Evolution of multiple sex-chromosomes associated with dynamic genome reshuffling in *Leptidea* wood-white butterflies

Atsuo Yoshido, Jindra Šíchová, Kristýna Pospíšilová, Petr Nguyen, Anna Voleníková, Jan Šafář, Jan Provazník, Roger Vila, František Marec

Received: 11 March 2020 / Revised: 21 May 2020 / Accepted: 21 May 2020 / Published online: 9 June 2020

© The Author(s) 2020. This article is published with open access

Abstract

Sex-chromosome systems tend to be highly conserved and knowledge about their evolution typically comes from macroevolutionary inference. Rapidly evolving complex sex-chromosome systems represent a rare opportunity to study the mechanisms of sex-chromosome evolution at unprecedented resolution. Three cryptic species of wood-white butterflies—*Leptidea juvernica*, *L. sinapis* and *L. reali*—have each a unique set of multiple sex-chromosomes with 3–4 W and 3–4 Z chromosomes. Using a transcriptome-based microarray for comparative genomic hybridisation (CGH) and a library of bacterial artificial chromosome (BAC) clones, both developed in *L. juvernica*, we identified Z-linked *Leptidea* orthologs of *Bombyx mori* genes and mapped them by fluorescence in situ hybridisation (FISH) with BAC probes on multiple Z chromosomes. In all three species, we determined synteny blocks of autosomal origin and reconstructed the evolution of multiple sex-chromosomes. In addition, we identified W homologues of Z-linked orthologs and characterised their molecular differentiation. Our results suggest that the multiple sex-chromosome system evolved in a common ancestor as a result of dynamic genome reshuffling through repeated rearrangements between the sex chromosomes and autosomes, including translocations, fusions and fissions. Thus, the initial formation of neo-sex chromosomes could not have played a role in reproductive isolation between these *Leptidea* species. However, the subsequent species-specific fissions of several neo-sex chromosomes could have contributed to their reproductive isolation. Then, significantly increased numbers of Z-linked genes and independent neo-W chromosome degeneration could accelerate the accumulation of genetic incompatibilities between populations and promote their divergence resulting in speciation.

Introduction

Sex chromosomes (XY/XX in male heterogamety and WZ/ZZ in female heterogamety) are known to play an important role in fundamental evolutionary processes, such as sex determination, inheritance of sex-specific traits, adaptation and speciation. A large contribution of the X chromosome to reproductive isolation (the so-called ‘large X-effect’) is well established, especially in *Drosophila* species (Presgraves 2008). In some organisms with female heterogamety, Z-linked genes or traits also contribute significantly to speciation and adaptation, referred to as the ‘large Z-effect’ (Qvarnström and Bailey 2009). Sex-limited Y or W chromosomes are frequently associated with sex-specific traits. For example, in some organisms, these chromosomes carry primary sex-determining signals (Bachtrog et al. 2014). In addition, several studies suggest that the Y chromosome plays a role in reproductive isolation of some organisms (Sweigart 2010; Campbell et al. 2012).
chicken W chromosome affects female fertility traits as well (Moghadam et al. 2012). Due to these properties, turnover of sex chromosomes by rearrangements with autosomes may facilitate adaptation and promote speciation (Kitano et al. 2009; Kitano and Peichel 2012; Nguyen et al. 2013; Graves 2016). Moreover, the sex chromosomes themselves are the source of intralocus and intragenomic conflicts that may even cause the turnover of sex chromosomes (Mank et al. 2014). Neo-sex chromosomes and multiple sex-chromosomes originating from fusions and/or translocations between sex chromosomes and autosomes, occur in species with both XY and WZ systems (Marec et al. 2010; Pennell et al. 2015). Exceptionally, repeated sex chromosome-autosome translocations can generate even more complex multiple sex-chromosomes like X1–Y1.5/X1.5X1–5 in platypus (Rens et al. 2004). Thus, the evolution and molecular differentiation of sex chromosomes are among the most intriguing questions of evolutionary genetics. Accumulating evidence suggests that sex chromosomes of moths and butterflies (Lepidoptera), the largest group of animals with holokinetic chromosomes and female heterogamy, also play a disproportionate role in speciation compared with autosomes. In a number of lepidopteran species, Z-linked genes significantly contribute to the formation of prezygotic and/or postzygotic reproductive barriers between different strains and closely related species (Sperling 1994; Naisbit et al. 2002; Presgraves 2002; Dopman et al. 2005; Kost et al. 2016). In addition, recent results suggest that a sex-linked inversion promotes divergence and facilitates speciation by suppressing recombination between Z chromosomes of two strains of the European corn borer moth, Ostrinia nubilalis (Wadsworth et al. 2015). Besides the common WZ/ZZ (♀/♂) sex chromosomes, WZ1Z2 and W1W2Z multiple sex-chromosome systems were found only in several distantly related lepidopteran taxa (Traut et al. 2007; Marec et al. 2010; Sahara et al. 2012). However, neo-sex chromosomes formed by fusion of both W and Z chromosomes with autosomes are much more common in Lepidoptera than previously thought (Nguyen et al. 2013; Mongue et al. 2017; Carabajal Paladino et al. 2019). Transfer of the whole set of autosomal genes under Z-linkage, as in multiple or neo-sex chromosomes, can greatly contribute to ecological specialisation, reproductive isolation and speciation in Lepidoptera (Yoshido et al. 2011a; Nguyen et al. 2013; Carabajal Paladino et al. 2019). This is demonstrated by the neo-W chromosome of the African queen butterfly, Danaus chrysippus, which drives speciation across the hybrid zone by linking the colour pattern and male killing caused by an endosymbiotic bacterium, Spiroplasma ixodeti (Smith et al. 2016; Traut et al. 2017).

Wood-white butterflies of the genus Leptidea (Pieridae) constitute an excellent model for studying the role of chromosome rearrangements in speciation. Especially three species with mainly Western Palaearctic distribution—L. juvenica, L. sinapis and L. reali—represent one of the most striking examples of cryptic diversity in Eurasian butterflies (Dincă et al. 2011). This triplet of species has evolved strong pre-mating reproductive barriers in their sympatric and allopatric populations (Friberg et al. 2008; Dincă et al. 2013). In addition, chromosome numbers vary greatly between and even within the species due to multiple chromosome fusions and fissions (Dincă et al. 2011; Lukhtanov et al. 2011, 2018; Šichová et al. 2015). Genome sequencing showed that the genome assembly of L. sinapis (643 Mb) is one of the largest in Lepidoptera studied so far, and variations in genome size between and within Leptidea species have been documented (Talla et al. 2017).

Previous studies showed that each of the four Leptidea species examined have a unique, species-specific and complex system of multiple sex-chromosomes: W1–Z1.4/Z1–Z1.4 in L. juvenica (♀/♂), W1–Z1–3/Z1–3Z1–3 in L. sinapis, W1–Z1–3/Z1–3Z1–3 in L. reali and W1–Z1–3/Z1–3 in L. amurensis (Šichová et al. 2015, 2016). Despite fluctuating chromosome numbers, even between siblings of individual species, the sex-chromosome systems seem stable in each species. Thus, these closely related species provide a unique opportunity to address the role of sex-chromosome rearrangements in the formation of reproductive barriers between their populations. However, little is known about the composition and origin of these multiple sex-chromosomes, because in such complex systems it is difficult to identify all sex chromosomes simply from genome assembly.

In this study, we cytogenetically identified Z chromosomes in three cryptic Leptidea species (L. juvenica, L. sinapis and L. reali) and reconstructed the evolution of their multiple sex-chromosome systems by comparative mapping of sex-linked genes. For this purpose we have developed a couple of genomic tools in L. juvenica, namely a female transcriptome-based microarray for comparative genomic hybridisation (CGH) and a bacterial artificial chromosome (BAC) library from females. BAC probes containing Leptidea orthologs of Bombyx mori genes identified all Z chromosomes in three Leptidea species. Furthermore, we analysed several BAC clones derived from the W chromosomes of L. juvenica, which allowed us to identify female-specific sequences and W-linked genes of autosomal origin.

Materials and methods

Specimens

Adult specimens of Leptidea juvenica and L. sinapis were collected in several localities in the Czech Republic whereas
L. reali was collected in the Montseny area near Barcelona, Spain. The taxonomic determination of specimens used was verified by morphometric analysis of their genitalia and sequencing of a mitochondrial gene, cytochrome c oxidase subunit 1, as described in Šichová et al. (2015). Fertilised females were individually kept in plastic containers to lay eggs. Hatched larvae of all three species were reared on one of their host plants, Lotus corniculatus, at room temperature and a natural day/night regime.

RNA sequencing and female transcriptome assembly in Leptidea juvernica

Total RNA was extracted from a homogenised female larva of L. juvernica (gut removed) using an RNA Blue reagent (Top-Bio, Prague, Czech Republic) according to the manufacturer’s protocol. An mRNA-seq library was constructed and sequenced using the Illumina HiSeq2000 platform by EMBL Genomics Core Facility (Heidelberg, Germany). Generated raw 100-bp paired-end reads were checked by FastQC (Andrews 2010) and trimmed and quality filtered by Trimmomatic version 0.30 (LEADING:5 TRAILING:5 SLIDINGWINDOW:4:20; Bolger et al. 2014). Transcriptome sequence was then assembled de novo by SOAPdenovo-trans-127mer with multiple odd k-mer sizes ranging from 21 to 81 (Xie et al. 2014) and Trinity (Haas et al. 2013). The resulting assemblies were merged and redundancy was removed by the EvidentialGene pipeline (Gilbert 2013).

Array-CGH analysis in Leptidea juvernica

To identify sex-linked genes in L. juvernica, microarray-based comparative genomic hybridisation (array-CGH) was performed according to the method described in Baker and Wilkinson (2010) (for details, see Supplementary Methods). Briefly, we searched for Leptidea orthologs of Bombyx mori genes in the EvidentialGene output using HaMSiR (Ebersberger et al. 2009). The Leptidea orthologs were used to design 60-mer oligonucleotide probes for custom-made microarray slides using Agilent Technologies eArray design wizard. DNA digestion, labelling and array-CGH were performed by GenLabs (Prague, Czech Republic) following a protocol for Agilent oligonucleotide array-based CGH for genomic DNA analysis.

Construction of BAC library in Leptidea juvernica females

A BAC library was constructed from female pupae of L. juvernica. The procedure followed the modified protocol described previously (Šafář et al. 2010). Briefly, high molecular weight DNA isolated from female pupae and embedded in agarose plugs was partially digested with restriction enzyme HindIII. The digested DNA was separated by pulsed field gel electrophoresis (two size-selection steps); 100–300-kb fragments were then isolated from the gel and ligated into a dephosphorylated vector pAGIBAC5. Recombinant BACs were transformed into E. coli strain MegaX DH10B T1 (Invitrogen, Carlsbad, CA, USA) by electroporation. In total, 36,864 clones were picked by an automatic robotic station Q-BOT, ordered in 384-well microwell plates, and stored at −80 °C. The average insert size in BAC clones was a~125 kb. A master copy of the BAC library, named LjufhA, is stored at the Centre of Plant Structural and Functional Genomics in Olomouc, Czech Republic. The master copy was used for preparation of two working copies. Subsequently, clones from a working copy of the LjufhA BAC library were used to prepare 3 DNA pools. Briefly, a liquid handling system Biomek NX (Beckman Coulter, Brea, CA, USA) was used for pooling 384 clones of each plate (‘plate pool’), for pooling rows from 384-well plates (‘row pool’), and for pooling columns from 384-well plates (‘column pool’). DNA from pooled clones was isolated by standard alkaline lysis and ordered in 96-well microwell plates for further PCR screening.

BAC screening for FISH mapping

Sequence information of Leptidea orthologs of B. mori genes was obtained from the transcriptome assembly of L. juvernica females. By blast searching gene models in KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/) against the L. juvernica transcriptome, selected Leptidea orthologs of B. mori genes were identified. We then designed primer sets to identify BAC clones containing the respective Leptidea orthologs from the BAC library (Supplementary Table S1). For BAC screening, we used a 3-step PCR-based screening method as described previously (Yasukochi 2002; Yoshido et al. 2015). Each reaction mixture was composed of 1.0 μl of template BAC-DNA pools, 10 pmol of each primer, 0.25 U of OneTaq DNA Polymerase and 2.0 μl of 5× OneTaq Standard Reaction Buffer (New England BioLabs, Ipswich, MA, USA). PCR amplifications were conducted under the following conditions: initial denaturation for 5 min at 94 °C, 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55–60 °C, elongation for 30–180 s at 68 °C and final elongation for 1 min at 72 °C. PCR products were loaded on 1.0–2.0% agarose gel in TAE buffer. Gels were stained with ethidium bromide and photographed under UV light.

BAC-FISH mapping

All required procedures are described in detail in Supplementary Methods. Spread preparations of meiotic chromosomes were obtained from gonads (ovaries and testes) and
mitotic chromosomes from wing imaginal discs of the last instar larvae as described in Šíchová et al. (2015) and Yoshido et al. (2015). For mapping of gene orthologs on a particular chromosome, several rounds of two-colour BAC-FISH were carried out following the procedure and reprobing protocol described in Yoshido et al. (2015). Extracted BAC-DNAs were labelled by nick translation using a mixture of DNase I and DNA polymerase I with either aminoallyl-dUTP-Cy3 or fluorescein-12-dUTP and BAC probes hybridised to chromosomes using the BAC-FISH mapping protocol (Yoshido et al. 2015).

**Analysis of BAC clones derived from W chromosomes and W homologues of Z-linked orthologs**

To find BAC clones derived from W chromosomes in *L. juvernica*, we screened the BAC library using FISH-based screening (Supplementary Fig. S1). Extracted BAC-DNAs of two clones derived from W chromosomes of *L. juvernica* (see ‘Results’) were digested with restriction enzyme HindIII, and several digested DNA fragments (700–5000 bp) were sub-cloned into pJET1.2/blunt Cloning Vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA). The plasmids obtained were transformed into *Escherichia coli* DH5α strain. Plasmid DNAs were extracted using the NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany) and sequenced using universal primers. Analysis of W homologues of Z-linked orthologs of the *Uch5l* and *Gst8* genes was performed by PCR and sequencing as described in Supplementary Methods.

**Quantitative real-time PCR (qPCR)**

To compare a relative gene dose of *Leptidea* orthologs between females and males, we performed quantitative real-time PCR (qPCR) using gDNAs as templates according to the method described in Nguyen et al. (2013) with slight modifications. For qPCR, gDNAs were extracted separately from three females and three males of *L. juvernica*, *L. sinapis*, and *L. reali* by standard phenol–chloroform procedure. Target and reference genes were analysed simultaneously in three different specimens for each sex. The *Leptidea* ortholog of the *B. mori* *RpsS3* gene was used as an autosomal reference gene. Primer sets for respective target and reference genes are listed in Supplementary Table S1 (asterisk) or Table S5. The qPCR was performed using a reaction mixture composed of 0.5–20 ng of template gDNA, 0.5 pmol of each primer and 5 μl of Xceed qPCR SG 2× Mix Lo-Rox (Institute of Applied Biotechnologies, Prague, Czech Republic). To calculate the amplification efficiency (*E*), 4 points of 4 times dilution series were used. The obtained data were processed using CFX Manager Software (Bio-Rad). The PCR reaction was carried out using the C1000 Thermal cycler CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The female-to-male relative dose ratio of the target gene was determined by comparison with the autosomal reference gene using a formula of Pfaff (2001) and statistically analysed by unpaired two-tailed *t* test for unequal variances.

**Results**

**Array-CGH in Leptidea juvernica**

To identify candidate orthologs located on *L. juvernica* Z chromosomes, we carried out array-CGH (Fig. 1). After filtering, *L. juvernica* log2 ratio values of male-to-female signal intensities were obtained for 4252 orthologs. The values averaged across four replicas clearly showed a bimodal distribution with an autosomal peak centred at −0.1 (Fig. 1a). Using a cut-off value of 0.5, we identified 454 putative Z-linked orthologs. Chromosomal location of their *B. mori* counterparts revealed that the identified *L. juvernica* Z-linked orthologs contain vast majority of genes assigned to *B. mori* Z-chromosome (chr. 1) and are further enriched on *B. mori* chr. 7, chr. 8, chr. 11, chr. 17 and chr. 24 (Fig. 1b).

**BAC-FISH mapping of Z1 chromosome**

We identified 19 BAC clones containing *Leptidea* orthologs of 17 *B. mori* Z-linked genes (Supplementary Table S1) and mapped them to *L. juvernica* chromosomes. Fourteen out of nineteen prepared BAC probes mapped to a single bivalent in *L. juvernica* males (Fig. 2a and Supplementary Table S1). This bivalent is evidently a Z-chromosome pair, since two representative probes out of these 14, BAC 62N7 and 91P9, containing orthologs of *Masc* and *ket* genes, respectively, mapped both to the same element of the sex-chromosome multivalent in *L. juvernica* females (Fig. 5a). We named this element as *Z1* chromosome. One of the probes, BAC 91J4 containing the *Leptidea* ortholog of *B. mori* *Prm*, hybridised not only to *Z1* but also to one autosomal bivalent, suggesting duplication of the *Z1* region carrying this gene (Supplementary Fig. S2a). The remaining four probes, BAC 91C3 containing the *Pgd* ortholog, 96D19, 66L7 and 62O17 containing each different size-fragment of the *Tpi* ortholog (Supplementary Table S1), mapped to autosomal bivalents in *L. juvernica* females (Supplementary Text 1 and Supplementary Fig. S2b, c). These results suggest that the orthologs of these two *B. mori* Z-linked genes, *Pgd* and *Tpi*, are not Z-linked in *L. juvernica* and the *Tpi* ortholog is not a single copy gene.
in the genome. Gene movement out of the L. juvernica Z₁ chromosome, such as Pgd translocation and Tpi translocation followed by duplication, is better documented by results of array-CGH, which showed that only 17 of the 197 tested orthologs (i.e., 8.6%) of B. mori Z-linked genes are autosomal (Fig.1b). Regarding Pgd, the autosomal location of this ortholog is fully consistent with a −0.0125 CGH log₂ ratio of the male-to-female signal intensities. The orthologs of Prm and Tpi were not included in the microarray.

FISH mapping of 14 BAC probes containing Leptidea orthologs of B. mori Z-linked genes showed that the probes did not cover the whole length of the L. juvernica Z₁ chromosome (Fig. 2a, asterisk). Thus, we tested BAC clones containing putative sex-linked Leptidea orthologs of B. mori autosomal genes identified by array-CGH (Fig. 1b). Three BAC probes, 19D3, 94M23 and 92J7, containing orthologs of B. mori chr. 17 genes, also mapped to Z₁ in L. juvernica males (Fig. 2b). However, three BAC probes, 66E20, 65E15 and 93F8, containing putative autosomal orthologs of B. mori chr. 17 genes, mapped to two autosomes in L. juvernica males (Fig. 2, underlined genes and Supplementary Fig. S3a). These results suggest that the Z₁ chromosome of L. juvernica consists of Leptidea orthologs of most of the B. mori Z-linked genes and part of the B. mori chr. 17 genes.

To verify the conservation of the Z₁ chromosome in Leptidea, Z₁-derived BAC probes were cross-hybridised to chromosomes of two other species, L. sinapis and L. reali. In L. sinapis, 17 Z₁-derived BAC probes mapped to a single bivalent in males and even in the same order as in the Z₁ chromosome of L. juvernica (Fig. 2c, d). Three representative BAC probes out of these 17, 62N7, 91P9 and 92J7, mapped to the sex-chromosome multivalent in L. sinapis females (Fig. 5c), thus confirming that the BAC-FISH-identified bivalent in L. sinapis males is a pair of Z₁ chromosomes that are orthologous to the Z₁ chromosome in L. juvernica. In contrast, 8 of the 17 Z₁-derived BAC probes of L. juvernica mapped to one bivalent and 9 probes to another bivalent in L. reali males (Fig. 2e). This result showed that in L. reali, the Z₁ of L. juvernica and L. sinapis split into two chromosomes in the region between the ap and ket orthologs (BAC probes 53C10 and 91P9, respectively) (Fig. 2). The order of 17 BAC loci in the respective bivalents was conserved in all three species. One of the BAC probes, 62N7, representing one of the two mapped bivalents and two BAC probes, 91P9 and 92J7, representing the other bivalent hybridised to the sex-chromosome multivalent in L. reali females (Fig. 5e). This result clearly showed that the mapped bivalents represent two of the four Z chromosomes in L. reali. We marked them as Z₁ and Z₄ chromosomes (Fig. 2).

**BAC-FISH mapping of Z₂ and Z₃ chromosomes**

To determine the origin of the other two Z chromosomes in L. juvernica, we performed FISH mapping of BAC clones containing putative sex-linked Leptidea orthologs of B. mori autosomal genes that were identified by array-CGH (Fig. 1b). Sixteen BAC probes containing orthologs of B. mori chr. 7, chr. 11 and chr. 24 genes mapped to a single bivalent in L. juvernica males and covered the entire length of the bivalent (Fig. 3a). One representative BAC probe (94J6) of these 16 hybridised to one chromosome in the
sex-chromosome multivalent in *L. juvernica* females (Fig. 5a). Thus, the male bivalent identified by BAC-FISH corresponds to another pair of four Z chromosomes in *L. reali*. Upper panel: male pachytene chromosomes showing hybridisation signals of Cy3- or fluorescein-labelled 14 BAC probes containing *Leptidea* orthologs of *Bombyx mori* Z-linked and 3 BAC probes of chromosome 17-linked genes. Chromosomes were stained with DAPI (grey). Bar = 10 μm. Asterisk indicates the beginning of region orthologous to *B. mori* chr. 17. (a, b) *L. juvernica*. (c, d) *L. sinapis*. (e) *L. reali*. Lower panel: schematic illustrations of comparative gene mapping between three *Leptidea* species and *B. mori*. The underlined genes on *B. mori* chromosomes indicate orthologs that mapped to autosomes in *Leptidea* species (Supplementary Fig. S3a).
7, chr. 11 and chr. 24 genes indeed map to autosomes in *L. juvernica* males (see the underlined genes in Fig. 3 and Supplementary Fig. S3b, c). Based on the above results, we concluded that the *Z*₂ chromosome of *L. juvernica* consists of three segments corresponding to parts of *B. mori* chr. 11, chr. 7 and chr. 24 (Fig. 3, lower panel).

All 16 BAC probes that mapped to the *Z*₂ chromosome of *L. juvernica* also mapped to the respective bivalent in *L. sinapis* and *L. reali* males. In addition, the order of mapped probes was also fully conserved in all three species (Fig. 3b, c). The representative BAC 94J6 probe of these 16 also hybridised to one chromosome of the sex-chromosome multivalent in *L. sinapis* and *L. reali* females (Fig. 5c, e), confirming that it is indeed a *Z*₂ chromosome in both species.

Eight BAC probes containing orthologs of *B. mori* chr. 8 genes mapped to two bivalents in *L. juvernica* males (Fig. 4a). One of the BAC probes (62A6), representing one of the two bivalents and another BAC probe (69P11), representing the other bivalent hybridised to the sex-chromosome multivalent in *L. juvernica* females (Fig. 5a), suggesting that both bivalents correspond to two of four
Z chromosomes in this species. We marked them as Z3 and Z4 chromosomes (Fig. 4, lower panel). To get more markers on Z3, we searched in the assembled genome sequence of L. sinapis (Talla et al. 2017) for a scaffold containing an ortholog of the m5u-mt gene located on B. mori chr. 8 (Fig. 4). We found that the scaffold_39 in the L. sinapis genome sequence consists not only of the m5u-mt gene ortholog but also of the ortholog of Ctatpase gene located on B. mori chr. 15 (Supplementary Table S2). This finding allowed us to select and test six BAC clones containing orthologs of B. mori chr. 15 genes (Supplementary Table S1). Three BAC probes of these six, 41J22, 17I6 and 19O6, mapped to the Z3 bivalent in L. juvernica males (Fig. 4a). We also confirmed that two and three BAC probes containing putative autosomal orthologs of B. mori chr. 8 and chr. 15 genes, respectively, mapped to autosomes in L. juvernica males (see the underlined genes in Fig. 4 and Supplementary Fig. S3c). Taken together, we conclude that the Z3 chromosome of L. juvernica consists of two segments, one corresponding to a part of chr. 8 and one to a part of chr. 15 in B. mori, and Z4 contains the other part corresponding to B. mori chr. 8.
All ten BAC probes that mapped to Z₃ and Z₄ chromosomes in *L. juvernica* mapped to each single bivalent in *L. sinapis* and *L. reali* males (Fig. 4b, c). The fact that both Z₃ and Z₄ of *L. juvernica* contain orthologs of *B. mori* chr. 8.

Fig. 5 BAC-FISH analyses of multiple sex-chromosomes in three *Leptidea* species. Upper panel (a–f): FISH mapping of BAC clones representing respective Z chromosomes in female and male pachytene chromosomes. BAC clones (see Supplementary Table S1) mapping to different Z chromosomes are marked with a different colour. Chromosomes were stained with DAPI (grey). Bar = 10 μm. (a) BAC-FISH image and schematic drawing of the sex-chromosome multivalent in female pachytene of *L. juvernica*. (b) Male pachytene complement of *L. juvernica*. (c) BAC-FISH image and schematic drawing of the sex-chromosome multivalent in female pachytene of *L. sinapis*. (d) Male pachytene complement of *L. sinapis*. (e) BAC-FISH image and schematic drawing of the sex-chromosome multivalent in female pachytene of *L. reali*. (f) Male pachytene complement of *L. reali*. Lower panel (a’–f’): schematic illustrations of multiple sex-chromosomes in three *Leptidea* species based on BAC-FISH results shown in upper panel. (a’) *L. juvernica* female. (b’) *L. juvernica* male. (c’) *L. sinapis* female. (d’) *L. sinapis* male. (e’) *L. reali* female. (f’) *L. reali* male. Z and W chromosomes are coloured grey and black, respectively. The phylogenetic relationships and estimated divergence times (My, million years) between the species are shown below the lower panel (Šíchová et al. 2015; Talla et al. 2017, 2019a).
indicates that they arose by fission (see ‘Discussion’). The order of ten BAC loci remained unchanged in *L. sinapis* and *L. reali* (Fig. 4b, c). We also confirmed that two representative BAC probes of these ten, 62A6 and 69P11, hybridised to one chromosome of the sex-chromosome multivalent in *L. sinapis* and *L. reali* females (Fig. 5c, e). This chromosome was designated as Z₃ in *L. sinapis* and *L. reali*.

To confirm that the Z chromosomes shown in Figs. 2–4 are indeed different individual chromosomes, we performed BAC-FISH mapping of six representative probes in males of each *Leptidea* species. These six BAC probes mapped to four chromosomes (Z₁–Z₄) in *L. juvernica* (Fig. 5b), three chromosomes (Z₁–Z₃) in *L. sinapis* (Fig. 5d) and four chromosomes (Z₁–Z₄) in *L. reali* (Fig. 5f), respectively. Thus, we successfully identified all Z chromosomes in all three *Leptidea* species.

**Analysis of molecular differentiation in W chromosomes**

BAC clones derived from W chromosomes of *L. juvernica* were identified by FISH-based screening (Supplementary Text 2 and Supplementary Figs S1, S4). The W-BAC 1B2 probe painted part of the sex-chromosome multivalent in *L. juvernica* females (Fig. 6a, b) and another W-BAC clone 1J4 hybridised to a specific region of the sex-chromosome multivalent in *L. juvernica* females (Fig. 6c). The 1B2 probe also painted part of the sex-chromosome multivalent in other two *Leptidea* species, whereas hybridisation signals of the 1J4 probe were not detected in either of these species (Supplementary Fig. S5).

Sequence analysis of sub-cloned W-BAC clones 1B2 and 1J4 and blast searches showed that most of the sub-cloned sequences were part of transposable elements, but two sub-clones (Nos 11 and 18) were part of the *Leptidea* ortholog of the *B. mori* Uch5l gene (Supplementary Table S3 and Supplementary Fig. S6a), which mapped to the Z₃ chromosome in *L. juvernica* (Fig. 4). This result suggests that the two sub-cloned sequences are part of a Whomologue of the Z₃-linked ortholog of the Uch5l gene.

We searched for W-specific sequences of the Uch5l ortholog by PCR with a set of designed primers (Supplementary Fig. S6a and Supplementary Table S1). PCR amplified two distinct fragments (1400 and 2095 bp) from female gDNA of *L. juvernica*, whereas only a single fragment was amplified from male gDNA (Fig. 6d). These PCR results also showed that the BAC clone 62A6, which mapped to the Z₃ chromosome (Fig. 4), contains a common fragment of both sexes (Fig. 6d, white asterisks) and that the W-BAC clones 1B2 and 70F8, the latter identified by
further BAC library screening, contain a female-specific fragment (Fig. 6d, red asterisks). The female-specific fragment (Lj_Uch5l_W) and the common fragment (Lj_Uch5l_Z) share sequences in their exons, but differ quite considerably in their introns (Supplementary Fig. S6b and Supplementary Table S4). The differences between introns are mainly due to randomly occurring insertions in the Lj_Uch5l_W intron (Supplementary Figs S6c, S7). The female-specific fragment was also amplified from gDNAs of L. sinapis and L. reali females and most of their sequences were well conserved in all three species, except for several insertions or deletions (Supplementary Fig. S8).

We designed a primer set based on the female-specific sequence in the intron of the Uch5l ortholog (Supplementary Table S5 and Supplementary Fig. S6b) to confirm the female-specific insertion. PCR with the female-specific primer set amplified a 341-bp fragment from female gDNAs of all three species ( Supplementary Fig. S6c, asterisks), whereas no 341-bp fragments were detected in male gDNAs.

We found a sex-specific polymorphism in the Leptidea ortholog of the Gst8 gene, located near the Uch5l gene on B. mori chr. 8 and we showed that the ortholog has a W-linked copy. PCR with a set of designed primers (Supplementary Fig. S9a and Supplementary Table S1) amplified a single fragment from male gDNA of L. juvernica (Fig. 6e, white asterisks), whereas several distinct fragments were amplified from female gDNA (Fig. 6e, red asterisks). We identified clone 20K23 containing a fragment of the Gst8 ortholog that was common to both sexes (Fig. 6e). The 20K23 probe mapped to the same region of the Z3 chromosome in L. juvernica as the 62A6 probe containing the Uch5l ortholog (Supplementary Fig. S9b). These results suggest that the fragment amplified from both sexes is derived from the Z3 chromosome. In contrast, the BAC clone 72H23 contained several distinct female-specific fragments of the Gst8 ortholog (Fig. 6e, red asterisks) and the 72H23 probe painted most of the W chromosomes in L. juvernica females (Supplementary Fig. S9c). Sequence analysis of the common fragment and the female-specific fragment of the Gst8 ortholog revealed several female-specific (W-specific) deletions in the introns and the occurrence of at least three distinct copies of the Gst8 ortholog on the L. juvernica W chromosomes (Supplementary Text 3 and Supplementary Fig. S9d).
To estimate the copy number of _Leptidea_ orthologs of the _Uch5l_ and _Gst8_ genes on the W chromosome(s) and to assess if other Z3- and Z4-linked orthologs still remain covered on the W chromosome(s), we compared the relative gene dose of these orthologs between female and male gDNAs in the _Leptidea_ species studied by qPCR (Fig. 7; Supplementary Fig. S10 and Supplementary Tables S6–S8). Because Z1- and Z2-linked orthologs have been identified as sex-linked genes by array-CGH in _L. juvernica_ (Figs. 1–3), female-to-male relative gene dose ratios of these orthologs should be 1:2. Accordingly, we confirmed a twofold difference between the female and male gene dose in all tested Z2-linked orthologs by qPCR in all three species (Supplementary Fig. S10). Results of qPCR with Z3- and Z4-linked orthologs showed that the female-to-male relative gene dose ratios of the _Uch5l_ and _Gst8_ orthologs are -5.7:1 and 2.8:1 in _L. juvernica_ (Fig. 7a), 7.3:1 and 3.1:1 in _L. sinapis_ (Fig. 7b) and 8:1 and 4.1:1 in _L. reali_ (Fig. 7c), respectively. Female-to-male relative gene dose ratios of orthologs of the _Clatpase_, _m5u-mt_ and _Ann1_ genes in all three species, _Lgr_ and _Dbadrh_ genes in _L. juvernica_, and the S3-12 gene in _L. reali_ were 1:2 (Fig. 7 asterisks and Supplementary Tables S6–S8). In contrast, we found no significant difference between the female and male gene doses in orthologs of the _Rpp0_, _Frl_ and _Smc_ genes in all three species, _Top2-bp_ and S3-12 genes in _L. juvernica_, S3-12, _Lgr_ and _Dbadrh_ genes in _L. sinapis_, and the _Dbadrh_ gene in _L. reali_ (Fig. 7 n.s. and Supplementary Tables S6–S8), suggesting that their orthologs also remained intact on the W chromosomes. Female-to-male relative gene dose ratios of the _Top2-bp_ gene ortholog were 1:1 in _L. juvernica_ but ~1.6:1 in _L. sinapis_ and _L. reali_. Results of qPCR showed a tenfold difference between the female and male gene dose of the _Lgr_ ortholog in _L. reali_ (Fig. 7c).

**Discussion**

In this work, we have deciphered the evolutionary origin of complex multiple sex-chromosomes in three cryptic _Leptidea_ species. BAC-FISH mapping of Z-linked genes clearly showed that the multiple sex-chromosomes arose by several translocations and fusions between the ancestral WZ pair and autosomes, followed by several fissions (Figs. 2–4). The resulting 3–4 Z chromosomes are composed each of 2–3 conserved syntenic blocks, in which the gene order remained well conserved in all three species. However, the Z chromosomes in individual species were differentiated by further rearrangements, either by fusion or fission (Fig. 5). In accordance with phylogenetic relationships between the three species (Dincă et al. 2011; Lukhtanov et al. 2011; Šíchová et al. 2015; Fig. 5 in this study), the Z1 of _L. juvernica_ and _L. sinapis_ most likely split into two Z chromosomes (Z1 and Z4) in _L. reali_ after _L. reali_ and _L. sinapis_ diverged from their common ancestor because both Z1 and Z4 of _L. reali_ contain genes of the original Z-chromosome in the ancestral lepidopteran karyotype (Van’t Hof et al. 2013; Ahola et al. 2014). The Z3 of _L. sinapis_ and _L. reali_ corresponds to Z3 and Z4 chromosomes in _L. juvernica_ (Fig. 4). Since both Z3 and Z4 of _L. juvernica_ contain orthologs of _B. mori_ chr. 8 genes that are located on one autosome in the ancestral lepidopteran karyotype, the two Z chromosomes most likely originated by fission of Z3 chromosome after _L. juvernica_ diverged from _L. sinapis_ plus _L. reali_. These findings strongly suggest that the sex-chromosome constitution in the common ancestor of these three _Leptidea_ species was similar to that of _L. sinapis_ (Fig. 5).

Conserved synteny blocks and conserved order of genes in the multiple Z chromosomes of _Leptidea_ species studied are in line with current knowledge of the genome architecture in Lepidoptera. Most lepidopteran species have a chromosome number equal to or near the ancestral number of _n_ = 31, and syteny of genes, including their order, is relatively well conserved even among distant species (Van’t Hof et al. 2013; Ahola et al. 2014; Yasukochi et al. 2016). The model species for Lepidoptera, _B. mori_, has a haploid chromosome number of _n_ = 28 and its karyotype evolved by three chromosome fusions from the ancestral _n_ = 31, except for a few inversions and translocations (Van’t Hof et al. 2013; Ahola et al. 2014; Yasukochi et al. 2016). A similar explanation applies to the karyotype evolution in other lepidopteran species with reduced chromosome numbers (Pringle et al. 2007; Yasukochi et al. 2009; Yoshido et al. 2011b). Our results, together with earlier reported variation in chromosome numbers in the genus _Leptidea_ (Dincă et al. 2011; Lukhtanov et al. 2011; Šíchová et al. 2015, 2016), suggest dynamic genome rearrangements that occurred not only in sex chromosomes but also in autosomes during karyotype evolution before _Leptidea_ species diverged from a common ancestor. Consequently, the _Leptidea_ species do not exhibit a conserved macro-synteny of genes compared with the putative ancestral karyotype of Lepidoptera (Van’t Hof et al. 2013; Ahola et al. 2014). The recent genome assembly of another pierid butterfly, _Pieris napi_, showed conserved microsynteny blocks of genes in autosomes but broken macrosynteny blocks, also suggesting dynamic genome rearrangements (Hill et al. 2019).

Based on the above findings and our results, we proposed a hypothetical scenario of the evolution of multiple sex-chromosomes in the three _Leptidea_ species studied (Fig. 8). Given the similar constitution of sex chromosomes, we infer that major genome reshuffling occurred in the common ancestor of these _Leptidea_ species. As a result, proto-WZ chromosomes in ancestral females originated by
translocation of a part of the autosome orthologous to *B. mori* chr. 17 (Fig. 8a, white parts of proto-WZ) onto the ancestral pair of WZ sex-chromosomes and two pairs of autosomes, referred to as proto-Z2 and proto-Z3, arose by fusion of several synteny blocks of the ancestral lepidopteran karyotype (Fig. 8a). At that time, ancestral males already had the current structure of Z1, Z2 and Z3 chromosomes as shown in Fig. 5d, d’ for *L. sinapis*, but proto-Z2 and proto-Z3 were still autosomes in both sexes. In the next step, proto-Z2 and proto-Z3 were translocated onto the proto-W chromosome and their unfused homologues thus turned from autosomes to Z2 and Z3 sex-chromosomes (Fig. 8b). In the resulting neo-W chromosome, the originally autosomal parts gradually degraded due to the absence of recombination in female meiosis (Fig. 8c). According to the degeneration of genes on the neo-W chromosomes demonstrated by the qPCR results (Fig. 7 and Supplementary Fig. S10), the proto-Z2 translocation onto the proto-W chromosome is most probably older than the proto-Z3 translocation. In the final step, two fissions occurred in the neo-W (Fig. 8d), resulting in a multiple sex-chromosome constitution with three W (W1–3) and three Z (Z1–3) chromosomes, as found in *L. sinapis* females (Fig. 8e). Subsequently, during the process of speciation, several further rearrangements of sex chromosomes contributed to the differentiation of closely related species. Fission of one of the W chromosomes and fission of the Z1 chromosome led to the W1–4Z1–4 constitution, as found in *L. reali*, whereas the Z3 fission resulted in the W1–3Z1–4 constitution, as found in *L. juvernica* (Fig. 8e). In this work, we showed that most homologues of Z2-linked genes decayed in the W chromosomes of all three species (Supplementary Fig. S10), while some homologues of Z3- and Z4-linked genes are still preserved (Fig. 7). However, it can be expected that degeneration of the originally autosomal parts of the W chromosomes, corresponding to Z3 and Z4 chromosomes, will proceed through the accumulation of transposable elements, duplications, insertions and/or deletions (Fig. 6d–e; Supplementary Figs S6–S9 and Supplementary Table S3). Especially qPCR results strongly suggest that two *Uch5I* and *Gst8* orthologs in all three species, and thus in their common ancestor, and *Lgr* ortholog in *L. reali* were amplified on the W chromosome(s) during the W chromosome degeneration (Fig. 7, double asterisks; cf. Bachtrog et al. 2019). Nevertheless, the finding of well-preserved homologues of genes of autosomal origin in the W chromosomes supports the proposed hypothetical scenario of evolution of multiple sex-chromosomes in *Lepidoptera* species.

In Lepidoptera, the correspondence of ‘ancestral’ Z-linked genes is highly conserved across the phylogenetic tree (Beldade et al. 2009; Yasukochi et al. 2009; Nguyen et al. 2013; Van’t Hof et al. 2013; Daliková et al. 2017a; Fraisse et al. 2017), but the gene order may be changed by intrachromosomal rearrangements (Yasukochi et al. 2009; Van’t Hof et al. 2013). A similar picture emerged in this study for *Leptidea* species. Despite many chromosomal rearrangements in their genomes, most ‘ancestral’ Z-linked genes mapped to the Z1 (and Z4 in *L. reali*) chromosome, except for a few genes (maximum 9%; see array-CGH results in Fig. 1b), but the gene order of ancestral Z-linked genes was changed compared with the Z chromosome of *B. mori* (Fig. 2). Lepidopteran Z chromosomes are known to play an important role in sex determination (Kiuchi et al. 2014), adaptation and speciation (Presgraves 2002; Dopman et al. 2005). The fact that the synteny block of the ‘ancestral’ Z-linked genes escaped the dynamic reorganisation of *Leptidea* genomes and remained conserved is
consistent with the key role of Z chromosomes in the evolution of Lepidoptera.

In most lepidopteran species, the maternally inherited W chromosome is composed of heterochromatin and, due to the absence of recombination, is characterised by extensive genetic erosion and accumulation of repetitive DNA sequences (Abe et al. 2005; Fuková et al. 2007; Marec et al. 2010; Yoshido et al. 2016; Dalfiková et al. 2017b). Evolutionary mechanisms of this genetic erosion have been demonstrated in the Y chromosomes of species with male heterogamy, especially in the *Drosophila* genus (Bachtrog 2005, 2013; Charlesworth et al. 2005). In evolutionary old and well-differentiated W chromosomes, homologous sequences of the ‘ancestral’ Z-linked genes were rarely found (Gottter et al. 1999; Van’t Hof et al. 2013). The multiple W chromosomes in three *Leptidea* species represent in fact evolutionary strata (Wright et al. 2016) of different ages and levels of differentiation that contain each a segment of autosomal origin (Fig. 8). Taking into account the estimated divergence times, which range from 1–2 million years in *L. sinapis* vs. *L. reali* to 2.5–3.5 million years in *L. juvernica* vs. the two other species (Talla et al. 2017, 2019a), these W chromosomes can be considered evolutionarily young and allow us to compare the level of their differentiation. We identified W homologues of Z1- and Z4-linked genes in all three species with some evidence of ongoing molecular differentiation, such as insertions, deletions and duplications (Fig. 6 and Supplementary Figs S6–S9). Moreover, about half of the examined Z3- and Z4-linked genes still had their homologues on the neo-W chromosomes in all three species (Fig. 7). The level of differentiation of these neo-W chromosomes thus roughly corresponds to the differentiation of the 1 million years old *Drosophila miranda* neo-Y chromosome, in which more than 40% of the genes initially present on the ancestral neo-Y chromosome became pseudogenes or was completely lost (Bachtrog 2013). However, we found no evidence of W homologues of Z1- and Z2-linked genes, suggesting advanced molecular differentiation of the relevant W chromosomes (Supplementary Fig. S10) comparable to the neo-W chromosome of *Danaus* species, which is believed to be older than 5 million years (Mongue et al. 2017). A relatively young neo-W chromosome with incomplete degeneration of the originally autosomal segment was also described in a wild silkmoth, *S. cynthia walkerii* (Yoshido et al. 2016). On the contrary, in evolutionary old neo-sex chromosomes that arose by fusion of ancestral sex-chromosomes with an autosome, it is difficult to demonstrate the neo-W chromosome due to progressive degeneration and the absence of homology (Mongue et al. 2017; Picq et al. 2018; Carabajal Paladino et al. 2019).

We reconstructed the evolution of multiple sex-chromosomes in three cryptic *Leptidea* species and identified species-specific differences in their composition. Could differences in sex-chromosome constitutions between these species have contributed to their speciation? Our results suggest that multiple sex-chromosome systems predated the formation of *Leptidea* species studied. Thus, the initial sex-chromosome turnover could not have played a role in reproductive isolation between these species. However, subsequent structural rearrangements leading to a species-specific number of multiple sex-chromosomes, namely fissions of several neo-sex chromosomes, could have significantly contributed to reproductive isolation due to mis-segregation of these neo-sex chromosomes in hybrids. In addition, different rates of neo-W chromosome degeneration (Fig. 7) could have also contributed to the divergence of *Leptidea* populations (Filatov 2018). A recent analysis of the whole-genome sequence data did not find any traces of post-divergence gene flow between these species (Talla et al. 2019a). These results suggest well-established reproductive barriers between these species, which include strong pre-mating reproductive isolation because females only accept conspecific males (Friberg et al. 2008; Dincă et al. 2013). The three *Leptidea* species showed a considerably lower level of genome-wide diversity than most other butterflies examined, indicating reduced effective population sizes (Talla et al. 2019b). They also showed a significantly reduced genetic diversity on the ‘ancestral’ Z-linked genes (corresponding to Z1 in this study, multiple Z-chromosomes were not considered) and a significantly higher level of genetic differentiation on the ‘ancestral’ Z-linked genes in comparison with the ‘ancestral’ autosomal genes (Talla et al. 2019a). Especially the latter results are consistent with the so-called ‘Faster-Z effect’, which means that the sex-linked genes are subjected to a faster rate of evolution (Mank et al. 2010). In *Leptidea* species, we showed that different sets of originally autosomal genes have become Z-linked. This greatly increased the number of Z-linked genes, which could significantly accelerate the accumulation of genetic incompatibilities between populations, thus contributing to their divergence and subsequent speciation (cf. Turelli and Begun 1997).

**Data availability**

The raw reads generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) database under the accession number SRR10381488 (Bioproject PRJNA586890). Other datasets generated in this study are available in the Dryad repository (https://doi.org/10.5061/dryad.h70rxwddw). The custom Python script used for analysis of array-CGH data is available at https://github.com/avolenikova/CGH_scripts.
Acknowledgements We are very grateful to Jaroslav Doležel for his great support of this research and for enabling the use of facilities at the Centre of Plant Structural and Functional Genomics, Institute of Experimental Botany CAS, Olomouc, Czech Republic. Our thanks go to Marie Korchová for excellent technical assistance and Radomíra Tušková for the preparation of microplates with BAC clones of the Lepidoptera juvenile library. We also wish to thank Magda Zrzavá, Zdeněk Hanč and Zdeněk Faltýnek Fric for their help in collecting adult specimens of L. juvenica and L. sinapis. Last but not least, we are grateful to the editor and three anonymous reviewers for their valuable comments on this manuscript. Access to computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum provided under the programme ‘Projects of Large Research, Development, and Innovations Infrastructures’ (CESNET LM2015042), is greatly appreciated. This research was funded by grant 14-2276SS and follow-up grant 17-13713S of the Czech Science Foundation (CSF). Publishing was supported by CSF grant 20-13784S given to FM. PN was supported by CSF grants 17-17211S and 20-20650Y. RV acknowledges support from project CGL2016-76322-P (AEI/FEDER, UE).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

References

Abe H, Mita K, Yasukochi Y, Oshiki T, Shimada T (2005) Retrotransposable elements on the W chromosome of the silkworm, Bombyx mori. Cytogeten Genome Res 110:144–151
Ahola V, Lehtonen R, Somervuo P, Salmela L, Koskinen P, Rastas P et al. (2014) The Glanville fritillary genome retains an ancient karyotype and reveals selective chromosomal fusions in Lepidoptera. Nat Commun 5:4737
Andrews S (2010) FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc
Bachtrog D (2005) Sex chromosome evolution: molecular aspects of Y chromosome degeneration in Drosophila. Genome Res 15:1393–1401
Bachtrog D (2013) Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. Nat Rev Genet 14:113–124
Bachtrog D, Mahajjan S, Bracewell R (2019) Massive gene amplification on a recently formed Drosophila Y chromosome. Nat Ecol Evol 3:1587–1597
Bachtrog D, Mank JE, Peichelm CL, Kirkpatrick M, Otto SP, Ashman T et al. (2014) Sex determination: why so many ways of doing it? PLoS Biol 12:e1001899
Baker RH, Wilkinson GS (2010) Comparative genomic hybridization (CGH) reveals a neo-X chromosome and biased gene movement in stalk-eyed flies (genus Teleopsis). PLoS Genet 6:e1001121
Beladze P, Saenko SV, Pul N, Long AD (2009) A gene-based linkage map for Bicyclus anynana butterflies allows for a comprehensive analysis of synteny with the lepidopteran reference genome. PLoS Genet 5:e1000366
Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120
Campbell P, Good JM, Dean MD, Tucker PK, Nachman MW (2012) The contribution of the Y chromosome to hybrid male sterility in house mice. Genetics 191:1271–1281
Carabajal Paladino LZ, Provazníková I, Berger M, Bass C, Aratçığe NS, López SN et al. (2019) Sex chromosome turnover in moths of the diverse superfamily Gelechioidea. Genome Biol Evol 11:1307–1319
Charlesworth D, Charlesworth B, Marais G (2005) Steps in the evolution of heteromorphic sex chromosomes. Heredity 95:118–128
Dalková M, Zrzavá M, Hladová I, Nguyen P, Šonský I, Fiegrová M et al. (2017a) New insights into the evolution of the W chromosome in Lepidoptera. J Hered 108:709–719
Dalková M, Zrzavá M, Kubíčková S, Mareč F (2017b) W-enriched satellite sequence in the Indian meal moth, Plodia interpunctella (Lepidoptera, Pyralidae). Chromosome Res 25:241–252
Dincă V, Lukhtanov VA, Talavera G, Vila R (2011) Unexpected layers of cryptic diversity in wood white Leptidea butterflies. Nat Commun 2:324
Dincă V, Wiklund C, Lukhtanov VA, Kodandaramaiah U, Nørén K, Dapporto L et al. (2013) Reproductive isolation and patterns of genetic differentiation in a cryptic butterfly species complex. J Evol Biol 26:2095–2106
Dopman EB, Perez L, Bogdanowicz SM, Harrison RG (2005) Consequences of reproductive barriers for genealogical discordance in the European corn borer. Proc Natl Acad Sci USA 102:14706–14711
Ebersberger I, Strauss S, von Haeseler A (2009) HaMStR: profile hidden Markov model based search for orthologs in ESTs. BMC Evol Biol 9:157
Filatov DA (2018) The two “rules of speciation” in species with young sex chromosomes. Mol Ecol 27:3799–3810
Fraise C, Picard MAL, Vicoso B (2017) The deep conservation of the Lepidoptera Z chromosome suggests a non-canonical origin of the W. Nat Commun 8:1486
Friberg M, Vongvanich N, Borg-Karlsson A-K, Kemp DJ, Merilaita S, Wicklund C (2008) Female mate choice determines reproductive isolation between sympatric butterflies. Behav Ecol Sociobiol 62:873–886
Fuková I, Traut W, Vítková M, Nguyen P, Kubíčková S, Mareč F (2007) Probing the W chromosome of the coding moth, Cydia pomonella, with sequences from microdissected sex chromatin. Chromosoma 116:135–145
Gilbert D (2013) Gene-omes built from mRNA seq not genome DNA. 7th Annual Arthropod Genomics Symposium. Notre Dame. F1000Research 5:1695. https://doi.org/10.7490/f1000research.1112594.1
Gotter AL, Levine JD, Reppert SM (1999) Sex-linked period genes in the silkmoth, Antheraea pernyi: implications for circadian clock regulation and the evolution of sex chromosomes. Neuron 24:953–965
Graves JAM (2016) Did sex chromosome turnover promote divergence of the major mammal groups? Bioessays 38:734–743

Springer Nature
Haas BJ, Papanicolaou A, Yassou M, Grabherr M, Blood PD, Bowden J et al. (2013) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc 8:1494–1512

Hill J, Rastas P, Hornet EA, Neethiraj R, Clark N, Morehouse N et al. (2019) Unprecedented reorganization of holocentric chromosomes provides insights into the enigma of lepidopteran chromosome evolution. Sci Adv 5:eaau3648

Kitano J, Peichel CL (2012) Turnover of sex chromosomes and speciation in fishes. Environ Biol Fishes 94:549–558

Kitano J, Ross JA, Mori S, Kume M, Jones FC, Chan YF et al. (2009) A role for a neo-sex chromosome in stickleback speciation. Nature 461:1079–1083

Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H, Arai Y et al. (2012) Neo-sex chromosomes in Spodoptera frugiperda. BMC Evol Biol 12:30

Kost S, Heckel DG, Yoshido A, Marec F, Groot AT (2016) A Z-linked sterility locus causes sexual abstinence in hybrid females and facilitates speciation in Spodoptera frugiperda. Evolution 70:1418–1427

Lukhtanov VA, Dincá V, Friberg M, Šichová J, Olofsson M, Vila R et al. (2018) Versatility of multivalent orientation, inverted meiosis, and rescued fitness in holocentric chromosomal hybrids. Proc Natl Acad Sci USA 115:9610–9619

Lukhtanov VA, Dincá V, Talavera G, Vila R (2011) Unprecedented within-species chromosome numbercline in the Wood White butterfly Leptidea sinapis and its significance for karyotype evolution and speciation. BMC Evol Biol 11:109

Mank JE, Hosken DJ, Wedell N (2014) Conflict on the sex chromosomes: cause, effect, and complexity. Cold Spring Harb Perspect Biol 6:a017715

Mank JE, Vicoso B, Berlin S, Charlesworth B (2010) Effective population size and the faster-X effect: empirical results and their interpretation. Evolution 64:663–674

Marcé F, Sahara K, Traut W (2010) Rise and fall of the W chromosome in Lepidoptera. In: Goldsmith MR, Marec F (eds) Molecular biology and genetics of the Lepidoptera. CRC Press, Boca Raton, FL, USA, p 49–63

Moghadam HK, Pointer MA, Wright AE, Berlin S, Mank JE (2012) W chromosome expression responds to female-specific selection. Proc Natl Acad Sci USA 109:8207–8211

Mongue AJ, Nguyen P, Voleníková A, Walters JR (2017) Neo-sex chromosomes: evidence from mapping in Heliconius melpomene. Genetics 177:417–426

Qvarnström A, Bailey RI (2009) Speciation through evolution of sex-linked genes. Heredity 102:4–15

Rens W, Grützner F, O’Brien PCM, Fairclough H, Graves JAM, Ferguson-Smith MA (2004) Resolution and evolution of the duck-billed platypus karyotype with an X1Y1X2Y2X3Y3 male sex chromosome constitution. Proc Natl Acad Sci USA 101:16257–16261

Šafar J, Simková H, Kubaláková M, Čiháliková J, Suchánková P, Bartoš J et al. (2010) Development of chromosome-specific BAC resources for genomics of bread wheat. CytoGenet Genome Res 129:211–223

Sahara K, Yoshihito A, Traut W (2012) Sex chromosome evolution in moths and butterflies. Chromosome Res 20:83–94

Šichová J, Ohno M, Dincá V, Watanabe M, Sahara K, Marec F (2016) Fusions, fissions, and translocations shaped the karyotype and multiple sex chromosome constitution of the northeast-Asian wood white butterfly, Leptidea amurensis. Biol J Linn Soc 118:457–471

Šichová J, Voleníková A, Dincá V, Nguyen P, Vila R, Sahara K et al. (2015) Dynamic karyotype evolution and unique sex determination systems in Leptidea wood white butterflies. BMC Evol Biol 15:89

Smith DAS, Gordon JJ, Traut W, Herren J, Collins S, Martins DJ et al. (2016) A neo-W chromosome in a tropical butterfly links colour pattern, male-killing, and speciation. Proc Biol Sci 283:20160821

Sperling FAH (1994) Sex-linked genes and species differences in Lepidoptera. Can Entomol 126:807–818

Sweigart AL (2010) Simple Y-autosomal incompatibilities cause hybrid male sterility in reciprocal cross between Drosophila virilis and D. americana. Genetics 184:779–787

Talla V, Johansson A, Dincá V, Vila R, Friberg M, Wklund C et al. (2019a) Lack of gene flow: narrow and dispersed differentiation islands in a triplet of Leptidea butterfly species. Mol Ecol 28:3756–3770

Talla V, Soler L, Kawakami T, Dincá V, Vila R, Friberg M et al. (2019b) Dissecting the effects of selection and mutation on genetic diversity in three wood white (Leptidea) butterfly species. Genome Biol Evol 11:2875–2886

Talla V, Suß A, Kalsoom F, Dincá V, Vila R, Friberg M et al. (2017) Rapid increase in genome size as a consequence of transposable element hyperactivity in wood-white (Leptidea) butterflies. Genome Biol Evol 9:2491–2505

Traut W, Ahola V, Smith DAS, Gordon JJ, ffrench-Constant RH (2017) Karyotypes versus genomes: the nymphaoid butterflies Melitaea cinxia, Danaus plexippus, and D. chrysippus. CytoGenet Genome Res 153:46–53

Traut W, Sahara K, Marec F (2007) Sex chromosomes and sex determination in Lepidoptera. Sex Dev 1:332–346

Turelli M, Begun DJ (1997) Haldane’s rule and X-chromosome size in Drosophila. Genetics 147:1799–1815

Van’t Hof AE, Nguyen P, Dalkivk P, Edmonds N, Marec F, Saccheri IJ (2013) Linkage map of the peppered moth, Biston betularia (Lepidoptera, Geometridae): a model of industrial melanism. Heredity 110:283–295

Wadsworth CB, Li X, Dopman EB (2015) A recombination suppressor contributes to ecological speciation in OSTRINIA moths. Heredity 114:593–600

Wright AE, Dean R, Zimmer F, Mank JE (2016) How to make a sex chromosome. Nat Commun 7:12087

Yasukochi Y (2002) PCR-based screening for bacterial artificial chromosome libraries. Methods Mol Biol 192:401–410

Yasukochi Y, Ohno M, Shibata F, Jouraku A, Nakano R, Ishikawa Y et al. (2016) A FISH-based chromosome map for the European corn borer yields insights into ancient chromosomal fusions in the silkworm. Heredity 116:75–83
Yasukochi Y, Tanaka-Okuyama M, Shibata F, Yoshido A, Marec F, Wu C et al. (2009) Extensive conserved synteny of genes between the karyotypes of Manduca sexta and Bombyx mori revealed by BAC-FISH mapping. PLoS One 4:e7465

Yoshido A, Marec F, Sahara K (2016) The fate of W chromosomes in hybrids between wild silkmoths, Samia cynthia ssp.: no role in sex determination and reproduction. Heredity 116:424–433

Yoshido A, Sahara K, Marec F, Matsuda Y (2011a) Step-by-step evolution of neo-sex chromosomes in geographical populations of wild silkmoths, Samia cynthia ssp. Heredity 106:614–624

Yoshido A, Sahara K, Yasukochi Y (2015) Silk moths (Lepidoptera). In: Sharakhov IV (ed) Protocols for cytogenetic mapping of arthropod genomes. CRC Press, Boca Raton, FL, USA, p 219–256

Yoshido A, Yasukochi Y, Sahara K (2011b) Samia cynthia versus Bombyx mori: comparative gene mapping between a species with a low-number karyotype and the model species of Lepidoptera. Insect Biochem Mol Biol 41:370–377

Xie Y, Wu G, Tang J, Luo R, Patterson J, Liu S et al. (2014) SOAPdenovo-Trans: de novo transcriptome assembly with short RNA-Seq reads. Bioinformatics 30:1660–1666