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Analysis of structure and origin of multiple sex chromosomes in *Leptidea* wood white butterflies

Bachelor thesis

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Annotation

Previous studies have shown a dynamic karyotype evolution and the presence of complex sex chromosome systems with 3-4 W chromosomes and 3-6 Z chromosomes in *Leptidea* wood white butterflies. To dissect the evolutionary history of multiple Z chromosomes of *Leptidea* species, we used identified and selected bacterial artificial chromosome (BAC) clones containing orthologous genes of *Bombyx mori* chromosome Z and 17, isolated them from the available BAC library and used them as probes for physical mapping by BAC-FISH, first in *L. juvernica*, then in the closely related *L. sinapis*. In both *Leptidea* species, the majority of BAC clones corresponding to the linkage group Z of the *B. mori* reference genome hybridized to one chromosome of the complicated sex chromosome multivalent. Thus, we named it as Z_1 chromosome. Location of all Z-derived BAC clones was identical in both species suggesting a conserved synteny and gene order between *L. juvernica* and *L. sinapis* Z_1 chromosome. Moreover, our findings indicate that the Z_1 chromosome is probably the ancestral Z chromosome in the genus *Leptidea*. Results of BAC-FISH mapping with clones corresponding to the linkage group 17 of the *B. mori* reference genome revealed the fusion/translocation event between an ancestral Z chromosome and the chromosome corresponding to *B. mori* chromosome 17 and supported a previous hypothesis about the role of chromosomal rearrangements in the formation of multiple sex chromosomes in *Leptidea* butterflies.

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Contents

1. General introduction

1.1. Lepidoptera

Lepidoptera, moths and butterflies, is the second largest and one of the most speciose orders of insect. Many species are economically important in a positive or negative way. Some of them are beneficial and utilized instead of herbicides to reduce weeds, e.g. the cactus moth (*Cactoblastis cactorum*) introduced from Argentina to Australia in 1925 to control several North American and South American species of *Opuntia* (Habeck *et al.* 1998), others are of great economic importance, such as silkworm (*Bombyx mori*), whose larvae enclose themselves in a cocoon made of raw silk (Resh and Cardé 2003). On the other hand, many lepidopteran species are pests feeding on particular plants with potential to cause serious damage in agriculture, for example, members of families Totricidae, Noctuidae and Pyralidae (Resh and Cardé 2003). Having a great impact on human living, moths and butterflies have become important and interesting model systems in various fields of research, such as ecology, physiology, evolution, phylogeny, molecular biology and genetics.

1.1.1. Lepidopteran karyotype

For a long time, cytogenetics of moths and butterflies remained unclear mainly due to the nature of their chromosomes. Lepidopteran chromosomes are uniform in shape, much smaller than vertebrate chromosomes and devoid of any morphological features for their identification. Moreover, the chromosomes are holokinetic as they lack a localized distinct primary constriction, the centromere (Carpenter *et al.* 2005). For these particular reasons, chromosome counting was the only applicable approach used in early cytogenetic research (Mediouni *et al.* 2004, Fuková *et al.* 2005).

Most species have haploid chromosome number n=28-32, and the modal chromosome number of n=31 occurs from basal to advanced lepidopteran clades (Suomalainen 1969, Robinson 1971, De Prins and Saitoh 2003). Based on karyotype surveys, it has been established that the ancestral number of chromosomes in haploid lepidopteran genome is n=31 (Suomalainen 1969, Lukhtanov 2000). Moreover, the putative ancestral chromosome number is supported by comparative genome mapping (Baxter *et al.* 2011, Sahara *et al.* 2013, Van't Hof *et al.* 2013, Ahola *et al.* 2014). Recent studies also suggest an extraordinary karyotype stability and high degree of conserved synteny of genes between the silkworm *B. mori* and other species of the clade Bombycoidea, *Manduca sexta* (Sahara *et al.* 2007), as well as representatives of not so closely related Papilionoidea, namely *Heliconius melpomene* (Yasukochi *et al.* 2006, Pringle *et al.* 2007) and *Bicyclus anynana* (Beldade *et al.* 2009), then three species of Noctuidea, *Helicoverpa armigera*, *Mamestra brassicae*, and *Spodoptera frugiperda* (d'Alençon *et al.* 2010, Sahara *et al.* 2013), and last but not

least, the diamondback moth *Plutella xylostella*, which belongs to Yponomeutoidea (Baxter *et al.* 2011). These results point to evolutionary stability of whole lepidopteran genomic regions.

On the other hand, some lepidopteran taxa deviate from the modal and also ancestral chromosome number of n=31. The smallest chromosome number of n=5 was found in neotropic butterfly *Hypothyris thea (*Nymphalidae; Brown *et al.* 2004), and the Arizona giant-skipper, *Agathymus aryxna* (Hesperiidae; De Prins and Saitoh 2003). Probably the greatest interspecific karyotype variation not only among Lepidoptera, but also in the animal kingdom, was found in blue butterflies (Lycaenidae: Polyommatidae) with haploid chromosome numbers ranging from n=10 to n=222-226 (Kandul *et al.* 2004, Lukhtanov 2015). The latter, observed in the Atlas blue, *Polyommatus atlanticus*, is the highest chromosome number described so far in non-polyploid eukaryotes (Lukhtanov 2015). It has been assumed, that such karyotype variation is caused by holokinetic nature of lepidopteran chromosomes facilitating chromosomal rearrangements, i.e. fissions and fusions, rather than by polyploidy (Wrensch *et al.* 1994, Lukhtanov *et al.* 2005).

1.1.2. Sex chromosomes in Lepidoptera

Lepidopteran species together with their sister group caddisflies (Trichoptera) have a chromosomal mechanism of sex determination, in which females are heterogametic with the sex chromosome constitution of either Z0 or WZ and males are homogametic with the sex chromosome constitution of ZZ. Female-specific W chromosome, if present, can be easily discerned according to its heterochromatic structure (e.g. Traut and Marec 1997, Fuková *et al.* 2005, Vítková *et al*. 2007, Šíchová *et al.* 2013). In comparison with the Z chromosome, the W chromosome is gene poor and consists mainly of non-coding repetitive sequences such as transposons (Sahara *et al.* 2003, Abe *et al.* 2005, Mediouni *et al.* 2004). In somatic interphase nuclei of females, the W chromosome forms a heterochromatin body (sex chromatin or W chromatin), which can serve as an indirect evidence for the presence of the W chromosome in the genome (Traut and Marec 1996). The absence of the W chromosome in Trichoptera and basal lineages of Lepidoptera suggests that the common ancestor of Lepidoptera and Trichoptera had a sex chromosome constitution of Z0/ZZ (reviewed by Traut *et al.* 2007, Marec *et al.* 2010, Sahara *et al.* 2012). According to the hypothesis described in Traut and Marec (1997), the W chromosome originated from an autosome, whose homolog fused with the ancestral Z chromosome. The lone and unpaired autosome became female limited and degenerated rapidly due to absence of crossing-over in lepidopteran females. Decay of genes, extensive invasion of repetitive elements such as transposons and morphological changes on this chromosome gave rise to the neo-W and neo-Z chromosomes. An alternative hypothesis of Lukhtanov (2000) suggests that the W chromosome arose from the supernumerary B-chromosome.

The Z chromosome differs from the W chromosome in chromatin structure, but the most important difference is the presence of transcriptionally active genes on the Z chromosome and recombination during meiotic division in males. However, for a long time, only a little was known about the Z chromosome in Lepidoptera (Koike *et al.* 2003) and most information was related to the model organism, silkworm (*B. mori*), whose genome was sequenced independently by two laboratories (Mita *et at.* 2004, Xia *et al.* 2004). Interesting data were published by Koike *et al.* (2003), who analyzed about 2% of the Z chromosome of *B. mori* using BAC contig covering 320-kb of chromosome. The analysis located 13 genes around *Bm kettin,* a homolog of *kettin* muscle protein gene in *Drosophila* (Suzuki *et al.* 1999), and many transposable elements. With the use of novel molecular-genetic and cytogenetic techniques such as fluorescence *in situ* hybridization (FISH) with probes derived from bacterial artificial chromosomes (BAC-FISH), scientists have focused on comparative genome mapping of Z-linked genes between phylogenetically distant species of Lepidoptera (Yasukochi *et al.* 2006, 2009, Sahara *et al.* 2013, Nguyen *et al.* 2013). The goal was to better understand the chromosomal evolution, mechanisms of speciation, as well as to obtain knowledge applicable in pest control technologies.

Although the majority of lepidopteran species have a WZ/ZZ sex chromosome system (Traut *et al.* 2007), variations of this standard system exist. These variations include secondary loss of the W chromosome or multiple sex chromosomes with following constitutions: W_1W_2Z/ZZ or $WZ_1Z_2/Z_1Z_1Z_2Z_2$. In Lepidoptera, multiple sex chromosomes forming trivalents are quite rare and have been found only in seven genera (F. Marec, personal communication). Multiple sex chromosomes with more than three elements are even less common in Lepidoptera. A curious sex determining system with 3-4 W chromosomes and 3-6 Z chromosomes was found in wood white butterflies of the genus *Leptidea* (Šíchová *et al.* 2015, 2016), a very promising model system for studying speciation.

1.2. *Leptidea* wood white butterflies

1.2.1. History, ecology and distribution

The discovery of a new cryptic species in the genus *Leptidea* (Pieridae) triggered an explosive investigation of their ecology, ethology, evolution and soon afterwards, *Leptidea* became a model system for studying the process of speciation by means of chromosomally-based suppressed recombination model, as well as clinal speciation (Lukhtanov *et al.* 2011, Dincă *et al.* 2011). At the end of the twentieth century, it was found that a common wood white butterfly with western Palearctic distribution, *L. sinapis*, consists of a pair of cryptic species: *L. sinapis* sensu stricto (Linnaeus, 1758) and *L. reali* (Reissinger, 1989; Réal 1998). The two species are indistinguishable by wing morphology, but can be separated based on their genitalia characters (Lorković 1993). The distinctness of these sibling species has also been confirmed by molecular analysis based on allozyme

markers and mitochondrial DNA (Martin *et al.* 2003). Moreover, several ecological and behavioral studies found other differences between *L. sinapis* and *L. reali*. These studies revealed that the premating reproductive barrier preventing between-species hybridization does occur, because females only accept conspecific males (Freese and Fiedler 2002, Friberg *et al.* 2008a). However, the possibility of occasional hybridization between *L. sinapis* females and *L. reali* males cannot be excluded (Verovnik and Glogovčan 2007). The two species have also partitioned in their niche preferences, differently in various parts of their sympatric distribution (Friberg *et al.* 2008b). Different habitat preferences also cause variety in voltinism and phenology between both species (Friberg 2008c). Despite their niche separation, both species have virtually identical host plants, i.e. meadow vetchling (*Lathyrus pratensis*) and/or common bird's-foot trefoil (*Lotus corniculatus*; Friberg *et al.* 2009).

Originally, it has been thought that the geographical distribution of *Leptidea reali* is almost as wide as that of *L. sinapis*. That idea was disproved after the discovery of a new *Leptidea* species, *L. juvernica* stat. nov. (Dincă *et al.* 2011). Dincă *et al.* (2011) used mitochondrial and nuclear markers, chromosome counts and morphological data to study the twin species, *L. sinapis* and *L. reali*. He discovered that *L. reali* actually hide a cryptic entity and named it cryptic wood white butterfly (*Leptidea juvernica* stat. nov). So, what was originally thought to be a species pair, *L. sinapis* - *L. reali*, is actually a triplet of closely related cryptic species. Measurement of genitalia for the three species was performed, but only *L. sinapis* was clearly differentiated, while *L. reali* and *L. juvernica* could not be reliably separated. This explains why *L. juvernica* remained unnoticed for so long (Dincă *et al*. 2011). While *L. sinapis* is widespread across the Europe and north-western Asia and can sometimes cohabitate with either *L. reali* or *L. juvernica*, the latter two species have not yet been found in sympatry. The distribution of *L. reali* is restricted to the western Mediterranean and *L. juvernica* occurs from Ireland to eastern Kazakhstan (Dincă *et al*. 2011). Both *L. reali* and *L. juvernica* are mostly allopatric except for parapatric population in southeastern France, where they are separated only by 87 kilometers. No evidence of hybridization between these species was found in this parapatry zone or elsewhere (Dincă *et al*. 2011).

1.2.2. *Leptidea* karyotype

As was mentioned in chapter 1.1.1., the majority of lepidopteran species have haploid chromosome number of n=31 (Suomalainen 1969, De Prins and Saitoh 2003, Robinson 1971). However, *Leptidea* butterflies exhibit exceptional inter- and intraspecific variability in chromosome numbers, with the tendency to increase during evolution (Dincă *et al.* 2011, Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016). The highest chromosome number was described in *L. duponcheli* with haploid chromosome number ranging from n=102 to n=104 (Lorković 1941; de Lesse 1960). High

chromosome numbers were also found in two *Leptidea* species with mainly Eastern Palearctic distribution, *L. morsei* with n=54 (Maeki 1958) and *L. amurensis* with observed differences in chromosome numbers between males and females, i.e. n=61 and 2n=118-119, respectively (Šíchová *et al.* 2016). However, the most extraordinary variability in chromosome numbers was found in *L. sinapis*, where Lukhtanov *et al*. (2011) described a chromosomal cline ranging from n=28 in Kazakhstan to n=53 in Spain. Such intraspecific variability in chromosome numbers is exceptional in animal kingdom and offers unique opportunity to study mechanisms involved in clinal speciation. Moreover, Šíchová *et al.* (2015) described a variable number of chromosomes in other two species with Western Palearctic distribution, *L. reali* (2n=51-55) and *L. juvernica* (2n=85-91).

There are two possible hypotheses explaining the intraspecific variability in chromosome numbers in *Leptidea* karyotypes. In some species, such variability is caused by B-chromosomes, i.e. additional chromosomes or supernumerary chromosomes (Bigger 1976, Lukhtanov 1992, Camacho *et al.* 2000). B-chromosomes consist mainly of repetitive elements and can occur only in some individuals from the given population. They can be easily detected during meiosis because they are usually small and form univalents, bivalents or multivalents (Jones *et al.* 2008). Although not essential for the population, B-chromosomes can accumulate by mitotic or meiotic drive (Jones *et al.* 2008). In general, we cannot exclude that B-chromosomes play a role in chromosome number variation found in *Leptidea*. Nevertheless, this possibility is rather unlikely as no B-chromosomes were found in Spanish population of *L. sinapis* with the highest chromosome numbers. Moreover, the number of chromosomes seemed to be stable both within and between individuals and no univalents were detected in any of the studied population (Lukhtanov *et al.* 2011). The alternative scenario ascribes such intraspecific variability in chromosome numbers in *Leptidea* to multiple chromosomal changes, mainly fusions and fissions (Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016). This idea is endorsed by an obvious pattern in karyotypes of all studied *Leptidea* species, i.e. the higher the chromosome number in a population, the smaller the size of the chromosomes. This regularity indicates that chromosomal fusions/fissions are the main mechanisms in karyotype evolution of all *Leptidea* species (Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016).

Intraspecific variability in *Leptidea* karyotypes was also confirmed by localization of two cytogenetic markers, i.e. clusters of RNA genes (major rDNA) and H3 histone genes (Šíchová *et al.* 2015, 2016). In *L. reali* and *L. amurensis*, the number and position of both cytogenetic markers were consistent within species. In *L. juvernica*, the number and location of rDNA clusters was identical between individuals, whereas the number and location of H3 histone genes differed even among the offspring of individual females. The most surprising result was obtained in *L. sinapis* with the highest variability in the number and location of both cytogenetic markers (Šíchová *et al.* 2015, 2016). Such variation in rDNA distribution is in agreement with the evolutionary dynamics of rDNA clusters that

was observed in other lepidopteran species (Nguyen *et al.* 2010). However, the interspecific and especially intraspecific variability in the number and location of H3 histone gene clusters is unexpected as this marker is conserved in the lepidopteran family Totricidae (Šíchová *et al.* 2013) as well as in other insect groups, for example grasshoppers and beetles (Cabrero *et al.* 2009, Cabral-de-Mello 2011). Such variability together with observed differences in chromosome numbers point to the dynamic karyotype evolution and stress the role of chromosomal rearrangements in the evolution of *Leptidea* karyotypes.

1.2.3. Multiple sex chromosomes in *Leptidea*

Profound inter- and intraspecific variability in chromosome numbers and localization of cytogenetic markers even among the progeny of individual females is in stark contrast with species-specific stability of sex chromosome constitution in *Leptidea* butterflies (Šíchová *et al.* 2015, 2016). Sex chromosomes of all so far studied *Leptidea* species form complicated multivalents with following constitutions: $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica*, $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis*, $W_1W_2W_3W_4$ $Z_1Z_2Z_3Z_4$ in *L. reali* and $W_1W_2W_3Z_1Z_2Z_3Z_4Z_5Z_6$ in *L. amurensis* (Šíchová *et al.* 2015, 2016). Multiple sex chromosomes with three or four elements are quite common in vertebrates including fishes (Kitano and Peichel 2012), amphibians (Schartl 2015), reptiles (Pokorná *et al.* 2014), and mammals (Gruetzner *et al.* 2006). Moreover, a recent study revealed multiple sex chromosome system newly in birds, i.e. penguins of genus *Pygoscelis* (Gunski *et al.* 2017). Trivalents or quadrivalents were also found in invertebrates, for example in true bugs (Hemiptera; Bardella *et al.* 2012), fleas (Siphonaptera; Thomas 1991) and grasshoppers (Orthoptera; Palacios-Gimenez *et al.* 2013). However, sex chromosome multivalents with more than four sex chromosomes are quite rare in animal kingdom. So far, they have been found only in several invertebrate species and in monotreme mammals, for example in the duck-billed platypus (*Ornithorhynchus anatinus*) with sex chromosome constitution ♀$X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$/♂$X_1X_2X_3X_4X_5Y_1Y_2Y_3Y_4Y_5$ or in echidna with constitution ♀$X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$/♂$X_1X_2X_3X_4X_5Y_1Y_2Y_3Y_4$. In both cases, the sex chromosome system arose by repeated sex chromosome-autosome translocations (Rens *et al.* 2004, 2007, Grützner *et al.* 2004). In invertebrate species, the most complex sex chromosome system with up to 19 chromosomes forming sex-linked chains or rings during meiosis was found in termites, *Kalotermes approximatus* (Syren and Luykx 1981). As was mentioned in chapter 1.1.2., sex chromosome multivalents are rare in Lepidoptera and have been found only in seven genera with constitutions either W_1W_2Z/ZZ or $WZ_1Z_2/Z_1Z_1Z_2Z_2$ (F. Marec, personal communication). The origin of these multivalents is ascribed to sex chromosome-autosome fusion or sex chromosome fission with the remaining chromosome becoming either W_2 or Z_2 chromosome (Marec *et al.* 2010). Therefore, multiple sex chromosomes with 3-4 W chromosomes and 3-6 Z

chromosomes found in *Leptidea* stand out for its complexity and are under close observation of recent studies.

Most likely, multiple sex chromosome system found in *Leptidea* wood white butterflies originated via complex chromosomal changes, i.e. translocations between an ancestral WZ pair and several autosomes (Šíchová *et al.* 2015, 2016). Generally, such chromosomal rearrangements may have negative effect on fertility and/or viability of individuals due to the unbalanced segregation of chromosomes during meiotic division (Baker and Bickham 1986). However, sex chromosome multiples have been found in all four studied *Leptidea* species (*L. juvernica, L. sinapis, L. reali,* and *L. amurensis*; Šíchová *et al*. 2015, 2016). The reason for such curiosity remains disputable. Several studies show that neo-sex chromosomes contribute to reproductive barriers between closely related species and thus reduce hybridization (Kitano and Peichel 2012, Yoshido *et al*. 2011). In Lepidoptera, detailed studies in geographic subpopulations of wild silkmoths *Samia cynthia* ssp. (Yoshido *et al*. 2011) and leaf-rollers of the family Tortricidae (Nguyen *et al.* 2013) point to the fact that the neo-sex chromosomes can contribute to the formation of reproductive barriers between populations and to speciation and radiation, respectively. Similarly, the formation of multiple sex chromosomes by means of chromosomal rearrangements in *Leptidea* butterflies increased the number of sex-linked genes and therefore could have accelerated the accumulation of genetic incompatibilities toward speciation as in the case of tortricid moths and *S. cynthia* subspecies (Šíchová *et al.* 2015, 2016). Thus, the principles of chromosomal changes underlying the formation of multiple sex chromosomes in *Leptidea* wood white butterflies are essential and their identification will help us to understand the role of sex chromosome multiples in the speciation process of this highly interesting model system.

2. Objectives

Leptidea wood white butterflies represent a new promising model system for the study on the origin and evolution of cryptic species as well as on speciation associated with chromosomal rearrangements (Dincă *et al*. 2011, Lukhtanov *et al.* 2011). These species complex exhibits an exceptional inter- and itraspecific variation in chromosome numbers as well as in location of otherwise conserved cytogenetic markers (Šíchová *et al*. 2015, 2016). This particularly regards to *L. sinapis* with an exceptional chromosomal cline ranging from n=28 in Kazakhstan to n=58 in Spain (Lukhtanov *et al.* 2011). More importantly, *Leptidea* butterflies possess a unique system of multiple sex chromosomes with 3-4 W chromosomes and 3-6 Z chromosomes originating by complex rearrangements of ancestral sex chromosomes and autosomes (Šíchová *et al*. 2015, 2016). Unlike the autosomes, the number of sex chromosomes is stable and species-specific, which suggests their significant role in the formation of reproductive barriers between the closely related *Leptidea* species. Thus, we propose to study sex chromosome multiples in *Leptidea* butterflies, to reconstruct their evolution and clarify their role in speciation.

The main purpose of this work is to perform a comparative analysis of multiple sex chromosomes in *L. juvernica* and *L. sinapis* using genomic tools recently established for *L. juvernica*, mainly a library of bacterial artificial chromosomes (BAC) of *L. juvernica* females. To reveal the evolutionary history of multiple Z chromosomes of *Leptidea* species, I will use BAC clones containing orthologous genes of *Bombyx mori* chromosome Z and 17, identified and selected by my colleagues, isolate them from the available BAC library and use them as probes for physical localization of the genes by fluorescence *in situ* hybridization (BAC-FISH), first in *L. juvernica*, then in closely related *L. sinapis*. Applicability of BAC-FISH for cross-hybridization in closely-related species was confirmed in other lepidopteran species (Yasukochi *et al*. 2009). However, it has to be tested in *Leptidea* wood whites. A physical map of sex-linked genes in *L. juvernica* and *L. sinapis* and its comparison with *B. mori* reference genome will be of great importance in identifying major mechanisms underlying the formation of multiple sex chromosomes in this closely related species pair and in reconstruction of their step-by-step evolution. Acquired knowledge will also clarify the speculation about the role of chromosomal rearrangements in *Leptidea* sex chromosome evolution and the potential contribution of multiple sex chromosomes in the formation of reproductive barriers between *Leptidea* species.

3. Material and methods

3.1. Sample collecting

Specimens of adult *Leptidea juvernica* and *L. sinapis* females were collected in the Czech Republic, in the surroundings of České Budějovice or Milovice and near Havraníky village in Podyjí National Park in South Moravia, respectively. In the laboratory, fertilized females were kept in plastic containers at room temperature to lay eggs on the host plant *Lotus corniculatus*, a feeding plant for hatched larvae. The genitalia of adult females were used for morphometric analysis and remaining bodies were frozen in 1.5 mL Eppendorf tubes in liquid nitrogen and stored in -80°C for further use.

3.2. Genitalia preparation and morphometric analysis

Female genitalia were dissected in physiological solution and the length of *ductus bursae* was measured under a stereomicroscope. The length of this element reliably discriminated *L. juvernica* from *L. sinapis*, as these two species cannot be distinguished from each other based just on wing patterns (Dincă *et al*. 2011).

3.3. Chromosome preparation

The chromosome preparations of both *Leptidea* species were prepared from fifth instar male and female larvae as described in Mediouni *et al*. (2004). Briefly, genitalia were dissected in physiological solution and fixed in Carnoy fixative (ethanol/chloroform/acetic acid, 6:3:1) for 15 min. Before fixation, testes were soaked in hypotonic solution (0.075M KCl) for 15 min and cleaned with tungsten needles. Genitalia were then macerated in a drop of 60% acetic acid and spread with tungsten needle on the slide using a heating plate at 45°C. The preparations were dehydrated in 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 selected genes

My colleagues from the Laboratory of Molecular Cytogenetics of the Institute of Entomology BC CAS established several genomic tools for the identification of genomic regions involved in the multiple sex chromosomes of *Leptidea* species. They generated and *de novo* assembled *L. juvernica* transcriptome to facilitate the identification of sex-linked genes by microarray-based comparative genomic hybridization (array-CGH; Baker and Wilkinson 2010). 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, CR (http://olomouc.ueb.cas.cz/category/dna-library/species/leptidea-juvernica). In our laboratory, we have available working copy of the BAC library and also matrix pool plates for PCR screening of the library (see Yasukochi 2002).

To dissect the evolutionary history of multiple Z chromosomes of *Leptidea* species, my colleagues identified orthologous sequences of genes exhibiting sex-linkage in *L. juvernica* and used these sequences to select BAC clones containing the orthologs by PCR screening of the *L. juvernica* BAC library. I isolated these particular BAC clones from the BAC library using Qiagen Plasmid Midi Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions.

3.5. Probe labeling

BAC-DNA labeling was carried out according to the protocol described in Kato *et al.* (2006) with slight modifications. Labeling 40 µl reaction contained 2 µg of unlabeled BAC DNA, 0.1 mM Cy3-dUTP (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). Reaction was incubated at 15°C for 5 h and then inactivated at 70°C for 10 min.

3.6. BAC-FISH mapping

Fluorescence *in situ* hybridization (FISH) with probes derived from bacterial artificial chromosomes (BAC-FISH) was performed following the procedure of Yoshido *et al.* (2005) with slight modifications. All clones selected from *L. juvernica* BAC library were at first hybridized separately on female chromosome preparations of *L. juvernica* and *L. sinapis* in order to prove their sex-linkage in chromosome complements. BAC clones that hybridized to the Z_1 chromosome of *L. juvernica* and *L. sinapis* were afterwards used to assemble the physical map of this chromosome in both species using two-color BAC-FISH method with reprobing protocol (Yoshido *et al.* 2005, Shibata *et al.* 2009).

To test the sex-linkage of selected BAC clones, female chromosome preparations of *Leptidea juvernica* and *L. sinapis* were removed from the freezer, passed through graded ethanol series (70%, 80% and 100%, 1 min each) and air-dried. The slides were denaturated at 68°C for 3 min 30 seconds in 70% formamide in 2× SSC. Hybridization cocktail for each slide contained 200 ng of Cy3-dUTP (Jena Bioscience) labeled BAC DNA, 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA) and 3 µg of unlabeled male genomic DNA (gDNA) in 10 µL of 50% formamide and 10% dextran sulfate in 2× SSC. The male gDNA of *L. juvernica* and *L. sinapis* 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. After incubation in a humid chamber at 37°C for 3 days, slides were washed at

62°C for 5 min in 0.1× SSC containing 1% Triton X-100. The slides were counterstained and mounted in antifade based on DABCO (Sigma-Aldrich) containing 0.5 μg/mL DAPI.

To assemble the physical map of Z_1 chromosome in *L. juvernica* and *L. sinapis* we used two-color BAC-FISH mapping on male pachytene chromosomes as described in Shibata *et al.* (2009) with some modifications. Briefly, male chromosome preparations were removed from the freezer, passed through graded ethanol series (70%, 80% and 100%, 1 min each) and 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 denaturated at 68°C for 3 min 30 seconds in 70% formamide in 2× SSC. The preparations were reprobed repeatedly with different probe mixtures containing three BAC clones labeled with Cy-3-dUTP (Jena Bioscience) and another two or three BAC clones labeled with Fluorescein-12-dUTP (Jena Bioscience). For the physical mapping we used BAC clones that hybridized on Z_1 chromosome in previous BAC-FISH experiments on *L. juvernica* and *L. sinapis* females. Each triplet contained two BAC clones from the opposite chromosomal ends and one from the middle of the Z_1 chromosome to make the signals easily distinguishable. Beside 200 ng of each probe labeled with Cy-3-dUTP (Jena Bioscience) and 500 ng of each probe labeled with Fluorescein-12-dUTP (Jena Bioscience), hybridization cocktail contained 25 μg of sonicated salmon sperm DNA and 6 μg of unlabeled male genomic DNA in 10 μL of 50% formamide and 10% dextran sulfate in 2x SSC. The mixture was denaturated at 90°C for 5 min and spread on each slide. After incubating in a humid chamber at 37°C for 3 days, slides were washed at 62°C for 5 min in 0.1× SSC containing 1% Triton X-100. The slides were counterstained and mounted in antifade based on DABCO (Sigma-Aldrich) containing 0.5 μg/mL DAPI. After each FISH round, the preparations were stripped off the cover slides carefully, washed 3 min in milliQ water and denaturated again as described above. The next hybridization cocktail mixture was applied directly on dehydrated and air-dried slides.

Male and female chromosome preparations from both BAC-FISH experiments were observed in a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Black-and-white images were captured with a cooled monochrome CCD camera XM10 equipped with cellSens Standard software version 1.9 (Olympus Europa Holding, Hamburg, Germany). Images were taken separately for each fluorescent dye and pseudocolored with various colors, i.e. red, green, yellow, violet, and orange, independently on the type of fluorescent dye that was used in particular experiment, in Adobe Photoshop, version 7.0.

4. Results

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

Identification of sex-linked genes by microarray-based comparative genomic hybridization (array-CGH) and PCR-based screening of BAC library of *L. juvernica* was done by my colleagues in the Laboratory of Molecular Cytogenetics. To facilitate the search for orthologous genes, they also used sequenced transcriptome of *L. juvernica*. In total, they identified and I isolated 21 BAC clones corresponding to 17 orthologous genes of the *Bombyx mori* Z chromosome and three orthologous genes of chromosome 17 (see Table 1). We focused on that chromosome because the results of array-CGH suggested that synteny blocks, which are sex-linked in *L. juvernica*, are enriched in the *B. mori* chromosome 17. In most cases, only one clone for each gene was selected except for three clones of *Triosephosphate isomerase* (*Tpi*), each carrying different part of gene sequence. Another exception was *tyrosine hydroxylase* (*THL*) and *Y box protein* (*Y-box*), both included in the same BAC clone. All isolated BAC clones I used for physical mapping on *L. juvernica* and *L. sinapis* chromosomes by BAC-FISH.

4.2. Sex-linkage analysis of selected BAC clones

All BAC-derived probes were at first hybridized separately on female pachytene oocytes of *L. juvernica* and *L. sinapis* in order to prove or disprove their sex-linkage. As described in Šíchová *et al.* (2015), female meiotic sex chromosomes form complicated multivalents with constitution $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica* and $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis* (Fig. 1), and can be easily recognized in the pachytene stage. In both species, the majority of selected BAC clones corresponding to the linkage group Z of the *B. mori* reference genome hybridized to one chromosome from the sex chromosome multivalent. Thus, we named it as Z_1 chromosome. Only one BAC clone, i.e. *Paramyosin* (*paramyosin*), hybridized not only to the Z_1 chromosome, but also to an autosome. Unfortunately, BAC clone *6-phosphogluconate dehydrogenase* (*6-PGD*) and all three clones of *Triosephosphate isomerase* (*Tpi*) hybridized to an autosome and therefore were excluded from further physical mapping. Results were identical for both *L. juvernica* and *L. sinapis.* The sex-linkage analysis helped us to estimate approximate position of individual BAC clones along the Z_1 chromosome, which was highly important for sorting BAC clones into groups for further physical mapping.

Table 1. List of *Leptidea juvernica* BAC clones isolated and mapped in this study.

		Leptidea juvernica		***Bombyx mori***		
Symbol	***B. mori* orthologs of selected gene**	**BAC clone**	**FISH**	**Gene ID**	**Chr. No.**	**Chromosome Position**
tan	*tan*	63E2	chromosome Z_1	BMgn002077	Z	460237-480848
apterous	*apterous*	53C10	chromosome Z_1	BMgn002127	Z	3487639-3516414
ABC-F2	*ATP-binding cassette sub-family F member 2*	90D1	chromosome Z_1	BMgn002004	Z	4621452-4632826
paramyosin	*Paramyosin*	9J14	chromosome Z_1+ autosome	BMgn000612	Z	5986799-6002013
kettin	*kettin*	91P9	chromosome Z_1	BMgn000622	Z	6513219-6533895
IDGF	*Imaginal disk growth factor*	93O2	chromosome Z_1	BMgn000648	Z	8533563-8553629
THL	*tyrosine hydroxylase*	66E6	chromosome Z_1	BMgn000563	Z	8795363-8803219
Y-box	*Y box protein*	66E6	chromosome Z_1	BMgn000526	Z	10855404-10857772
imp	*insulin-like growth factor 2 mRNA-binding protein1*	69D15	chromosome Z_1	BMgn000515	Z	11419210-11500018
period	*Period*	72D11	chromosome Z_1	BMgn000485	Z	12956618-13004501
Masc	*Masculinizer*	62N7	chromosome Z_1	BMgn012300	Z	15129206-15132406
SNF4	*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
Shaker	*Shaker*	19P21	chromosome Z_1	BMgn003851	Z	20911282-20921258
PAH (Henna)	*Phenylalanine hydroxylase (Henna)*	69F16	chromosome Z_1	BMgn003866	Z	21842454-21845665
Tpi	*Triosephosphate isomerase*	91C3, 66L7, 96D19	autosome	BMgn000559	Z	9023502-9027095
6-PGD	*6-phosphogluconate dehydrogenase*	62O17	autosome	BMgn012298	Z	15112863-15127673
KGM04993	*KGM04993*	94M23	chromosome Z_1	BMgn003962	17	18278392-18281478
RpL22	*Ribosomal protein L22*	19D03	chromosome Z_1	BMgn006986	17	14152955-14155752
asparaginase	*I-asparaginase*	92J07	chromosome Z_1	BMgn007025	17	11551636-11556300

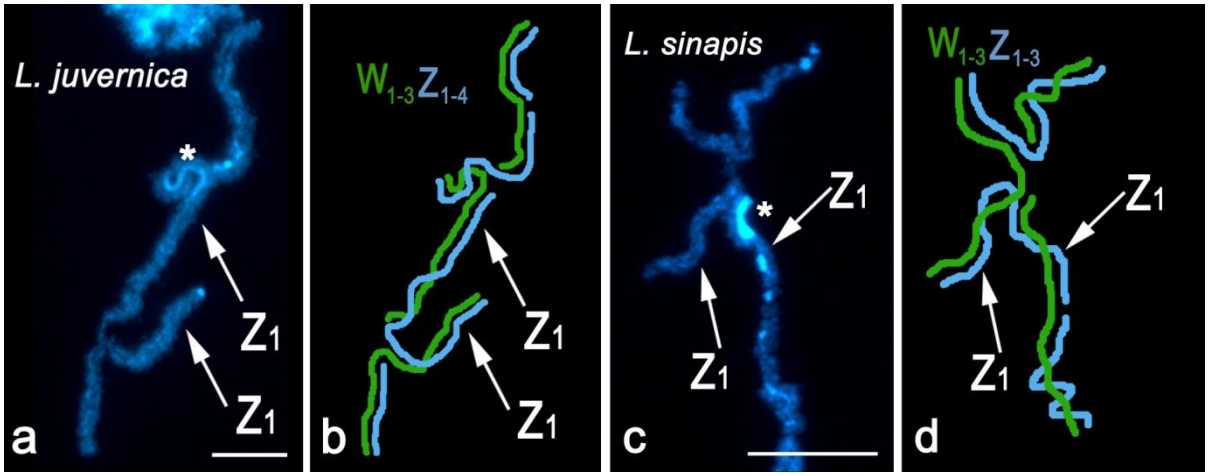

Figure 1. Female meiotic sex chromosome multivalents of *Leptidea juvernica* **(a, b)** and *L. sinapis* **(c, d)**. Chromosomes were counterstained with DAPI (light blue). **(a, c)** DAPI images, arrows indicate Z_1 chromosomes, asterisks indicate DAPI-positive heterochromatic blocks at the end of one W chromosome; **(b, d)** schematic drawings of the sex chromosome multivalent. Scale bar = 10 µm. (Šíchová *et al.* 2015)

4.3. Physical mapping of orthologous genes of *Bombyx mori* Z chromosome

For simplification, we performed physical mapping of sex-specific BAC clones carrying orthologs of *B. mori* Z chromosome genes on male pachytene chromosomes with Z_1Z_1 bivalent rather than on female pachytene oocytes with more complicated sex chromosome constitution. BAC clones were divided into four triplets (note that the genes *THL* and *Y-box* are included in the same BAC clone) and one couple and labeled with red and/or green fluorescent dies according to their position on the female Z_1 chromosome (Table 2). Each triplet contained two BAC clones from the opposite chromosomal ends and one from the middle of the Z_1 chromosome. Three runs of BAC-FISH with reprobing protocol were carried out on male chromosome preparations of *L. juvernica* and the physical map of all 15 Z_1 orthologous genes was assembled (Fig. 2a). Subsequently, the same BAC probes were cross-hybridized on male chromosome preparations of *L. sinapis* to reconstruct the physical map of Z_1 chromosome also in this closely related species (Fig. 2c). All BAC clones showed strong fluorescent signals even in *L. sinapis* and the localization of all individual clones was completely identical in both species. Thus, our experiments confirmed conserved synteny and gene order between *L. juvernica* and *L. sinapis* Z_1 chromosomes.

Furthermore, the physical map of Z_1 chromosome in *L. juvernica* and *L. sinapis* revealed one interesting feature of this chromosome. All BAC clones corresponding to the linkage group Z of the *B. mori* reference genome hybridized to two continuous parts of Z_1Z_1 bivalent leaving one chromosomal

end as well as the smaller section between two BAC clones, *Shaker* (*Shaker*) and *Period* (*period*), without any signal (Fig. 2a, c, asterisks).

Table 2. The labeling scheme for individual reprobing runs of BAC-FISH with BAC clones corresponding to the linkage group Z of the *B. mori* reference genome.

Run No.	Hybridized BAC clones	Labeling
1	*masc+ tan+ IDGF*	Cy-3-dUTP (red)
2	*SNF4+ apterous+ THL+ Y-box*	Cy-3-dUTP (red)
	LDH+ kettin+ imp	Fluorescein-12-dUTP (green)
3	*Henna+ period+ ABC-F2*	Cy-3-dUTP (red)
	Shaker+ paramyosin	Fluorescein-12-dUTP (green)

4.4. Physical mapping of orthologous genes of *Bombyx mori* chromosome 17

In order to identify other Z chromosomes from the complicated sex chromosome multivalent my colleagues identified and selected three BAC clones carrying orthologs of *B. mori* chromosome 17 genes, i.e. *KGM04993* (*KGM04993*), *Ribosomal protein L22* (*RpL22*), *I-asparaginase* (*asparaginase*), and I isolated them from the BAC library and hybridized them separately on female pachytene chromosomes of *L. juvernica* and *L. sinapis*. Surprisingly, all three BAC clones highlighted one end of the Z_1 chromosome, the part where no BAC clones corresponding to the linkage group Z of the *B. mori* reference genome hybridized in previous experiments. To confirm their location on Z_1 chromosome in both species, we performed BAC-FISH mapping on male pachytene chromosomes. Two runs of BAC-FISH with reprobing protocol included three orthologs of *B. mori* chromosome 17 and four previously localized orthologs of *B. mori* Z chromosome, namely *IDGF*, *imp*, *THL, Y-box* (Table 3). This experiment confirmed that all three orthologs of *B. mori* chromosome 17 are localized at the lower end of Z_1 chromosome in both species (Fig. 2b, d) and revealed a fusion or translocation event between the ancestral Z chromosome and an autosome corresponding to the *B. mori* chromosome 17.

Table 3. The labeling scheme for individual reprobing runs of BAC-FISH with BAC clones corresponding to the linkage groups Z and 17 of the *B. mori* reference genome.

Run No.	Hybridized BAC clones	Labeling
1	*Rpl 22+ IDGF*	Cy-3-dUTP (red)
	THL+ Y-box	Fluorescein-12-dUTP (green)
2	*KGM04993+ imp*	Cy-3-dUTP (red)
	asparginase	Fluorescein-12-dUTP (green)

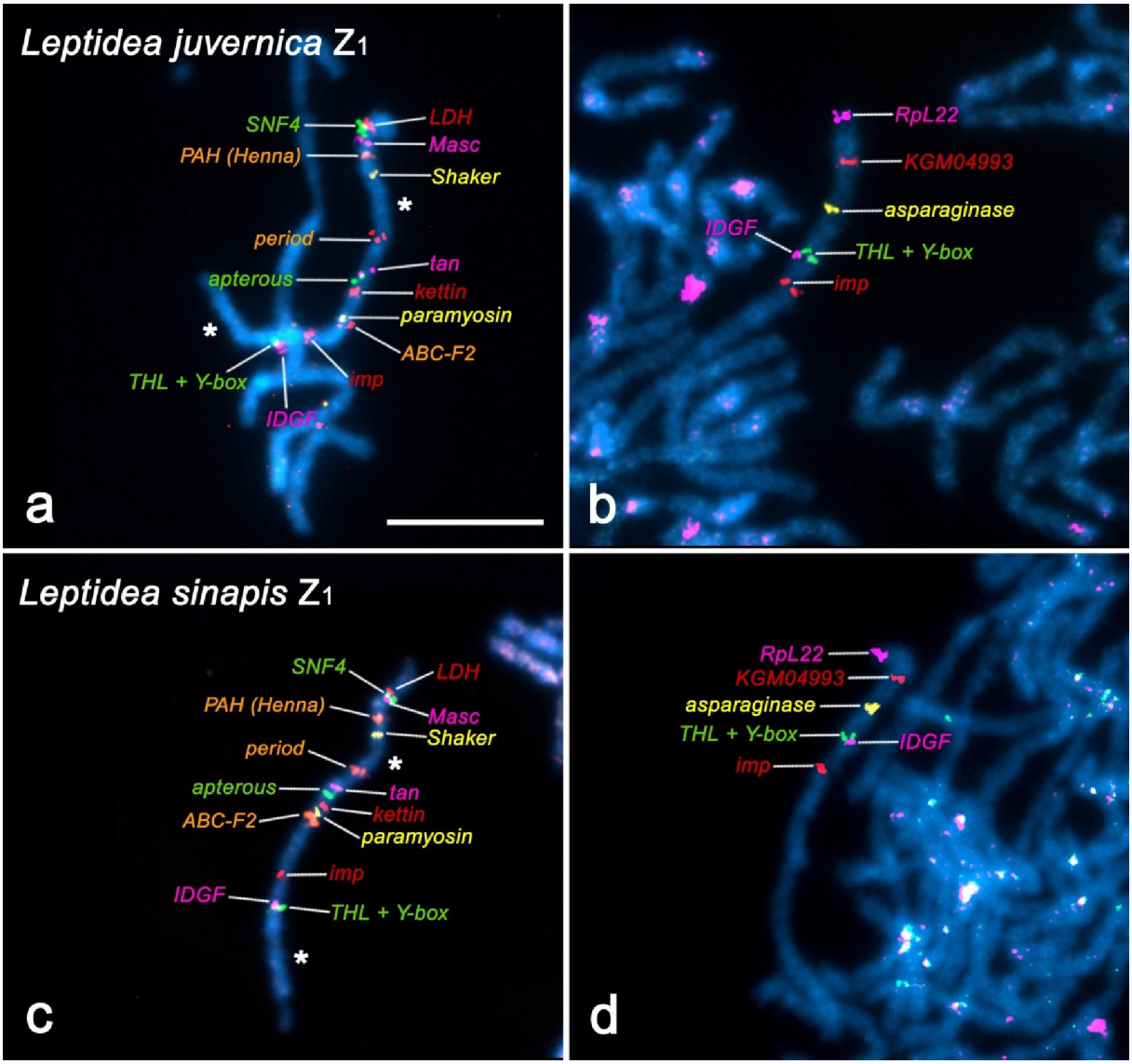

Figure 2. BAC-FISH mapping of genes on male chromosome preparations of *Leptidea juvernica* **(a, b)** and *L. sinapis* **(c, d)**. Hybridization signals of BAC probes (red, green, yellow, violet, orange) indicate the physical position of loci marked by gene symbol. Chromosomes were counterstained with DAPI (light blue). Asterisks indicate sections without any hybridization signals. **(a, c)** Three runs of BAC-FISH with reprobing protocol localized 15 orthologs of *Bombyx mori* Z-linked genes (*SNF4, PAH (Henna), period, apterous, ABC-F2, IDGF, LDH, Masc, Shaker, tan, kettin, paramyosin, imp, THL, Y-box*) to a single Z_1Z_1 bivalent. **(b, d)** Two runs of BAC-FISH with reprobing protocol localized four *B. mori* Z-linked orthologs (*imp, IDGF, THL, Y-box*) and three orthologs of chromosome 17 (*RpL22, KGM04993, asparginase*) on the same Z_1Z_1 chromosome bivalent. Scale bar = 10 µm.

5. Discussion

The unexpected discovery of cryptic diversity in wood white butterflies of the genus *Leptidea* made this species complex an interesting model for studying speciation by means of chromosomally-based suppressed recombination model, as well as clinal speciation (Dincă *et al*. 2011, Lukhtanov *et al.* 2011). Previous studies on karyotype evolution of three cryptic species from Western Palearctic, namely *L. juvernica*, *L. sinapis* and *L. reali*, and one species from Eastern Palearctic, *L. amurensis*, showed exceptional inter- and intraspecific variation in chromosome numbers and also in location of otherwise conserved cytogenetic marker (H3 histone genes; Lukhtanov *et al.* 2011, Šíchová *et al.* 2015, 2016). These results point to dynamic karyotype evolution and stress the role of chromosomal rearrangements, especially multiple chromosome fusions and fissions, in the speciation of *Leptidea* wood whites. More importantly, all analyzed *Leptidea* species possess a unique, species-specific sex determination system with 3-4 W chromosomes and 3-6 Z chromosomes originating by complex rearrangements of ancestral sex chromosomes and autosomes. Such complicated sex chromosome system is unique in Lepidoptera and should be counted as additional factor that might contribute to the evolution of *Leptidea* butterflies (Šíchová *et al.* 2015, 2016).

In this study, we carried out a comparative analysis of multiple sex chromosomes in two *Leptidea* species, *L. juvernica* and *L. sinapis*, using novel genomic tools recently established for *L. juvernica.* These are *de novo* assembled transcriptome to facilitate the identification of sex-linked genes by microarray-based comparative genomic hybridization (array-CGH) and a newly constructed library of bacterial artificial chromosomes (BAC) of *L. juvernica* females. To uncover the evolutionary history of Z chromosomes in *Leptidea* species, we identified orthologous sequences of genes exhibiting Z-linkage in *L. juvernica* and used them to select particular BAC clones containing the orthologs by PCR screening of the *L. juvernica* BAC library. Selected BAC clones were used as probes for physical localization of the genes by fluorescence *in situ* hybridization (BAC-FISH), first in *L. juvernica*, then in the closely related *L. sinapis*. The acquired knowledge allowed us to uncover chromosomal mechanism underlying the formation of multiple sex chromosomes in *Leptidea* butterflies, and helped us to reconstruct their step-by step evolution.

5.1. Physical mapping of Z_1 chromosome by BAC-FISH in *Leptidea* butterflies

We assembled the physical map of the Z_1 chromosome of *L. juvernica* and *L. sinapis* using BAC-FISH method with reprobing protocol. In Lepidoptera, this method seems to be efficient despite the nature and number of their chromosomes and had been previously applied in various karyotyping, cytogenetic mapping, and evolutionary studies (Yoshido *et al.* 2005, Sahara *et al.* 2007, Yasukochi *et al.* 2009, Nguyen *et al.* 2013). Using two-color BAC-FISH method, Yoshido *et*

al. (2005) identified each of the 28 pachytene bivalents in the karyotype of *Bombyx mori* (Bombycoidea; Bombycidae) by its labeling pattern and assigned each chromosome to one of the already established genetic linkage groups. Sahara *et al.* (2007) described a high degree of conserved synteny including conserved gene order between *B. mori* chromosome 15 and the corresponding chromosome in tobacco hornworm, *Manduca sexta* (Bombycoidea; Sphingidae), and a comparison of the *M. sexta* BAC physical map with the linkage map and genome sequence of *B. mori* by Yasukochi *et al.* (2009) confirmed this well conserved synteny and almost identical gene order in most of *B. mori* and *M. sexta* chromosomes. Moreover, the cross-hybridization of *M. sexta* BAC clones to the chromosomes of closely related convolvulus hawk moth, *Agrius convolvuli* (Bombycoidea; Sphingidae), demonstrated that BAC-FISH method is an efficient tool in karyotyping a wide range of related and genetically uncharacterized species (Yasukochi *et al.* 2009). Last but not least, Nguyen *et al.* (2013) used BAC-FISH method with selected probes to reconstruct a physical map of the codling moth, *Cydia pomonella* (Totricoidea; Totricidae), Z chromosome and confirmed a conserved synteny of Z-linked genes between this species and representatives of superfamilies Pyraloidea, Bombycoidea, and Papilionoidea.

In this work, we used BAC-FISH mapping with clones corresponding to the linkage group Z of the *B. mori* reference genome to assemble the physical map of Z_1 chromosome in *L. juvernica*. Moreover, we cross-hybridized these BAC clones to the pachytene chromosomes of closely related *L. sinapis* in order to test the applicability of this method in the reconstruction of evolutionary history of multiple sex chromosomes in *Leptidea* butterflies. In total, we performed three runs of BAC-FISH with reprobing protocol and localized 14 BAC clones carrying 15 orthologous genes of *B. mori* Z chromosome in both *Leptidea* species. Unfortunately, in previous experiments BAC clone *6-phosphogluconate dehydrogenase* (*6-PGD*) and three BAC clones of *Triosephosphate isomerase* (*Tpi*), hybridized to an autosome in both species, probably due to some translocation event during the evolution of *Leptidea* sex chromosomes. Thus, these four BAC clones were excluded from physical mapping. The final physical map of Z_1 chromosomes in both *L. juvernica* and *L. sinapis* contained 14 BAC clones carrying orthologs of *B. mori* Z chromosome genes. The position and order of all analyzed BAC clones was completely identical suggesting a conserved synteny and gene order between both studied species. Moreover, our findings indicate that part of the Z_1 chromosome is probably the ancestral Z chromosome in the genus *Leptidea*. However, further research and comparative analysis of the Z_1 chromosome in other *Leptidea* species is needed to confirm this hypothesis.

Physical map of Z_1 chromosomes in *L. juvernica* and *L. sinapis* revealed one interesting feature of this chromosome. All BAC clones corresponding to the linkage group Z of the *B. mori* reference genome hybridized to two continuous parts of the Z_1 chromosome leaving one chromosomal end as well as the smaller section between two BAC clones, *Shaker* (*Shaker*) and *Period*

(*period*), without any signal. It implies that another chromosome (or chromosomes) could be involved in the evolution of the Z_1 chromosome in *Leptidea* butterflies. The results of microarray-based comparative genomic hybridization (array-CGH) suggest that synteny blocks which are sex-linked in *L. juvernica* correspond to *B. mori* chromosome 1 (Z), 7, 8, 11, 17 and 24 (P. Nguyen, personal communication). In this study, we isolated three BAC clones carrying orthologous genes of *B. mori* chromosome 17 and mapped them to *L. juvernica* and *L. sinapis* sex chromosomes together with three BAC clones that hybridized to Z_1 chromosomes in previous experiments. Two runs of BAC-FISH with reprobing protocol localized all three orthologs of *B. mori* chromosome 17 at the end of *L. juvernica* and *L. sinapis* Z_1 chromosome, the part where no BAC clones hybridized in previous experiments. The position and gene order was again identical in both species. These data suggest that the Z_1 chromosomes in *L. juvernica* and *L. sinapis* arose by fusion between an ancestral Z chromosome and an autosome corresponding to *B. mori* chromosome 17. However, taking into account that only three BAC clones corresponding to the linkage group 17 of the *B. mori* reference genome were localized, we cannot exclude the possibility of partial translocation of an autosome to the Z_1 chromosome. Hopefully, the additional analysis with more clones of *B. mori* chromosome 17 orthologs will clarify this speculation.

Other interesting facts emerged from the comparison of *L. juvernica* and *L. sinapis* Z_1 chromosome maps with the *B. mori* reference genome (Fig. 3). As was mentioned in previous two paragraphs, except for BAC clones *6-phosphogluconate dehydrogenase* (*6-PGD*) and *Triosephosphate isomerase* (*Tpi*), all selected BAC clones carrying orthologs of *B. mori* Z chromosome hybridized to the Z_1 chromosome in both studied *Leptidea* species. These results suggest a high degree of conserved synteny of Z-linked genes between *B. mori* and *Leptidea* butterflies. However, different gene order between *B. mori* Z chromosome and *Leptidea* Z_1 chromosomes points to many intrachromosomal rearrangements underlying the formation of Z_1 chromosomes in *L. juvernica* and *L. sinapis.* Simply put, genes from the lower half of the *B. mori* Z chromosome [*period, Masc, SNF4, LDH, Shaker, PAH (Henna)*] were translocated to the upper end of *Leptidea* Z_1 chromosome, followed by genes from the other half of *B. mori* Z chromosome (*tan, apterous, ABC-F2, paramyosin, kettin, IDGF, THL, Y-box, imp*). The colinearity of individual genes remained conserved just in case of gene pair *tan* - *apterous* and *IDGF* - *THL+Y-box*. Furthermore, the mutual positions of *ABC-F2* - *kettin* and *Masc* - *LDH* suggest possible inversions in these regions. Similarly, the inverted gene order was observed in the case of *Shaker* and *PAH (Henna)*. Three BAC clones carrying orthologous genes of *B. mori* chromosome 17, namely *RpL22, KGM04993,* and *asparginase,* hybridized to the lower end of Z_1 chromosomes in both *Leptidea* species suggesting a partial translocation or fusion event between the ancestral Z chromosome and an autosome corresponding to *B. mori* chromosome 17. Moreover, a terminal

position of *RpL22* compared to its neighbor clone *KGM04993* suggests other intrachromosomal rearrangement, probably inversion.

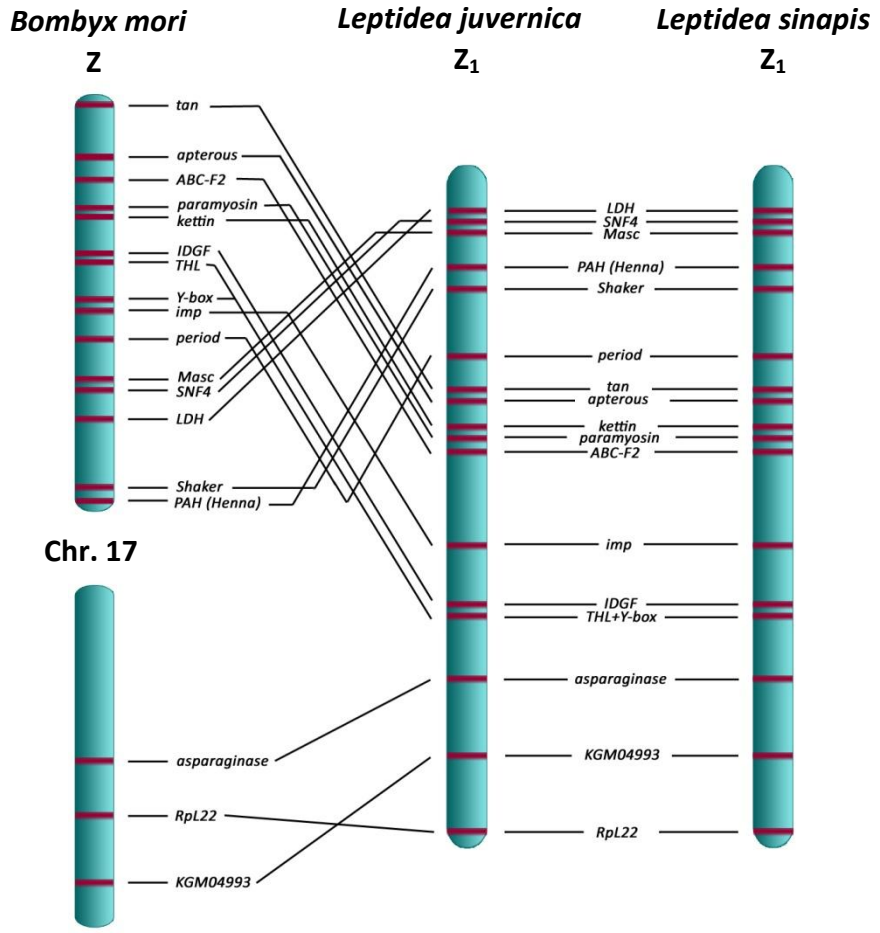

Figure 3. A gene-based scheme of Z_1 chromosomes of *Leptidea juvernica* and *L. sinapis* integrating all BAC-FISH mapping data and its comparison with the *Bombyx mori* chromosomes Z and 17. Location of *B. mori* genes were retrieved from KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). The mean relative positions of loci in *Leptidea* species were calculated from data obtained by measuring physical distances between hybridization signals and the chromosome end in Z_1Z_1 bivalents; the distances were then related to the total length of the Z chromosome. Note well conserved synteny and gene order between Z_1 chromosomes of both *Leptidea* species including orthologs of *B. mori* chromosome Z and 17. On the other hand, many intrachromosomal rearrangements differentiated Z_1 chromosomes of both *Leptidea* species and *B. mori* Z chromosome.

5.2. Chromosomal rearrangements, multiple sex chromosomes and speciation

Previous studies suggest that chromosomal rearrangements, especially multiple chromosome fusions, fissions and translocations, between the ancestral sex chromosomes and several autosomes, gave rise to multiple sex chromosomes in *Leptidea* butterflies (Šíchová *et al.* 2015, 2016). Our study has provided supporting evidence for the earlier prediction by observed fusion/translocation event between the Z_1 chromosome and an autosome corresponding to *Bombyx mori* chromosome 17*.* In general, fusions or translocations between sex chromosomes and autosomes increase the number of sex-linked genes. But question remains why is it so convenient for genes to be located on the sex chromosomes rather than on autosomes? Several studies on interspecific hybrids show that the replacement of one species's X chromosome for the other's has a larger effect on fitness of hybrids compared to similar substitution of an autosome, the so-called 'Large-X effect'. Masly and Presgraves (2007) substituted many chromosomal segments of one *Drosophila* species, *D. sechellia*, with those of closely related *D. mauritiana* and tracked which markers cause infertility in hybrids. Although X-linked and autosomal introgressions were of similar size, X-linked introgressions caused hybrid male sterility in 60% cases whereas autosomal introgressions only in 18%. These results proved that the X chromosome has a disproportionately larger effect on hybrid fitness compared to autosomes and is a hotspot for speciation genes, which prevent genetic exchanges between closely related species. Thus, higher density of X-linked genes can play a major role in postzygotic reproductive isolation and facilitate the divergence toward speciation (Presgraves 2008, Storchová *et al*. 2010). The role of the X chromosomes in postzygotic reproductive isolation was also documented across the central European portion of the hybrid zone between two house mouse subspecies, *Mus musculus domesticus* and *M. m. musculus* (Storchová 2004, Good *et al.* 2008, Macholán *et al.* 2007), where the gene flow was significantly higher on the autosomes than on X chromosomes (Good *et al.* 2008).

A similar large effect of homogametic sex chromosomes on hybrid fitness, the so-called 'Large-Z effect', was also explored in organisms with heterogametic females, e.g. birds (Sætre *et al*. 2003, Ellegren 2009, Storchová *et al*. 2010). A comparative study of two closely related bird species of nightingales, namely *Luscinia luscinia* and *L. megarhynchos*, showed that interspecific introgression of Z-linked loci is reduced compared to autosomal ones (Storchová *et al.* 2010). Analogous results have been demonstrated in sympatric populations of two naturally hybridizing *Ficedula* flycatchers (Sætre *et al.* 2003) as well as across the hybrid zone between *Passerina* buntings, *P. cyanea* and *P. amoena* (Carling and Brumfield 2008, 2009). Large effect of the Z chromosome on divergence of populations and speciation is also expected in butterflies, another organism with heterogametic females (Sperling 1994, Prowell 1998). In Lepidoptera, involvement of the Z chromosome in reproductive isolation has been suggested in two *Heliconius* butterflies, i.e. *Heliconius cydno* and *H. melpomene* (Naisbit *et al.* 2002). Linkage analysis using the Z-linked *triose-*

phosphate isomerase locus revealed a 'Large-Z effect' on female hybrid sterility that varied among crosses from the production of normal but infertile eggs to a complete failure to develop ovarioles.

One possible explanation of "Large-X" and "Large-Z effect" is rapid divergence of X/Z-linked coding sequences compared to autosomal ones, the so-called "Fast-X" or "Fast-Z effect" (Presgraves 2008, Mank *et al*. 2010). Simply put, new recessive mutations are not masked by standard allele in the heterozygous state and thus immediately preferred by selection. Another important factor that affects the efficacy of selection is the effective population size (N_e) of the X/Z chromosomes and autosomes. In organisms with XY sex chromosome constitution, males have only one copy of the X chromosome, the effective population size of the X chromosome (N_{eX}) is equal to three-quarters of the autosomal effective population size (N_{eA}). However, N_{eX} is influenced by differences between the sexes in the variance of reproductive success. A higher variance in reproductive success increases N_{eX} in species with male heterogametic sex and decreases N_{eZ} in species with female heterogametic sex (Vicoso and Charlesworth 2009).

Results of recent studies suggest that chromosomal changes that give rise to multiple sex chromosomes could play a major role in the formation of reproductive barriers between populations (Yoshido *et al.* 2011, Kitano and Peichel 2012, Yoshido *et al*. 2013,). Kitano and Peichel (2012) have shown that neo-X chromosomes resulting from a fusion between the ancestral sex chromosome and an autosome can contribute to the reproductive isolation between closely related species of stickleback fish. This is consistent with the idea that the turnover of sex chromosomes may actually promote speciation. Moreover, in Lepidoptera, step-by-step evolution of the neo-sex chromosomes by repeated sex chromosome-autosome fusions was described in subpopulations of wild silkmoth *Samia cynthia* spp., where four allopatric populations differentiated in chromosome constitutions as well as in chromosome numbers (Yoshido *et al.* 2005, 2011, 2013). Most likely, a common ancestor of this species complex had a standard constitution of WZ/ZZ chromosomes with diploid chromosome number of 2n=28/28 and the same chromosome constitution and number was observed in *S. c. pryeri.* The loss of the W chromosome resulted in Z0/ZZ system with 2n=27/28 in *S. c. ricini*, whereas fusion of sex chromosomes with autosomes gave rise to neo-Wneo-Z/neo-Zneo-Z in *S. c. walkeri* with 2n=26/26, and neo-WZ_1Z_2/$Z_1Z_1Z_2Z_2$ in *S. cynthia* subsp. indet. with 2n=25/26. Thus, it has been suggested, that sex chromosome rearrangements in *S. cynthia* populations contribute to formation of reproductive barriers and facilitate the divergence toward speciation (Yoshido *et al.* 2011). A detailed study on sex chromosome evolution in leaf-rollers of the family Totricidae points to the fact that the chromosome rearrangements leading to multiple sex chromosomes may also contribute to radiation in some lepidopteran representatives (Nguyen *et al.* 2013). Physical mapping of the Z chromosome in the codling moth, *C. pomonella*, revealed the fusion between the Z chromosome and an autosome corresponding to the linkage group 15 of the *B. mori* reference

genome. The fusion originated in a common ancestor of the main tortricid subfamilies, Olethreutinae and Tortricinae, and caused sex-linked inheritance of two genes responsible for insecticide resistance and clusters of genes involved in detoxification of plant secondary metabolites (Nguyen *et al.* 2013). As mentioned before, sex linkage can be highly beneficial for recessive alleles, as they spread faster in population due to the hemizygosity in females (Orr 2010). Therefore, increased number of sex-linked genes, especially those beneficial conferring resistance, may have contributed to the spectacular radiation of tortricid moths (Nguyen *et al.* 2013). Previous studies suggest that *Leptidea* butterflies possess derived neo-sex chromosomes that originated as a result of complex chromosomal rearrangements (Šíchová *et al.* 2015, 2016). Our data provide an evidence that the Z_1 chromosome in *L. juvernica* and *L. sinapis* originated by fusion/translocation event between the ancestral Z chromosome and an autosome corresponding to *B. mori* chromosome 17. Such chromosomal rearrangements increased the number of sex-linked genes and thus could have accelerated the accumulation of genetically based incompatibilities toward divergence and speciation of this genus as in the case of above-mentioned leaf-rollers of the family Tortricidae and geographical subspecies of *S. cynthia*.

In future, we propose to identify and isolate more BAC clones carrying orthologs of *B. mori* chromosome 17 genes and hybridize them on pachytene chromosomes of *L. juvernica* and *L. sinapis*. This could help us to decide whether the fusion or translocation event was the main force in the formation of Z_1 chromosome in both *Leptidea* species. Using BAC clones corresponding to the linkage group Z and 17 of the *B. mori* reference genome we plan to assemble the physical map of Z_1 chromosome in closely related *Leptidea* species with known sex chromosome constitution, namely *L. reali* and *L. amurensis*. Moreover, the results of array-CGH helped us to identify synteny blocks which are sex-linked in *L. juvernica* and correspond to *B. mori* chromosome 1 (Z), 7, 8, 11, 17 and 24. Together with sequenced transcriptome and available BAC library we plan to identify, select and isolate BAC clones from these candidate chromosomes and assemble the physical maps of other Z chromosomes that are involved in complicated sex chromosome multivalents in *Leptidea* butterflies. We also propose to identify sex chromosome constitution in two basal *Leptidea* species, *L. duponcheli* and *L. morsei*, as the knowledge of sex chromosomes in these species is essential to elucidate step-by-step evolution of multiple sex chromosome systems in genus *Leptidea.*

6. Conclusion

In this work, we performed a detailed physical mapping analysis of *Bombyx mori* sex-linked genes in two closely related *Leptidea* species, namely *L. juvernica* and *L. sinapis*. Using fluorescence *in situ* hybridization with clones derived from bacterial artificial chromosomes (BAC-FISH), we localized 17 orthologous genes of *B. mori* Z chromosome and three orthologous genes of *B. mori* chromosome 17 on *L. juvernica* pachytene chromosomes. Moreover, we cross-hybridized these BAC clones to the pachytene chromosomes of closely related *L. sinapis* in order to test the applicability of this method in the reconstruction of evolutionary history of multiple sex chromosomes in *Leptidea* butterflies. Except for BAC clone *6-phosphogluconate dehydrogenase* (*6-PGD*) and three clones of *Triosephosphate isomerase* (*Tpi*), all selected BAC clones carrying orthologs of *B. mori* Z chromosome hybridized to one chromosome of the sex chromosome multivalent in both *Leptidea* species. Thus, we named it as a Z_1 chromosome. BAC-FISH method with reprobing protocol enabled us to assemble a physical map of the Z_1 chromosome containing 15 orthologous genes of the *B. mori* Z chromosome in both *Leptidea* species. The identical position and colinearity of all BAC clones suggest a conserved synteny and gene order between Z_1 chromosomes in *L. juvernica* and *L. sinapis*. These findings also indicate that part of the Z_1 chromosome is most likely the ancestral Z chromosome in the genus *Leptidea*.

The results of BAC-FISH mapping with clones corresponding to the linkage group 17 of the *B. mori* reference genome revealed the fusion or translocation event between the ancestral Z chromosome and an autosome corresponding to *B. mori* chromosome 17. These data provided supporting evidence for the role of chromosomal rearrangements in the evolution of multiple sex chromosomes in this genus. Moreover, the comparison of *L. juvernica* and *L. sinapis* Z_1 chromosome map with *B. mori* reference genome revealed a high degree of conserved synteny of Z-linked genes between *B. mori* and *Leptidea* butterflies. It is in agreement with previously observed evolutionary stability of the whole genomic regions in Lepidoptera. However, the different gene order between *B. mori* Z chromosome and *Leptidea* Z_1 chromosomes points to many intrachromosomal rearrangements underlying the formation of Z_1 chromosomes in *Lepidea* butterflies. Taken together, our study supports the previous hypothesis about the role of chromosomal rearrangements in the formation of multiple sex chromosomes in *Leptidea* and points to the importance of multiple sex chromosomes in species divergence and formation of reproductive barriers in this highly interesting group of butterflies.

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