reads in individual clusters was compared between males and females using R version 4.0.3 in RStudio version 1.4.1103 (https://www.rstudio.com). Only clusters that consisted of at least 0.01% of the analysed reads and showed statistically significant differences ($P < 0.05$,two sample *t*-test with unequal variance) were considered. The annotation of the repeats was based on the automatic annotation of RepeatExplorer using Metazoa v3 database. Annotation of selected repeats was further confirmed by protein-based repeat masking in RepeatMasker 4.1.2[36] (https://www.repeatmasker.org).

PCR and cloning

Based on the RepeatExplorer results, we designed primers (Table S1) for a selected contig from cluster 32 and the assembly of three contigs covering most of the cluster 79 using Geneious Prime version 2021.1.1. (https://www.geneious.com). These primers were used in a standard 25 µl PCR mix containing 1× Ex*Taq* buffer (TaKaRa, Otsu, Japan), 2U DNA Ex*Taq* Polymerase (TaKaRa), 200 µM of each nucleotide, 1 µM of each primer and 50 ng of gDNA with the following profile: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min 30 s; final extension was at 72°C for 3 min. The results were checked on a standard 1.5% agarose gel in 1× TAE buffer. Gels were stained with ethidium bromide for 20 min and photographed under UV light. To avoid cloning problems due to the size of the PRW Bel-Pao sequence, we generated two shorter overlapping PCR products (using primer pairs PRW_F1 + PRW_R1 and PRW_F2 + PRW_R2; Table S1). All products were purified using ExoSAP-IT (ThermoFisher Scientific) and cloned into a vector using pGEM-T Easy Vector System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Plasmid DNA was isolated using the NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The identity of the fragment was verified by Sanger sequencing in SEQme (Dobříš, Czech Republic) with universal M13 primers.

Coverage analysis

To obtain more information about the representation of selected fragments in the genomes of both sexes, we mapped the trimmed, filtered and paired reads (see above) to the cloned consensus sequences in Geneious Prime version 2021.1.1. using the built-in algorithm. Mapping was done separately for male

and female reads (three combined samples from each sex) using the 95% identity threshold, and the mapped reads were manually sorted to ensure accurate results. We also estimated copy number based on total coverage.

Probe labelling and fluorescence *in situ* hybridization (FISH)

PRW LINE-like mapping was done in Jaén, Spain. 1 µg of PRW LINE-like DNA was labelled by nick translation using the biotin-NT-mix (Roche, Basel, Switzerland). The probe was then precipitated along with 50 µg yeast RNA and 50 µg salmon sperm DNA and redissolved in 50% formamide in 2× SSC. Fluorescence *in situ* hybridization (FISH) was performed according to the published protocol [37] with listed modifications[38].

PRW Bel-Pao mapping was done in České Budějovice, Czech Republic. Both fragments (I and II, which do not contain the deleted region - see below) were labelled by PCR containing 0.04 mM each of dATP, dCTP and dGTP; 0.014 mM dTTP, 0.025 mM biotin-16-dUTP (Jena Bioscience, Jena, Germany), 1× Ex*Taq* buffer (TaKaRa), 1 – 10 ng plasmid DNA, 1 µM of each primer and 2U DNA Ex*Taq* Polymerase (TaKaRa) under the same conditions as described above. FISH was performed according to the published protocol[39], with signal amplification using Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc, West Grove, PA, USA) at a dilution of 1:1000 with washing blocking buffer.

Results

Characterization of repeats in the genome of *P. rhomboidaria*

Total genome coverage after read processing was 2.38× for females and 2.24× for males, corresponding to approximately 0.77× coverage depth/per sample, based on a rough estimate of genome size 1C ~ 0.646 pg performed by flow cytometry in the laboratory of Petr Koutecký according to the published protocol[32].

The RepeatExplorer analysis clustered about 44% of input reads, indicating the relative representation of repeats in the genome.

Of all the identified clusters, 220 (representing ~ 24% of the input reads) were subjected to automatic annotation and further analysis, as each of these clusters represented at least 0.01% of the examined reads. However, the automatic annotation was not able to classify most of the top 220 clusters and clusters corresponding to 87% of the input reads remained unknown. Among the annotated clusters, about 0.91% of the input reads were satellites. As for the classified mobile elements, the prevailing groups were long interspersed nuclear elements (LINE) with 1.33% of the reads and long terminal repeats (LTR) with 0.65% of the reads.

Characterization of selected sequences

Out of the top 220 clusters, 12 clusters were statistically significantly female enriched and 3 clusters were male enriched (Fig. S2). Since the maximum male enrichment was 1.37×, any female-enriched cluster where the female-to-male ratio was below this value was considered a false positive. In the end, we obtained 10 clusters representing putative W repeats (Fig. S2). The average female enrichment in these clusters was 6.48× and the maximum enrichment was 197.21×. Most of the putative W repeats were annotated as retrotransposons (Table 1). The two most abundant W repeats represented by cluster 32 (PRW LINE-like) and cluster 79 (PRW Bel-Pao) were further analysed.

Table 1

Putative W chromosome enriched repeat clusters. Cluster numbers, their enrichment in the female genome compared to the male genome, their representation in the female and male genomes in % and their annotation according to RepeatMasker. Further examined clusters in bold.

Cluster No.	Female enrichment	Female genome (%)	Male genome (%)	Annotation
32	**3.57 ×**	**0.31**	**0.09**	**LINE and/or DNA/Transib**
79	**197.21 ×**	**0.11**	**0.001**	**LTR/Bel-Pao**
93	5.66 ×	0.07	0.01	LTR/Ty3-Gypsy
97	1.84 ×	0.05	0.03	LTR/Ty3-Gypsy
127	3.91 ×	0.04	0.01	LTR/Ty3-Gypsy
161	58.43 ×	0.03	0.001	Unknown
178	13.13 ×	0.02	0.002	LTR/Ty3-Gypsy
182	1.94 ×	0.02	0.009	Unknown
199	1.9 ×	0.01	0.008	LTR/Bel-Pao
207	1.6 ×	0.01	0.008	LINE

Based on the RepeatExplorer results, cluster 32 (corresponding to PRW LINE-like) was enriched 3.57× in the female data, where it represented about 0.31% of the genome, while in males it was only about 0.09% (Table 1, Fig. 1). Since the RepeatExplorer results are based on data with very low coverage, we mapped all processed reads to the cloned consensus sequence to obtain more accurate information on the abundance of this repeat. The results showed significantly higher coverage in females (774.6×) than in males (39.2×). We also estimated copy number using total coverage information, which was 325 copies in females and 17 copies in males, making up to an 19-fold difference.

Automatic annotation by RepeatExplorer classified the cluster 32 repeat as a retrotransposon from the LINE element group, based on the homology of the two identified reverse transcriptase (RT) domains. The RepeatMasker-based annotation was more complicated. In addition to the two LINE RTs from the LINE/CR1 and LINE/L2 subgroups, a DNA transposon similar to Transib was also discovered in part of this cluster. Considering the relatively complicated structure of the graph corresponding to cluster 32 (Fig. S3) and the annotation corresponding to the partial sequences of several different mobile elements, this repeat is probably an old, non-functional and degenerate copy of a LINE element combined with a DNA transposon. The part of this cluster that was cloned and used for FISH and coverage analysis corresponds to part of the LINE/CR1 element.

Database search in NCBI BLASTN[40] revealed 72% identity with a non-LTR retrotransposon T1Q-like reverse transcriptase gene of *Abraxas sylvata* (GenBank Acc. No. HQ284333.1), another moth from the family Geometridae. All other relevant hits without closer annotation came from the Tree of Life project[41] and ranged from 99% sequence identity in *P. rhomboidaria* to 70% identity in many related species.

Cluster 79 (corresponding to PRW Bel-Pao) was the most female-enriched repeat cluster identified by RepeatExplorer (197.21×). The results show that this repeat is almost absent in the male genome (0.001%), while it covers more than 0.1% of the female genome suggesting that it is mainly localised on the W chromosome (Fig. 1). This assumption was confirmed by read mapping, which revealed even greater imbalance between the sexes, as coverage was 840× in females and only 2.5× in males. We also found a less frequent version of this element with a 213-bp long deletion. After mapping the reads to this "deleted" region, the coverage dropped to 698.5× in females, while it remained the same in males. The estimated copy number is 353 copies in females, out of which 60 have the deleted region, and 1–2 copies in males.

Both RepeatMasker and RepeatExplorer classified this cluster 79 repeat as an LTR transposon from the Bel-Pao group, based on the group-specific antigen (GAG), integrase (INT) and protease (PROT) domains. Although the graph of this repeat was linear (Fig. S4), indicating a less variable sequence of this repeat between copies, we could not recover all crucial protein-coding domains for this type of element, and the long terminal repeats were also missing. Based on these results, this repeat is probably also an old, non-functional and truncated copy of the mobile element.

NCBI BLASTN search yielded hits for *P. rhomboidaria* (98% identity) and for other Geometridae species, including the W chromosome of *Crocallis elinguaria* (73%) and the W chromosomes of two other Lepidoptera species, *Clostera curtula* (Notodontidae, 70%) and *Hydraecia micacea* (Noctuidae, 68%), with all sequences coming from the Tree of Life project[41].

All examined sequences examined (both contigs and cloned fragments) were deposited in GenBank (Table 2).

Table 2
Accession numbers of studied sequences.

Name	Origin	Accession number
PRW LINE-like	clone	OP361285
PRW Bel-Pao part I	clone 1	OP361282
PRW Bel-Pao part II	clone 2	ON815295.1
PRW Bel-Pao part I with deletion	clone 3	OP361284
PRW Bel-Pao consensus	assembly of clone 1 and 2	OP361283
PRW Bel-Pao consensus with deletion	assembly of clone 3 and 2	OP410344
PRW LINE-like contig from RE	*in silico* RepeatExplorer	OP410342
PRW Bel-Pao contig consensus from RE	assembly of 3 *in silico* RepeatExplorer contigs	OP410343

PCR amplification of selected repeats from gDNA

Both examined fragments were amplified by PCR (in the case of PRW Bel-Pao, we used the entire non-divided product from primer F1 to primer R2), using equal amounts of male and female gDNA. While the PCR products were present in both sexes, the female band was much stronger (Fig. 2). These results suggest that both elements are mainly present on the W chromosome.

FISH mapping

To verify the predicted location of selected fragments, we carried out physical mapping using FISH. Indeed, both probes labelled the W chromosome (Fig. 3A, 4A), which was also identified by its heterochromatinization and corresponding DAPI positivity (Fig. 3B, 4B). In the case of PRW LINE-like (Fig. 3), the probe showed significant signal accumulation on the W chromosome, and multiple small scattered signals were also detected on other chromosomes, which were equally abundant in both sexes. In PRW Bel-Pao (Fig. 4), the probe hybridized exclusively on the W chromosome, which was labelled almost along its entire length, except for a small gap at one of the chromosomal ends. Accordingly, no signal was detected in males (Fig. 4D).

Discussion

The genome size of *P. rhomboidaria* was estimated to 0.646 pg (corresponding to 631 Mb), which is similiar to another geometrid species *Operophtera brumata* (645 Mb[42]) and falls within the range of

known genome sizes in Lepidoptera from 273 Mb in *Danaus plexippus* (Nymphalidae) to 1.9 Gb in *Euchlaena irraria* (also Geometridae)[43]. However, the vast majority of species tend to have smaller genomes, similar to *B. mori* with 0.53 pg (corresponding to 518 Mb)[44]. The proportion of repetitive DNA in the genome of *P. rhomboidaria* was estimated to 44%; however, its actual volume is expected to be higher based on the genome size and repeat proportion in genomes of other Lepidoptera. For comparison, repeats in *D. plexippus* make up only 10.2% of the genome[45], while in *B. mori* they comprise up to 46.8%[46] and in *O. brumata* even more than half of the genome (53.5%)[42]. This discrepancy in *P. rhomboidaria* is likely caused by the RepeatExplorer analysis, which ommits microsatellites and ancient diversified copies of mobile elements[47]. Although most of the repetitive sequences in *P. rhomboidaria* remain unclassified, similarly to genomes of other Lepidoptera the most abundant class of repeats are transposable elements. For instance, LINEs are particularly abundant in the two species of Geometridae, *P. rhomboidaria* (this study) and *O. brumata*[42].

In this study, we characterized 10 putative W-enriched repeats. Most of them were classified as retrotransposons, which is also a common feature of the W chromosome in other Lepidoptera species [5,7,10]. Their identification was done by comparative analysis with the RepeatExplorer software, a novel approach for Lepidoptera as is only has been used for mapping satellite sequences in Crambidae[8], and for identification and characterization of W-enriched repetitive sequences in *Diatraea saccharalis* (Crambidae)[48].

All these findings suggest that transposable elements are the main repeats with a tendency to colonize the heteromorphic sex chromosomes, as also observed in e.g. papaya[49] or spinach[50] and recently reviewed[51]. In particular, retrotransposons are the most abundant types of repeats in general due to their copy-and-paste mechanism of replication. Therefore, their abundant occurrence on the W chromosome in Lepidoptera is not surprising. In fact, they seem to successfully exploit the inability of host genomes to prevent such expansion in the non-recombining sex chromosomes like Y and W. Since most genes that would be under selection have been lost, the accumulation of transposable elements probably reflects the strongly reduced efficiency of natural selection on these chromosomes[52,53], which then serve as a graveyard for these and similar types of repeats[54,55]. This is especially true for the W chromosomes of Lepidoptera, as meiotic recombination is completely absent in females[56–58].

Moreover, the colonization of the W chromosomes of Lepidoptera by a particular type of retrotransposon is probably a rapid process with a random outcome, occurring independently even in closely related taxa[8]. However, it seems that at least for some retrotransposons, the W chromosome becomes the final destination, as they often genetically erode. This happens mainly through their recurrent uncoordinated insertions into already existing elements, which may destroy their functional domains. This, of course, also complicates their annotation.

PRW LINE-like element is a possible example of such an event. The complexity of the graph of the cluster 32 analysis with RepeatExplorer suggests a derived type of repeat with ambiguous annotation, as it

contains two RT domains from different LINE elements and one DNA transposon Transib domain. Indeed, it is possible that there were originally several types of transposable elements that were repeatedly inserted into each other during their accumulation in the W chromosome (as in *B. mori*[59]), and this new 'patchwork' sequence could potentially amplify independently as a novel repeat (as suggested in legumes[60]).

Regarding the PRW LINE-like relative copy number differences between males and females, the RepeatExplorer analysis showed a 3.57× enrichment in females, while coverage analysis in Geneious revealed an 19× enrichment in females. These discrepancies are most likely due to the fact that RepeatExplorer analysed the entire cluster generated *in silico*, while only the cloned fragment (annotated as LINE CR1 by RepeatMasker) was used for Geneious read mapping. Since the RepeatExplorer graph may actually compile more types of monomers, it is possible that some of them are not accumulated on the W chromosome.

FISH mapping revealed a significant accumulation of the cloned PRW LINE-like sequence on the W chromosome. In addition, its presence was also confirmed on other chromosomes, as many scattered signals were detected in both males and females. These results are also consistent with the PCR results, which showed products in both sexes, although the female gDNA products were considerably stronger.

In the case of PRW Bel-Pao, it was classified as an LTR Bel-Pao element both by RepeatExplorer automatic annotation and RepeatMasker protein search. This element is probably also non-functional and genetically eroded, as it lacks the RT domain, both long terminal repeats and in case of the less frequent version also the PROT domain. The female enrichment of this transposon is very clear in both RepeatExplorer (197.21×) and coverage analysis in Geneious (176×, presuming 2 copies in male genome) mapping results. The difference is negligible and most likely technical, as the RepeatExplorer analysis is affected by a smaller sample size and a lower required percentage of identity between reads (90% vs 95% in Geneious). Based on the coverage differences between the full-size fragment and the deleted region, we estimate that about 17% of copies carry the deletion.

The PCR results strongly support the bioinformatic data. Two PCR products were found in both sexes, differing in volume and length. They correspond to a shorter, less abundant version carrying the deletion and a longer, more abundant full-length sequence. These results, combined with the coverage analysis data, therefore confirm that both deleted and non-deleted versions of this sequence are present on and off the W chromosome. Since it is unlikely that the original non-deleted version would mutate independently on different chromosomes, we hypothesize that the derived deleted version have migrated either to or from the W chromosome via an alternative transposition mechanism, such as ectopic recombination or with another mobile element.

As expected, the PCR products from the female gDNA were considerably stronger than those from the male gDNA. Accordingly, the FISH mapping revealed striking differences between the sexes and provided the final physical evidence for the accumulation of this repeat on the W chromosome. We did not observe

hybridization signals on other chromosomes, but the probe highlighted almost the entire length of the W chromosome except for its terminal part, which did not contain heterochromatin. In our previous work, we showed results of comparative genomic hybridization (CGH), which revealed clear differences between the signals of the female gDNA probe in this terminal part and the heterochromatic part, proving that the latter part consists mainly of female-enriched sequences[28]. Thus, the PRW Bel-Pao repeat is probably one of the key components of the CGH results. Moreover, by colonizing the W chromosome, this repeat significantly contributes to its progressive differentiation.

To conclude, we validated a novel approach to finding differences in repeat content using RepeatExplorer. Our bioinformatic results correlated very well with PCR and cytogenetic mapping, demonstrating the connectivity and mutual indispensability of these methods and providing a comprehensive insight into biological reality. Most importantly, we have identified and examined two types of retrotransposons that have accumulated on the W chromosome of *P. rhomboidaria*. We have thus contributed another piece to the puzzle of sex chromosome differentiation and evolution in Lepidoptera.

Declarations

Acknowledgemens

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Author contributions

M.H. conceived the study, designed experiments, performed research and wrote the first draft. M.D. analyzed the bioinformatic data. E.M. and M.Z. provided supervision and consulting. F.M. and P.L. contributed expertise in cytogenetics and provided partial funding. All authors contributed to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Data Availability

All examined sequences have been deposited into the GenBank with accession numbers provided in the Table 2 of Results section.

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Figures

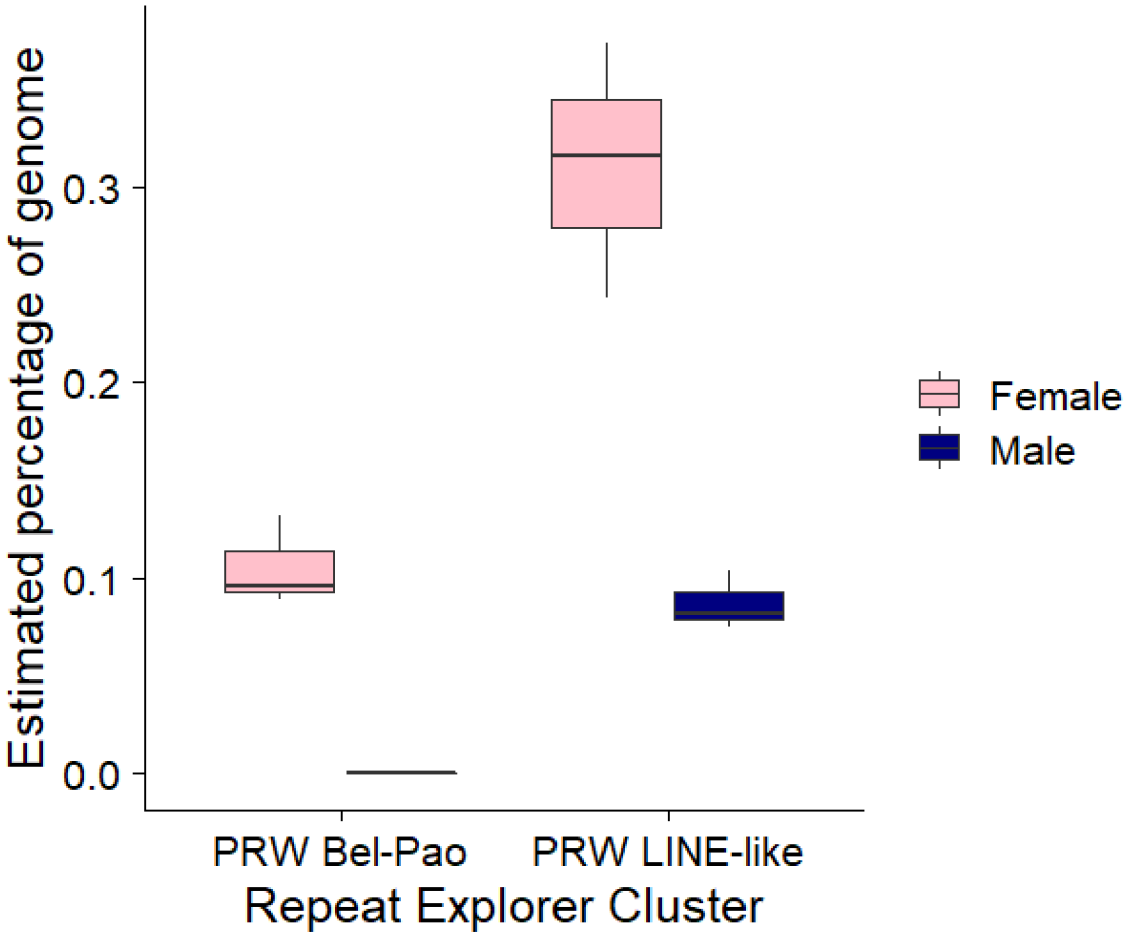

Figure 1

Boxplot of estimated genome percentages of the two most abundant putative W repeats based on RepeatExplorer results from 3 females and 3 males of *Peribatodes rhomboidaria*. Median, interquartile range, and smallest and largest values in 1.5× interquartile range are shown.

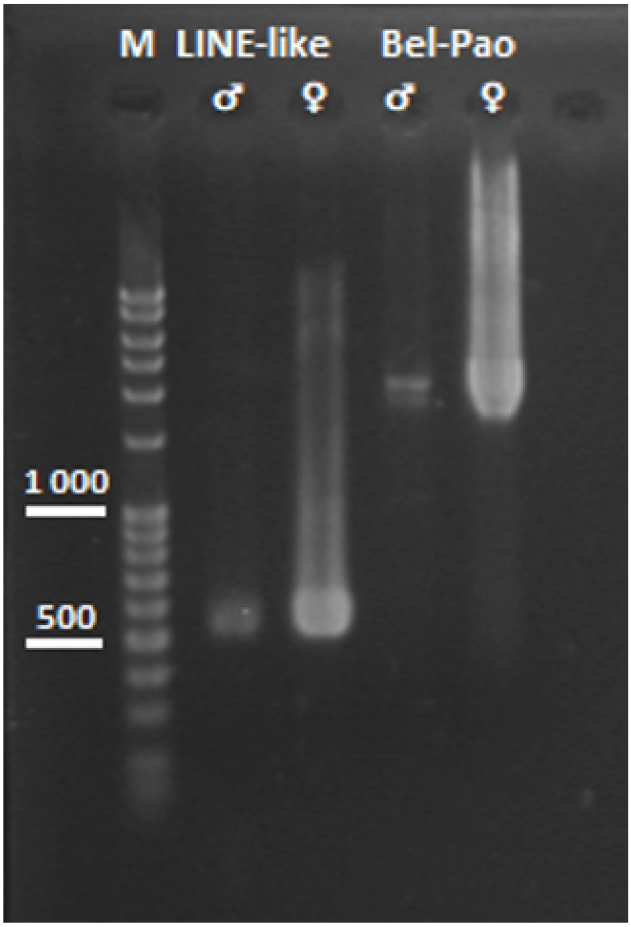

Figure 2

PCR amplification from genomic DNA of both sexes of *Peribatodes rhomboidaria*. Although the products are present in all samples, they are visibly stronger in females. Legend (from left): M - marker (100 bp DNA ladder, Invitrogen), PRW LINE-like male, PRW LINE-like female (540 bp), PRW Bel-Pao male, PRW Bel-Pao female (2118 bp; note a smaller band of 1905 bp carrying the deletion).

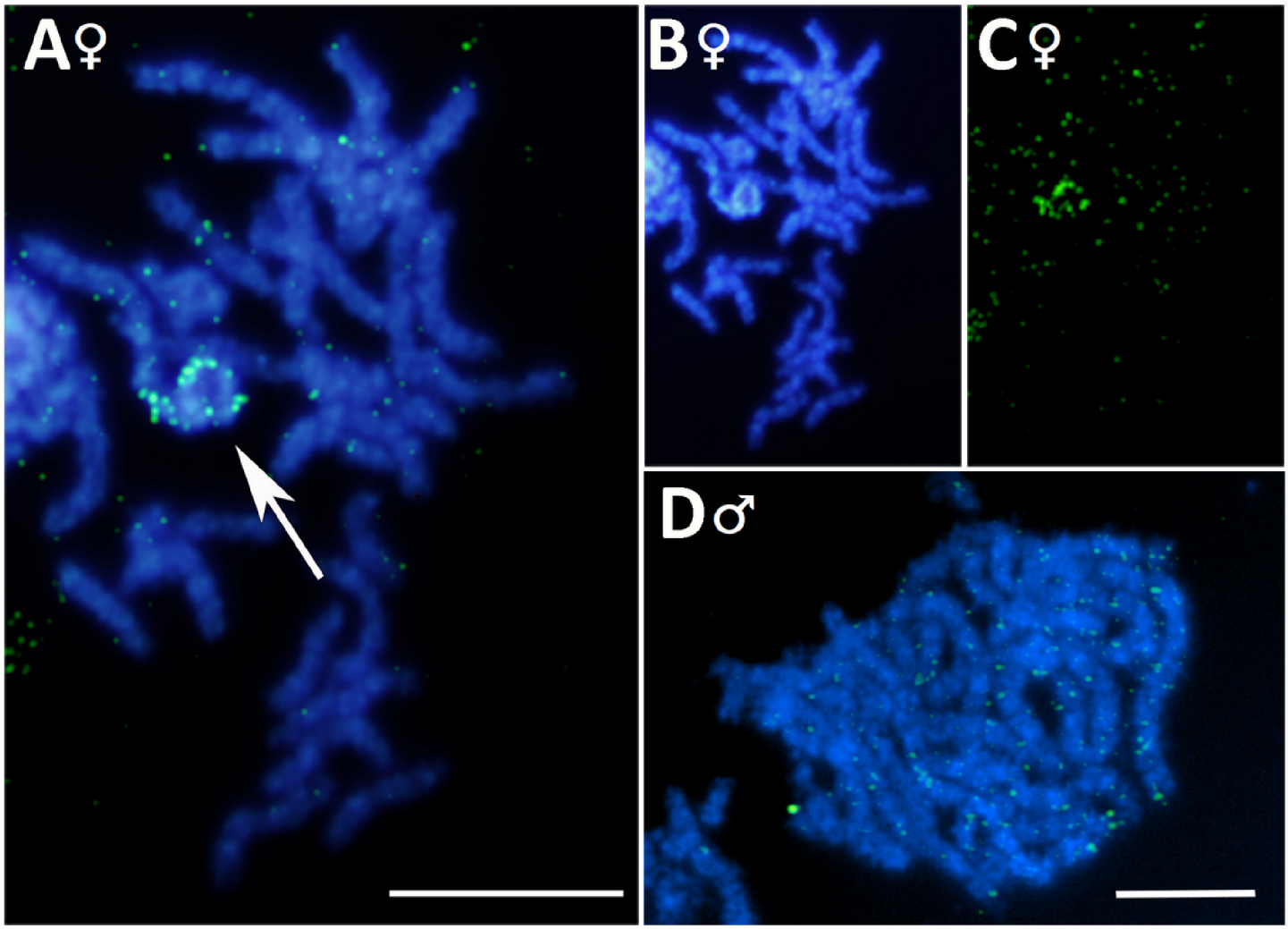

Figure 3

FISH mapping of PRW LINE-like on pachytene nuclei of *Peribatodes rhomboidaria*. (A-C) Female chromosomes with DAPI-positive W chromosome (arrow) accumulating hybridization signals and showing multiple scattered signals on other chromosomes, also seen in males (D). Legend: A,D – merged image of DAPI staining and probe (green), B - DAPI staining, C - probe (green). Bar = 10 µm.

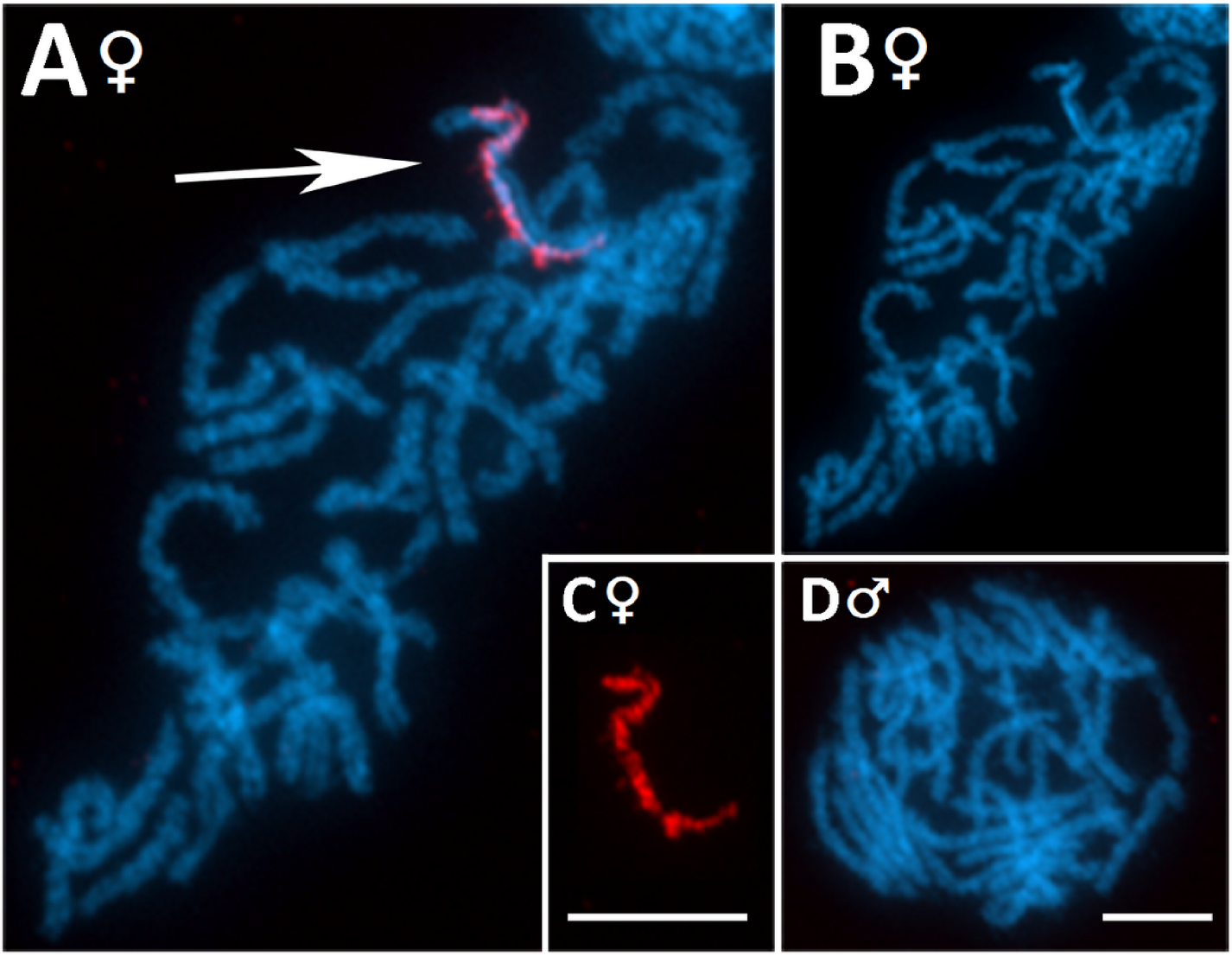

Figure 4

FISH mapping of PRW Bel-Pao on pachytene nuclei of *Peribatodes rhomboidaria*. (A-C) Female chromosomes with DAPI-positive W chromosome (arrow)showing strong hybridization signals, while no signals are visible on other chromosomes and on male pachytene chromosomes (D). Legend: A,D - merged image of DAPI staining and probe (red), B - DAPI staining, C - probe (red) - detail of the W chromosome. Bar = 10 μm.

Supplementary Files

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Supplementary tables

Table S1. Primers used for PCR amplification of selected contigs.

Primer name	Primer sequence	Product
PRWline_F	CGGAACACCTGAAAGAGCGA	PRW LINE-like (540 bp)
PRWline_R	GCATGTGTCCAGTCTCCTTG	
PRW_F1	CGAATCCGTCCCCCTACTCT	PRW Bel-Pao part I (1394 bp)
PRW_R1	ATACAGCCACAAGTCCCACG	
PRW_F2	GCAATTCTTCTTGCTCCCAAA	PRW Bel-Pao part II (1466 bp)
PRW_R2	GCTCCAGTATTTGAAGGCGG	

Supplementary figures

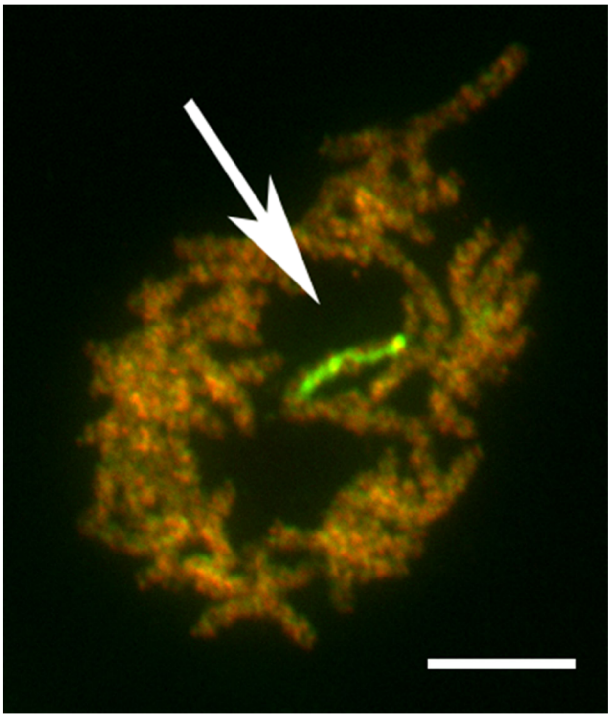

Figure S1. Female pachytene nucleus of *Peribatodes rhomboidaria* after comparative genomic hybridization (CGH). Male (red signals) and female (green signals) genomic DNAs were hybridized to female meiotic chromosomes. The W chromosome is strongly highlighted by the female probe, suggesting the presence of W-specific or highly W-enriched sequences. Arrow indicates the WZ bivalent. Bar = 10 µm.

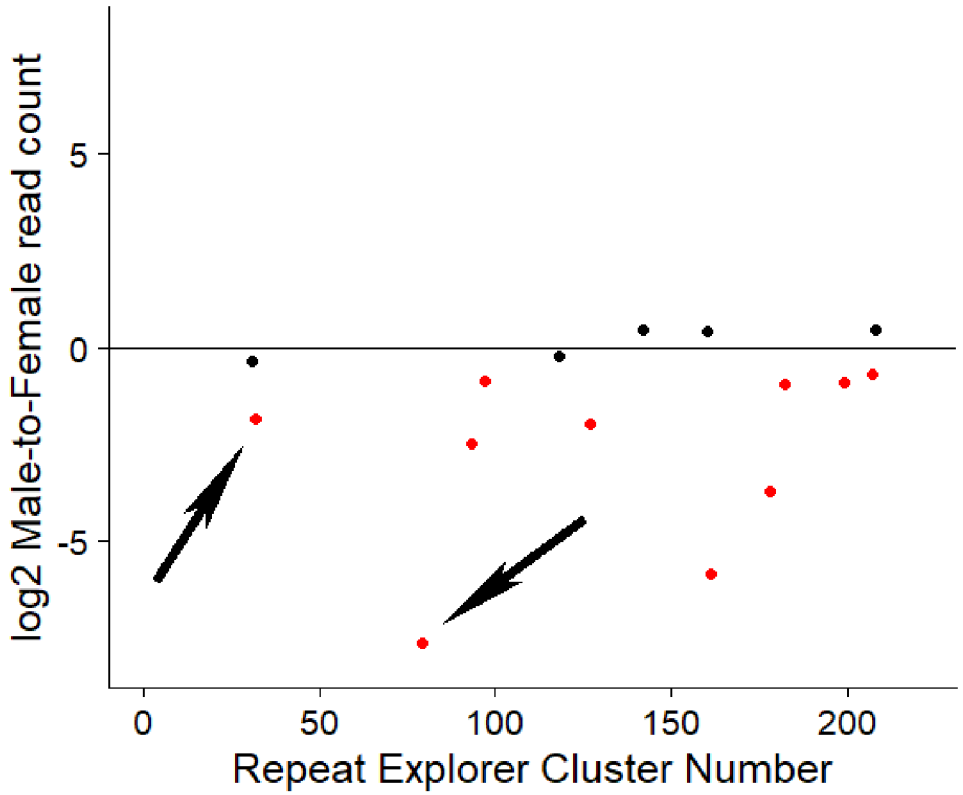

Figure S2. RepeatExplorer results of comparative analysis. Only clusters with statistically significant results between male and female read counts are shown (*t*-test, $P < 0.05$). Red dots represent clusters classified as putative W repeats. Clusters 32 and 79, which were further analysed are marked by arrows.

Figure S3. PRW LINE-like RepeatExplorer graph. Graph layout for cluster 32 generated by RepeatExplorer2. Nodes represent individual illumina reads and edges their sequence overlap.

Figure S4. PRW Bel-Pao RepeatExplorer graph. Graph layout for cluster 79 generated by RepeatExplorer2. Nodes represent individual illumina reads and edges their sequence overlap.

4. Summary of Results & Discussion

4.1. W chromosome evolution in Lepidoptera

Unlike most insect orders, caddisflies (Trichoptera) and moths and butterflies (Lepidoptera) share the female heterogamety system. All examined Trichoptera and basal Lepidoptera have Z0/ZZ (female/male) sex chromosomes, with the only exception of Hepialidae, where the W chromosome was reported (Kawazoé 1987; Voleníková 2015). Hence, the occurrence of the W chromosome is typical for the advanced Lepidoptera (Ennis 1976; Traut & Marec 1996; Marec & Novák 1998; Lukhtanov 2000). Classical theories on the W chromosome origin in Lepidoptera dated its emergence in the group Euheteroneura, which comprises Ditrysia (i.e. advanced moths and butterflies) and Tischeriidae. The common origin of W chromosome in these groups was presumed based on their close phylogenetic relationship. While in Tischeriidae the presence of sex chromatin and W chromosome is based on information from a single examined species *Tischeria ekebladella* (Lukhtanov 2000; Dalíková et al. 2017a), in Ditrysia it has been repeatedly confirmed in the vast majority of studied species (Traut & Mosbacher 1968; Ennis 1976; Traut & Marec 1996; Šíchová et al. 2013; Zrzavá et al. 2018; Hejníčková et al. 2021).

However, a growing body of evidence started to challenge the hypothesis of common W chromosome origin and led to a postulation of the new one, which favors two independent W chromosome origins in Tischeriidae and in Ditrysia. This assumption is supported by the preserved homology of the Z chromosome in these groups (Dalíková et al. 2017a; Fraïsse et al. 2017). Nevertheless, the main argument lies in the fact that some basal groups of Ditrysia – namely Psychidae and Tineidae - probably lack the W chromosome. In Dalíková et al. (2017a), we investigated sex chromatin and sex chromosomes in *Tineola bisselliella* (Tineidae) and confirmed the Z0/ZZ system. Regarding Psychidae, the long-term ignored studies on their karyotypes implied the existence of the Z0/ZZ system based on odd chromosome numbers in females (Seiler 1919; Seiler 1922; Seiler 1960; Narbel-Hofstetter 1961). Hypothetically, such chromosome number difference between sexes could be produced e.g. by a fusion of the W chromosome with an autosome. Hence, our study Hejníčková et al. (2019) provided the essential evidence by investigating sex chromosome systems in three species of Psychidae, showing that they indeed lack the W chromosome.

These findings further support the hypothesis of a non-canonical origin of the W chromosomes in Euheteroneura group, for example by the adoption of a B chromosome (Dalíková et al. 2017a; Fraïsse et al. 2017). To shed more light on this field it will be necessary to conduct research on sex chromosomes in Meesiidae (basal Ditrysia), from which no data is currently available, and possibly also in other species of Psychidae and Tineidae to obtain more reliable sample. Assuming that the basal families of Ditrysia lack the W chromosome, this feature is most likely their ancestral state rather than a secondary trait, which would require multiple independent losses of the W chromosome.

In addition, it would be interesting to further investigate sex chromosomes in Hepialidae, where the W chromosome was reported (Kawazoé 1987; Voleníková 2015). If true, this would mean at least the third independent emergence of the W chromosome in Lepidoptera. In order to understand the full context of the W chromosome evolution, it will also be necessary to investigate the other non-ditrysian groups of Lepidoptera, such as Eriocraniidae, Incurvariidae or Nepticulidae, which supposedly lack sex chromatin (Lukhtanov 2000). Therefore, the absence of the W chromosome in these groups is expected, although this option cannot be excluded without a thorough cytogenetic analysis (see below).

4.2. Sex chromatin and W chromosome features in Geometridae

Sex chromatin has been used as a marker of the W chromosome presence for decades, and its occurrence across lepidopteran species partially shaped the W chromosome evolution theories in the past. Typically, a normal sex chromatin body reflects the presence of a "classical" heterochromatin-rich W chromosome, as seen e.g. in *Ephestia kuehniella* (Traut et al. 1986) or *Cydia pomonella* (Fuková et al. 2005), while no sex chromatin is present in Z0/ZZ species (Lukhtanov 2000; Marec & Novák 1998). Hence, if no sex chromatin is observed, the absence of the W chromosome is expected. However, several studies have shown that there are certain factors influencing the appearance of the sex chromatin body, which may eventually cause an erroneous conclusions of the sex chromosome constitution in the examined species. For instance, chromosomal rearrangements/fusions involving the W chromosome caused disintegration of sex chromatin in mutant strains of *E. kuehniella* (Rathjens 1974; Traut et al. 1986; Marec & Traut 1994), and in the wild populations of the

clouded Apollo *Parnassius mnemosyne* (Vlašánek et al. 2017), the vapourer moth *Orgyia antiqua* (Yoshido et al. 2005b) and the wood white butterflies *Leptidea* (Šíchová et al. 2015). Further, sex chromatin aberrations were observed in highly transcriptionally active nurse and follicle cells during previtellogenesis in *E. kuehniella* (WZ/ZZ, Guelin 1994). The W chromosome is also present in the lupine ghost moth, *Phymatopus californicus,* which lacks sex chromatin completely (Voleníková 2015). On the other hand, a regular sex chromatin body was recorded in the Monarch butterfly, *Danaus plexippus*, despite the presence of a neo-W chromosome which originated via W-autosome fusion (Mongue et al. 2017).

In our study Hejníčková et al. (2021), we showed more examples of sex chromatin aberrations and examined their link to the W chromosome status in 8 species of Geometridae. To my knowledge, this is the most comprehensive study on sex chromosomes in a single family of Lepidoptera. The results covered the whole spectrum of possible outcomes, ranging from the classical combination of a regular sex chromatin body in WZ females, to a plethora of other aberrant variants of sex chromatin bodies and complicated neo-W or multivalent systems to the lack of sex chromatin in Z0 females. Although this study did not provide a universal answer as to which features of the W chromosome affect the form of sex chromatin body, we did observe certain patterns. Our data in combination with the available literature data suggest that sex chromatin formation is influenced by the actual level of differentiation of the W chromosome and, especially in the case of neo-W chromosomes, possibly by the relative proportion of the euchromatic segment to heterochromatin. To produce a proper sex chromatin body, the W-chromosome DNA probably needs to be compacted by some kind of a higher-order mechanism, which could be facilitated for instance by epigenetic markers such as phosphorylation (Kataoka et al. 2016), and/or certain types of sequences such as LINE elements (Loda et al. 2017). Naturally, presence of these features on the W chromosome may greatly differ between species.

Hence, we believe that sex chromatin is a valuable marker that can provide tentative information about the condition of the W chromosome. However, it must be interpreted with caution and the actual sex chromosome status always needs to be verified.

Sex chromosomes in general are expected to vary between species to a certain degree (e.g. Reinhold and Engqvist 2013; Ansai et al. 2022), nevertheless the variability in sex chromosome constitution we detected within the family of Geometridae is astonishing. Although similar

level of diversity has been observed across lepidopteran families (Yoshido et al. 2006) and even in a single genus (Šíchová et al. 2015; Zrzavá et al. 2018), in this study we recorded two cases of intraspecific polymorphism, differing both in W chromosome and sex chromatin features. These findings emphasize the importance of a thorough investigation in several populations of the examined species, and also the necessity to inspect several species from an examined lepidopteran family before drawing any conclusions.

4.3. Retrotransposon accumulation on the W chromosome

Sex-specific chromosomes such as Y or W are known to accumulate various types of repetitive sequences (Charlesworth & Charlesworth 2000; Traut et al. 2013). In the case of Lepidoptera, the content of the W chromosome is still largely unknown. Moreover, considering the enormous variability of W chromosomes across examined species it is now obvious that they must significantly differ from each other in molecular composition. Their most prominent shared feature is the high content of mobile elements, especially retrotransposons, which has been shown e.g. in *B. mori* (Abe et al. 2005; Abe et al. 2010) and *C. pomonella* (Fuková et al. 2007). Their ample occurrence is largely facilitated by their copy-paste replication mechanism, which enables them to expand rapidly, and by the absence of recombination in female meiosis, which would normally limit such expansion.

As expected, the types of retrotransposons that colonize the W chromosome vary from species to species. While in some of them the W chromosome accumulates all kinds of retrotransposons which occur in the genome, it is occupied by a particular type of sequences in others. We confirmed this observation based on CGH results in Geometridae (Hejníčková et al. 2021), when we studied two WZ/ZZ species with cytogenetically different W chromosomes. In *E. atomaria,* the results indicated that common repetitive sequences are accumulating on the W chromosome, while in *P. rhomboidaria* the W chromosome seems to contain female-enriched repeats. We searched for the differences in repetitive DNA between both sexes in these species in order to identify W-chromosome enriched repeats. As expected, we found very few matches in *E. atomaria*, and the only promising sequence which was mapped turned out to be highly ubiquitous (Kubínová 2022). In contrast, we identified 10 putative W-enriched sequences in *P. rhomboidaria* (Hejníčková et al. 2022, preprint), of

which the two most abundant sequences were selected for further investigation. The results showed that they are indeed highly enriched on the W chromosome, especially the PRW Bel-Pao element which covers its entire length, except for its terminal euchromatic part. These findings are consistent with previous research and emphasize the importance of retrotransposons in the W chromosome evolution in Lepidoptera.

Finally, it is tempting to speculate that it applies also *vice versa* – the W chromosomes could in fact play a role in the evolution of retrotransposons. Typically, the accumulated sequences lose their functional domains and thus the ability of transposition. However, their abundance combined with the absence of meiotic recombination could theoretically facilitate their structural reshuffling which could produce new transposable elements. W chromosomes thus represent a wasteland of opportunities.

5. Synthesis and future perspectives

Why are the W chromosomes in Lepidoptera so variable? In my humble opinion, it seems that one of the underlying forces of diversification are indeed retrotransposons, which replicate and rearrange in a random manner and successfully exploit the absence of female meiotic recombination to colonize the W chromosome. The colonization is probably a dynamic process with an unpredictable outcome, therefore the result differs greatly even if the original "input" (e.g. the proto-W chromosome) is equal. The actual sequential composition of the W chromosome also affects its ability to form and maintain sex chromatin body, however, the exact machinery remains elusive.

The evolution of W chromosomes is probably even more stochastic than originally thought. It is now clear that we need to study each lepidopteran family thoroughly, sample several populations, and also focus on the sequence features of the sex chromosomes to obtain the overall picture. This is now possible thanks to long-read sequencing technologies which may help to uncover the mechanisms of the W chromosome nascence. In most of the examined species of Ditrysia, no sequential homology was found between W and Z (Fu et al. 2018; Wan et al. 2019; Lewis et al. 2021), consistent with their proposed B-chromosome adoption (Dalíková et al. 2017a). However, recent data from two species of Crambidae (also Ditrysia) suggest that the W chromosome emerged from the Z chromosome (Dai et al. 2022; preprint) This would mean that the theory on non-canonical W chromosome origin in Lepidoptera is in fact even more complicated, since multiple independent versions of the W chromosome with various emergence mechanisms may exist across the ditrysian clade. Thus, the rise of a new W chromosome may be actually a relatively frequent event.

Such a statement is not exaggerated in view of the amazing W chromosome variability across Lepidoptera. On the one hand, it seems logical that an uncoordinated accumulation of random retrotransposons leads to the emergence of different W chromosomes, which then follow unpredictable evolutionary trajectories. On the other hand, there are still not enough high-quality chromosome-level assemblies from both sexes which would allow to confirm or disprove this claim, so it should be taken with a grain of salt.

All these findings shed new light on the role of the W chromosome in the sex determination of Ditrysia. While it is known to be a sex-determining chromosome in some species (Kiuchi et

al. 2014; Visser 2021), it is irrelevant (Yoshido et al. 2016) or absent (Yoshido et al. 2006; Hejníčková et al. 2021) in others. In conclusion, the evolution, content and the importance of the W chromosome in Lepidoptera remain to be studied.

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WORK EXPERIENCE

LABORATORY OF MOLECULAR CYTOGENETICS, CZECH ACADEMY OF SCIENCES (2016-NOW).

Master thesis, dissertation (in progress), lab manager (supplies, chemicals, services), student mentoring, research: PCR, FISH, CGH, DNA and RNA isolation, bioinformatic tools, BASH programming language, command line

FACULTY OF SCIENCES, UNIVERSITY OF SOUTH BOHEMIA (2018-NOW).

Teaching and organization of practical courses: Genetics, Cytogenetics, and Advanced Methods in Molecular Biology. Organisation of popularisation events for pupils: MOLBIB, TSSB.

LABORATORY OF POPULATION AND EVOLUTION GENETICS, CZECH ACADEMY OF SCIENCES (2013-2016)

Bachelor thesis: DNA isolation, PCR, barcoding, basic population statistics + bioinformatics

ENGLISH LECTOR IN LITE LANGUAGE SCHOOL (2013-2017)

English courses for small groups (A1-B2 level), courses in companies (e.g., Madeta, TSE), private lessons

SKILLS

Advanced knowledge of methods of molecular biology + bioinformatics

English: C1-C2, Spanish: B1, German: A1

Driving license A + B

User knowledge of MS Office

Trade certificate in farming / private horse stable owner

SOFT SKILLS

Highly organized, great communication skills, fast learner, independent, able to supervise and lead, extroverted and friendly

PUBLICATIONS/GRANTS

Hejníčková et al. 2022: Exploring the W chromosome: accumulation of retrotransposons contributes to sex chromosome differentiation in the willow beauty *Peribatodes rhomboidaria* (Lepidoptera: Geometridae). Under review in Scientific Reports, preprint on Research Square.

Kubínová 2022: Analýza a mapování repetivní DNA u tmavoskvrnáče vřesového, *Ematurga atomaria* (Lepidoptera: Geometridae). Bachelor thesis (supervision).

Hejníčková et al. 2021: Degenerated, Undifferentiated, Rearranged, Lost: High Variability of Sex Chromosomes in Geometridae (Lepidoptera) Identified by Sex Chromatin. Cells 10: 2230.

Provazníková et al. 2021: Large-scale comparative analysis of cytogenetic markers across Lepidoptera. Scientific Reports 9 (1):12214.

Hejníčková et al. 2019: Absence of W Chromosome in Psychidae Moths and Implications for the Theory of Sex Chromosome Evolution in Lepidoptera. Genes 10(12): 1016.

Dalíková et al. 2017: New Insights into the Evolution of the W Chromosome in Lepidoptera. Journal of Heredity 108(7):709-719.

Grant Agency of University of South Bohemia 2020: Repetitive sequences in W chromosome evolution: cytogenomics as a tool for unravelling sex chromosome dynamics and diversity in Geometridae (Lepidoptera). Project funding.

EDUCATION

RERUM NATURALIUM DOCTOR (RNDr.), FACULTY OF SCIENCES, UNIVERSITY OF SOUTH BOHEMIA (2018)

New insights into the evolution of the W chromosome in Lepidoptera.

MASTER DEGREE *SUMMA CUM LAUDAE*, GENETIC ENGINEERING, FACULTY OF SCIENCES, UNIVERSITY OF SOUTH BOHEMIA (2018)

Analysis of the karyotype of bagworms (Psychidae, Lepidoptera) by means of classical and molecular cytogenetics.

BACHELOR DEGREE, BIOLOGY, FACULTY OF SCIENCES, UNIVERSITY OF SOUTH BOHEMIA (2015)

Nucleotide variability in *enol-1* gene of entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema feltiae*.

CAMBRIDGE LANGUAGE CERTIFICATE CAE (2011)

CONFERENCES

Hejníčková et al. (2022) W chromosome enriched sequences in *P. rhomboidaria*. Molecular biology and genetics of the lepidoptera, Kolympari, Crete, Greece. Oral presentation.

Hejníčková et al. (2020) Unexpected sex chromosome variability in loopers (Geometridae) uncovered by sex chromatin marker. The Molecular Basis and Evolution of Sexual Dimorphism, Heidelberg, Germany. Virtual Poster.

Hejníčková et al. (2019) Sex chromatin identifyes atypical W chromosomes in looper moths. European Cytogenomics Conference, Salzburg, Austria. Poster and oral presentation.

INTERNATIONAL EXPERIENCE

UNIVERSITY OF JAÉN, JAÉN, SPAIN (JULY 2021)

Internship in the laboratory of Dr. Pedro Lorite, department of Experimental Biology

UNIVERSITY OF TARTU, TARTU, ESTONIA (JULY 2019)

Internship in the laboratory of Dr. Toomas Tammaru, Institute of Ecology and Earth Sciences

NOVOGENIA, SALZBURG, AUSTRIA (SEP.-DEC. 2016)

Internship in commercial laboratory supported by the ERASMUS +

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W chromosomes in Lepidoptera: evolution, diversity and molecular features
Ph.D. Thesis (2022)

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