University of South Bohemia in České Budějovice

Faculty of Science

Reconstruction of the evolution of multiple sex chromosomes in *Leptidea* wood white butterflies

Master thesis

Bc. Kristýna Pospíšilová

Supervisor: **prof. RNDr. František Marec, CSc.**

České Budějovice 2020

Master thesis

Pospíšilová K (2020) Reconstruction of the evolution of multiple sex chromosomes in *Leptidea* wood white butterflies. Master thesis, in English. Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Pp. 50.

Annotation

Having a crucial role in many evolutionary processes, such as sex determination, speciation and adaptation, sex chromosomes tend to be highly conserved. Rapidly evolving sex chromosome systems offer a special opportunity to study the evolution of the sex chromosomes in miraculous resolution. Butterflies of genus *Leptidea* possess a unique species-specific sex chromosome system with 3–4 W and 3–4 Z chromosomes. Using novel genomic tools established for *L. juvernica*, namely transcriptome-based microarray for comparative genomic hybridization (array-CGH) and a library of bacterial artificial chromosome (BAC) clones, we assembled the physical maps of Z chromosomes in three cryptic *Leptidea* species (*L. juvernica*, *L. sinapis*, and *L. reali*) by fluorescence *in situ* hybridization (FISH) of BAC clones containing orthologs of *Bombyx mori* genes. In all three species, we identified the ancestral Z chromosome and synteny segments of autosomal origin and reconstructed the step-by-step evolution of multiple sex chromosomes. We propose that the multiple sex chromosome system originated in the common ancestor of *Leptidea* species by means of multiple chromosomal rearrangements, especially translocations, fusions and fissions, between the sex chromosomes and autosomes. Thus, the turnover of neo-sex chromosomes could not be the main engine driving speciation in this genus. Instead, we propose that subsequent differentiation of the sex chromosome multiples in each species together with enlarged number of Z-linked genes could play a crucial role in accumulation of genetic incompatibilities facilitating subsequent divergence and speciation in the *Leptidea* species studied.

Financial support

This work was funded by the Grant Agency of the Czech Republic, grant 17-13713S.

Declaration

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my master thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with afore mentioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 29.4.2020

.....

Kristýna Pospíšilová

Acknowledgements

I would like to give my biggest thanks to the head of our laboratory and my supervisor, František Marec, for giving me the opportunity to participate on the research and become a member of the Beaver Lab Crew. I appreciate his infinite kindness, patience, open-minded attitude, willingness to help whenever needed and his great sense of humor. I also wish to thank to Atsuo Yoshido for his help with the practical part of the research and for all valuable advice he gave. It was a pleasure to cooperate. I also thank to Petr Nguyen and Anička Voleníková for performing the microarray and analyzing the results of array-CGH. Last but not least, I thank to Jindra Tušerová (née Šíchová) for guiding my first steps in the lab when teaching me all the essentials, and all the other people I work with for supporting me and creating a friendly and relaxed atmosphere in our lab.

Special thanks (in Czech)

Největší poděkování patří mé skvělé rodině, mým úžasným rodičům za podporu ve všem, o co se pokouším, za ochotu mě vždy vyslechnout a pomoci v každé situaci, a za inspiraci a motivaci při dosahování mých i našich společných cílů. Děkuji i mé sestře Tereze, se kterou tvoříme dokonalý tým, ať už pochodujeme po ledovci, lezeme po stěně nebo regenerujeme v ledové vodě. Bez Vás tří by život nebyl zdaleka tak veselý, radostný a barevný, jako je teď. Velké poděkování patří i všem mým přátelům, zejména Láďovi Landkamerovi, kteří se pokusili resuscitovat můj notebook, jenž zkolaboval při psaní magisterské práce a vzal si s sebou i všechna data. V neposlední řadě děkuji i mé druhé rodině z lezecké stěny, ze které stále padáme a společně hledáme nové cesty vzhůru. Tak, jako je tomu i v životě.

Contents

1. General introduction

1.1. Lepidoptera

Moths and butterflies (Lepidoptera) are the second largest order of insects only to the Coleoptera (beetles), with about 160 000 species worldwide (Nieukerken *et al.* 2011). Their massive phylogenetic diversity is associated with the diversification of flowering plants along with the diversification of their predators and parasitoids (Grimaldi and Engel 2005). This co-evolution led to many morphological, physiological and behavioral innovations among interacting organisms and made Lepidoptera an important subject for investigation in the history of life. Ecologically, Lepidoptera plays various and important roles in terrestrial ecosystems. They are pollinators as they feed on nectar hidden inside flowers, they serve as a primary source of food for insectivores, and they also act as hosts for numerous insect parasitoids (Goldstein 2017). From the economic standpoint, many species are prosperous, such as the silkworm (*Bombyx mori*), whose larvae make their cocoons out of silk (Resh and Cardé 2003), whilst others can be destructive agricultural pests. Moths and butterflies have also figured in religion and spirits of many cultures as their appearance symbolize the beauty and fragility of human soul (Hearn 1904). Thanks to immense diversity, richness and strong impact on human living, Lepidoptera have become an intensively studied taxa in various fields of research, e.g. ecology, phylogeny, evolution, physiology, molecular biology, genetics, and genomics.

1.1.1. Lepidopteran karyotype

Until recently, the genetics of moths and butterflies remained blurred mostly due to the character of their chromosomes. Lepidopteran chromosomes are very tiny, usually much smaller than chromosomes of flies and mammals, uniform in shape and deficient in many morphological traits allowing their identification. Besides, chromosomes are holokinetic without primary constriction, the centromere (Carpenter *et al.* 2005). Given these unusual characteristics, genetic studies were for long limited only to chromosome counting which did not provide very deep insights into the lepidopteran cytogenetics (Mediouni *et al.* 2004, Fuková *et al.* 2005).

Chromosome number in most lepidopteran karyotypes tend to be stable among species and ranges between n=29–31 (Robinson 1971). This points to the chromosomal conservatism, a state in which all closely related taxa share identical number of chromosomes (Lukhtanov 2014). The modal chromosome number of n=31 is widespread across the lepidopteran phylogenetic tree and based on karyotype studies (Suomalainen 1969, Lukhtanov 2000), the ancestral chromosome number of chromosomes in haploid genome has been established on n=31 as well. This assumption was also supported by comparative genome mapping of distinct species (Baxter *et al.* 2011, Sahara *et al.* 2013, Van't Hof *et al.* 2013, Ahola *et al.* 2014) suggesting extraordinary karyotype stability, well conserved

synteny of genes, and similar gene order on chromosome level. Using fluorescence *in situ* hybridization of bacterial artificial chromosomes (BAC-FISH), or molecular linkage analysis of genes, the conserved synteny was described between *Bombyx mori*, the model organism in Lepidoptera, and other representative of Bombycoidea, *Manduca sexta* (Sahara *et al.* 2007, Yasukochi *et al*. 2009), then two species of Papilionoidea, *Heliconius melpomene* (Yasukochi *et al.* 2006, Pringle *et al.* 2007) and *Bicyclus anynana* (Beldade *et al.* 2009), as well as three representatives of Noctuidea, *Helicoverpa armigera*, *Mamestra brassicae*, and *Spodoptera frugiperda* (d'Alençon *et al.* 2010, Sahara *et al.* 2013), further on the representative with a low-number karyotype of Saturniidae, *Samia cynthia* ssp. (Yoshido *et al.* 2011b), and also in the diamondback moth *Plutella xylostella* (Yponomeutoidea; Baxter *et al.* 2011).

On the other hand, several studies reported a substantial variation in chromosome number between n=5 to n=226 indicating chromosomal instability of lepidopteran karyotypes (Brown *et al.* 2004, Lukhtanov 2015). Moreover, the amount of DNA remains almost consistent among different species, implying that species with lower chromosome numbers have longer chromosomes. For this reason, lepidopteran karyotypes are believed to have evolved by means of complex chromosomal rearrangements, especially fusions and fissions (White 1973). Such reshuffling events may be facilitated by holocentric nature of chromosomes possessing extended kinetochore activity along their length. Moreover, they are considered less harmful than in organisms with monocentric chromosomes (Marec *et al.* 2010). Besides, genome rearrangements are associated with formation of reproductive barriers between species and thus with speciation and radiation (Yoshido *et al*. 2011a, Nguyen *et al.* 2013). As both phenomena – chromosome conservatism and chromosome instability – are combined in moths and butterflies, this group of organisms offers a unique opportunity for studying the process of chromosome evolution.

1.1.2. Sex chromosomes in Lepidoptera

Moths and butterflies have sex chromosome system, in which females are heterogametic with WZ chromosomes, whereas males are homogametic with a pair of ZZ chromosomes. The basal lineages of Lepidoptera together with their sister order Trichoptera lack the W chromosome, suggesting that the common ancestor of these sister clades had the sex chromosome constitution of Z0/ZZ (female/male) and the W chromosome was acquired secondarily (reviewed by Traut *et al.* 2007, Marec *et al.* 2010, Sahara *et al.* 2012). The W chromosome, if present, is usually smaller or of a similar size as the Z chromosome, but can be easily differentiated from its partner through its heterochromatic structure as it consists mostly of repetitive DNA sequences such as transposable elements (e.g. Sahara *et al.* 2003, Abe *et al.* 2005, Traut *et al.* 2013) and only a few genes (Gotter *et al*. 1999, Van't Hof *et al*. 2013). Until recently, only two genes having homologs on the opposite sex chromosomes have been described, namely *laminin A* on the W chromosome of *Biston betularia* (Van't Hof *et al.* 2013), and

period gene having two variants on the W chromosome of *Antheraea pernyi*. However, one of the variants of *period* produces only a truncated protein, and the other antisense RNA transcript (Gotter *et al.* 1999). The first protein-coding gene found exclusively on the lepidopteran W chromosome was only recently described in *Helicoverpa armigera* by Deng *et al.* (in press). In female polyploid somatic interphase nuclei, multiple copies of the W chromosome form a spherical heterochromatin body (sex chromatin or W chromatin) providing an indirect proof for the W chromosome presence in the karyotype (Traut and Marec 1996). Two hypotheses have been proposed on the origin of the W chromosome. The first model suggests the evolution of the W chromosome via Z chromosome-autosome fusion, generating one lone and unpaired autosome, which became female restricted and started to degenerate due to achiasmatic meiosis and absence of recombination in females. Consequently, the loss of genes and invasion of repetitive sequences such as transposons gave rise to the W chromosome (Traut and Marec 1997). An alternative hypothesis of Lukhtanov (2000) proposes the secondary acquisition of the W chromosome via recruitment of a supernumerary chromosome (so-called B chromosome), which carried female sex-determining genes and performed female-specific functions. This hypothesis has been also supported by comparative genomics analysis (Fraïsse *et al.* 2017) and sex chromosome analysis (Dalíková *et al.* 2017a) in representatives of non-ditrisian and basal ditrisian lineages. Moreover, the latter study indicated two independent origins of the W chromosome in family Tischeriidae and in advanced Ditrysia, which was later supported by the study of Hejníčková *et al.* (2019). Although these new studies provided compelling evidence for the origin of the W chromosome from a B chromosome, we cannot exclude the general hypothesis suggesting de novo origin of the sex chromosomes from a pair of autosomes (Wright *et al.* 2016). This theory suggests that one of the autosomes (the proto-W chromosome) acquired a female sex determining gene, molecularly degenerated due to restricted recombination and became the female-limited W chromosome.

In contrast to the W chromosome, the Z chromosome is usually similar to autosomes, contains vast number of transcriptionally active genes and undergoes the process of recombination in males. Until the development of modern cytogenetic techniques, such as the use of pachytene chromosomes for research and fluorescence *in situ* hybridization (FISH), only little was known about lepidopteran Z chromosomes (Traut *et al.* 2007). The first breakthrough which shed light on the Z chromosome and also on the autosomes was the sequenced genome of silkworm *Bombyx mori* (Mita *et al.* 2004, Xia *et al.* 2004) and the identification of all 28 pachytene bivalents in its karyotype by two-color BAC-FISH method (Yoshido *et al.* 2005). Since that time, extensive comparative analysis of autosomal and Z-linked genes have been conducted on phylogenetically distant lepidopteran species (Yasukochi *et al.* 2006, 2009, Sahara *et al.* 2013, Nguyen *et al.* 2013, Van't Hof *et al.* 2013) with the aim to elucidate the

evolution of lepidopteran karyotype as well as to apply the acquired knowledge in pest control technologies.

As was mentioned above, the majority of moths and butterflies have a WZ/ZZ sex chromosome constitution. However, the variations of this standard system occur including the secondary loss of the W chromosome and multiple sex chromosome systems with either W_1W_2Z/ZZ or $WZ_1Z_2/Z_1Z_1Z_2Z_2$ chromosomes (Traut *et al.* 2007). Moreover, an unusual system of sex determination with 3–6 Z chromosomes and 3–4 W chromosomes was discovered in four wood white butterflies of the genus *Leptidea.* (Šíchová *et al.* 2015, 2016), for which they have become an interesting model system for the study of evolution of multiple sex chromosomes and their possible role in speciation.

1.2. *Leptidea* wood white butterflies

1.2.1. History, distribution and ecology

Butterflies of the genus *Leptidea* Billberg, 1820 (Pieridae) include several, at least nine, Palearctic species. Despite more than two decades of extensive research, the entire taxonomic diversity of *Leptidea* butterflies was only recently brought to light. At the end of the 20th century, a common wood white butterfly *L. sinapis* (Fig. 1, left) with Western Palearctic distribution, was found out to hide a cryptic entity, *L. reali* (Réal 1988; Fig. 1, middle). These two sibling species are inseparable based on their wing pattern but can be reliably distinguished by genitalia morphology (Lorković 1993). The existence of sibling species *L. sinapis* – *L. reali* was also proved by molecular analysis based on mitochondrial DNA and allozyme markers (Martin *et al.* 2003). These findings triggered even more detailed investigation of the species pair, and as a consequence, Dincă *et al.* (2011) discovered another cryptic species in this genus. They used morphological data, chromosome counts and nuclear markers to study the species pair *L. sinapis* – *L. reali* and found out that *L. reali* also hides a cryptic species, now referred to as *L. juvernica* stat. nov. (Dincă *et al.* 2011; Fig. 1, right). Genitalia measurements of all three species reliably differentiated *L. sinapis* from the other two species, but measurements of *L. juvernica* and *L. reali* were overlapping. This explains why *L. juvernica* remained unseen for such a long time. So what was originally considered as one species is now a triplet of closely related and cryptic species, *L. juvernica*, *L. sinapis* and *L. reali*. This breakthrough immensely increased the popularity of *Leptidea* butterflies and enhanced the surveys on their ecological interactions and behavior dependent on environmental conditions, both important for conservational efforts (O'Neill and Montgomery 2018). More importantly, wood whites have become the promising model system for studying the evolution of cryptic species through speciation (Dincă *et al.* 2013, Friberg *et al.* 2013).

Figure 1. Cryptic *Leptidea* wood white butterflies. *L. sinapis* (left)[1], *L. reali* (middle)[2], and *L. juvernica* (right)[3].

After the discovery of cryptic triplet, a new research was needed to clarify the distribution of individual species. The species with the widest distribution, *L. sinapis*, occurs from western Spain and Ireland to eastern Kazakhstan (Dincă *et al.* 2011, 2013), and further east reaching Lake Baikal in Russia (Sinev 2008). *L. juvernica* and *L. reali* have mostly allopatric distributions, but both are known to be sympatric with *L. sinapis* in parts of their ranges (Dincă *et al.* 2013). *L. reali* is restricted to southwestern Europe (Spain, southern France, and Italy), whereas *L. juvernica* is widespread from Ireland and France to eastern Kazakhstan (Dincă *et al.* 2011, 2013), mountain massif Tian Shan in Kyrgyzstan, northwestern China (Bolshakov 2006) and the Republic of Tuva in Russia (Sinev 2008). In France, populations of *L. juvernica* and *L. reali* are parapatric and separated only by 87 kilometers. However, no introgression between these species was documented in this parapatry area or elsewhere (Dincă *et al*. 2011, 2013, see below).

As many European butterflies suffered significant population declines (Thomas 1995, Bickford *et al.* 2007), recent studies also focus on the distinctions in ecology of cryptic species, which may play an important role in conservational efforts (Clarke *et al.* 2011, O'Neill and Montgomery 2018). Although *L. sinapis* with *L. juvernica* and *L. sinapis* with *L. reali* occur sympatrically in Europe, each species exhibits different habitat preferences and niche specialization depending on its geographical location. In the Czech Republic, *L. sinapis* occurs in xerotermic areas, whilst *L. juvernica* is a generalist living in most habitat types (Beneš *et al.* 2003). Likewise, in Poland, *L. sinapis* has more limited distribution comprising of xerotermic habitats and woodlands, whereas *L. juvernica* is a generalist inhabiting broad range of habitats (Sachanowicz *et al.* 2011). On the contrary, in Sweden, *L. juvernica* occurs only sporadically in open meadows, while *L. sinapis* is known as generalist inhabiting also forests (Friberg *et al.* 2008b). Similarly, in the Balkan Peninsula, populations of *L. sinapis* do not exhibit any habitat selectivity, whereas of *L. juvernica* vary from habitat generalists in the west to habitat specialists in the east, inhabiting humid mountain habitats (Shtinkov 2016). In the British Isles, habitat

1. Mgr. František Šaržík; https://www.biolib.cz/cz/image/id266452/
2. Zdeněk Chalupa; https://www.biolib.cz/cz/image/id118647/
3. David Černoch; http://davidcernoch.hostuju.cz/album_motyli/Pieridae%20-%20belaskoviti/index.html

preferences of *L. sinapis* and *L. juvernica* are clearly differentiated. In Britain, populations of *L. sinapis* are confined to plantation woodlands (Clarke *et al.* 2011), including open woodland paths and ridges (Warren *et al.* 1986, Thomas 2010). In Ireland, *L. sinapis* is also bound to woods and protected scrubby areas with limestone bedrock (Nash *et al.* 2012). As regards *L. juvernica*, which has so far been found only in Ireland (O'Neill and Montgomery 2018), its populations usually prefer open areas like flowering grasslands, marsh edges, deserted stone quarries and also sand dunes (Thompson and Nelson 2006). As for the species pair *L. sinapis* – *L. reali* cohabiting in Western Mediterranean, *L. sinapis* is a habitat generalist, whereas *L. reali* is considered a habitat specialist confined to dryer meadows (Stefanescu *et al.* 2010, Friberg *et al.* 2013). However, additional research is needed to bring more detailed data. Despite the differences in habitat preferences, all species are oligophagous, feeding on a limited range of larval host-plants of the family Fabaceae, including the meadow vetchling (*Lathyrus pratensis*), the big trefoil (*Lotus pedunculatus*), and the common bird's-foot trefoil (*Lotus corniculatus*) (Warnock 2008, Friberg and Wiklund 2009, Clarke *et al.* 2011).

Sympatric distribution of *Leptidea* cryptic species also raised speculations about their genetic isolation and possible introgression between these species. According to Mallet (2005), the introgression is quite common trend among sympatrically living and closely related butterfly species as approximately 16% of 440 European butterfly species are capable of hybridization with at least one other species in natural conditions. Despite decreased fertility or complete sterility of interspecific hybrids, such hybridization can lead to gene flow in hybrid zones (Mavárez *et al.* 2006, Descimon and Mallet 2009). As for the *Leptidea* species, interspecific hybrids or product of their backcrosses have been reported between *L. juvernica* and *L. sinapis* in Slovenia (Verovnik and Glogovčan 2007) and Novosibirsk province (Kosterin *et al.* 2007, Ivonin *et al.* 2009). However, the results of Dincă *et al.* (2013) suggest that between-species hybridization in *Leptidea* butterflies is very uncommon event, as none of 66 heterospecific courtships between Swedish *L. juvernica*, Spanish *L. sinapis* and Spanish *L. reali* ended in mating. Moreover, biochemical and behavioural prezygotic barriers maintained by female acceptance of only conspecific males have been demonstrated (Friberg *et al.* 2008a, Dincă *et al.* 2013). Therefore, to determine the roots and evolution of *Leptidea* butterflies, scientists started digging in their genetics and cytogenetics.

1.2.2. *Leptidea* karyotype

As was mentioned before, most of the lepidopteran species have 31 chromosomes in haploid genome (Suomalainen 1969, Robinson 1971, De Prins and Saitoh 2003). Nevertheless, butterflies of the genus *Leptidea* show evidence of extraordinary inter- and intraspecific variability in chromosome counts with a tendency to increase during speciation (Dincă *et al.* 2011, Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016). The highest number of chromosomes was found in the Eastern wood white, *L.*

duponcheli, with the chromosome number between n=102 to n=104 in haploid genome (Lorković 1941, De Lesse 1960). Large numbers of chromosomes were also found in two species with mostly Eastern Palearctic dispersal, *L. morsei* with n=54 chromosomes (Maeki 1958) and *L. amurensis* with different chromosome numbers in males (n=61) and females (2n=118–119; Šíchová *et al.* 2016). Similarly, variability in chromosome counts between and even within species was also reported in a triplet of cryptic species with Western Palearctic occurrence. In *L. juvernica,* chromosome number ranges between 2n=85 to 2n=91, whereas karyotype of *L. reali* seems to be more stable, with 2n=51–55 chromosomes (Šíchová *et al.* 2015). In contrast, an odd variability in chromosome numbers was described in *L. sinapis*, where Lukhtanov *et al*. (2011) discovered a chromosomal cline with tendency to increase number of chromosomes from n=28 in Kazakhstan to n=53 in Spain. Such intraspecific chromosome number variability offers an exceptional opportunity to study mechanisms underlying the clinal speciation.

Two possible hypotheses may explain the chromosome number variation between closely related species. The first theory is dealing with the story of B chromosomes, or so-called supernumerary chromosomes, additional chromosomes, or selfish chromosomes (Bigger 1976, Lukhtanov 1992, Camacho *et al.* 2000). Most of B chromosomes are mainly or entirely heterochromatic and may be present only in several individuals from particular population. During meiosis, they can be found as univalents, bivalents or multivalents, but never paired with normal chromosomes, so-called A chromosomes (Jones *et al.* 2008). Although not essential for survival of species, B chromosomes are inhabitants of nucleus, where they may interact with A chromosomes (Jones 2012). Moreover, B chromosomes can act as selfish elements since they accumulate in numbers by various processes of mitotic or meiotic drive (Jones *et al.* 2008). Generally, we cannot reject that B chromosomes take part in chromosome number variability in *Leptidea* species as they were detected in the Idaho population of checkerspot butterfly, *Euphydryas colon* (Nymphalidae; Pearse and Ehrlich 1979), as well as in *Pieris napi* and *P. rapae* (Bigger 1976), both representatives of the same family Pieridae like *Leptidea* species. However, this scenario is rather doubtful, since no B chromosomes were detected in either the Spanish *L. sinapis* population with the highest chromosome counts or in any of the populations studied (Lukhtanov *et al.* 2011). The alternative hypothesis proposes that intraspecific chromosome number variation arose from complex chromosomal rearrangements, like fusions and fissions (Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016, Lukhtanov *et al.* 2018). This hypothesis was supported by described pattern in karyotypes of so far *Leptidea* species studied, i.e. the smaller the size of the chromosomes, the higher the number of chromosomes in a population (Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016).

Both interspecific and intraspecific variability in karyotypes of *Leptidea* butterflies were also proven by location of clusters of ribosomal DNA (rDNA) and H3 histone genes (Šíchová *et al.* 2015,

2016). In two species, *L. amurensis* and *L. reali*, the location and number of both cytogenetic markers were conserved within species. In *L. juvernica*, the number and position of rDNA clusters were consistent, however, number and position of H3 histone genes varied even among the offspring of individual females. The highest variability in location and number of both markers between and within the progeny of individual females was observed in *L. sinapis* (Šíchová *et al.* 2015, 2016). Variability in distribution of rDNA clusters is in line with previous evolutionary studies in other lepidopteran species (Nguyen *et al*. 2010). On the contrary, instability in number and position of H3 histone gene clusters is rather surprising, as they are largely conserved in the leafroller moths of the family Totricidae (Šíchová *et al.* 2013), and among other groups of insects, e.g. grasshoppers (Cabrero *et al.* 2009), spittlebugs, leafhoppers, and treehoppers (Anjos *et al.* 2018), and beetles (Cabral-de-Mello 2011). Taken together, these results point at dynamic genome rearrangements as the main forces shaping karyotype of *Leptidea* butterflies (Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016).

1.2.3. *Leptidea* multiple sex chromosomes

The inter- and intraspecific variability in number of chromosomes as well as in number and position of cytogenetic markers in *Leptidea* karyotypes contrasts with the remarkable stability of their multiple sex chromosome systems (Šíchová *et al.* 2015, 2016). Previous studies of so far examined *Leptidea* species showed that each species have a unique sex chromosome constitution with ♀$W_{1–3}Z_{1–4}$/♂$Z_{1–4}Z_{1–4}$ in *L. juvernica*, ♀$W_{1–3}Z_{1–3}$/♂$Z_{1–3}Z_{1–3}$ in *L. sinapis*, ♀$W_{1–4}Z_{1–4}$/♂$Z_{1–4}Z_{1–4}$ in *L. reali*, and ♀$W_{1–3}Z_{1–6}$/♂$Z_{1–6}Z_{1–6}$ in *L. amurensis* (Šíchová *et al.* 2015, 2016). Sex chromosome trivalents or quadrivalents were found in several vertebrate groups, for example in fishes (Kitano and Peichel 2012, De Oliveira *et al.* 2018), amphibians (Schartl 2015), reptiles (Pokorná *et al.* 2014, Rovatsos *et al.* 2019), birds (Gunski *et al.* 2017) and mammals (Gruetzner *et al.* 2006). Sex chromosome multiples with three or four elements were also found in numerous invertebrate groups, like for example in mollusks (Vitturi *et al.* 1993), or spiders (Maddison 1982, Král *et al.* 2019), and many insect groups, e.g. fleas (Siphonaptera; Thomas 1991), true bugs (Hemiptera; Bardella *et al.* 2012), grasshoppers (Orthoptera; Palacios-Gimenez *et al.* 2013), and others (see Blackmon *et al.* 2017). Nevertheless, sex chromosome multivalents comprising more than four chromosomes are considered quite unique among vertebrates as they have been so far found only in monotreme mammals such as duck-billed platypus (*Ornithorhynchus anatinus*) with the ♀$X_{1–5}X_{1–5}$/♂$X_{1–5}X_{1–5}$ sex chromosome system or in echidna with the ♀$X_{1–5}$/♂$X_{1–5}Y_{1–4}$ constitution. In both organisms, the origin of multiple sex chromosomes is ascribed to chromosomal rearrangements between sex chromosomes and autosomes (Rens *et al.* 2004, 2007, Grützner *et al.* 2004). In invertebrates, the most complicated sex chromosome system was found in the termite *Kalotermes approximates* with 19 chromosomes creating sex-linked rings or chains in meiosis (Syren and Luykx 1981). As for the order Lepidoptera, the overview comprising 40 lepidopteran

species with identified sex chromosomes reported 12 species with multiple sex chromosome constitution with either W_1W_2Z/ZZ or $WZ_1Z_2/Z_1Z_1Z_2Z_2$ chromosomes (Traut *et al.* 2007). Yet, such complicated sex chromosome systems with 3–4 W and 3–6 Z chromosomes in the genus *Leptidea* have not been found in any other lepidopteran taxa and have made *Leptidea* butterflies a promising model system for studying the sex chromosome evolution.

The evolution of multiple sex chromosomes in *Leptidea* wood whites is assigned to chromosomal rearrangements between sex chromosomes and autosomes, specifically fusions of ancestral WZ pair with several autosomes (Šíchová *et al.* 2015, 2016). In general, chromosomal rearrangements often lead to reduced fitness of individuals due to unbalanced segregation of multivalents during meiotic division (Baker and Bickham 1986). This particularly relates to monocentric chromosomes undergoing canonical meiosis with segregation of homologous chromosomes during meiosis I followed by sister chromatid segregation in meiosis II. However, the extended kinetochore activity of holocentric chromosomes and their different orientation in metaphase I enables to invert the order of meiotic events and substitute the critical phase of reductional segregation of homologous chromosomes by less risky equational segregation of sister chromatids (Lenormand *et al.* 2016). According to Lukhtanov *et al.* (2018), the inverted meiosis is likely to prevent the unbalanced chromosome segregation and thus reduce the harmful effects of chromosomal rearrangements. These findings explain observed high reproductive fitness of *L. sinapis* intraspecific hybrids despite chromosome number variability in their karyotypes. Moreover, complex chromosomal rearrangements may facilitate the dynamic karyotype evolution associated with ecological specialization, reproductive isolation, and speciation (Lukhtanov *et al.* 2018). This idea was supported by number of studies among lepidopteran species, e.g. the African queen butterfly, *Danaus chrysippus* (Smith *et al.* 2016, Traut *et al.* 2017), wild silkmoths *Samia cynthia* ssp. (Yoshido *et al*. 2011a), leafroller moths of the family Tortricidae (Nguyen *et al.* 2013) and representatives of five families within curved-horn moths, Gelechioidea (Carabajal Paladino *et al.* 2019). Neo-sex chromosomes have also been suggested to promote divergence in vertebrate groups, e.g. in fishes (Kitano *et al.* 2009, Kitano and Peichel 2012) and mammals (Graves 2016). Our initial analysis of multiple sex chromosomes in genus *Leptidea* also indicates the turnover of the sex chromosomes by rearrangements with autosomes. Our results showed that *L. juvernica* and *L. sinapis* Z_1 chromosomes arose by fusion or translocation of part of an autosome orthologous to *B. mori* chromosome 17 and the ancestral Z chromosome (Pospíšilová 2018). This finding adds to accumulating evidence about the role of chromosomal rearrangements shaping lepidopteran karyotypes. Moreover, it increased our motivation to clarify the structure and origin of multiple sex chromosomes in *Leptidea*, as well as their speculative contribution to reproductive barriers between species and subsequent speciation.

2. Objectives

The unexpected discovery of cryptic diversity in wood white butterflies of the genus *Leptidea* has made these species an intensively studied model complex of organisms from various perspectives of research. Despite being target of ecological, behavioural, conservational and genetic studies, still little is known about their evolution and speciation. Considerable inter- and intra-specific chromosome number variability and inconsistency in position of usually conserved cytogenetic markers (Šíchová *et al*. 2015, 2016) point to a dynamic genome reshuffling underlying the formation of *Leptidea* karyotypes. Besides, so far studied *Leptidea* species exhibit a unique, species-specific sex chromosome constitution comprising of 3–4 W chromosomes and 3–6 Z chromosomes most likely originating in complex chromosomal rearrangements between ancestral sex chromosomes and autosomes (Šíchová *et al*. 2015, 2016). In contrast to variability in chromosome numbers, the constitution of multiple sex chromosomes is species-specific and indicates their great contribution to the creation of reproductive barriers between *Leptidea* species. The role of chromosomal rearrangements in the formation of *Leptidea* neo-sex chromosomes has been supported by our previous research in *L. juvernica* and *L. sinapis*, which Z_1 chromosome originated in fusion/translocation between the ancestral Z chromosome and an autosome orthologous to *B. mori* chromosome 17 (Pospíšilová 2018). Thus, we proposed to examine the remaining Z chromosomes in *L. juvernica* and *L. sinapis*, and also Z chromosomes in the third cryptic species, *L. reali*, to elucidate the role of multiple sex chromosomes in the evolution and speciation of *Leptidea* butterflies.

The main aim of this work was to perform comparative sex chromosome analysis in three Western Palearctic species, namely *L. juvernica*, *L. sinapis*, and *L. reali*, using genomic tools developed for *L. juvernica*, which are female transcriptome-based microarray for comparative genomic hybridization (array-CGH) and a bacterial artificial chromosome (BAC) library from *L. juvernica* females. BAC clones containing orthologs of *B. mori* genes, identified and selected by my colleagues, were isolated from BAC library and used as probes for physical mapping of genes by fluorescence *in situ* hybridization (BAC-FISH). A physical map of Z-linked genes and its comparison with the *B. mori* reference genome uncovered the chromosomal rearrangements underlying the process of formation of *Leptidea* multiple sex chromosomes. Moreover, the acquired knowledge helped to clarify the speculation about the role of multiple sex chromosomes in the creation of reproductive barriers between *Leptidea* species.

3. Material and methods

3.1. Sample collecting

Adult specimens of *Leptidea juvernica* and *L. sinapis* females were collected by my colleagues and myself in the Czech Republic, namely *L. juvernica* in the vicinity of České Budějovice and near the towns Milovice (district Nymburk) and Jistebnice (district Tábor), and *L. sinapis* near Havraníky village in the Podyjí National Park in South Moravia, near the village Kamýk nad Vltavou (district Příbram) and in the quarry Vyšný (district Český Krumlov). Adult female specimens of the third species, *L. reali*, were collected and supplied by Roger Vila in the Montseny Massif north of Barcelona, Spain. In the laboratory, fertilized females were kept individually in plastic containers at room temperature and normal day/night regime to lay eggs on one of their host plants, *Lotus corniculatus*, a feeding plant for newly-hatched larvae. Remaining bodies of all collected individuals were frozen and stored in 1.5 mL Eppendorf tubes in liquid nitrogen in –80°C, except for their genitalia, which were immediately used for morphometric analysis.

3.2. Genitalia preparation and morphometric analysis

Female genitalia were dissected in saline solution and inspected under a stereomicroscope. The *ductus bursae* length reliably distinguished *Leptidea sinapis* from both *L. juvernica* collected in the Czech Republic and *L. reali* collected in Spain, since these cohabiting species cannot be differentiated from each other based only on wing patterns (Dincă *et al*. 2011). Besides, my colleagues performed species level identification based on the analysis of two DNA markers, the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene and the nuclear internal transcribed spacer 2 (*ITS2*) sequence, both confirming the taxonomical identity of specimens used in the present study.

3.3. Chromosome preparation

The pachytene preparations of all three *Leptidea* species were carried out from fifth instar female and male larvae according to Mediouni *et al*. (2004). Briefly, gonads were dissected in saline solution, swollen in hypotonic solution (75mM KCl) for 10–15 min, torn to pieces with tungsten needles, and fixed in Carnoy fixative (ethanol/chloroform/acetic acid, 6:3:1) for 10–20 min. Ovaries were fixed in Carnoy fixative directly after dissection in physiological solution. Gonads were then macerated with tungsten needles in a drop of 60% acetic acid and spread on the slide placed on a heating plate at 45°C. The preparations were passed through a graded ethanol series (70%, 80% and 100%, 1 min each) and stored at –80°C for further use.

3.4. Identification of BAC clones containing genes of interest

The identification of genomic regions involved in *Leptidea* multiple sex chromosomes required the use of several genomic tools established for *L. juvernica*. My colleagues from the Laboratory of Molecular Cytogenetics of the Institute of Entomology BC CAS generated and *de novo* assembled *L. juvernica* female transcriptome to enable the identification of sex-linked genes by microarray-based comparative genomic hybridization (array-CGH; Baker and Wilkinson 2010). Raw reads of genes expressed in a female larva are deposited in the NCBI Sequence Read Archive (SRA) database under the accession number SRR10381488 (Bioproject PRJNA586890) and the Pyhton script used for the analysis of array-CGH is available at https://github.com/anicka-v/aCGH_scripts. A library of bacterial artificial chromosomes (BAC) of *L. juvernica* has been prepared by J. Šafář in the Centre of Plant Structural and Functional Genomics, IEB CAS, Olomouc, Czech Republic. In our laboratory, we have a sister copy of the BAC library and matrix pool plates for PCR screening of the BAC library (see Yasukochi 2002). To conduct the comparative analysis of *Leptidea* Z chromosomes, my colleagues identified orthologous sequences of sex-linked genes in *L. juvernica* by array-CGH and used them to select BAC clones carrying the gene orthologs by PCR screening of the *L. juvernica* BAC library. I extracted these individual BAC DNAs from the BAC library using the Qiagen Plasmid Midi Kit (Qiagen, Düsseldorf, Germany) or the NucleoBond Xtra Midi plasmid purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocols.

3.5. Labeling BAC probes

Isolated BAC-DNA was labeled according to Kato *et al.* (2006) with slight modifications. Labeling 40 µl reaction consisted of 2 µg of unlabeled BAC DNA, 0.1 mM aminoallyl-dUTP-Cy3 (Jena Bioscience, Jena, Germany) or fluorescein-12-dUTP (Jena Bioscience), 0.05 mM dATP, dCTP, dGTP, and 0.01 mM dTTP, 1x NT Buffer (0.05 M Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.005% BSA), 0.01 M mercaptoethanol, 40 U DNA polymerase I (ThermoFisher Scientific, Waltham, Massachusetts, USA) and 0.01 U DNase I (ThermoFisher Scientific). Labeling reaction was incubated at 15°C for 4 h 30 min or 5 h and then inactivated at 70°C for 10 min.

3.6. BAC-FISH mapping

Fluorescence *in situ* hybridization (FISH) with bacterial artificial chromosome-derived probes (BAC-FISH) was used for mapping gene orthologs on a particular chromosome. The pachytene preparations of *Leptidea juvernica*, *L. sinapis* and *L. reali* were repeatedly reprobed with different probe coctails containing two or three BAC clones labeled with aminoallyl-dUTP-Cy3 (Jena Bioscience) and another two or three BAC clones labeled with fluorescein-12-dUTP (Jena Bioscience). Each couple

or triplet of BAC clones contained BACs located in different parts of a particular chromosome (i.e. at opposite chromosomal ends and the central region of the chromosome) to differentiate the signals easily. Several rounds of two-colored BAC-FISH were performed according to the reprobing protocol of Yoshido *et al.* (2014) with slight modifications.

Chromosome preparations were removed from the freezer, passed through a graded ethanol series (70%, 80% and 100%, 1 min each), air-dried, fixed for 5 min in freshly prepared 4% formaldehyde in 2× SSC (pH=8) and washed twice in 2× SSC for 3 min. The slides were then denaturated at 68–70°C for 3 min 30 s in 70% formamide in 2× SSC. Hybridization mixture for each slide contained 200 ng of each probe labeled with aminoallyl-dUTP-Cy3 (Jena Bioscience) and 500 ng of each probe labeled with fluorescein-12-dUTP (Jena Bioscience), 3–10 µg of unlabelled sonicated male gDNA of the respective species (extracted from larvae by standard phenol-chloroform procedure), and 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich) in 10 µl hybridization buffer of 50% formamide, 10% dextran sulphate (Sigma-Aldrich) in 2× SSC. The male gDNA of each species was amplified by GenomiPhi HY DNA Amplification Kit (GE Healthcare, Milwaukee, WI, USA) and sonicated using a Sonopulus HD 2070 (Bandelin Electric, Berlin, Germany). Hybridization mixture was denaturated at 90°C for 5 min and spread on each slide. Slides were incubated in a humid chamber at 37°C for 3 days, and then washed at 62°C for 5 min in 0.1× SSC containing 1% Triton X-100. The slides were stained and mounted in antifade based on DABCO (1,4-diazabicyclo (2.2.2)-octane, Sigma-Aldrich) containing 0.5 µg/mL DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich). After each FISH round, the cover slides were removed carefully, and preparations were denaturated again as described above after 3 min washing in milliQ water. The next hybridization coctail was applied straight on dehydrated and air-dried slides.

3.7. Microscopy and image processing

All chromosome preparations were inspected in a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Digital black-and-white images were recorded with a cooled monochrome CCD camera XM10 equipped with cellSens Standard software version 1.9 (Olympus Europa Holding, Hamburg, Germany). Images were taken individually for each fluorescent dye (blue DAPI, red aminoallyl-dUTP-Cy3, and green fluorescein-12-dUTP) and pseudocolored with various colors, i.e. light blue for DAPI, green, red, orange, yellow and violet, independently on probe labeling that was applied in a particular experiment, in Adobe Photoshop, version 7.0.

4. Results

4.1. Identification, selection and isolation of *Leptidea juvernica* BAC clones

My colleagues in the Laboratory of Molecular Cytogenetics performed microarray-based comparative genomic hybridization (array-CGH) to identify sex-linked synteny blocks in *L. juvernica* females. The results indicated that *L. juvernica* Z chromosomes contain majority of genes allocated to *Bombyx mori* Z chromosome and are enriched with genes assigned to five *B. mori* autosomes, i.e. chromosomes 7, 8, 11, 17, and 24. My colleagues also performed PCR-based screening method of *L. juvernica* BAC library to find BAC clones containing genes of interest. Sequences of *Leptidea* orthologs of *B. mori* genes were obtained from the transcriptome assembly of *L. juvernica* females. In total, my colleagues identified 454 putative Z-linked orthologs of *B. mori* genes. All the above results are part of a joint publication (Yoshido *et al.*, in prep.). Based on the results of array-CGH and PCR screening of the BAC library, I isolated 66 BAC clones containing 17 orthologs of *B. mori* Z chromosome genes, six orthologs of *B. mori* chromosome 17 (see Table 1), ten orthologs of *B. mori* chromosome 7, 11 orthologs of *B. mori* chromosome 11, six orthologs of *B. mori* chromosome 24 (Table 2), six orthologs of *B. mori* chromosome 15, and 13 orthologs of *B. mori* chromosome 8 (Table 3). In most cases, one BAC clone was selected for each orthologous gene and contained just one gene. Only exceptions were *Enolase* (*Eno*) with two BAC clones (70D8, 90E24) and *Triosephosphate isomerase* (*Tpi*) with three BAC clones (91C3, 66L7, 96D19), each containing different parts of the gene sequence. Other exceptions were *tyrosine hydroxylase* (*Th*) and *Y box protein* (*Ybp*), both found in the same BAC clone (66E6), *Annexin IX isoform B* (*AnnIXB*), *pixie* (*pixie*), *putative Copper homeostasis protein cutC-like protein* (*CUTClp*) found in BAC clone 94J6*, Ubiquitin carboxyl-terminal hydrolase 5-like isoform 1* (*Uch5l*), *hypothetical protein KGM_12964* (KGM12964), and *S3-12-like protein* (*S3-12*) in BAC clone 62A6, and similarly, *Leucine-rich repeat G protein-coupled receptor precursor* (*Lrg*) and *hypothetical protein KGM_00143* (*KGM00143*) both found in two similar BAC clones (69P11, 90N21). All selected BAC clones were used for the sex chromosome analysis in *L. juvernica*, *L. sinapis*, and *L. reali* by BAC-FISH.

Table 1. List of *Leptidea juvernica* BAC clones carrying orthologs of *Bombyx mori* chromosomes Z and 17 mapped in Pospíšilová (2018) and this study.

		Leptidea juvernica		***Bombyx mori***		
Symbol	***B. mori* orthologs of selected gene**	**BAC clone**	**BAC-FISH mapping**	**Gene ID**	**Chr. No.**	**Chromosome position**
Tan	*Tan*	63E2	chromosome Z_1	BMgn002077	Z	460237-480848
ap	*apterous*	53C10	chromosome Z_1	BMgn002127	Z	3487639-3516414
ABCF2	*ATP-binding cassette sub-family F member 2*	90D1	chromosome Z_1	BMgn002004	Z	4621452-4632826
Prm	*Paramyosin*	9J14	chromosome Z_1+ autosome	BMgn000612	Z	5986799-6002013
ket	*kettin*	91P9	chromosome Z_1	BMgn000622	Z	6513219-6533895
Idgf	*Imaginal disk growth factor*	93O2	chromosome Z_1	BMgn000648	Z	8533563-8553629
Th	*tyrosine hydroxylase*	66E6	chromosome Z_1	BMgn000563	Z	8795363-8803219
Ybp	*Y box protein*	66E6	chromosome Z_1	BMgn000526	Z	10855404-10857772
Imp	*IGF-II mRNA-binding protein*	69D15	chromosome Z_1	BMgn000515	Z	11419210-11500018
per	*period*	72D11	chromosome Z_1	BMgn000485	Z	12956618-13004501
Masc	*Masculinizer (hypothetical protein KGM_08818 [Danaus plexippus])*	62N7	chromosome Z_1	BMgn012300	Z	15129206-15132406
SNF4Ay	*SNF4/AMP-activated protein kinase gamma subunit*	90K6	chromosome Z_1	BMgn012310	Z	15595590-15678086
Ldh	*L-lactate dehydrogenase*	95B19	chromosome Z_1	BMgn012336	Z	17338625-17350610
Shkr	*Shaker*	19P21	chromosome Z_1	BMgn003851	Z	20911282-20921258
Hn	*phenylalanine hydroxylase (henna)*	69F16	chromosome Z_1	BMgn003866	Z	21842454-21845665
Tpi	*Triosephosphate isomerase*	91C3, 66L7, 96D19	autosome	BMgn000559	Z	9023502-9027095
Pgd	*6-phosphogluconate dehydrogenase*	62O17	autosome	BMgn012298	Z	15112863-15127673
Treh	*Trehalase*	66E20	autosome	BMgn005664	17	1555037-1558124
Rrb	*putative regulator of ribosome biosynthesis*	65E15	autosome	BMgn005564	17	2388316-2390458
eIF3D	*eukaryotic translation initiation factor 3 subunit D*	93F8	autosome	BMgn005592	17	4290839-4299895
ASPG	*l-asparaginase*	92J7	chromosome Z_1	BMgn007025	17	11551636-11556300
RpL22	*Ribosomal protein L22*	19D3	chromosome Z_1	BMgn006986	17	14152955-14155752
KGM04993	*hypothetical protein KGM_04993 [Danaus plexippus]*	94M23	chromosome Z_1	BMgn003962	17	18278392-18281478

Table 2. List of *Leptidea juvernica* BAC clones carrying orthologs of *Bombyx mori* chromosomes 7, 11, and 24 mapped in this study.

		Leptidea juvernica		***Bombyx mori***		
Symbol	***B. mori* orthologs of selected gene**	**BAC clone**	**BAC-FISH mapping**	**Gene ID**	**Chr. No.**	**Chromosome position**
EH-dp1	*EH domain-containing protein 1*	72H14	chromosome Z_2	BMgn009992	7	67746-96791
KGM21114	*hypothetical protein KGM_21114 [Danaus plexippus]*	91D03	chromosome Z_2	BMgn010027	7	1455398-1458517
Dl	*Delta*	72I17	chromosome Z_2	BMgn010195	7	6075871-6111563
tRNAmt	*tRNA methyltransferase*	53K21	chromosome Z_2	BMgn010207	7	7001096-7096151
AnnIXB	*Annexin IX isoform B*	94J6	chromosome Z_2	BMgn010130	7	8615817-8626114
pixie	*pixie*	94J6	chromosome Z_2	BMgn010129	7	8632389-8643051
CUTClp	*putative Copper homeostasis protein cutC-like protein*	94J6	chromosome Z_2	BMgn010253	7	8644140-8647781
Cad	*Cadherin*	95N3	autosome	BMgn010267	7	9747774-9767857
Gcy	*Guanylate cyclase*	41B20	autosome	BMgn010091	7	11414173-11492005
unc50l	*unc-50-like protein*	42F24	autosome	BMgn008674	7	14646799-14653293
KGM08377	*hypothetical protein KGM_08377 [Danaus plexippus]*	70A22	chromosome Z_2	BMgn001710	11	76987-81275
Zf228	*putative zinc finger protein 228*	71H18	chromosome Z_2	BMgn001744	11	891908-909137
Cyp450	*Cytochrome P450*	15C11	chromosome Z_2	BMgn001753	11	1264947-1272550
Pisd	*Phosphatidylserine decarboxylase*	65P23	chromosome Z_2	BMgn001766	11	1795166-1796032
KGM01846	*hypothetical protein KGM_01846 [Danaus plexippus]*	96H3	chromosome Z_2	BMgn001655	11	2284069-2284887
Cpsf5	*Cleavage and polyadenylation specific factor 5*	67C21	chromosome Z_2	BMgn001806	11	3052181-3055687
Osbp	*Oxysterol binding protein*	13L18	chromosome Z_2	BMgn001810	11	3139662-3192524
Gtp-bp	*putative GTP-binding protein*	19O4	chromosome Z_2	BMgn011993	11	5217859-5228534
RpL18	*Ribosomal protein L18*	95K24	autosome	BMgn011620	11	9399516-9401192
Dmc1	*Dmc1 homolog*	43I3	autosome	BMgn011811	11	11626450-11635244
KGM19656	*hypothetical protein KGM_19656 [Danaus plexippus]*	43L15	autosome	BMgn012129	11	16517488-16577040
FBX28	*putative F-box protein 28*	65L03	chromosome Z_2	BMgn008185	24	1656904-1669541
CPH35	*putative cuticle protein CPH35*	93A18	chromosome Z_2	BMgn000083	24	7542521-7548473
Tmc7	*Transmembrane channel-like protein 7*	90C15	chromosome Z_2	BMgn000078	24	8061419-8067032
KGM02279	*hypothetical protein KGM_02279 [Danaus plexippus]*	49I8	autosome	BMgn009578	24	11525730-11537948
Sui1	*Protein translation factor SUI1 homolog*	94G12	autosome	BMgn003805	24	16081319-16082543
O-fut2	*Protein-O-fucosyltransferase 2*	49E5	autosome	BMgn012194	24	17459857-17461971

Table 3. List of *Leptidea juvernica* BAC clones carrying orthologs of *Bombyx mori* chromosomes 8 and 15 mapped in this study.

		Leptidea juvernica		***Bombyx mori***		
Symbol	***B. mori* orthologs of selected gene**	**BAC clone**	**BAC-FISH mapping**	**Gene ID**	**Chr. No.**	**Chromosome position**
RpS5	*Ribosomal protein S5*	9M16	autosome	BMgn007710	15	7586843-7587692
RpS8	*Ribosomal protein S8*	96L18	autosome	BMgn003397	15	13998108-14000483
RpP1	*Ribosomal protein P1*	89C2	autosome	Gene009141	15	14486757-14488166
Ctatpase	*putative cation-transporting atpase*	41J22	chromosome Z_3	BMgn003317	15	15694441-15712206
RpP0	*Ribosomal protein P0*	17I6	chromosome Z_3	BMgn003309	15	16146287-16150050
Top2-bp1	*DNA topoisomerase 2 binding protein 1*	19O6	chromosome Z_3	BMgn003443	15	16724374-16748667
Trp	*Translocation protein Sec62*	95D23	autosome	BMgn005270	8	4673007-4693568
Eno	*Enolase*	70D8, 90E24	autosome	BMgn005493	8	11726013-11734127
m5u-mt	*RNA m5u methyltransferase*	17J5	chromosome Z_3	BMgn005286	8	13114526-13123939
Uch5l	*Ubiquitin carboxyl-terminal hydrolase 5-like isoform 1*	62A6	chromosome Z_3	BMgn009941	8	15351712-15371974
KGM12964	*hypothetical protein KGM_12964 [Danaus plexippus]*	62A6	chromosome Z_3	BMgn009934	8	15372779-15374302
S3-12	*putative plasma membrane associated protein, S3-12-like protein*	62A6	chromosome Z_3	BMgn009933	8	15377476-15398398
Frl	*Formin-like protein CG32138-like isoform 1*	70B3	chromosome Z_4	BMgn009881	8	16772354-16799660
Lgr	*Leucine-rich repeat G protein-coupled receptor precursor*	69P11, 90N21	chromosome Z_4	BMgn009886	8	17100494-17156828
KGM00143	*hypothetical protein KGM_00143 [Danaus plexippus]*	69P11, 90N21	chromosome Z_4	BMgn009852	8	17158743-17162365
tra2	*transformer 2*	90N21	chromosome Z_4	BMgn009888	8	17184249-17192522
Ann1	*Annexin isoform 1*	67E14	chromosome Z_4	BMgn009900	8	17604017-17616920
Dbadrh	*putative DEAD box ATP-dependent RNA helicase*	65A14	chromosome Z_4	BMgn009910	8	18103635-18113027
Smc	*Structural maintenance of chromosomes 1A*	22K17	chromosome Z_4	BMgn009835	8	18335387-18355471

4.2. Physical mapping of Z_1 chromosome

In our previous study (Pospíšilová 2018), we assembled the physical map of Z_1 chromosome in *Leptidea juvernica* and *L. sinapis*. Firstly, we separately hybridized 18 BAC-derived probes corresponding to *Bombyx mori* chromosome Z-linkage group and three BAC probes corresponding to *B. mori* chromosome 17-linkage group on female pachytene preparations of *L. juvernica* and *L. sinapis* in order to verify their sex-linkage in sex chromosome multivalents that are formed during meiosis (Fig. 6a, c, in this study; BAC clones 92J7, 91P9, 62N7 shown only, yellow signals). The constitution of species-specific multivalents consisting of 3–4 W and 3–4 Z chromosomes was described in Šíchová *et al.* (2015) for *L. juvernica*, *L. sinapis*, and *L. reali*, and thus simplified the identification of individual W and Z chromosomes in the present study. In *L. juvernica* and *L. sinapis* females, the majority of BAC clones hybridized to one chromosome in the sex chromosome multivalent, therefore named as Z_1 chromosome. One exception was BAC clone carrying ortholog of *Paramyosin* (*Prm*) gene that hybridized to the Z_1 chromosome, and also to an autosome. In addition, BAC clone *6-phosphogluconate dehydrogenase* (*Pgd*) and three clones of *Triosephosphate isomerase* (*Tpi*) provided discrete hybridization signals on autosomes and therefore were excluded from physical mapping of Z_1 chromosome (results not shown). For simplification, BAC-FISH mapping of the Z_1 chromosome of *L. juvernica* and *L. sinapis* was performed on male pachytene preparations with the ZZ bivalents rather than on female preparations with complex sex chromosome multivalents. In both species, several rounds of BAC-FISH localized 14 BAC clones carrying orthologs of *B. mori* chromosome Z and three BAC clones carrying orthologs of chromosome 17 on a single Z_1Z_1 bivalent in the identical order. These results thus confirmed the conserved synteny and the conserved gene order between Z_1 chromosomes in these two species (Fig. 2a, b, c, d; Pospíšilová 2018).

In this study, similar approach was used when assembling the physical map of *L. reali* Z_1 chromosome. Basically, BAC clones that proved sex-linkage in *L. juvernica* and *L. sinapis* were divided into five triplets (genes *THL* and *Y-box* are in the same BAC clone) and one couple and labeled with green and/or red fluorescent dyes (Table 4). Three rounds of BAC-FISH reprobing were performed on *L. reali* male pachytene preparations. Surprisingly, BAC probes mapped to two distinct chromosomes (Fig. 2e). Eight orthologs of *B. mori* Z chromosome (*Ldh*, *SNF4Ay*, *Masc*, *Hn*, *Shkr*, *per*, *Tan*, *ap*) were localized on one bivalent, whereas remaining seven orthologs of *B. mori* Z chromosome (*ket*, *par*, *ABCF2, Imp, Idgf, Th, Ybp*) together with three orthologs of *B. mori* chromosome 17 (*ASPG*, *KGM04993*, *RpL22*) were localized on another bivalent (Fig. 2e). The gene order remained consistent with *L. juvernica* and *L. sinapis.* To confirm that marked bivalents are Z chromosomes, representative BAC clones for each bivalent, i.e. 62N7 (*Masc)* for the first bivalent and 91P9 (*ket*) plus 92J7 (*ASPG*) for the second bivalent were hybridized on female *L. reali* pachytene preparations. All three BAC clones marked their positions in the sex chromosome multivalent (Fig. 6e, yellow signals). Therefore, these

results indicate that the Z_1 chromosomes of *L. juvernica* and *L. sinapis* split into two chromosomes in *L. reali* in the region between the *ap* and *ket* genes (Figs. 2e and 7), which are both located on *B. mori* chromosome Z. Thus, these chromosomes were named Z_1 and Z_4 chromosomes in *L. reali*.

Besides, our results indicate that the original Z_1 chromosome arose via fusion or translocation between the ancestral Z chromosome and an autosome orthologous to *B. mori* chromosome 17 (Fig. 7). To exclude one of these options, we selected three more BAC clones 66E20 (*Treh*), 65E15 (*Rrb*), and 93F8 (*elFD3*) corresponding to *B. mori* chromosome 17-linkage group. These BAC clones mapped to two autosomes in *L. juvernica* males (Fig. 3a, purple signals; Fig. 7, underlined genes), which suggests that the Z_1 chromosome arose by translocation of an autosomal segment, corresponding to part of chromosome 17 in *B. mori*, onto the Z chromosome.

Table 4. The labeling plan for individual BAC-FISH reprobing runs in *Leptidea reali* with BAC clones corresponding to linkage groups Z and 17 of the *Bombyx mori* reference genome.

Run No.	Hybridized BAC clones	Labeling
1	*Ldh+ ket+ Imp* *SNF4Ay+ ap+ Th_Ybp*	aminoallyl-dUTP-Cy3 (red) fluorescein-12-dUTP (green)
2	*Masc+ Tan+ Idgf* *Hn + ABCF2*	aminoallyl-dUTP-Cy3 (red) fluorescein-12-dUTP (green)
3	*Shkr+ Prm+ KGM04993* *per+ ASPG+ RpL22*	aminoallyl-dUTP-Cy3 (red) fluorescein-12-dUTP (green)

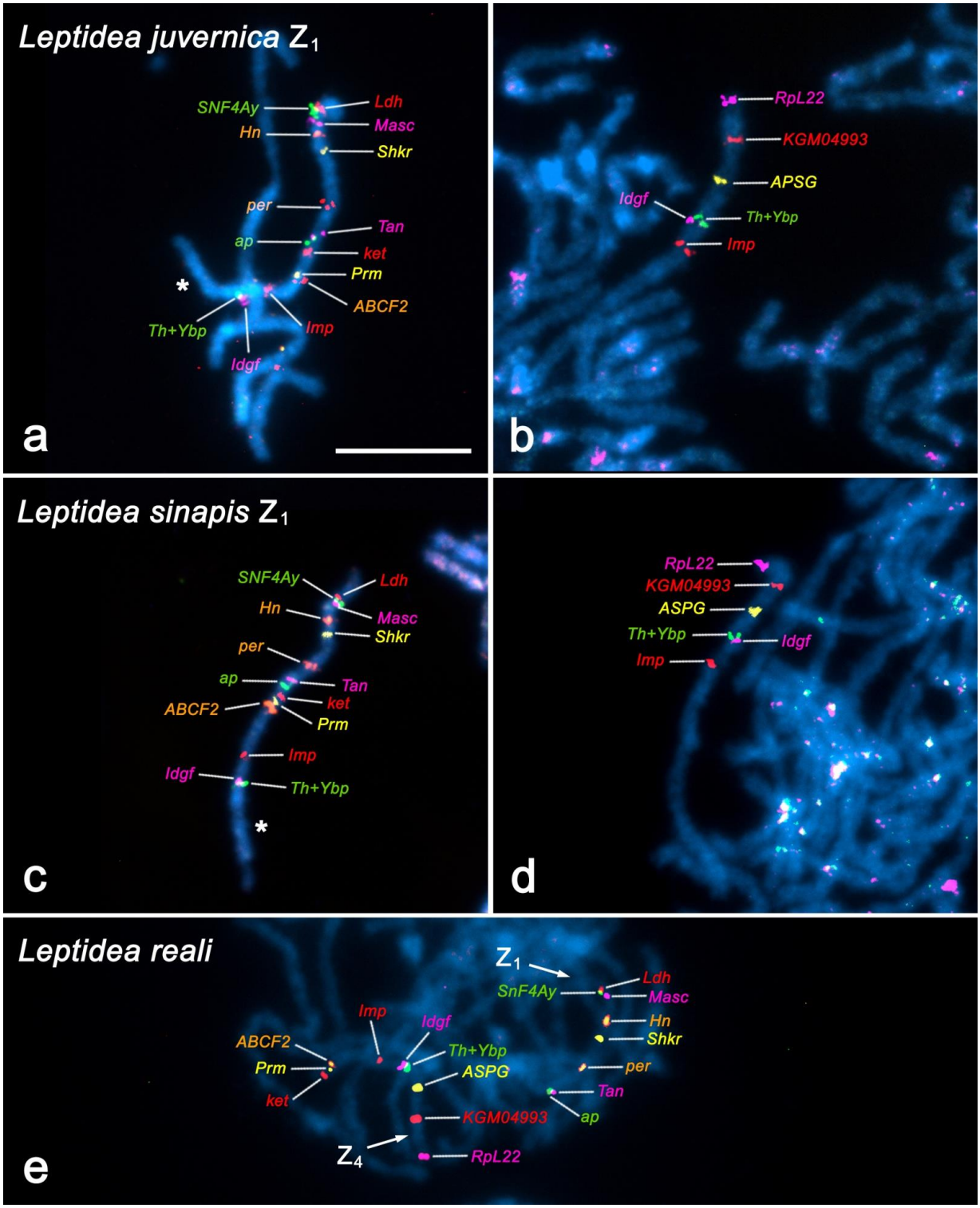

Figure 2. BAC-FISH mapping of Z_1 chromosome in male pachytene preparations of *Leptidea juvernica* **(a, b)**, *L. sinapis* **(c, d)** (Pospíšilová 2018), and Z_1 and Z_4 chromosomes in *L. reali* **(e)**.BAC probe hybridization signals (red, green, orange, yellow, violet) indicate the physical position of loci labeled by gene symbols. Chromosomes were stained with DAPI (blue). Asterisks show sections without any signals. **(a, c)** Hybridization signals of 14 BAC probes containing *Leptidea* orthologs of *Bombyx mori* chr. Z on a single Z_1Z_1 bivalent. **(b, d)** Hybridization signals of three BAC probes containing *Leptidea* orthologs of *B. mori* chr. Z (*Imp*, *Idgf*, *Th_Ybp*) together with three BAC probes containing orthologs of *B. mori* chr. 17 (*ASPG*, *KGM04993*, *RpL22*) on a single Z_1Z_1 bivalent (Pospíšilová 2018). **(e)**

Hybridization signals of eight BAC probes containing *Leptidea* orthologs of *B. mori* chr. Z on a single Z_1Z_1 bivalent and hybridization signals of six BAC probes containing *Leptidea* orthologs of *B. mori* chr. Z (*ket- Th_Ybp*) together with three BAC probes containing orthologs of *B. mori* chr. 17 (*ASPG*, *KGM04993*, *RpL22*) on a single Z_4Z_4 bivalent. Scale bar = 10 µm.

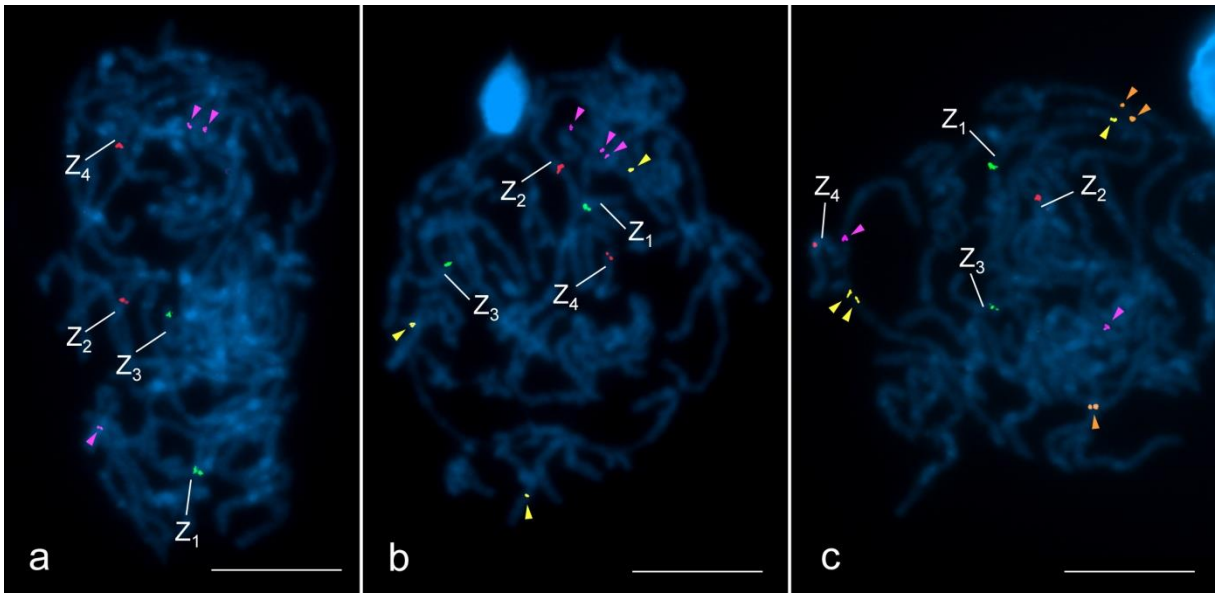

Figure 3. FISH mapping of Z-derived and autosome-derived BAC probes in male pachytene chromosomes of *Leptidea juvernica*. BAC probe hybridization signals (red, green, yellow, violet) indicate the physical position of BAC clones marked by arrowheads. Chromosomes were stained with DAPI (blue). Representative BAC probes, 92J7 (green) for Z_1, 94J6 (red) for Z_2, 62A6 (green) for Z_3, and 69P11 (red) for Z_4, were used to identify respective Z chromosomes. Z_1 vs. Z_3 and Z_2 vs. Z_4 were distinguished by different lengths of these chromosomes. (**a**) Three BAC probes, 66E20, 65E15, and 93F8 (purple), containing *Leptidea* orthologs of *B. mori* chr. 17 mapped to autosomes. (**b**) Three BAC probes, 95K24, 43I3, and 43L15 (yellow), containing *Leptidea* orthologs of *B. mori* chr. 11 and three BAC probes, 49I8, 94G12, and 49E5 (purple), containing *Leptidea* orthologs of *B. mori* chr. 24 mapped to autosomes. (**c**) Three BAC probes, 95N3, 41B20, and 42F24 (orange), containing *Leptidea* orthologs of *B. mori* chr. 7, two BAC probes, 95D23 and 70D8 (purple), containing orthologs of *B. mori* chr. 8 and three BAC probes, 9M16, 96L18, and 89C2 (yellow), containing orthologs of *B. mori* chr. 15 mapped to autosomes. Scale bar = 10 µm.

4.3. Physical mapping of Z_2 chromosome

To clarify the origin of the other Z chromosomes in *Leptidea juvernica,* my colleagues selected, and I isolated BAC clones carrying sex-specific *Leptidea* orthologs of *Bombyx mori* autosomes, which were previously identified by array-CGH (Yoshido *et al.*, in prep.). Physical mapping of BAC clones containing seven orthologous genes of *B. mori* chromosome 7, eight orthologs of *B. mori* chromosome 11, and three orthologs of *B. mori* chromosome 24 was carried out on *L. juvernica* male pachytene preparations with ZZ bivalents. All 16 BAC clones were split into four triplets and two couples and labeled with green and/or red fluorescent dyes (Table 5). Three rounds of BAC-FISH reprobing localized all 16 BAC clones to a single bivalent (Fig. 4a) and covered the full length of the bivalent. Subsequently, one representative BAC clone 94J6 (*AnnIXB_pixie_CUTCIp*) of these 16 clones was hybridized on *L. juvernica* female pachytene preparations and proved its sex linkage in the sex chromosome multivalent (Fig. 6a, purple signal). This experiment confirmed that the bivalent identified in the previous BAC-FISH mapping in *L. juvernica* males is another pair of Z chromosomes. Thus, we marked this chromosome as the Z_2 chromosome.

To uncover the evolution of other Z chromosomes in closely related species, *L. sinapis* and *L. reali,* all 16 BAC clones were cross-hybridized on pachytene chromosome preparations of males in both species. Three rounds of BAC-FISH following the labeling scheme as described above localized all BAC clones on a single bivalent (Fig. 4b, c). Moreover, the order of the individual genes remained conserved in all three *Leptidea* species. Subsequently, one representative BAC probe 94J6 (*AnnIXB_pixie_CUTCIp*) of 16 BAC clones was hybridized on *L. sinapis* and *L. reali* female preparations and proved sex linkage in the sex chromosome multivalent (Fig. 6c, e, purple signals). Therefore, this chromosome was also named as Z_2 chromosome in both species.

For deeper insight into the evolution of *Leptidea* Z_2 chromosome, we selected and isolated nine more BAC clones containing three orthologs of *B. mori* chromosome 7 [95N3 (*Cad*), 42B20 (*Gcy*), 42F24 (*unc50l*)], three orthologs of *B. mori* chromosome 11 [95K24 (*RpL18*), 43I3 (*Dmc1*), 43L15 (*KGM19656*)], and three orthologs of *B. mori* chromosome 24 [49I8 (*KGM02279*), 94G12 (*Sui1*), 49E5 (*O-fut2*)]. Several BAC-FISH rounds on *L. juvernica* male preparations localized all nine probes on autosomes (Fig. 3c, orange signals, 3b, yellow and purple signals, respectively; Fig. 8, underlined genes). These results suggest that *Leptidea* Z_2 chromosome most probably originated by translocations of three autosomes as it contains segments corresponding to parts of *B. mori* chromosomes 7, 11, and 24 (Fig. 8).

Table 5. The labeling plan for individual BAC-FISH reprobing runs in *Leptidea juvernica*, *L. sinapis*, and *L. reali* with BAC clones corresponding to linkage groups 7, 11 and 24 of the *Bombyx mori* reference genome.

Run No.	Hybridized BAC clones	Labeling
1	*tRNAmt+ KGM08377+ Cpsf5*	aminoallyl-dUTP-Cy3 (red)
	AnnIXB_pixie_CUTCIp+ Cyp450+ Gtp-bp	fluorescein-12-dUTP (green)
2	*EH-dp1+ Zf228+ FBX28*	aminoallyl-dUTP-Cy3 (red)
	KGM21114+ KGM01846+ Tmc7	fluorescein-12-dUTP (green)
3	*Dl+ Pisd*	aminoallyl-dUTP-Cy3 (red)
	Osbp + CPH35	fluorescein-12-dUTP (green)

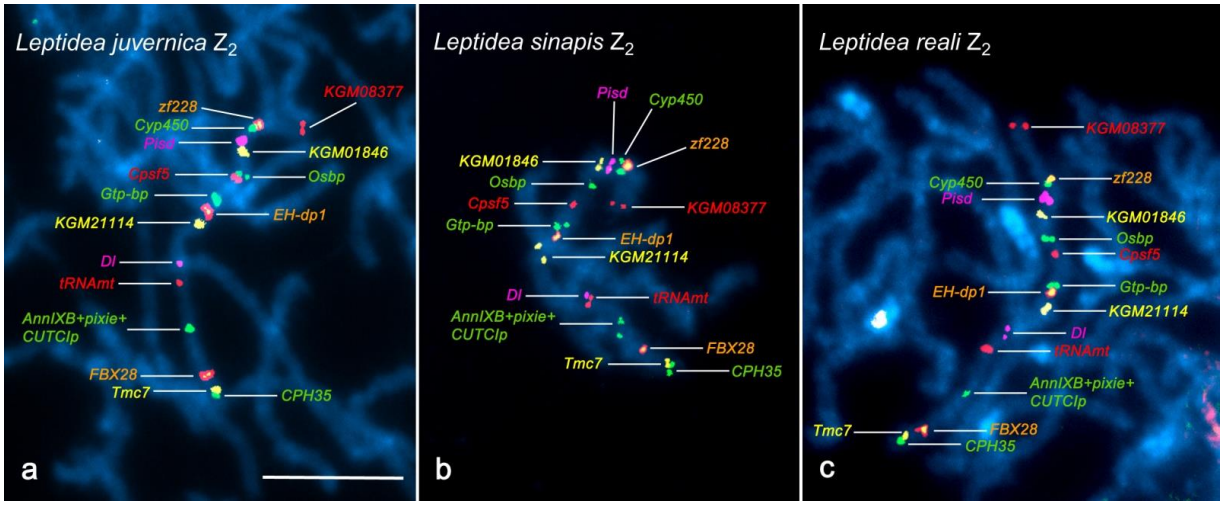

Figure 4. BAC-FISH mapping of Z_2 chromosome in male pavhytene chromosome preparations of *Leptidea juvernica* **(a)**, *L. sinapis* **(b)**, and *L. reali* **(c)**. BAC probe hybridization signals (red, green, orange, yellow, violet) indicate the physical position of loci labeled by gene symbols. Chromosomes were stained with DAPI (blue). Hybridization signals of 16 BAC probes containing eight *Leptidea* orthologs of *Bombyx mori* chr. 11 (*KGM08377-Gtp-bp*), seven orthologs of *B. mori* chr. 7 (*EH-dp1- CUTCIp*) and three orthologs of *B. mori* chr. 24 (*FBX28, Tmc7, CPH35*) on single Z_2Z_2 bivalent. Scale bar = 10 µm.

4.4. Physical mapping of Z_3 chromosome

To uncover the origin of remaining *Leptidea juvernica* Z chromosomes, my colleagues focused on other *Bombyx mori* autosomes. The results of array CGH suggested that *B. mori* chromosomes 8 could also contain synteny segments, which are sex-linked in *L. juvernica* (Yoshido *et al.*, in prep.). For this reason, my colleagues selected, and I isolated eight BAC clones carrying 11 orthologs of *B. mori* chromosome 8 genes. BAC-FISH reprobing procedure on *L. juvernica* male pachytene preparations localized these eight BAC clones on two distinct bivalents (Fig. 5a). One bivalent carried seven orthologous genes (*Frl*, *Lgr*, *KGM00143*, *tra2*, *Ann1*, *Dbadrh*, *Smc*), whereas the second bivalent carried four orthologs (*m5u-mt*, *Uch5I*, *KGM12964*, *S3-12*) of *B. mori* chromosome 8. To verify, that both bivalents are sex chromosomes, BAC clone 62A6 (*Uch5I_KGM12964_S3-12*) representing one of the two bivalents, and BAC clone 69P11 (*Lgr_KGM00143*), representing the other bivalent, were hybridized on *L. juvernica* female pachytene preparations. Indeed, both BAC clones marked their positions on two different Z chromosomes in the sex chromosome multivalent (Fig. 6a, green and red signals, respectively). Therefore, we named these chromosomes as Z_3 and Z_4 chromosomes. However, the probe signals in the previous experiment did not cover the full length of the male Z_3Z_3 bivalent. This indicated that more chromosomes could be engaged in the evolution of Z_3 chromosome. To detect the origin of this unlabeled part of the chromosome, my colleagues inspected the assembled genome of *L. sinapis* (Talla *et al.* 2017) and found out that the gene ortholog of *m5u-mt* in *L. sinapis* genome is in the same scaffold as the ortholog of *Ctatpase* gene located on *B. mori* chromosome 15. This finding allowed us to select and isolate six BAC clones containing six orthologs of *B. mori* chromosome 15 (*RpS5*, *RpS8*, *RpP1*, *Ctatpase*, *RpP0*, *Top2-bp1*). Three BACs (*Ctatpase*, *RpP0*, *Top2-bp1*) of these six clones were co-localized on *L. juvernica* Z_3 chromosome together with three BAC clones containing four orthologs of *B. mori* chromosome 8 mentioned before (Fig. 5a). This result suggests that *L. juvernica* Z_3 chromosome most probably originated by chromosomal rearrangements between autosomes. Our hypothesis was confirmed by another BAC-FISH experiment, which localized two BAC clones containing orthologs of *B. mori* chromosome 8 [95D23 (*Trp*), 70D8 (*Eno*)] and three BAC clones containing orthologs of *B. mori* chromosome 15 [9M16 (*RpS5*), 96L18 (*RpS8*), 89C2 (*RpP1*)] on autosomes in *L. juvernica* males (Fig. 3c, purple and yellow signals, respectively; Fig. 9, underlined genes). Taken together, we conclude that *L. juvernica* Z_3 chromosome consists of two segments corresponding to parts of *B. mori* chromosomes 8 and 15, whereas *L. juvernica* Z_4 chromosome contains the other part of *B. mori* chromosome 8 (Fig. 9).

To elucidate the evolution of the remaining *L. sinapis* and *L. reali* Z chromosomes, 10 BAC clones, which proved sex-linkage in *L. juvernica* Z_3 and Z_4 chromosomes, were hybridized on male chromosome preparations of *L. sinapis* and *L. reali*. BAC clones were divided into two triplets and two

pairs and labeled with green and/or red fluorescent dyes (Table 6). Two rounds of BAC-FISH reprobing localized all BAC probes on a single bivalent in the identical order as in *L. juvernica* (Fig. 5b, c). Subsequently, we confirmed that this bivalent is a pair of Z chromosomes as two BAC clones 62A6 (*Uch5l_KGM12964_S3-12*) and 69P11 (*Lgr_KGM00143*) hybridized to the sex chromosome multivalents in *L. sinapis* and *L. reali* females (Fig. 6c, e, green and red signals). Thus, we marked this chromosome as the Z_3 chromosome in both species. Besides, our results indicate that *L. juvernica* Z_3 and Z_4 chromosomes most probably originated by fission of the original Z_3 chromosome as they both contain orthologs of *B. mori* chromosome 8 genes.

Table 6. The labeling plan for individual BAC-FISH reprobing runs in *Leptidea sinapis* and *L. reali* with BAC clones corresponding to linkage groups 8 and 15 of the *Bori mori* reference genome.

Run No.	Hybridized BAC-clones	Labeling
1	*Top2-bp1+ Uch5l_KGM12964_S3-12+ Frl*	aminoallyl-dUTP-Cy3 (red)
	Ctatpase+ Lgr_KGM00143_tra2+ Smc	fluorescein-12-dUTP (green)
2	*RpP0+ Ann1*	aminoallyl-dUTP-Cy3 (red)
	m5u-mt+ Dbadrh	fluorescein-12-dUTP (green)

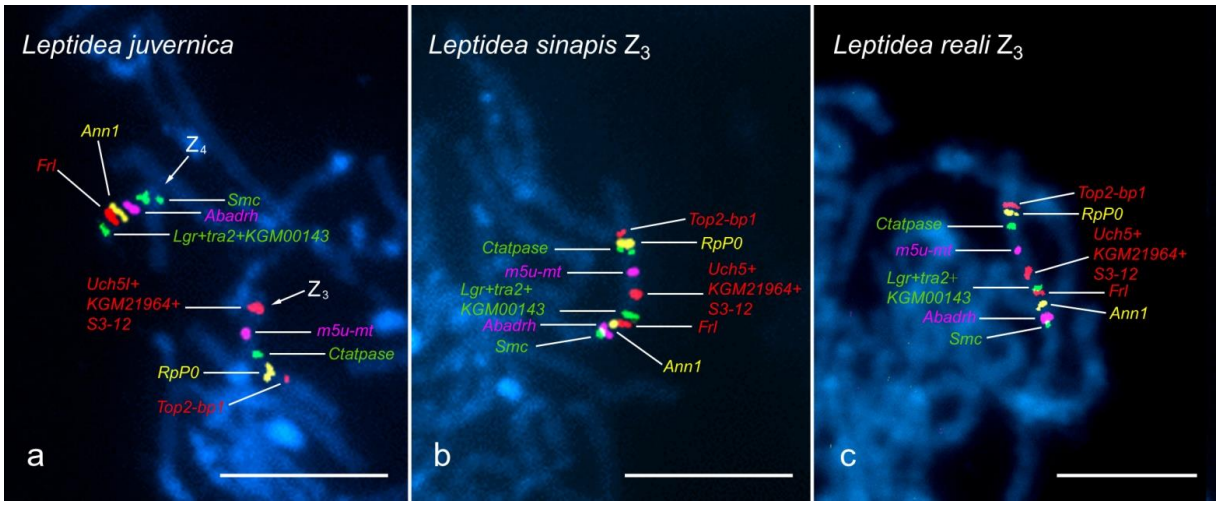

Figure 5. BAC-FISH mapping of Z_3 and Z_4 chromosomes in male pachytene preparations of *Leptidea juvernica* **(a)**, and Z_3 chromosome in *L. sinapis* **(b)**, and *L. reali* **(c)**. BAC probe hybridization signals (red, green, orange, yellow, violet) indicate the physical position of loci labeled by gene symbols. Chromosomes were stained with DAPI (blue). Hybridization signals of 10 BAC probes containing 11 *Leptidea* orthologs of *Bombyx mori* chr. 8 (*Smc- m5u-mt*), and three orthologs of *B. mori* chr. 15 (*Top2-bp1, RpP0, Ctatpase*). Scale bar = 10 µm.

4.5. Simultaneous identification of individual Z chromosomes by BAC-FISH

To confirm that our previous experiments indeed identified different Z chromosomes in *Leptidea juvernica*, *L. sinapis*, and *L. reali*, we performed another BAC-FISH experiment on male and female pachytene preparations of each *Leptidea* species. Six BAC clones representing respective Z chromosomes were chosen and labeled based on their estimated positions on Z chromosomes (Table 7) to make individual Z chromosomes easily distinguishable. BAC clones were divided into three pairs and labeled with green and/or red fluorescent dyes (Table 8). Two rounds of BAC-FISH reprobing localized BAC signals on four different chromosomes (Z_1–Z_4) in *L. juvernica* (Fig. 6a, b), three chromosomes (Z_1–Z_3) in *L. sinapis* (Fig. 6c, d), and four chromosomes (Z_1–Z_4) in *L. reali* (Fig. 6e, f), respectively. Therefore, we successfully identified all Z chromosome bivalents in males and all Z chromosomes in female multivalents in three *Leptidea* species studied.

Table 7. List of BAC clones used for identification of individual Z chromosomes in *Leptidea juvernica*, *L. sinapis*, and *L. reali* and their expected position on Z chromosomes in *Leptidea* species.

BAC clone	Gene symbol	*L. juvernica*	*L. sinapis*	*L. reali*
62N7	*Masc*	Z_1	Z_1	Z_1
91P9	*ket*	Z_1	Z_1	Z_4
92J7	*ASPG*	Z_1	Z_1	Z_4
94J6	*AnnIXB* *pixie* *CUTCIp*	Z_2	Z_2	Z_2
62A6	*Uch5I* *KGM12964* *S3-12*	Z_3	Z_3	Z_3
69P11	*Lgr* *KGM00143*	Z_4	Z_3	Z_3

Table 8. The labeling plan for individual BAC-FISH reprobing runs in *Leptidea juvernica*, *L. sinapis*, and *L. reali.*

Run No.	Hybridized BAC-clones	Labeling
1	91P9 (*ket*) +69P11 (*Lgr_KGM00143*)	aminoallyl-dUTP-Cy3 (red)
	62A6 (*Uch5I_KGM12964_S3-12*) +92J7 (*ASPG*)	fluorescein-12-dUTP (green)
2	62N7 (*Masc*)	aminoallyl-dUTP-Cy3 (red)
	94J6 (*AnnIXB_pixie_CUTCIp*)	fluorescein-12-dUTP (green)

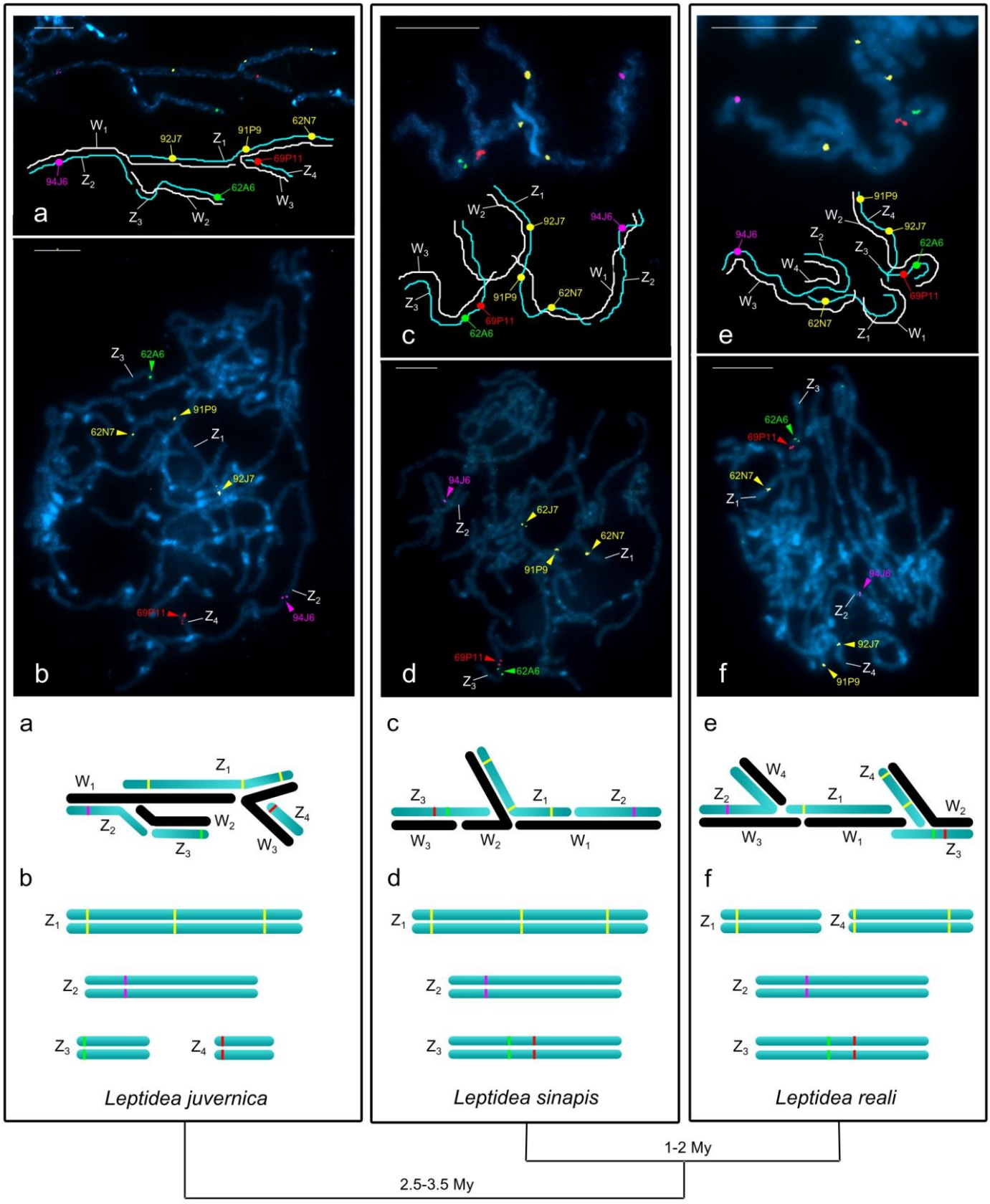
a
b
c
d
e
f
W1
Z1
92J7
91P9
62N7
69P11
Z4
94J6
Z2
62A6
W3
Z3
W2
W4
62J7
a
c
e
b
d
f
Leptidea juvernica
Leptidea sinapis
Leptidea reali
1-2 My
2.5-3.5 My

Figure 6. BAC-FISH analyses of multiple sex chromosomes in *Leptidea juvernica*, *L. sinapis*, and *L. reali*. Upper panel: BAC-FISH mapping of clones representing individual Z chromosomes in female and male pachytene chromosomes. BAC probe hybridization signals (red, green, yellow, violet) indicate the physical position of loci labeled by BAC clone symbols. Chromosomes were stained with DAPI (blue). Scale bar = 10 µm. (**a**) BAC-FISH image and schematic drawing of pachytene sex chromosome multivalent of *L. juvernica* female with $Z_{1–4}/W_{1–3}$ chromosome constitution. (**b**) Male pachytene complement of *L. juvernica* with four different ZZ bivalents. (**c**) BAC-FISH image and schematic drawing of pachytene sex chromosome multivalent of *L. sinapis* female with $Z_{1–3}/W_{1–3}$ chromosome constitution. (**d**) Male pachytene complement of *L. sinapis* with three different ZZ bivalents. (**e**) BAC-FISH image and schematic drawing of pachytene sex chromosome multivalent of *L. reali* female with $Z_{1–4}/W_{1–4}$ chromosome constitution. (**f**) Male pachytene complement of *L. reali* with four different ZZ bivalents. Lower panel: schematic illustrations of multiple sex chromosomes in three *Leptidea* species based on BAC-FISH results shown in upper panel. Z and W chromosomes are colored light blue and black, respectively. The phylogenetic relationships of the three *Leptidea* species are shown below the lower panel (Šíchová *et al.* 2015) with the estimated time of divergence (My= million year; Talla *et al.* 2017).

5. Discussion

The discovery of unexpected layers of cryptic diversity in *Leptidea* butterflies (Dincă *et al.* 2011) put this species complex under a thorough inspection of ecologists, ethologists, conservationists, and also geneticists. Previous studies showed, that a triplet of cryptic species with mainly Western Palearctic distribution, namely *L. juvernica*, *L. sinapis*, and *L. reali*, evolved strong prezygotic reproductive barriers in their sympatric and allopatric populations (Friberg *et al.* 2008b, Dincă *et al.* 2013). More importantly, these three species and one Eastern Palearctic species, *L. amurensis*, differs considerably in chromosome counts and also in the position of usually conserved cytogenetic markers, H3 histone genes. (Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016). Such variability is ascribed to multiple chromosomal rearrangements, specifically fusions and fissions, in the evolution of *Leptidea* karyotypes. In addition, each of four studied species has a species-specific and complex system of multiple sex chromosomes comprising of 3–4 W chromosomes and 3–6 Z chromosomes originating most probably in rearrangements between sex chromosomes and several autosomes. Although chromosome numbers vary in *Leptidea* species, even among the progeny of individual female, the sex chromosome constitutions seem to be stable in each species (Šíchová *et al.* 2015, 2016). Therefore, wood white butterflies provide an excellent opportunity to study the role of chromosome rearrangements in formation of reproductive barriers between species. However, despite the two decades of intensive research on *Leptidea* butterflies, still a little is known about the structure and evolution of their multiple sex chromosomes which seems to play an important role in speciation in this genus.

In this study, we performed comparative analysis of 3–4 Z chromosomes in three cryptic *Leptidea* species, namely *L. juvernica*, *L. sinapis*, and *L. reali*, and reconstructed the evolution of their multiple sex chromosome systems using genomic tools developed for *L. juvernica*. These are a female transcriptome-based microarray for comparative genomic hybridization (array-CGH) and a bacterial artificial chromosome (BAC) library from *L. juvernica* females. Orthologous sequences of *B. mori* genes exhibiting Z-linkage in *L. juvernica* were used to select BAC clones carrying the *Leptidea* orthologs by PCR screening of the *L. juvernica* BAC library. BAC-derived probes identified all Z chromosomes in all three *Leptidea* species by fluorescence *in situ* hybridization (BAC-FISH).

5.1. BAC-FISH mapping of Z chromosomes in *Leptidea* butterflies

In this work, we dissected the evolutionary history of multiple sex chromosomes in three cryptic *Leptidea* species by assembling the physical maps of individual Z chromosomes using BAC-FISH reprobing procedure. We used this method although DNA sequencing and genome assembly are nowadays the most frequently used techniques and the number and quality of insect genomes published is growing rapidly. Next-generation sequencing has progressed through 3rd to 4th generation sequencing, facilitating high-quality assemblies of even very complicated genomes (Heather and Chain 2016). Although sequencing technology has already been used for such high-quality genome assembly in several lepidopteran species, like for example in two strains of a serious polyphagous pest, the fall armywarm *Spodoptera frugiperda* (Noctuidae; Gouin *et al.* 2017), and in a well-known pest of pome fruit, the codling moth *Cydia pomonella* (Tortricidae; Wan *et al.* 2019), the traditional cytogenetic technique of BAC-FISH seemed to be more appropriate for our research. In such complex sex chromosome systems of *Leptidea* sp., it would be probably too difficult to identify all sex chromosomes simply from the genome assembly. Moreover, despite the nature of lepidopteran chromosomes disabling their easy identification, BAC-FISH reprobing has proved to be efficient as it has been successfully used in karyotyping, cytogenetic mapping, as well as in evolutionary analyses (Yoshido *et al.* 2005, Sahara *et al.* 2007, Yasukochi *et al.* 2009, Nguyen *et al.* 2013).

BAC-FISH mapping of *Leptidea* Z-linked genes showed that species-specific multivalents arose by repeated translocations and fusions between the ancestral WZ pair and several autosomes. The resulting Z chromosomes are compiled each from 2 or 3 conserved synteny segments, in which the gene order remained conserved in all three studied species. Nevertheless, the Z chromosomes in individual species were differentiated by subsequent chromosomal rearrangements, most probably by fissions, resulting in species-specific sex chromosome systems. Our previous study (Pospíšilová 2018) revealed that *L. juvernica* and *L. sinapis* Z_1 chromosomes arose by fusion or translocation between the ancestral Z chromosome and an autosome orthologous to *B. mori* chromosome 17. Besides, the results confirmed conserved synteny and gene order between Z_1 chromosomes in these two species. However, a comparison with the *B. mori* reference genome uncovered many intrachromosomal rearrangements underlying the formation of *Leptidea* Z_1 chromosomes. To elucidate the origin of Z_1 chromosome also in the third cryptic species, we performed BAC-FISH mapping with the same BAC clones on pachytene preparations of *L. reali*. Surprisingly, the results revealed, that Z_1 chromosome of *L. juvernica* and *L. sinapis* most probably split into two different chromosomes (Z_1 and Z_4) in *L. reali*, as the BAC probes labeled two distinct chromosomes in *L. reali* karyotype (Fig. 2). The fission appeared in the region between genes *apterous* and *kettin*, which are both Z-linked in *B. mori* (Fig. 7). Thus, we assume, that the fission most probably occurred after *L. reali* diverged from their common ancestor as both Z_1 and Z_4 of *L. reali* carry genes of the ancestral lepidopteran Z chromosome (Van't Hof *et al.* 2013,

Ahola *et al.* 2014). In addition, our results confirmed well conserved synteny and gene order between *L. juvernica* and *L. sinapis* Z_1 chromosomes and *L. reali* Z_1 and Z_4 chromosomes, since no intrachromosomal rearrangements have been observed (Fig. 7). Additionally, we proved that Z_1 arose via translocation (not fussion), because several BAC clones containing orthologs of *B. mori* chromosome 17 hybridized to autosomes in *L. juvernica* (Fig. 3; Fig. 7, underlined genes).

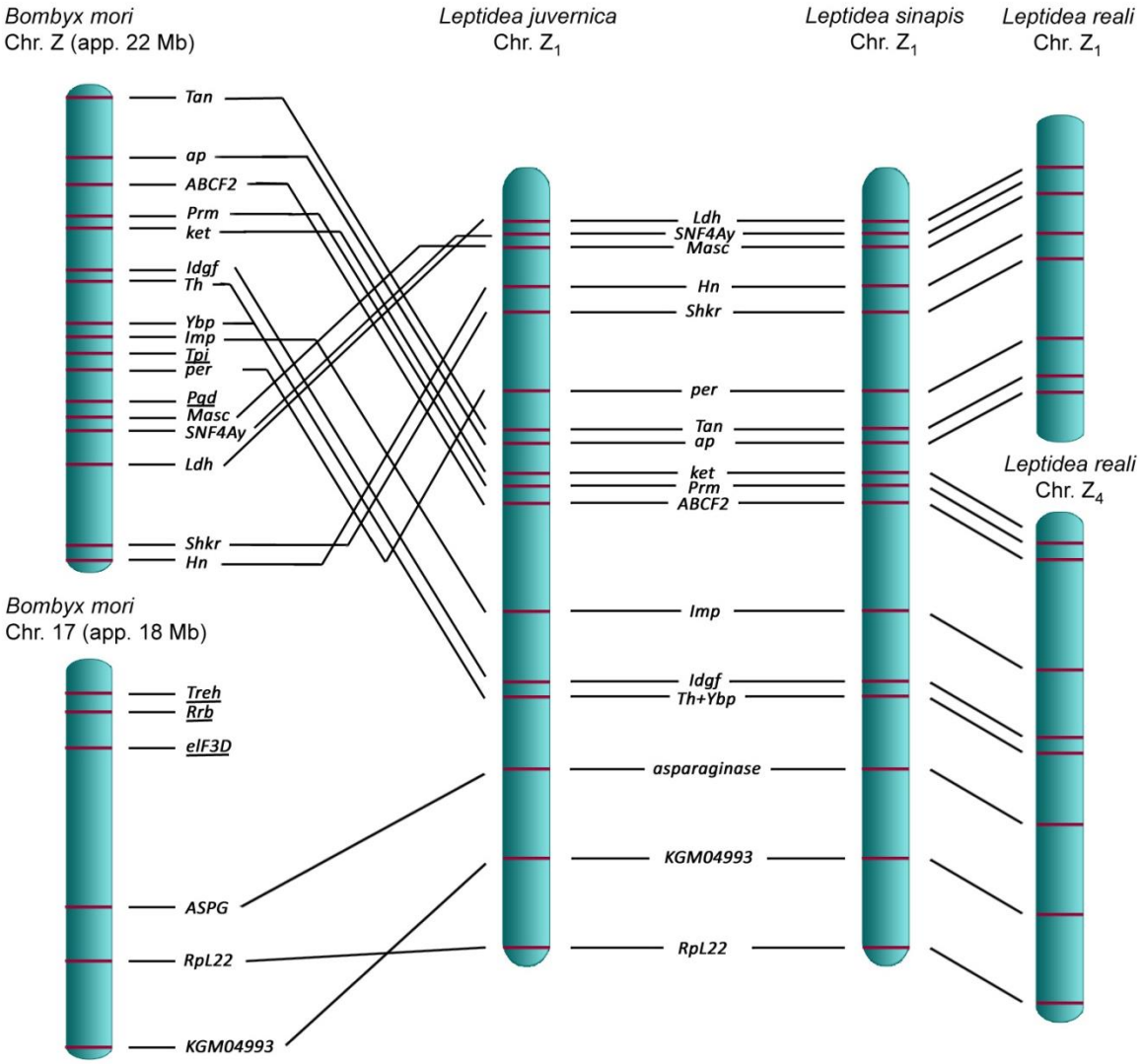

Figure 7. Schematic interpretation of *Leptidea juvernica* and *L. sinapis* Z_1 chromosomes and *L. reali* Z_1 and Z_4 chromosomes based on BAC-FISH mapping results and comparison of these chromosomes with *Bombyx mori* chr. Z and 17. Positions of *B. mori* genes were obtained from KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). Note the fission of Z_1 chromosome in *L. reali* into Z_1 and Z_4 chromosomes and conserved synteny and gene order between Z chromosomes of all three *Leptidea* species. On the contrary, complex intrachromosomal rearrangements distinguished *Leptidea* Z chromosomes and *B. mori* chr. Z and 17. The underlined genes in *B. mori* chromosomes indicate orthologous genes that mapped to autosomes in *L. juvernica*.

As for the other Z chromosomes in *L. juvernica*, *L. sinapis* and *L. reali*, BAC-FISH mapping revealed that Z_2 chromosome is the only one conserved in all three species (Fig. 4). This chromosome is composed of three segments corresponding to *B. mori* chromosomes 7, 11, and 24 and most probably evolved by translocations between autosomes as some of BAC clones containing *B. mori* chromosome 7, 11, and 24-linked genes hybridized to autosomes in *L. juvernica* (Fig. 3; Fig. 8, underlined genes). Moreover, the synteny and gene order remained consistent between all three *Leptidea* species. Subsequently, we compared physical maps of the Z_2 chromosome with corresponding *B. mori* autosomes. We found that the co-linearity of individual genes (i.e. the gene order) remained conserved except for the inverted pairs *Cpsf5-Osbp* and *CPH35-Tmc7* differentiating *Leptidea* Z_2 chromosome and *B. mori* chromosomes 11 and 24, respectively (Fig. 8). On the contrary, BAC-FISH mapping of *Leptidea* Z_3 chromosome revealed sex chromosomal rearrangements differentiating the three cryptic *Leptidea* species. We found that Z_3 chromosomes of *L. sinapis* and *L. reali* contain segments corresponding to *B. mori* chromosomes 8 and 15. However, BAC-FISH mapping with the same BAC probes identified two different chromosomes in the *L. juvernica* karyotype, now referred to as Z_3 and Z_4 chromosomes (Fig. 5). Since both chromosomes contain orthologous genes of *B. mori* chromosome 8, which are allocated on the same autosome in lepidopteran ancestral karyotype (Van't Hof *et al.* 2013, Ahola *et al.* 2014, Yasukochi *et al.* 2016), we suggest that Z_3 chromosome of *L. sinapis* and *L. reali* has undergone a fission in *L. juvernica* after its divergence from *L. sinapis* plus *L. reali*. Besides, we confirmed the conserved synteny and the gene order between all three *Leptidea* species and also with *B. mori* autosomes 8 and 15, the latter differentiating from *L. juvernica* Z_4 chromosome and *L. sinapis* and *L. reali* Z_3 chromosomes only by one inversion (Fig. 9). As in previous experiments, several BAC clones carrying orthologs of *B. mori* 8 and 15-linked genes hybridized to autosomes in *L. juvernica* (Fig. 3; Fig. 9, underlined genes) and confirmed the origin of Z_3 chromosomes by translocations between autosomes. Taken together, these findings point to dynamic chromosomal rearrangements shaping *Leptidea* karyotypes and suggest that the ancestral sex chromosome constitution of *Leptidea* butterflies was similar to that of *L. sinapis*.

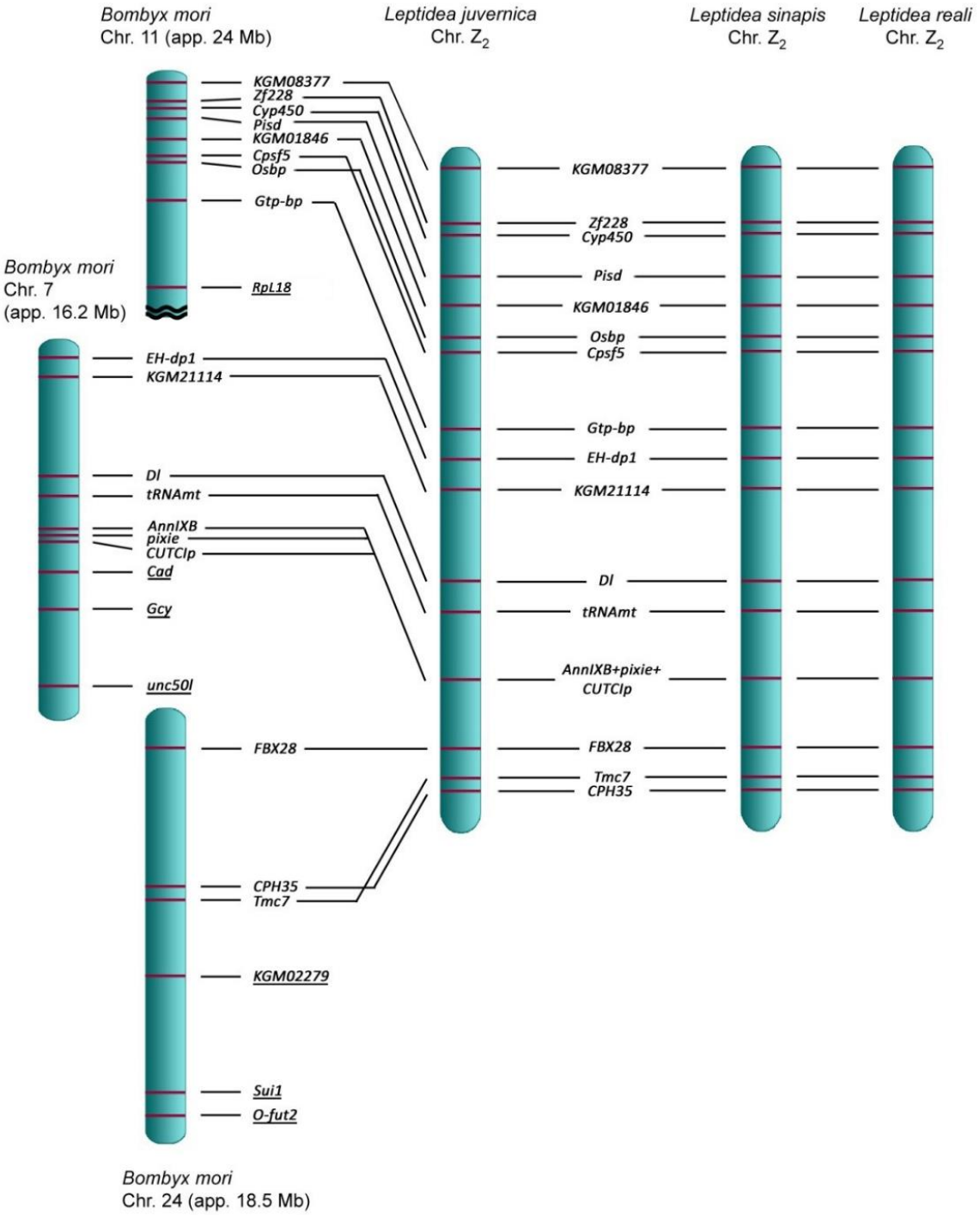

Figure 8. Schematic interpretation of Z_2 chromosomes of *Leptidea juvernica*, *L. sinapis* and *L. reali* based on BAC-FISH mapping results and their comparison with *Bombyx mori* chr. 7, 11 and 24. Positions of *B. mori* genes were obtained from KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). The synteny and gene order between Z_2 chromosomes of *Leptidea* species and *B. mori* chr. 7, 11, and 24 remained conserved except for the inversions of genes *Cpsf5* with *Osbp* and *CPH35* with *Tmc7* differentiating *Leptidea* Z_2 chromosomes from *B. mori* chr. 11 and 24, respectively. The underlined genes in *B. mori* chromosomes indicate orthologous genes that mapped to autosomes in *L. juvernica*.

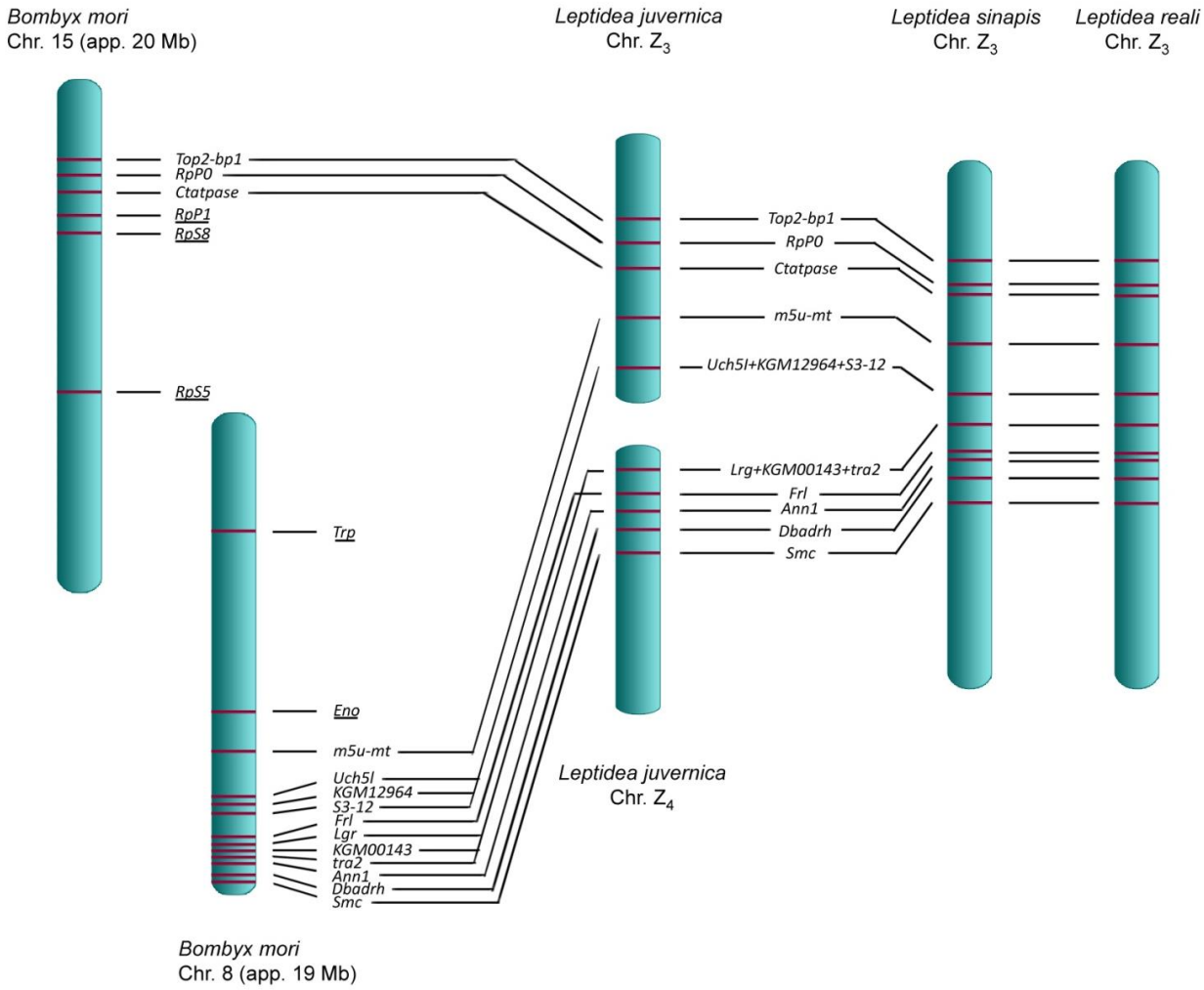

Figure 9. Schemetic interpretation of *Leptidea juvernica* Z_3 and Z_4 chromosomes and Z_3 chromosome of *L. sinapis* and *L. reali* based on BAC-FISH mapping data and comparison of these chromosomes with *Bombyx mori* chr. 8 and 15. Positions of *B. mori* genes were obtained from KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). Note the fission of Z_3 chromosome of *L. sinapis* and *L. reali* into Z_3 and Z_4 chromosomes in *L. juvernica*. The synteny of genes and gene order remained conserved between all three *Leptidea* species. Only two BAC clones carrying orthologous genes *Lrg_KGM00143_tra2* and *Frl*, mapped in inverted order in all *Leptidea* species and thus differentiated *Leptidea* chromosomes from *B. mori* chr. 8. The underlined genes in *B. mori* chromosomes indicate orthologous genes that mapped to autosomes in *L. juvernica*.

Conserved synteny blocks and consistent gene order in the Z chromosomes of so far studied *Leptidea* species correspond with the current knowledge of the genome structure in Lepidoptera. As was mentioned in the chapter 1.1.1., the modal and the ancestral chromosome number of chromosomes in the haploid genome is n=31. Comparative genome mapping also showed remarkable karyotype stability, conserved synteny and gene order among distantly related lepidopteran species (Baxter *et al.* 2011, Sahara *et al.* 2013, Van't Hof *et al.* 2013, Ahola *et al.* 2014, Yasukochi e*t al.* 2016). Number of studies also found conserved gene content in the ancestral Z chromosome across the lepidopteran phylogenetic tree (Beldade *et al.* 2009, Yasukochi *et al.* 2009, Nguyen *et al.* 2013, Van't Hof *et al.* 2013, Dalíková *et al.* 2017a, Fraïsse *et al.* 2017), but reported broken order of genes resulting from large-scale intrachromosomal rearrangements (Yasukochi *et al.* 2009, Van't Hof *et al.* 2013). *Leptidea* Z chromosomes seem to possess similar characteristics. Despite many chromosomal rearrangements between sex chromosomes and autosomes in their genome, the vast majority of ancestral Z-linked genes remained Z-linked in *Leptidea* as well. The results of array-CGH demonstrated that only approximately 9% of those genes escaped from *Leptidea* Z_1 (and Z_4 in *L. reali*) chromosomes (Yoshido *et al.*, in prep.). An interesting fact is that the order of the ancestral Z-linked genes in *L. juvernica* and *L. sinapis* Z_1 chromosomes and *L. reali* Z_1 and Z_4 chromosomes was much more rearranged compared to genes with autosomal origin. This curiosity may be explained by different evolutionary rates and dynamics of the sex chromosomes compared to autosomes as evolutionary processes such as mutation, random genetic drift, selection, and genomic conflict act more rapidly on sex chromosomes than on autosomes (Johnson and Lachance 2012). However, the fact that the set of the ancestral Z-linked genes, which are known to be involved in the sex determination (Kiuchi *et al.* 2014), adaptation and speciation (Presgraves 2002, Dopman *et al.* 2005), avoided the dynamic reorganization of *Leptidea* genomes and remained sex-linked is in accordance with the key role of Z chromosomes in the evolution of Lepidoptera.

5.2. Turnover of multiple sex chromosomes

The origin of neo-sex chromosomes via fusion of the ancestral sex chromosome(s) with one or more autosomes has been documented in number of lepidopteran taxa. However, the autosomal parts of the neo-sex chromosomes differ between lepidopteran groups. The study of Yoshido *et al.* (2011a, b) on geographical subspecies of wild silkmoths, *Samia cynthia* ssp. (Saturniidae), revealed that neo-Z chromosome in Sapporo population of *S. c. walkeri* (neo-Wneo-Z) originated by fusion between the ancestral Z chromosome and two autosomes corresponding to *B. mori* chromosome 8 and 12. Similarly, the neo-Z_1 chromosome in Nagano population of *S. cynthia* subsp. indet. (neo-WZ_1Z_2) carried part of the ancestral Z chromosome and two autosomes corresponding to *B. mori* chromosome 8 and 12, whereas neo-Z_2 chromosome carried parts of *B. mori* chromosome 11 and 24. Another study of

Nguyen *et al.* (2013) showed that Z chromosome in the codling moth, *Cydia pomonella* (Tortricidae), and other tortricids arose by fusion between the ancestral Z chromosome and an autosome orthologous to *B. mori* chromosome 15. Similar picture emerged from the comparison of another tortricid representative, *Choristoneura fumiferana*, whose neo-Z chromosome also contains parts of the ancestral Z chromosome and an autosome orthologous to *B. mori* chromosome 15 (Picq *et al.* 2018), thus supporting the results of Nguyen *et al.* (2013). Besides, sex chromosome-autosome fusions have been also spotted across the large superfamily Gelechioidea (Carabajal Paladino *et al.* 2019). Recent studies also revealed interesting data on neo-sex chromosomes in *Danaus* butterflies. Mongue *et al.* (2017) provided an evidence for a Z chromosome-autosome fusion in the monarch butterfly, *D. plexippus,* involving an autosome homologous to chromosome 21 in *Melitaea cinxia*, which largely corresponds to *B. mori* chromosome 16 (Ahola *et al.* 2014). Several studies also suggested that the fussion event occurred in the common ancestor of *Danaus* genus (Ahola *et al.* 2014, Mongue *et al.* 2017, Traut *et al.* 2017). This idea was supported by the study of Traut *et al.* (2017) on the W chromosome of *D. plexippus*, and closely related *D. chrysippus*. Despite the degradation of the W chromosome, they confirmed the fusion event between the original W and an autosome homologous to *B. mori* chromosome 16, whose partner subsequently fused with the Z chromosome as was described in Mongue *et al.* (2017). In addition, the more recent fusion event was discovered in the African hybrid population of *D. chrysippus*, generating the sex chromosome constitution of neo-WZ_1Z_2 (Traut *et al.* 2017). The study of Martin *et al.* (2020) revealed that the neo-W chromosome in the hybrid population of *D. chrysippus* carries a region from chromosome 15 containing a color patterning allele of the so-called BC supergene. Moreover, the presence of the BC supergene is linked with the infection of maternally inherited male-killer endosymbiont *Spiroplasma ixodetis* driving female-biased sex-ratios. The fussion between the neo-W and chromosome 15 explains reduced chromosome number in *D. chrysippus* females (n=29) compared to males (n=60) in this population (Traut *et al.* 2017), and the sex chromosome constitution of the neo-WZ_1Z_2, as the remaining unfused partner of chromosome 15 most probably became the Z_2 chromosome.

Our study on three cryptic *Leptidea* species adds to accumulating evidence for turnover of the sex chromosomes by fusions between sex chromosomes with one or more autosomes. Nevertheless, the evolution of multiple sex chromosomes in these species is much more complicated. Multiple sex chromosomes of three *Leptidea* species examined in this study are composed of ancestral Z chromosome and parts of six different autosomes. Moreover, the physical mapping of BAC clones, which did not prove Z-linkage in *Leptidea* butterflies, showed that even BAC clones carrying orthologous genes from the same *B. mori* autosome are located on different autosomes in *L. juvernica* karyotype (Fig. 3). These results, together with profound variability in chromosome numbers in *Leptidea* species, indicate that not only sex chromosomes, but also autosomes have undergone a

dynamic reshuffling in the evolution of *Leptidea* karyotypes before *Leptidea* species diverged from a common ancestor. Thus, *Leptidea* butterflies do not possess a conserved macrosynteny, i.e. synteny involving a large number of genes, compared to the putative ancestral karyotype of Lepidoptera (Van't Hof *et al.* 2013, Ahola *et al.* 2014). Our results are in agreement with the genome assembly of two pierid species, *Pieris rapae* (Shen *et al.* 2016, Nallu *et al.* 2019) and *P. napi* (Hill *et al.* 2019), which revealed extensive series of chromosomal rearrangements resulting in broken macrosynteny blocks leaving conserved only small microsynteny segments. However, it would be too doubtful to assume that crumbled macrosynteny is a hallmark of Pieridae, as *Pieris* and *Leptidea* are very loosely related within this large family comprising of 85 genera (Wahlberg *et al.* 2014).

Based on our results, we suggested a hypothetical step-by-step evolution of multiple sex chromosomes in the three cryptic wood whites studied. We assume that large-scaled rearrangements occurred in the common ancestor of these *Leptidea* species (Fig. 10a), since they possess a similar constitution of multiple sex chromosomes. The proto-WZ chromosomes in ancestral females arose from the translocation of a part of an autosome orthologous to *B. mori* chromosome 17 to the ancestral pair of WZ sex chromosomes. Simultaneously, fusions of three autosomal blocks homologous to *B. mori* chromosomes 7, 11, and 24, and fusions of two autosomal blocks homologous to *B. mori* chromosomes 8 and 15 gave rise to two pairs of autosomes, referred to as proto-Z_2 and proto-Z_3, respectively (Fig. 10b). Ancestral males already possessed the composition of Z_1, Z_2, and Z_3 chromosomes, but proto-Z_2 and proto-Z_3 were still autosomes in both sexes. Subsequently, in ancestral females, proto-Z_2 and proto-Z_3 fused with proto-W chromosome and their unfused homologues become Z_2 and Z_3 sex chromosomes (Fig. 10c). In the new-born neo-W chromosome, the original parts of autosomes gradually degraded thanks to the suppressed recombination in female meiosis (Fig. 10d). Finally, two fissions in neo-W (Fig. 10e) resulted in formation of multiple sex chromosomes with three W ($W_{1–3}$) and three Z ($Z_{1–3}$) chromosomes (Fig. 10f). Hence, the constitution of sex chromosome multivalent in the common ancestor of *Leptidea* species cryptic triplet was similar to that in *L. sinapis* (Fig. 10g). Afterwards, several chromosomal rearrangements differentiated sex chromosome constitution of *Leptidea* butterflies. In *L. juvernica*, fission of the Z_3 chromosome resulted in $W_{1–3}Z_{1–4}$ constitution (Fig. 10h), whereas fission of one of the W chromosome and fission of the Z_1 chromosome led to the $W_{1–4}Z_{1–4}$ constitution, as found in *L. reali* (Fig. 10i). Thus, each of the cryptic wood whites has a species-specific sex chromosome constitution. To confirm that neo-W chromosomes indeed originated in sex chromosome-autosome fusion, my colleagues also tried to find homologous sequences on *Leptidea* W chromosomes. Despite its extensive genetic erosion and accumulation of repetitive DNA sequences, which is typical for lepidopteran W chromosome (Abe *et al.* 2005, Fuková *et al.* 2007, Marec *et al.* 2010, Yoshido *et al.* 2016, Dalíková *et al.* 2017b), my colleagues identified W-homologs of Z-linked orthologs and characterized their molecular differentiation (Yoshido *et al.*, in

prep.). This finding supports the evidence for the origin of multiple sex chromosomes by chromosome rearrangements in the common ancestor of *Leptidea* species.

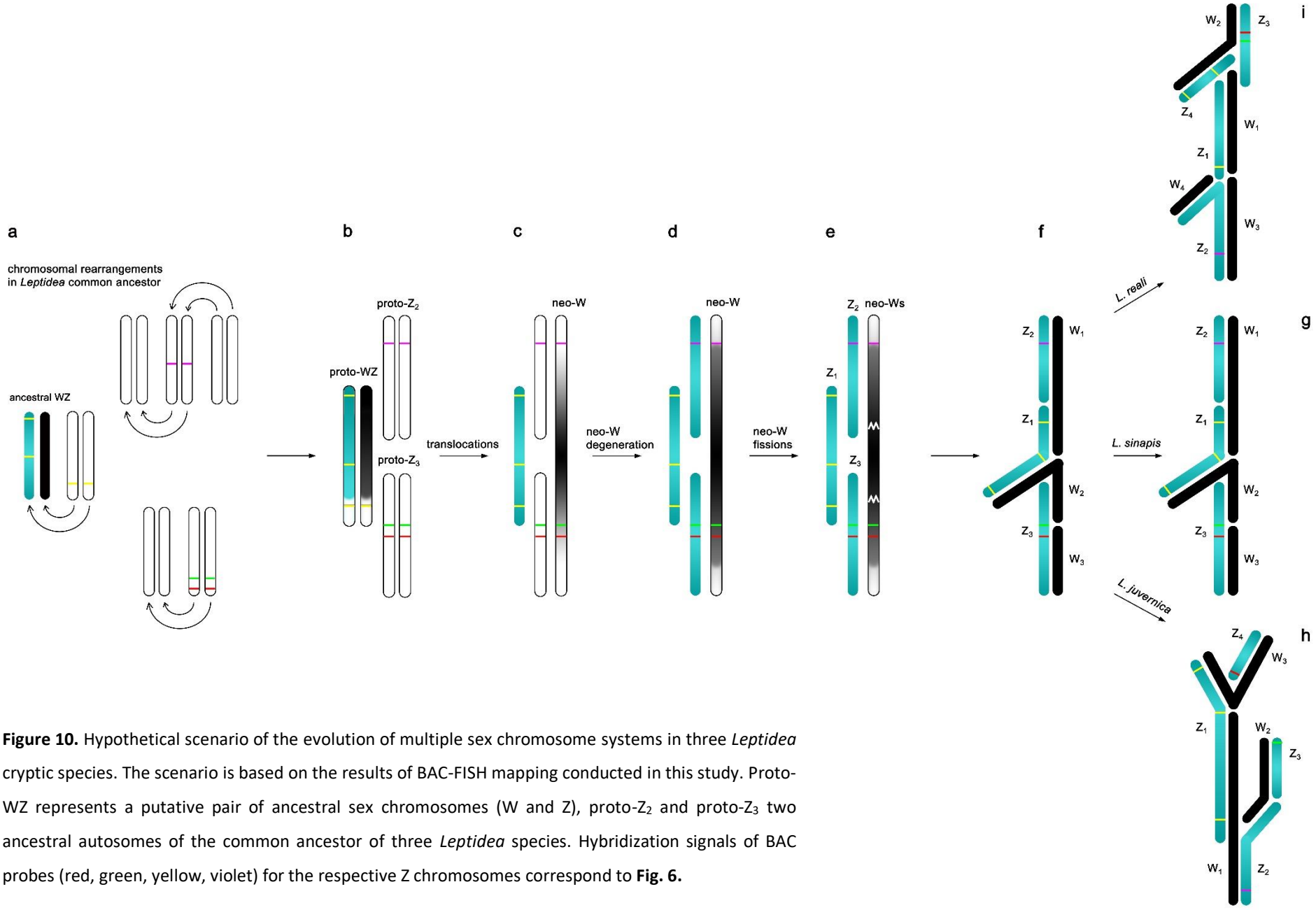

Figure 10. Hypothetical scenario of the evolution of multiple sex chromosome systems in three *Leptidea* cryptic species. The scenario is based on the results of BAC-FISH mapping conducted in this study. Proto-WZ represents a putative pair of ancestral sex chromosomes (W and Z), proto-Z_2 and proto-Z_3 two ancestral autosomes of the common ancestor of three *Leptidea* species. Hybridization signals of BAC probes (red, green, yellow, violet) for the respective Z chromosomes correspond to **Fig. 6.**

5.3. The role of chromosomal rearrangements in speciation

Chromosomal rearrangements between sex chromosomes and autosomes increase the number of genes under sex-linkage. The question is why it is so beneficial for genes to be sex-linked? This curiosity can be explained by so-called 'Large-X effect'. Based on introgression analysis of hybrid incompatibilities, the 'Large-X effect' proposes that X chromosome has much larger impact on hybrid fitness in comparison with autosomes (Masly and Presgraves 2007) and is a hotspot for speciation genes preventing genetic flow between closely related species (Storchová *et al.* 2004, Good *et al.* 2008, Macholán *et al.* 2007). Consequently, higher portions of X-linked genes can facilitate postzygotic reproductive isolation and speciation (Presgraves 2008, Storchová *et al*. 2010). A similar effect was also discerned in organisms with female heterogamety, e.g. birds and butterflies, referred to as 'large Z-effect' (Sætre *et al*. 2003, Ellegren 2009, Storchová *et al*. 2010). For example, the contribution of the Z chromosome to the adaptation and speciation was explored in leafrollers of the family Totricidae (Nguyen *et al.* 2013, Picq *et al.* 2018). Due to the fusion of the ancestral Z chromosome and an autosome orthologous to *B. mori* chromosome 15, genes accountable for the insecticide resistance and genes included in the detoxification of plant secondary metabolites have become sex-linked and contributed to radiation and speciation in tortricid moths (Nguyen *et al.* 2013, Picq *et al.* 2018). Similarly, the role of neo-sex chromosomes in speciation was also discussed in the superfamily Gelechioidea. The Z chromosome in this large group of moths is enriched with originally autosomal gene clusters of UDP-glucosyltransferases, which are responsible for the detoxification of plant secondary metabolites as in above mentioned Tortricidae (Carabajal Paladino *et al.* 2019). However, not only Z chromosome, but also the W chromosome can greatly contribute to ecological specialization, reproductive isolation, and speciation in Lepidoptera. This was shown on the neo-W chromosome of the African queen butterfly, *Danaus chrysippus*, which is responsible for genetic separation of two incipient species across the hybrid zone by linking the color pattern and male-killing caused by an endosymbiotic bacterium, *Spiroplasma ixodeti* (Smith *et al.* 2016, Traut *et al.* 2017, Martin *et al.* 2020).

Our study provides the evidence for sex chromosome-autosome fusions resulting in species-specific composition of multiple sex chromosomes in three cryptic *Leptidea* species. It is tempting to speculate that differences in the constitution of multiple sex chromosomes between these species played a key role in their isolation. However, our data shows that the multiple sex chromosome system preceded the evolution of *Leptidea* species studied. Therefore, the sex chromosome turnover could not have been the main engine driving the formation of reproductive barriers between species. This idea is supported by recent genome-wide sequence analysis, which did not detect any post-divergence gene flow among three cryptic wood whites (Talla *et al.* 2019) and confirmed previously reported well-established pre-mating barriers maintained by female acceptance of only conspecific males (Friberg *et*

al. 2008b, Dincă *et al.* 2013). All species also showed significantly reduced genetic diversity on the 'ancestral' Z chromosome (corresponding to part of Z_1 in this study, multiple Z chromosomes were not considered) and higher level of genetic differentiation of the ancestral Z chromosome compared to autosomes (Talla *et al.* 2019). One possible explanation for rapid divergence of Z-linked coding sequences in comparison with autosomal ones is the so-called 'Fast-Z effect' (Presgraves 2008, Mank *et al*. 2010). Briefly, in heterozygotes, new recessive mutations are not concealed by standard allele and thus are immediately preferred by selection. Consequently, sex-linked genes undergo a faster evolution compared to autosomes (Mank *et al.* 2010). In *Leptidea*, multiple sex chromosomes originated in chromosomal rearrangements between the ancestral Z chromosomes and several autosomes. Although sex chromosome turnover could not be a key factor in the formation of reproductive barriers between species, chromosomal rearrangements increased the number of Z-linked genes and thus might have driven the accumulation of genetic incompatibilities facilitating subsequent divergence and speciation in *Leptidea* butterflies.

In future, with the use of BAC library and sequenced transcriptome of *L. juvernica*, we plan to perform BAC-FISH mapping also in the Eastern Palearctic species, *L. amurensis*, with ♀$W_{1–3}Z_{1–6}$/♂$Z_{1–6}Z_{1–6}$ sex chromosome constitution. Furthermore, we suggest to identify the sex chromosome systems in two basal *Leptidea* species, *L. morsei* and *L. duponcheli*, as knowledge of the sex chromosome constitutions in these two species is crucial for understanding the piecemeal evolution of multiple sex chromosome systems in *Leptidea* wood white butterflies.

6. Conclusions

In this study, we performed a detailed comparative analysis of 3–4 Z chromosomes in three cryptic *Leptidea* species, namely *L. juvernica*, *L. sinapis*, and *L. reali*, and reconstructed the evolution of their species-specific multiple sex chromosome systems. Fluorescence *in situ* hybridization with clones derived from bacterial artificial chromosomes (BAC-FISH) clearly showed that Z chromosomes arose by translocations between the ancestral WZ pair and six different autosomes in the common ancestor of genus *Leptidea*. Each Z chromosome consists of 2 or 3 conserved segments, in which the collinearity of genes remained conserved between all three species studied. However, after the divergence of these cryptic species, the Z chromosomes differentiated by several fissions resulting in a unique species-specific sex chromosome constitution. In addition, the comparison of *Leptidea* Z chromosomes with *B. mori* reference genome uncovered a high level of conserved synteny blocks between *B. mori* and *Leptidea* butterflies. This finding is consistent with the current knowledge of the karyotype stability and conserved gene content in the 'ancestral' Z chromosome and the autosomes across the lepidopteran phylogenetic tree. Nevertheless, the gene order of *Leptidea* Z-linked genes was different from the gene order of *B. mori*, especially in the ancestral Z_1 chromosome in *L. juvernica* and *L. sinapis*, and Z_1 plus Z_4 in *L. reali* indicating dynamic intrachromosomal rearrangements.

Taken together, our study brought evidence for the origin of multiple sex chromosomes by means of complex chromosomal rearrangements between sex chromosomes and autosomes. In addition, we reconstructed the step-by-step evolution of multiple sex chromosome system, which preceded the formation of *Leptidea* species studied and thus could not be the main engine driving speciation in this genus. We propose, that chromosomal rearrangements increasing the number of Z-linked genes could themselves play a crucial role in accumulation of genetic incompatibilities facilitating subsequent divergence and speciation in *Leptidea* butterflies.

7. References

Abe H, Mita K, Yasukochi Y, Oshiki T, Shimada T (2005) Retrotransposable elements on the W chromosome of the silkworm, *Bombyx mori*. *Cytogenet Genome Res* **110:** 144–151.

Ahola V, Lehtonen R, Somervuo P, Salmela L, Koskinen P, Rastas P, Välimäki N, Paulin L, Kvist J, Wahlberg N, Tanskanen J, Hornett EA, Ferguson LC, Luo S, Cao Z, de Jong MA, Duplouy A, Smolander O-P, Vogel H, McCoy RC, Qian K, Chong WS, Zhang Q, Ahmad F, Haukka JK, Joshi A, Salojärvi J, Wheat CW, Grosse-Wilde E, Hughes D, Katainen R, Pitkänen E, Ylinen J, Waterhouse RM, Turunen M, Vähärautio A, Ojanen SP, Schulman AH, Taipale M, Lawson D, Ukkonen E, Mäkinen V, Goldsmith MR, Holm L, Auvinen P, Frilander MJ, Hanski I (2014) The *Glanville fritillary* genome retains an ancient karyotype and reveals selective chromosomal fusions in Lepidoptera. *Nat Commun* **5:** 4737.

Anjos A, Paladini A, Evangelista O, Cabral-de-Mello DC (2018) Insights into chromosomal evolution of Cicadomorpha using fluorochrome staining and mapping 18S rRNA and H3 histone genes. *J Zool Syst Evol Res* **57:** 314–322.

Baker RJ, Bickham JW (1986) Speciation by monobrachial centric fusions. *Proc Natl Acad Sci U S A* **83:** 8245–8248.

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.

Bardella VB, Dias AL, Giuliano-Caetano L, Ribeiro JRI, Da Rosa R (2012) Sex chromosome differentiation in *Belostoma* (Insecta: Heteroptera: Belostomatidae). *Genet Mol Res* **11:** 2476–2486.

Baxter SW, Davey JW, Johnston JS, Shelton AM, Heckel DG, Jiggins CD, Blaxter ML (2011) Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. *PLoS ONE* **6:** e19315.

Beldade P, Saenko SV, Pul N, Long AD (2009) A gene-based linkage map for *Bicyclus anynana* butterflies allows for a comprehensive analysis of synteny with the lepidopteran reference genome. *PLoS Genet* **5:** e1000366.

Beneš J, Konvička M, Vrabec V, Zámečník J (2003) Do the sibling species of small whites, *Leptidea sinapis and L. reali* (Lepidoptera, Pieridae) differ in habitat preferences? *Biologia-Bratislava* **58:** 943–952.

Bickford D, Lohman D, Sodhi N, Ng P, Meier R, Winker K, Ingram K, Das I (2007) Cryptic species as a window on diversity and conservation. *Trends Ecol Evol* **22:** 148–155.

Bigger TRL (1976) Karyotypes of three species of Lepidoptera including an investigation of B-chromosomes in *Pieris*. *Cytologia* **41:** 261–282.

Blackmon H, Ross L, Bachtrog D (2017) Sex Determination, Sex Chromosomes, and Karyotype Evolution in Insects. *J Hered* **108:** 78–93.

Bolshakov LV (2006) New subspecies of *Leptidea reali* Reissinger, 1989 (Lepidoptera: Pieridae) from the mountain regions of Middle Asia. *Eversmannia* **5:** 6–10.

Brown KS Jr, von Schoultz B, Suomalainen E (2004) Chromosome evolution in Neotropical Danainae and Ithomiinae (Lepidoptera). *Hereditas* **141:** 216–236.

Cabral-de-Mello DC, Oliveira SG, de Moura RC, Martins C (2011) Chromosomal organization of the 18S and 5S rRNAs and histone H3 genes in *Scarabaeinae coleopterans*: insights into the evolutionary dynamics of multigene families and heterochromatin. *BMC Genet* **12:** 88.

Cabrero J, López-León MD, Teruel M, Camacho JPM (2009) Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. *Chromosome Res* **17:** 397–404.

Camacho JPM, Sharbel TF, Beukeboom LW (2000) B-chromosome evolution. *Phil Trans R Soc Lond B* **355:** 163–178.

Carabajal Paladino LZ, Provazníková I, Berger M, Bass C, Aratchige NS, López SN, Marec F, Nguyen P (2019) Sex chromosome turnover in moths of the diverse superfamily Gelechioidea. *Genome Biol Evol* **11:** 1307–1319.

Carpenter JE, Bloem S, Marec F (2005) Inherited sterility in insects. In: Dyck VA, Hendrichs J, Robinson AS (eds.) *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management.* Springer, Dordrecht, The Netherlands. Pp. 115–146.

Clarke S, Green D, Joy J, Wollen K, Butler I (2011) *Leptidea sinapis* (wood white butterfly) egg-laying habitat and adult dispersal studies in Herefordshire. *J Insect Conserv* **15:** 23–35.

d'Alençon E, Sezutsu H, Legeai F, Permal E, Bernard-Samain S, Gimenez S, Gagneur C, Cousserans F, Shimomura M, Brun-Barale A, Flutre T, Couloux A, East P, Gordon K, Mita K, Quesneville H, Fournier P, Feyereisen R (2010) Extensive synteny conservation of holocentric chromosomes in Lepidoptera despite high rates of local genome rearrangements. *Proc Natl Acad Sci U S A* **107:** 7680–7685.

Dalíková M, Zrzavá M, Hladová I, Nguyen P, Šonský I, Flegrová M, Kubíčková S, Voleníková A, Kawahara AY, Peters RS, Marec F (2017a) New insights into the evolution of the W chromosome in Lepidoptera. *J Hered* **108:** 709–719.

Dalíková M, Zrzavá M, Kubíčková S, Marec F (2017b) W-enriched satellite sequence in the Indian meal moth, *Plodia interpunctella* (Lepidoptera, Pyralidae). *Chromosome Res* **25:** 241–252.

De Lesse H (1960) Spéciation et variation chromosomique chez les Lépidoptères Rhopalocères. *Annales des sciences naturelles. Zoologie et biologie animale* **2:** 1–223.

De Oliveira EA, Sember A, Bertollo LAC Yano CF, Ezaz T, Moreira-Filho O, Hatanaka T, Trifonov V, Liehr T, Al-Rikabi ABH, Ráb P, Pains H, Cioffi MB (2018) Tracking the evolutionary pathway of sex chromosomes among fishes: characterizing the unique XX/XY1Y2 system in *Hoplias malabaricus* (Teleostei, Characiformes). *Chromosoma* **127:** 115–128.

De Prins J, Saitoh K (2003) Karyology and sex determination. In: Kristensen NP (ed.) *Lepidoptera, Moths and Butterflies: Morphology, Physiology, and Development.* Walter de Gruyter, Berlin, Germany. Pp. 449–468.

Deng ZY, Zhang Y, Zhang M, Huang J, Ni X, Li X (in press) Characterization of the first W-unique protein-coding gene for gender determination in *Helicoverpa armigera*. *Front Genet* (in press).

Descimon H, Mallet J (2009) Bad species. In: Settele J, Shreeve TG, Konvicka M, Dyck VH (eds.) *Ecology of Butterflies in Europe.* Cambridge University Press, Cambridge, UK. Pp. 219–249.

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, Norén K, Dapporto L, Wahlberg N, Vila R, Friberg M (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 U S A* **102:** 14706–14711.

Ellegren H (2009) Genomic evidence for large-Z effect. *Proc R Soc Lond B Biol Sci* **276:** 361–366.

Fraïsse 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, Wiklund C (2009) Host plant preference and performance of the sibling species of butterflies *Leptidea sinapis* and *Leptidea reali*: a test of the trade-off hypothesis for food specialisation. *Oecologia* **159:** 127–13.

Friberg M, Leimar O, Wiklund C (2013) Heterospecific courtship, minority effects and niche separation between cryptic butterfly species *J Evol Biol* **26:** 971–979.

Friberg M, Olofsson M, Berger D, Karlsson B, Wiklund C (2008b) Habitat choice precedes host plant choice-niche separation in a species pair of a generalist and a specialist butterfly. *Oikos* **117:** 1337–1344.

Friberg M, Vongvanich N, Borg-Karlson AK, Kemp DJ, Merilaita S, Wiklund C (2008a) Female mate choice determines reproductive isolation between sympatric butterflies. *Behav Ecol Sociobiol* **62:** 873–886.

Fuková I, Nguyen P, Marec F (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. *Genome* **48:** 1083–1092.

Fuková I, Traut W, Vítková M, Nguyen P, Kubíčková S, Marec F (2007) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. *Chromosoma* **116:** 135–145.

Goldstein PZ (2017) *Diversity and significance of Lepidoptera: A phylogenetic perspective.* In: Foottit RG, Adler PH (eds.) *Insect biodiversity: Science and society.* Blackwell Publishing, Oxford, UK, 2nd ed. Pp. 463–495.

Good JM, Dean MD, Nachman MW (2008) A complex genetic basis to X-linked hybrid male sterility between two species of house mice. *Genetics* **179:** 2213–2228.

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.

Gouin A, Bretaudeau A, Nam K, Gimenez S, Aury JM, Duvic B, Hilliou F, Durand N, Montagné N, Darboux I, Kuwar S, Chertemps T, Siaussat D, Bretschneider A, Moné Y, Ahn SJ, Hänniger S, Grenet AG, Neunemann D, Maumus F, Luyten I, Labadie K, Xu W, Koutroumpa F, Escoubas JM, Llopis A, Maïbèche-Coisne M, Salasc F, Tomar A, Anderson AR, Khan SA, Dumas P, Orsucci M, Guy J, Belser C, Alberti A, Noel B, Couloux A, Mercier J, Nidelet S, Dubois E, Liu NY, Boulogne I, Mirabeau O, Le Goff G, Gordon K, Oakeshott J, Consoli FL, Volkoff AN, Fescemyer HW, Marden JH, Luthe DS, Herrero S, Heckel DG, Wincker P, Kergoat GJ, Amselem J, Quesneville H, Groot AT, Jacquin-Joly E, Nègre N, Lemaitre C, Legeai F, d'Alençon E, Fournier P (2017) Two genomes of highly polyphagous lepidopteran pests (*Spodoptera frugiperda*, Noctuidae) with different host-plant ranges. *Sci Rep* **7:** 11816.

Graves J (2016) Did sex chromosome turnover promote divergence of the major mammal groups? *Bioessays* **38:** 734–743.

Grimaldi D, Engel MS (2005) *Evolution of the Insects*. Cambridge University Press, Cambridge, UK. Pp. 755.

Gruetzner F, Ashley T, Rowell DM, Graves JAM (2006) How did the platypus get its sex chromosome chain? A comparison of meiotic multiples and sex chromosomes in plants and animals. *Chromosoma* **115:** 75–88.

Grützner F, Rens W, Tsend-Ayush E, El Mogharbel N, O'Brien PC, Jones RC, Ferguson-Smith MA, Marshall Graves JA (2004) In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature* **432:** 913–917.

Gunski RJ, Cañedo AD, Garnero ADV, Ledesma MA, Coria N, Montalti D, Degrandi TM (2017) Multiple sex chromosome system in penguins (Pygoscelis, Spheniscidae). *Comp Cytogenet* **11:** 541–552.

Hearn L (1904*) Kwaidan: Stories and Studies of Strange Things*. Houghton Mifflin Company, New York. Pp. 240.

Heather JM, Chain B (2016) The sequence of sequencers: the history of sequencing DNA. *Genomics* **107:** 1–8.

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.

Hill J, Rastas P, Hornett EA, Neethiraj R, Clark N, Morehouse N, de la Paz Celorio-Mancera M, Cols JC, Dircksen H, Meslin C, Keehnen N, Pruisscher P, Sikkink K, Vives M, Vogel H, Wiklund C, Woronik A, Boggs CL, Nylin S, Wheat CW. Unprecedented reorganization of holocentric chromosomes provides insights into the enigma of lepidopteran chromosome evolution. *Sci Adv* (2019) **5:** eaau3648.

Ivonin VV, Kosterin OE, Nikolaev SL (2009) Butterflies (Lepidoptera, Diurna) of Novosibirsk Province, Russia. 1. Hesperiidae, Papiliondae, Pieridae. *Euroasian Ecol Entomol* **8:** 85–104.

Johnson NA, Lachance J (2012) The genetics of sex chromosomes: evolution and implications for hybrid incompatibility. *Ann N Y Acad Sci* **1256:** E1–E22.

Jones RN (2012) B chromosomes in plants. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology: Official Journal of the Societa Botanica Italiana* **146:** 727–737.

Jones RN, Gonzalez-Sanchez M, Gonzalez-Garcia M, Vega JM, Puertas MJ (2008) Chromosomes with a life of their own. *Cytogenet Genome Res* **120:** 265–280.

Kato A, Albert P, Vegy J, Bircher J (2006) Sensitive fluorescence *in situ* hybridization signal detection in maize using directly labelled probes produced by high concentration DNA polymerase nick translation. *Boitech Histochem* **81:** 71–78.

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, Absher DM, Grimwood J, Schmutz J, Myers RM, Kingsley DM, Peichel CL (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, Ishihara G, Kawaoka S, Sugano S, Shimada T, Suzuki Y, Suzuki MG, Katsuma S (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* **509:** 633–636.

Kosterin OE, Sergeev MG, Dubatolov VV (2007) Butterflies (Lepidoptera, Diurna). In: Zhimulev IF (ed.) *Nature of Academy Town: 50 Years After.* Novosibirsk, Russia. Pp. 105133 (in Russian).

Král J, Forman M, Kořínková T, Reyes Lerma AC, Haddad C, Musilová J, Řezáč M, Ávila Herrera IM, Thakur S, Dippenaar-Schoeman AS, Marec F, Horová L, Bureš P (2019) Insights into the karyotype and genome evolution of haplogyne spiders indicate a polyploid origin of lineage with holokinetic chromosomes. *Sci Rep* **9:** 3001.

Lenormand T, Engelstädter J, Johnston SE, Wijnker E, Haag CR (2016) Evolutionary mysteries in meiosis. *Philos Trans R Soc Lond B Biol Sci* **371:** 20160001.

Lorković Z (1941) Die Chromosomenzahlen in der Spermatogenese der Tagfalter. *Chromosoma* **2:** 155–191.

Lorković Z (1993) *Leptidea reali* Reissinger, 1989 (=lorkovicii Real 1988), a new European species (Lepid., Pieridae). *Nat Croatica* **2:** 1–26.

Lukhtanov VA (1992) Karyotype evolution and systematics of higher taxa of Pieridae (Lepidoptera) of the world. *Entomol Rev* **71:** 57–82.

Lukhtanov VA (2000) Sex chromatin and sex chromosome systems in nonditrysian Lepidoptera (Insecta). *J Zoolog Syst Evol Res* **38:** 73–79.

Lukhtanov VA (2014) Chromosome number evolution in skippers (Lepidoptera, Hesperiidae). *Comp Cytogenet* **8:** 275–291.

Lukhtanov VA (2015) The blue butterfly *Polyommatus (Plebicula) atlanticus* (Lepidoptera, Lycaenidae) holds the record of the highest number of chromosomes in the non-polyploid eukaryotic organisms. *Comp Cytogenet* **9:** 683–690.

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

Lukhtanov VA, Dincă V, Friberg M, Šíchová J, Olofsson M, Vila R, Marec F, Wiklund C (2018) Versatility of multivalent orientation, inverted meiosis, and rescued fitness in holocentric chromosomal hybrids. *Proc Natl Acad Sci U S A* **115:** E9610–E9619.

Macholán M, Munclinger P, Šugerková M, Dufková P, Bímová B, Božíková E, Zima J, Piálek J (2007) Genetic analysis of autosomal and X-linked markers across a mouse hybrid zone. *Evolution* **61:** 746–771.

Maddison WP (1982) XXXY sex chromosomes in males of the jumping spider genus *Pellenes* (Araneae: Salticidae). *Chromosoma* **85:** 23–37.

Maeki K (1958) On the cytotaxonomical relationship in *Leptidea* (Lepidoptera-Rhopalocera). *Jpn J Genet* **33:** 283–285.

Mallet J (2005) Hybridization as an investigation of the genome. *Trends Ecol Evol* **20:** 229–237.

Mank JE, Nam K, Ellegren H (2010) Faster-Z evolution is predominantly due to genetic drift. *Mol Biol Evol* **27:** 661–670.

Marec 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. Pp. 49–63.

Martin J, Gilles A, Descimon H (2003) Species concepts and sibling species: the case of *Leptidea sinapis* and *Leptidea reali.* In: Boggs CL, Watt WB, Ehrlich PR (eds.) *Butterflies: Ecology and Evolution Taking Flight.* Chicago University Press, Chicago, USA. Pp. 459–476.

Martin SH, Singh KS, Gordon IJ, Omufwoko KS, Collins S, Warren IA, Munby H, Brattström O, Traut W, Martins DJ, Smith DAS, Jiggins CD, Bass C, ffrench-Constant RH (2020) Whole-chromosome hitchhiking driven by a malekilling endosymbiont. *PLoS Biol* **18:** e3000610.

Masly JP, Presgraves DC (2007) High-resolution genome-wide dissection of the two rules of speciation in *Drosophila*. *PLoS Biol* **5:** e243.

Mavárez J, Salazar CA, Bermingham E, Salcedo C, Jiggins CD, Linares M (2006) Speciation by hybridization in *Heliconius* butterflies. *Nature* **441:** 868–871

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

Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T Yamada T, Kanamori H, Namiki N, Kitagawa M, Yamashita H, Yasukochi Y, Kadono-Okuda K, Yamamoto K, Ajimura M, Ravikumar G, Shimomura M, Nagamura Y, Shin-I T, Abe H, Shimada T, Morishita S, Sasaki T (2004) The genome sequence of silkworm, *Bombyx mori*. *DNA Res* **11:** 27–35.

Mongue AJ, Nguyen P, Voleníková A, Walters JR (2017) Neo-sex chromosomes in the monarch butterfly, *Danaus plexippus*. *G3* **7:** 3281–3294.

Nallu S, Hill JA, Don K, Sahagun C, Zhang W, Meslin C, Snell-Rood E, Clark NL, Morehouse NI, Bergelson J, Wheat CW, Kronforst MR (2019) The molecular genetic basis of herbivory between butterflies and their host plants. *Nat Ecol Evol* **2:** 1418–1427.

Nash D, Boyd T, Hardiman D (2012) *Ireland's butterflies: a review.* The Dublin Naturalist's Field Club, Dublin, UK. Pp. 272.

Nguyen P, Sahara K, Yoshido A, Marec F (2010) Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). *Genetica* **138:** 343–354.

Nguyen P, Sýkorová M, Šíchová J, Kůta V, Dalíková M, Čapková Frydrychová R, Neven LG, Sahara K, Marec F (2013) Neo-sex chromosomes and adaptive potential in tortricid pests. *Proc Natl Acad Sci U S A* **110:** 6931–6.

Nieukerken EJv, Kaila L, Kitching IJ, Kristensen NP, Lees DC, Minet J, Mitter C, Mutanen M, Regier JC, Simonsen TJ, Wahlberg N, Yen SH, Zahiri R, Adamski D, Baixeras J, Bartsch D, Bengtsson BÅ, Brown JW, Bucheli SR, Davis DR, De Prins J, De Prins W, Epstein ME, Gentili-Poole P, Gielis C, Hättenschwiler P, Hausmann A, Holloway JD, Kallies A, Karsholt O, Kawahara AY, Koster JC, Kozlov MV, Lafontaine JD, Lamas G, Landry JF, Lee S, Nuss M, Park KT, Penz C, Rota J, Schintlmeister A, Schmidt BC, Sohn JC, Solis MA, Tarmann GM, Warren AD, Weller S, Yakovlev RV, Zolotuhin VV, Zwick A (2011) Order Lepidoptera. In: Zhang Z-Q (ed.) *Animal Biodiversity: An Outline of Higher-Level Classification and Survey of Taxonomic Richness. Zootaxa* **3148:** 212–221.

O'Neill J, Montgomery I (2018) Demographics and spatial ecology in a population of cryptic wood white butterfly *Leptidea juvernica* in Northern Ireland. *J Insect Conserv* **2**: 499–510.

Palacios-Gimenez OM, Castillo ER, Martí DA, Cabral-de-Mello DC (2013) Tracking the evolution of sex chromosome systems in Melanoplinae grasshoppers through chromosomal mapping of repetitive DNA sequences. *BMC Evol Biol* **13:** 167.

Pearse FK, Ehrlich PR (1979) B chromosome variation in *Euphydryas colon* (Lepidoptera: Nymphalidae). *Chromosoma* **73:** 263–274.

Picq S, Lumley L, Šíchová J, Laroche J, Pouliot E, Brunet BMT, Levesque RC, Sperling FAH, Marec F, Cusson M (2018) Insights into the structure of the spruce budworm (*Choristoneura fumiferana*) genome, as revealed by molecular cytogenetic analyses and a high-density linkage map. *G3* **8:** 2539–2549.

Pokorná M, Altmanová M, Kratochvíl L (2014) Multiple sex chromosomes in the light of female meiotic drive in amniote vertebrates. *Chromosome Res* **22:** 35–44.

Pospíšilová K (2018) Analysis of structure and origin of multiple sex chromosomes in *Leptidea* wood white butterflies. Bc. Thesis, in English. Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Pp. 30.

Presgraves DC (2002) Patterns of postzygotic isolation in Lepidoptera. *Evolution* **56:** 1168–1183.

Presgraves DC (2008) Sex chromosomes and speciation in *Drosophila*. *Trends Genet* **24:** 336–343.

Pringle EG, Baxter SW, Webster CL, Papanicolaou A, Lee SF, Jiggins CD (2007) Synteny and chromosome evolution in the Lepidoptera: Evidence from mapping in *Heliconius melpomene*. *Genetics* **177:** 417–426.

Réal P (1988) Lépidoptères nouveaux principalement jurassiens. *Mém Comité de Liaison Rech Ecofaunist* **4:** 1–28.

Rens W, Grützner F, O'Brien PC, Fairclough H, Graves JA, Ferguson-Smith MA (2004) Resolution and evolution of the duck-billed platypus karyotype with an $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ male sex chromosome constitution. *Proc Natl Acad Sci U S A* **101:** 16257–16261.

Rens W, O'Brien PC, Grützner F, Clarke O, Graphodatskaya D, Tsend-Ayush E, Trifonov VA, Skelton H, Wallis MC, Johnston S, Veyrunes F, Graves JAM, Ferguson-Smith MA (2007) The multiple sex chromosomes of platypus and echidna are not completely identical and several share homology with the avian Z. *Genome Biol* **8:** R243.

Resh VH, Cardé RT (2003) *Encyclopedia of Insects.* Academic Press, Elsevier Science. Pp. 631–664.

Robinson R (1971) *Lepidoptera Genetics*. Pergamon Press, Oxford, UK. Pp. 687.

Rovatsos M, Altmanová M, Augstenová B, Mazzoleni S, Velenský P, Kratochvíl L (2019) ZZ/ZW Sex Determination with Multiple Neo-Sex Chromosomes is Common in Madagascan Chameleons of the Genus *Furcifer* (Reptilia: Chamaeleonidae). *Genes (Basel)* **10:** 1020.

Sætre GP, Borge T, Lindroos K, Haavie J, Sheldon BC, Primmer C, Syvänen AC (2003) Sex chromosome evolution and speciation in *Ficedula* flycatchers. *Proc Biol Sci* **270:** 53–59.

Sachanowicz K, Wower A, Buszko J (2011) Past and present distribution of the cryptic species *Leptidea sinapis* and *L. reali* (Lepidoptera: Pieridae) in Poland and its implications for the conservation of these butterflies. *Eur J Entomol* **108:** 235–242.

Sahara K, Yoshido A, Kawamura N, Ohnuma A, Abe H, Mita K, Oshiki T, Shimada T, Asano S, Bando H, Yasukochi Y (2003) W-derived BAC probes as a new tool for identification of the W chromosome and its aberrations in *Bombyx mori*. *Chromosoma* **112:** 48–55.

Sahara K, Yoshido A, Marec F, Fukova I, Zhang HB, Wu CC, Goldsmith MR, Yasukochi Y (2007) Conserved synteny of genes between chromosome 15 of *Bombyx mori* and a chromosome of *Manduca sexta* shown by five-color BAC-FISH. *Genome* **50:** 1061–1065.

Sahara K, Yoshido A, Shibata F, Fujikawa-Kojima N, Okabe T, Tanaka-Okuyama M, Yasukochi Y (2013) FISH identification of *Helicoverpa armigera* and *Mamestra brassicae* chromosomes by BAC and fosmid probes. *Insect Biochem Mol Biol* **43:** 644–653.

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

Schartl M (2015) Sex determination by multiple sex chromosomes in *Xenopus tropicalis*. *Proc Natl Acad Sci U S A* **112:** 10575–10576.

Shen J, Cong Q, Kinch LN, Borek D, Otwinowski Z, Grishin NV (2016) Complete genome of *Pieris rapae*, a resilient alien, a cabbage pest, and a source of anti-cancer proteins. *F1000Res* **5:** 2631.

Shtinkov N, Kolev Z, Vila R, Dincă V (2016) The sibling species *Leptidea juvernica* and *L. sinapis* (Lepidoptera, Pieridae) in the Balkan Peninsula: ecology, genetic structure, and morphological variation. *Zoology* **119:** 11–20.

Sinev SY (2008) *Catalogue of the Lepidoptera of Russia.* KMK Scientific Press, St. Petersburg-Moscow, Russia. Pp. 424 (in Russian).

Smith DAS, Gordon IJ, Traut W, Herren J, Collins S, Martins DJ, Saitoti K, Ireri P, ffrench-Constant R (2016) A neo-W chromosome in a tropical butterfly links colour pattern, male-killing, and speciation. *Proc R Soc B* **283:** 20160821.

Stefanescu C, Torre I, Jubany J, Páramo F (2010) Recent trends in butterfly populations from north-east Spain and Andorra in the light of habitat and climate change. *J Insect Conserv* **15:** 83–93.

Storchová R, Gregorová S, Buckiová D, Kyselová V, Divina P, Forejt J (2004) Genetic analysis of X-linked hybrid sterility in the house mouse. *Mamm Genome* **15:** 515–524.

Storchová R, Reif J, Nachman MW (2010) Female heterogamety and speciation: Reduced introgression of the Z chromosome between two species of nightingales. *Evolution* **64:** 456–471.

Suomalainen E (1969) Chromosome evolution in the Lepidoptera. *Chromosom T* **2:** 132–138.

Syren RM, Luykx P (1981) Geographic variation of sex-linked translocation heterozygosity in the termite *Kalotermes approximatus* Snyder (Insecta: Isoptera). *Chromosoma* **82:** 65–88.

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

Šíchová J, Ohno M, Dincă V, Watanabe M, Sahara K, Marec F (2016) Fissions, fusions, and translocations shaped the karyotype and multiple sex chromosome constitution in the northeast-Asian wood white butterfly, *Leptidea amurensis*. *Biol J Linnean Soc* **118:** 457–471.

Šíchová J, Voleníková A, Dincă V, Nguyen P, Vila R, Sahara K, Marec F (2015) Dynamic karyotype evolution and unique sex determination systems in *Leptidea* wood white butterflies. *BMC Evol Biol* **15:** 89.

Talla V, Johansson A, Dincă V, Vila R, Friberg M, Wiklund C, Backström N (2019) Lack of gene flow: Narrow and dispersed differentiation islands in a triplet of *Leptidea* butterfly species. *Mol Ecol* **28:** 3756–37700.

Talla V, Suh A, Kalsoom F, Dincă V, Vila R, Friberg M, Wiklund C, Backström N (2017) Rapid increase in genome size as a consequence of transposable element hyperactivity in wood-white (*Leptidea*) butterflies. *Genome Biol Evol* **9**: 2491–2505.

Thomas C (1991) Cytogenetics of fleas (Siphonaptera: Pulicidae). 1. Rat fleas of the genus Xenopsylla. *Cytobios* **67:** 29–43.

Thomas J (1995) The conservation of declining butterfly populations in Britain and Europe: priorities, problems and successes. *Biol J Lin Soc* **56:**55–72.

Thomas J (2010) *Butterflies of Britain and Ireland.* British Wildlife Publishing, Oxford, UK. 3rd ed. Pp. 288.

Thompson R, Nelson B (2006) *The butterflies and moths of Northern Ireland.* Blackstaff Press, National Museums Northern Ireland, Belfast, UK. Pp. 428.

Traut W, Marec F (1996) Sex chromatin in Lepidoptera. *Q Rev Biol* **71:** 239–256.

Traut W, Marec F (1997) Sex chromosome differentiation in some species of Lepidoptera (Insecta). *Chromosome Res* **5:** 283–291.

Traut W, Ahola V, Smith DAS, Gordon IJ, ffrench-Constant RH (2017) Karyotypes versus Genomes: the nymphalid 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.

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

Van't Hof AE, Nguyen P, Dalíková M, 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.

Verovnik R, Glogovčan P (2007) Morphological and molecular evidence of a possible hybrid zone of *Leptidea sinapis* and *L. reali* (Lepidoptera: Pieridae). *Eur J Entomol* **104:** 667–674.

Vitturi R, Catalano E, Colombera D, Avila AL, Fucà A (1993) Multiple sex-chromosome system and other karyological characterizations of *Pterotrachea hippocampus* (Mollusca: Mesogastropoda). *Marine Biology* **115:** 581–585.

Wahlberg N, Rota J, Braby MF, Pierce NP, Wheat CW (2014) Revised systematics and higher classification of pierid butterflies (Lepidoptera: Pieridae) based on molecular data. *Zool Scr* **43:** 641–650.

Wan F, Yin C, Tang R, Chen M, Wu Q, Huang C, Qian W, Rota-Stabelli O, Yang N, Wang S, Wang G, Zhang G, Guo J, Gu L, Chen L, Xing L, Xi Y, Liu F, Lin K, Guo M, Liu W, He K, Tian R, Jacquin-Joly E, Franck P, Siegwart M, Ometto L, Anfora G, Blaxter M, Meslin C, Nguyen P, Dalíková M, Marec F, Olivares J., Maugin S, Shen J, Liu J, Guo J, Luo J, Liu B, Fan W, Feng L, Zhao X, Peng X, Wang K, Liu L, Zhan H, Liu W, Shi G, Jiang C, Jin J, Xian X, Lu S, Ye M, Li M, Yang M, Xiong R, Walters J. R, Li F (2019) A chromosome-level genome assembly of *Cydia pomonella* provides insights into chemical ecology and insecticide resistance. *Nat Commun* **10:** 4237.

Warnock N (2008) The ecology and conservation of *Leptidea reali* (Real's Wood White) in Northern Ireland. MSc thesis, Queen's University Belfast, UK. Pp. 88.

Warren M, Pollard E, Bibby T (1986) Annual and long-term changes in a population of the wood white butterfly *Leptidea sinapis*. *J Anim Ecol* **55:** 707–719.

White MJD (1973) *Animal Cytology and Evolution*. Cambridge University Press, Cambridge, UK. 3rd ed. Pp. 468.

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

Xia QY, Zhou ZY, Lu C, Cheng DJ, Dai F, Li B, Zhao P, Zha X, Cheng T, Chai C, Pan G, Xu J, Liu C, Lin Y, Qian J, Hou Y, Wu Z, Li G, Pan M, Li C, Shen Y, Lan X, Yuan L, Li T, Xu H, Yang G, Wan Y, Zhu Y, Yu M, Shen W, Wu D, Xiang Z, Yu J, Wang J, Li R, Shi J, Li H, Li G, Su J, Wang X, Li G, Zhang Z, Wu Q, Li J, Zhang Q, Wei N, Xu J, Sun H, Dong L, Liu D, Zhao S, Zhao X, Meng Q, Lan F, Huang X, Li Y, Fang L, Li C, Li D, Sun Y, Zhang Z, Yang Z, Huang Y, Xi Y, Qi Q, He D, Huang H, Zhang X, Wang Z, Li W, Cao Y, Yu Y, Yu H, Li J, Ye J, Chen H, Zhou Y, Liu B, Wang J, Ye J, Ji H, Li S, Ni P, Zhang J, Zhang Y, Zheng H, Mao B, Wang W, Ye C, Li S, Wang J, Wong GK, Yang H, Biology Analysis Group (2004) A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). *Science* **306:** 1937–1940.

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

Yasukochi Y, Ashakumary LA, Baba K, Yoshido A, Sahara K (2006) A second-generation integrated map of the silkworm reveals synteny and conserved gene order between lepidopteran insects. *Genetics* **173:** 1319–1328.

Yasukochi Y, Ohno M, Shibata F, Jouraku A, Nakano R, Ishikawa Y, Sahara K (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, Zhang H, Goldsmith MR, Sahara K (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, Bando H, Yasukochi Y, Sahara K (2005) The *Bombyx mori* karyotype and the assignment of linkage groups. *Genetics* **170:** 675–685.

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 (2014) Silk moths (Lepidoptera). In: Sharakhov IV (ed.) *Protocols for Cytogenetic Mapping of Arthropod Genomes.* CRC Press: Boca Raton, FL, USA. Pp. 219–256.

Yosido A, Šíchová J, Pospíšilová K, Nguyen P, Voleníková A, Šafář J, Provazník J, Vila R, Marec F (in prep.) Evolution of multiple sex chromosomes associated with dynamic genome reshuffling in *Leptidea* wood white butterflies. Manuscript in preparation.

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.