School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice
Faculty of Science

Contrasting patterns of karyotype and sex chromosome evolution in Lepidoptera

Ph.D. Thesis

Mgr. Jindra Šíchová

Supervisor: Prof. RNDr. František Marec, CSc.

Biology Centre of the Czech Academy of Sciences, Institute of Entomology

České Budějovice 2016

This thesis should be cited as:

Šíchová J (2016) Contrasting patterns of karyotype and sex chromosome evolution in Lepidoptera. Ph.D. Thesis. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 91 pp.

Annotation

It is known that chromosomal rearrangements play an important role in speciation by limiting gene flow within and between species. Furthermore, this effect may be enhanced by involvement of sex chromosomes that are known to undergo fast evolution compared to autosomes and play a special role in speciation due to their engagement in postzygotic reproductive isolation. The work presented in this study uses various molecular-genetic and cytogenetic techniques to describe karyotype and sex chromosome evolution of two groups of Lepidoptera, namely selected representatives of the family Tortricidae and *Leptidea* wood white butterflies of the family Pieridae. The acquired knowledge points to unexpected evolutionary dynamics of lepidopteran karyotypes including the presence of derived neo-sex chromosome systems that originated as a result of chromosomal rearrangements. We discuss the significance of these findings for radiation and subsequent speciation of both lepidopteran groups.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 01.03.2016 .....

Jindra Šíchová

This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre of the CAS, supporting doctoral studies in the Molecular and Cell Biology and Genetics study programme.

Přírodovědecká
fakulta
Faculty
of Science

Financial support

This work was supported by:

The Grant Agency of the ASCR, grant IAA600960925

The Czech Science Foundation, grants 523/09/2106 and 14-22765S

The Grant Agency of the University of South Bohemia, grants 137/2010/P, 059/2010/P, 063/2012/P and 052/2013/P

Acknowledgements

I would like to thank to my supervisor, František Marec, for accepting me into his laboratory, for his invaluable consultations whenever needed and for his support. I also thank my colleagues and friends because without them the lab wouldn´t be the same place. Especially, I thank to Leo, who brought the idea of drinking mate and having regular lab meetings. My special thanks go to Maruška, who helped me to rear millions of *Leptidea* butterflies, and to Roger and Vlad, who always helped me providing them. Last but not least, I would like to thank my family and Michal for neverending support and faith.

List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

1) 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**: 6931-6936 (IF = 9.674).

As a corresponding author I hereby confirm that Jindra Šíchová participated in experimental procedures including chromosome preparations, FISH experiments and data analysis.

.....

František Marec

2) Šíchová J, Nguyen P, Dalíková M, Marec F (2013) Chromosomal evolution in tortricid moths: conserved karyotypes with diverged features. *PLoS ONE* **8**: e64520 (IF = 3.234).

Hereby I declare that Jindra Šíchová conceived the study, designed experiments, prepared all chromosome preparations, performed FISH experiments including data analysis and decisively contributed to the first version of the manuscript.

.....

František Marec

3) 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 (IF = 3.368).

Hereby I declare that Jindra Šíchová conceived the study, designed experiments, prepared all chromosome preparations, performed FISH experiments including data analysis, wrote the first draft of the manuscript and decisively contributed to the revision of the manuscript.

.....

František Marec

4) 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 of the northeast-Asian wood white butterfly, *Leptidea amurensis*. *Biol J Linnean Soc* (published online 21 January 2016) DOI: 10.1111/bij.12756 (IF = 2.264).

Hereby I declare that Jindra Šíchová conceived the study, designed experiments, prepared all chromosome preparations, performed FISH experiments including data analysis, wrote the first draft of the manuscript and decisively contributed to the revision of the manuscript.

.....

František Marec

Contents

1. General introduction

The differences in genome architecture of closely related species, along with evidence that chromosomal rearrangements contribute to the formation of postzygotic barriers, suggest a causative role of chromosomal changes in speciation. Karyotypes of many related organisms often diverge from ancestral ones by chromosomal rearrangements (e.g. inversions, translocations, fusions, and fissions). The extent and rate of karyotype changes differ in various groups of organisms depending on physical characteristics of specific karyotypes and particular structures of chromosomes (Imai *et al*. 1986, Wrensch *et al*. 1994, Henikoff *et al*. 2001, Bureš and Zedek 2014). Moreover, the effect of chromosomal rearrangements on ecological specialization and speciation may be further enhanced by involvement of sex chromosomes (X and Y in male heterogametic systems, W and Z in female heterogametic systems) due to their disproportionate role in reproductive isolation. However, despite a century of research on the evolution of karyotypes, the extent to which chromosomal rearrangements might contribute to speciation remains obscure.

The work presented in this thesis describes the karyotype and sex chromosome evolution of selected lepidopteran representatives. Moths and butterflies, the insect order Lepidoptera, constitute one of the largest animal groups (van Nieukerken *et al*. 2011). Despite their importance, our knowledge of lepidopteran genome architecture and evolution remains insufficient. The thesis fills some of these gaps and helps us to understand the general course of chromosomal evolution in Lepidoptera.

1.1. Lepidopteran cytogenetics

The Lepidoptera represent the most well-known and easily recognizable order of insects. They have also proven invaluable model systems in various fields of research such as, for example, ecology, evolution, physiology, molecular biology, and developmental genetics. However, cytogenetics of the Lepidoptera had long been overlooked mainly due to difficulties in handling the lepidopteran chromosomes. Therefore, for years deeper knowledge of chromosome biology was limited to a few model species, such as the Mediterranean flour moth (*Ephestia kuehniella*) and the silkworm (*Bombyx mori*), the latter being of a great importance for sericulture. Lepidopteran species, together with their sister group caddisflies (Trichoptera), show several peculiar cytogenetic features by which they greatly differ from other insect groups (Goldsmith and Marec 2010). The most striking feature is the chromosomal mechanism of sex determination, in which female sex is determined by heterogametic constitution Z0 or WZ. The female-specific W chromosome, if present, is usually differentiated from its partner, the Z chromosome, and can be easily recognized through its heterochromatic nature (Traut and Marec 1997, Fuková *et al.* 2005, Vítková *et al.* 2007). The W chromosome is gene-poor and consists mainly of interspersed repetitive elements such as

transposons (Sahara *et al*. 2003, Mediouni *et al*. 2004, Abe *et al.* 2005, Vítková *et al.* 2007). In somatic interphase nuclei, W chromosome forms a heterochromatin body, the so-called sex chromatin or W chromatin, which is easily discernible in polyploid cells (Traut and Marec 1996). The absence of sex chromatin in trichopteran species and some representatives of basal Lepidoptera suggest that the common ancestor of Lepidoptera had a sex determination system Z0/ZZ and the W chromosome arise later in lepidopteran evolution (reviewed in Traut *et al*. 2007). Two hypotheses have been proposed to explain the evolutionary origin of lepidopteran W chromosomes. The first hypothesis suggests the fusion between an autosome and the ancestral Z chromosome. Homologous partner of this autosome became female-limited and due to the achiasmatic meiosis and absence of recombination in females, it started to degenerate giving rise to the neo-W and neo-Z chromosomes (Traut and Marec 1997). An alternative scenario proposes that the W chromosome originated from a supernumerary B chromosome, which started to pair with the ancestral Z chromosome (Lukhtanov 2000).

In advanced Lepidoptera, numerical variations of the standard WZ/ZZ sex chromosome system occur, including secondary loss of the W chromosome or multiple sex chromosome systems with three elements, either W_1W_2Z/ZZ, or $WZ_1Z_2/Z_1Z_1Z_2Z_2$. However, multiple sex chromosomes have been described only in seven genera from different lineages of the lepidopteran phylogenetic tree and are thought to have originated via sex chromosome-autosome fusions and fissions (reviewed in Traut *et al*. 2007, Marec *et al.* 2010, Sahara *et al.* 2012). These chromosomal rearrangements have played a major role in the evolution of neo-sex chromosomes of wild silkmoths, *Samia cynthia* ssp. (Saturniidae; Yoshido *et al.* 2005, 2011, 2013). In this species complex, geographical populations differ considerably in their sex chromosome constitution, which leads to a unique polymorphism in chromosome number. Four different sex chromosome constitutions were identified, i.e. WZ/ZZ in *S. c. pryeri* with 2n=28/28, Z0/ZZ in *S. c. ricini* with 2n=27/28, 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. It has been proposed that the common ancestor of *Samia cynthia* ssp. had a standard WZ/ZZ constitution with diploid chromosome number of 2n=28/28 and the neo-sex chromosomes in *S. c. walkeri* and *S. cynthia* subsp. indet. arose by repeated sex chromosome-autosome fusions. Taking into account the sex chromosomes can play a role in population and species divergence (Charlesworth *et al*. 1987), the variation in sex chromosome constitution in *S. cynthia* ssp. makes this species a promising model for studying speciation (Yoshido *et al.* 2011).

Other interesting features of lepidopteran karyotypes concern their chromosomes. Lepidopteran chromosomes are mostly small, uniform in shape, and lack any morphological trait that allows their identification (Mediouni *et al.* 2004, Fuková *et al.* 2005). Furthermore, Lepidoptera belongs among organisms with holokinetic chromosomes, i.e. they lack distinct primary constriction,

the centromere (Carpenter *et al*. 2005). Moreover, conventional diagnostic banding patterns or discrete size distributions are continuously failing to identify individual chromosomes, and thus for a long time cytogenetic research was limited to mere chromosome counts. The majority of moths and butterflies have haploid chromosome number of n=31 (Suomalainen 1969, Robinson 1971). Based on the phylogenetic surveys in the Lepidoptera, it has been inferred that the ancestral lepidopteran karyotype consists of n=31 chromosomes. Moreover, this ancestral chromosome number has been recently supported by comparative genome mapping (Baxter *et al*. 2011, Sahara *et al.* 2013, Van't Hof *et al.* 2013, Ahola *et al.* 2014).

The absence of centromere in holokinetic chromosomes seems to facilitate karyotype evolution via chromosomal rearrangements in Lepidoptera (Wrensch *et al*. 1994). However, recent studies of comparative genomics revealed a high degree of conserved synteny of genes between the silkworm *B. mori* (Bombycoidea) and several other lepidopteran species, including not only another representative of Bombycoidea, the tobacco hornworm *Manduca sexta* (Sahara *et al*. 2007, Yasukochi *et al*. 2009), but also two species of butterflies (Papilionoidea), *Heliconius melpomene* (Yasukochi *et al*. 2006, Pringle *et al*. 2007) and *Bicyclus anynana* (Beldade *et al*. 2009), two species of Noctuidea, *Helicoverpa armigera* and *Spodoptera frugiperda* (d'Alençon *et al*. 2010), a representative of Geometroidea, the peppered moth *Biston betularia* (Van't Hof *et al*. 2013), and a representative of a very distant basal group of Yponomeutoidea, the diamondback moth *Plutella xylostella* (Baxter *et al*. 2011). The conservation at the chromosomal level across the phylogenetic tree of Lepidoptera suggests evolutionary stability of whole genomic regions.

Extraordinary stability of lepidopteran karyotypes contrasts with dynamic karyotype evolution found in some lepidopteran groups. This especially applies to blue butterflies of the genus *Polyommatus* (Lycaenidae) with the highest within-genus chromosome number variation, i.e. from n=10 to n= ca224-226. The latter comes from the Atlas blue, *P. atlanticus*, and represents the highest chromosome number of all non-polyploid eukaryotes (Kandul *et al.* 2004, Lukhtanov 2015). Similar genome size of these closely related species suggests that the chromosome number variation is not caused by polyploidy but arose as a result of chromosome rearrangements, i.e. fusion and fission (Lukhtanov *et al.* 2005). Quite exceptional intraspecific variability of karyotypes was also described in wood white butterflies of the genus *Leptidea* (Pieridae), comprising several widespread Eurasian species (Dincă *et al*. 2011, Lukhtanov *et al*. 2011). In this genus, chromosome numbers vary greatly between and within species with a tendency to increase during speciation. Two species from predominantly Eastern Palaearctic, *L. morsei* and *L. amurensis*, have most likely constant chromosome number of n=54 and n=61, respectively (listed in Robinson 1971). On the contrary, three cryptic species of the Western Palaearctic have a variable number of chromosomes. While chromosome numbers range from n=40 to n=42 in *L. juvernica* and from n=26 to n=27 in *L. reali*

(Dincă *et al*. 2011), the most extraordinary variability was described by Lukhtanov *et al*. (2011) in *L. sinapis* with a chromosomal cline ranging from n=28 in Kazakhstan to n=53 in Spain. Such exceptional intra-population chromosome number polymorphism is rare for any animal or plant and provides a strong evidence for explosive within-species accumulation of multiple chromosomal rearrangements.

1.2. Chromosomal rearrangements and karyotype evolution

Traditional chromosomal theories of speciation suggest that chromosomal rearrangements play a major role in reproductive isolation through reducing reproductive fitness of heterozygous individuals (White 1978). These models resulted from the observation that 'heterokaryotypes' may have abnormalities in meiosis, which impair their fertility. However, the first chromosome speciation models were unsatisfactory and opinions varied dramatically from the view that chromosomal rearrangements play the primary role in the majority of speciation events (White 1978), to them being considered largely incidental by-products of speciation processes (Futuyama and Mayer 1980, Coyne and Orr 1998). In addition, the most prevalent and supported model of speciation, Bateson-Dobzhansky-Muller model (the so-called BDM model), was that the reproductive isolation is mostly completed without gene flow, i.e. in allopatry. The theoretical basis for BDM model is that hybrid sterility and inviability are caused by alternatively fixed alleles in two populations of a species that evolved in geographical isolation. If individuals meet again and are still able to mate, these incompatible alleles will generate unfit or unviable hybrids and thus, result in reproductive isolation (Orr and Presgraves 2000, Gavrilets 2003). The BDM model was so straightforward that it became widely respected and chromosome speciation was neglected.

Over the past decades, many overlapping models of chromosome speciation tried to explain how chromosomal rearrangements prevent gene flow (reviewed in Rieseberg 2001). The first chromosome speciation models were hybrid-sterility models in which heterozygotes for chromosomal rearrangements are partially or totally infertile, either due to segregation problems during meiosis or the generation of dysfunctional gametes originated as products of recombination. According to these models chromosome rearrangements cause heterozygote disadvantage (underdominance) and thus can serve as a genetic barrier to gene flow between two populations (Rieseberg 2001, Faria and Navarro 2010). However, hybrid-sterility models are inconsistent and lack solid empirical support (Lande 1985, Nachman and Myers 1989, Turelli *et al*. 2001). Recently, new experimental evidence has reinvigorated the development of theoretical models that offer an alternative explanation of how chromosomal rearrangements may facilitate speciation in the face of gene flow (Rieseberg 2001, Faria and Navarro 2010, Kawakami *et al*. 2011). These suppressed-recombination models are based on the reduction of recombination between chromosomes carrying different rearrangements. These regions of restricted recombination may facilitate the development

and maintenance of reproductive isolation by creating linkage disequilibrium along large parts of the genome, including alleles contributing to local adaptation or conferring barriers to gene flow. A speciation model of suppressed recombination was first presented by Coluzzi (1982) to explain speciation events within the species complex *Anopheles gambiae*, the most important vectors of malaria in sub-Saharan Africa, caused by dangerous malaria parasite, *Plasmodium falciparum*. Later, some new verbal supressed-recombination models were proposed to account for speciation in wild sunflowers (*Helianthus*; Rieseberg 2001), or closely related *Drosophila* species (Machado *et al*. 2002). However, the strongest evidence that chromosomal rearrangements have a causative role in speciation came from a correlation detected by Noor *et al*. (2001) in recently diverged sympatric pairs of *Drosophila* species and by Navarro and Barton (2003), who compared genes and DNA sequences between humans and chimpanzees.

Of all considered chromosomal rearrangements, the scope of chromosome speciation models was mainly limited to inversion polymorphism, which affects recombination especially strongly (Navarro *et al*. 1997, Brown *et al*. 2004, White *et al*. 2010). However, chromosome fusions and fissions also have the potential to limit gene flow and drive speciation (Baker and Bickham 1986, Basset *et al*. 2006). The majority of studies on the effects of chromosome fusion and fission have been done in organism with monocetric chromosomes that exhibit Robertsonian translocations, i.e. centric fusions (Bidau *et al*. 2001, Basset *et al*. 2006). Indeed, these studies confirmed the potential of Robertsonian fusions to suppress recombination in rearranged areas of the genome by coupling previously unlinked loci. In organisms with holokinetic chromosomes, i.e. chromosomes without centromere, the role of chromosome rearrangements is not so well understood. Theoretically, the absence of centromere might facilitate chromosome speciation via fusion and fission (Wrensch *et al*. 1994). In holokinetics, the kinetochore is extended over almost the entire length of the chromosome surface (Melters *et al*. 2012) and ensures that most chromosome breaks do not lead to the loss of fragments as it is typical in species with monocentric chromosomes. Instead, the fragments are inherited in a non-Mendelian fashion and may persist over numerous cell divisions (Marec *et al*. 2001). Holocentric chromosomes have been found in plants, few algae, nematodes and some arthropod orders (reviewed in Melters *et al*. 2012). In the model organism *Caenorhabditis elegans*, a strong evolutionary force seems to maintain karyotype stability (Dernburg 2001). On the contrary, holocentry is accompanied by extensive inter- and intraspecific karyotype variability in some plants and invertebrate species (Normark 1999, Kandul *et al*. 2004, Hipp 2007, Lukhtanov *et al*. 2011). In plants, the greatest non-polyploid interspecific variation in chromosome number was found in sedge genus *Carex*, where the chromosome number varies from n=6 to n=66. In this case, chromosome evolution proceeds almost exclusively by fusion and fission, without duplication of chromosomes (Hipp 2007). Moreover, recent studies in sedges confirmed that fusion and fission of holokinetic

chromosomes might actually limit gene flow and ultimately lead to speciation (Hipp *et al*. 2010). Nevertheless, the widest diversities of within-genus karyotypes are found in invertebrates, specifically in blue butterflies of the genus *Polyommatus* (Lycaenidae), which display the greatest interspecific variation in chromosome numbers known in the animal kingdom (Kandul *et al.*, 2004; Lukhtanov, 2015).

Chromosomal rearrangements are also important forces in the rise and differentiation of sex chromosomes. It is generally agreed that sex chromosomes evolved from a pair of autosomes which acquired a sex-determining function. Natural selection seems to favor the linkage of sexually antagonistic genes, i.e. genes beneficial to one sex but deleterious to the other, to a sex-determining region and restriction of recombination between originally homologous chromosomes by inversion. Level of recombination arrest can range from the sex determining region to the whole genome of heterogametic sex (Charlesworth 1991, Ellegren 2011). The restriction of recombination ultimately leads to degeneration of the sex-specific chromosomes, i.e. Y chromosome in XY/XX systems and the W in WZ/ZZ systems, by decay of genes and accumulation of repetitive sequences. Several mechanisms have been proposed to promote the degeneration process: Muller's ratchet, accumulation of deleterious hitchhiking mutations, and background selection (Charlesworth 1991). On the other hand, still recombining X and Z chromosomes are known to undergo faster adaptive evolution in comparison with the autosomes, the phenomenon known as the 'fast-X effect' or 'fast-Z effect' (Mank *et al.* 2007, 2010), and thus can play a major role in postzygotic reproductive isolation and facilitate the divergence toward speciation (Presgraves 2008, Ellegren 2009, Štorchová *et al*. 2010). Moreover, recent studies have contributed to the idea that chromosome rearrangements, i.e. sex chromosome-autosome fusions leading to neo-sex chromosomes, might actually promote speciation (Yoshido *et al*. 2011, Kitano and Peichel 2012).

2. Outline of research

It is known that chromosomal rearrangements play an important role in speciation by limiting gene flow within and between species. Furthermore, this effect may be enhanced by involvement of sex chromosomes that are known to undergo fast adaptive evolution and play a special role in speciation due to their engagement in postzygotic reproductive isolation. The work presented in this study uses various molecular-genetic and cytogenetic techniques to describe karyotype and sex chromosome evolution of two groups of Lepidoptera, namely selected representatives of the family Tortricidae and *Leptidea* wood white butterflies of the family Pieridae. The acquired knowledge contributes to the understanding of chromosomal evolution in Lepidoptera in general. The following sections will deal with individual experiments more deeply and explain how these fit into a broader context.

In paper one we performed a detailed comparative mapping of sex-linked genes in the codling moth, *Cydia pomonella* (Tortricidae), a well-known pome fruit pest. We used fluorescence in situ hybridization (FISH) with BAC-derived probes (BAC-FISH) to construct a physical map of the codling moth Z chromosome. The results obtained revealed a neo-Z chromosome that originated by fusion of an ancestral Z chromosome and an autosome corresponding to chromosome 15 of the *Bombyx mori* reference genome. Moreover, our findings suggest that the fusion originated in a common ancestor of two main tortricid subfamilies. We discuss the significance of these finding for radiation and subsequent speciation of tortricid moths.

In paper two we examined karyotypes of four other tortricid species by standard cytogenetic techniques and by mapping multigene families, i.e. major rRNA genes and histone genes. We also identified their sex chromosomes and determined the level of molecular differentiation of the sex chromosomes. We compared these cytogenetic characteristics with those of the codling moth, *Cydia pomonella*, in order to reconstruct karyotype evolution in the family Tortricidae. Our research was also motivated by the fact that two out of four analysed moths are candidates for the sterile insect technique (SIT) and the knowledge about their genome architecture will facilitate the application of this pest control technology.

In paper three we performed a detailed karyotype analysis of three closely related *Leptidea* species from the family Pyeridae, i.e. *L. juvernica*, *L. sinapis* and *L. reali*, by means of molecular cytogenetic techniques including FISH mapping of major ribosomal RNA (rRNA) and H3 histone genes, genomic in situ hybridization (GISH) combined with FISH with telomeric probes, and comparative genomic hybridization (CGH). Previous studies showed inter- and intraspecific variation in chromosome numbers in all three species. These results suggested a dynamic karyotype evolution and stressed the role of chromosomal rearrangements in the speciation of *Leptidea* butterflies. To

extend our knowledge and to verify these results, we determined exact chromosome numbers in both sexes, mapped the location of major rDNA and H3 histone genes, and analysed sex chromosome constitution in all three species. The obtained data helped us to identify causes of the karyotype variability and reveal sex determination systems with multiple sex chromosomes, 3-4 W and 3-4 Z, which is unique not only for the Lepidoptera but also for all organisms with female heterogamety.

In paper four we further explored the chromosomal particularities of *Leptidea* butterflies by examining the karyotype of an Eastern Palearctic species, *L. amurensis*, previously reported as a species with a constant chromosome number. A comparison of male and female mitotic chromosomes allowed us to determine more accurately the range of diploid chromosome numbers in this species. We also mapped major cytogenetic markers (rDNA and H3 histone genes) and analysed sex chromosome constitution by GISH combined with telomeric probes. The analysis of female meiotic chromosomes revealed a complex sex-chromosome multivalent with three W and six Z chromosomes. The mode of meiotic pairing of the sex chromosomes suggests that the multiple sex chromosomes originated through complex chromosomal rearrangements, such as fusion, fission and translocation, between ancestral sex chromosomes and autosomes.

3. Original publications

3.1. Paper I

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**: 6931-6936.

Abstract

Changes in genome architecture often have a significant effect on ecological specialization and speciation. This effect may be further enhanced by involvement of sex chromosomes playing a disproportionate role in reproductive isolation. We have physically mapped the Z chromosome of the major pome fruit pest, the codling moth, *Cydia pomonella* (Tortricidae), and show that it arose by fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 in the *Bombyx mori* reference genome. We further show that the fusion originated in a common ancestor of the main tortricid subfamilies, Olethreutinae and Tortricinae, comprising almost 700 pest species worldwide. The Z–autosome fusion brought two major genes conferring insecticide resistance and clusters of genes involved in detoxification of plant secondary metabolites under sex-linked inheritance. We suggest that this fusion significantly increased the adaptive potential of tortricid moths and thus contributed to their radiation and subsequent speciation.

Neo-sex chromosomes and adaptive potential in tortricid pests

Petr Nguyen[a,b], Miroslava Sýkorová[a,b], Jindra Šíchová[a,b], Václav Kůta[a,b], Martina Dalíková[a,b], Radmila Čapková Frydrychová[a], Lisa G. Neven[c], Ken Sahara[d], and František Marec[a,b,1]

[a]Laboratory of Molecular Cytogenetics, Institute of Entomology, Biology Centre of the Academy of Sciences of the Czech Republic, 370 05 České Budějovice, Czech Republic; [b]Department of Genetics, Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic; [c]US Department of Agriculture, Agricultural Research Service, Yakima Agricultural Research Laboratory, Wapato, WA 98951; and [d]Laboratory of Applied Entomology, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

Edited by May R. Berenbaum, University of Illinois at Urbana–Champaign, Urbana, IL, and approved March 12, 2013 (received for review November 22, 2012)

Changes in genome architecture often have a significant effect on ecological specialization and speciation. This effect may be further enhanced by involvement of sex chromosomes playing a disproportionate role in reproductive isolation. We have physically mapped the Z chromosome of the major pome fruit pest, the codling moth, *Cydia pomonella* (Tortricidae), and show that it arose by fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 in the *Bombyx mori* reference genome. We further show that the fusion originated in a common ancestor of the main tortricid subfamilies, Olethreutinae and Tortricinae, comprising almost 700 pest species worldwide. The Z–autosome fusion brought two major genes conferring insecticide resistance and clusters of genes involved in detoxification of plant secondary metabolites under sex-linked inheritance. We suggest that this fusion significantly increased the adaptive potential of tortricid moths and thus contributed to their radiation and subsequent speciation.

adaptive evolution | leaf-rollers | performance genes | sex chromosome–autosome fusion | sex-linkage

Karyotype differences observed between closely related species have stimulated long-standing debates over the role of chromosome rearrangements in speciation. Recently, new empirical evidence has inspired the development of theoretical models that offer an explanation of how changes in genome architecture may facilitate speciation in the face of gene flow. It has been suggested that selection can favor chromosome rearrangements that decrease the incidence of recombination between alleles contributing to local adaptations, which in turn can enhance fixation of karyotype differences within local populations (1). Of all such chromosomal rearrangements, the scope of these models is limited to inversion polymorphisms that directly suppress recombination. However, another significant mode of karyotype change that often leads to speciation is intraspecific differences in chromosome numbers, altered by chromosome fusions and fissions (2). These rearrangements have the potential to limit gene flow although their effect is presumably smaller (1). Indeed, chromosome fusions have been shown to influence recombination by decreasing the number of chiasmata via their interference and, more importantly, by coupling previously unlinked loci (3). Similar to chromosomal rearrangements, genetic linkage between traits contributing to reproductive and ecological isolation has been found to impede breakdown of linkage disequilibria following recombination (4–7).

Both linkage disequilibrium and chromosome rearrangements are important forces in the rise of sex chromosomes and their subsequent differentiation. Natural selection appears to favor the linkage of sexually antagonistic alleles to sex-determining loci and inversion-mediated suppression of recombination in sex-specific W or Y chromosomes (8). The lack of recombination ultimately causes degeneration of sex-specific chromosomes via accumulation of repetitive sequences and gene loss. In contrast, recombining X and Z chromosomes are known to undergo fast adaptive evolution and play a special role in speciation due to their involvement in postzygotic reproductive isolation (8–10). Furthermore, recent reports on the turnover of sex chromosomes have contributed to the idea that sex chromosome–autosome fusions might actually promote speciation (11).

Moths and butterflies (Lepidoptera) have a WZ/ZZ sex chromosome system with female heterogamety. Although sex chromosomes have been identified in only a handful of species, derived variants W_1W_2Z/ZZ and $WZ_1Z_2/Z_1Z_1Z_2Z_2$ were observed in nine genera, suggesting a relatively high incidence of neo-sex chromosomes in this species-rich group (12). Neo-sex chromosome evolution via multiple sex chromosome–autosome fusions was described in moths with highly derived karyotypes, *Orgyia antiqua* and *Orgyia thyellina* (Lymantriidae), and in geographical subspecies of *Samia cynthia* (Saturniidae) (13). Recently, it has been suggested that the sex chromosome rearrangements in *S. cynthia* populations may contribute to the formation of reproductive barriers and facilitate divergence toward speciation (14).

A previous study predicted a translocation of an autosome onto the Z chromosome in the family Tortricidae (15). To test this hypothesis, we performed comparative physical mapping of the Z chromosome in the major pome fruit pest, the codling moth, *Cydia pomonella* (Tortricidae: Olethreutinae), and found that a neo-Z chromosome formed following fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 in the *Bombyx mori* reference genome. Furthermore, we show that the fusion originated in a common ancestor of the main subfamilies Olethreutinae and Tortricinae, which comprise 97% of extant species of tortricids. We discuss the relevance of our findings for adaptive evolution and radiation of tortricid moths.

Results

BAC-FISH Mapping of the Codling Moth Z Chromosome. Partial sequences of 17 *C. pomonella* genes linked to the chromosomes Z and 15 in the reference genome of *B. mori* (Table S1) were cloned and deposited in GenBank (see Table S2 for accession numbers). These genes included three major genes linked to insecticide resistance (*ABCC2, Ace-1*, and *Rdl*), four enzyme-coding genes (*Idh-2, Ldh*, *Pgd*, and *Tpi*), and 10 protein-coding genes without enzymatic function (*ABCF2*, *apterous, kettin*, *mago*, *nanchung*, *Notch*, *RpL10*,

Author contributions: P.N., K.S., and F.M. designed research; P.N., M.S., J.Š., V.K., and M.D. performed research; R.Č.F., L.G.N., and K.S. contributed new reagents/analytic tools; P.N., M.S., J.Š., V.K., M.D., R.Č.F., and F.M. analyzed data; and P.N., L.G.N., and F.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. JQ771334, JQ771335, JQ771337, JQ771338, JQ771339, JQ771341, JQ771343, JQ771344, JQ771346, JQ771353, JQ771354, JQ771355, JQ771357, JQ771358, JQ771360–JQ771363, JQ771368, JQ771369, JX258662, JX258665–JX258668, and JX307647).

[1]To whom correspondence should be addressed. E-mail: marec@entu.cas.cz.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1220372110/-/DCSupplemental.

RpP0, *RpS5*, and *Shaker*). In the case of *Ace-1* gene, a comparison of the obtained sequence showed 100% identity with the corresponding part of *Ace-1* isolated earlier from *C. pomonella* susceptible strain [accession no. DQ267977 (16)]. Additionally, a partial sequence *of* the *C. pomonella* circadian gene *period* (*per*) was acquired from GenBank [accession no. JX996071 (17)]. Hybridization probes generated from the cloned gene fragments were used for screening of the *C. pomonella* bacterial artificial chromosome (BAC) library. Positive BAC clones were identified and confirmed by PCR for all genes except *Ace-1* (Table S3). For full gene names and their abbreviated symbols, see Table S1.

Fluorescent in situ hybridization (FISH) of BAC-derived probes on pachytene nuclei of the codling moth confirmed conserved synteny of all nine tested orthologs of the *B. mori* Z-linked genes. Eight of these orthologs mapped to about one half of a long pachytene bivalent (Fig. 1 *A* and *D*). Also, the gene

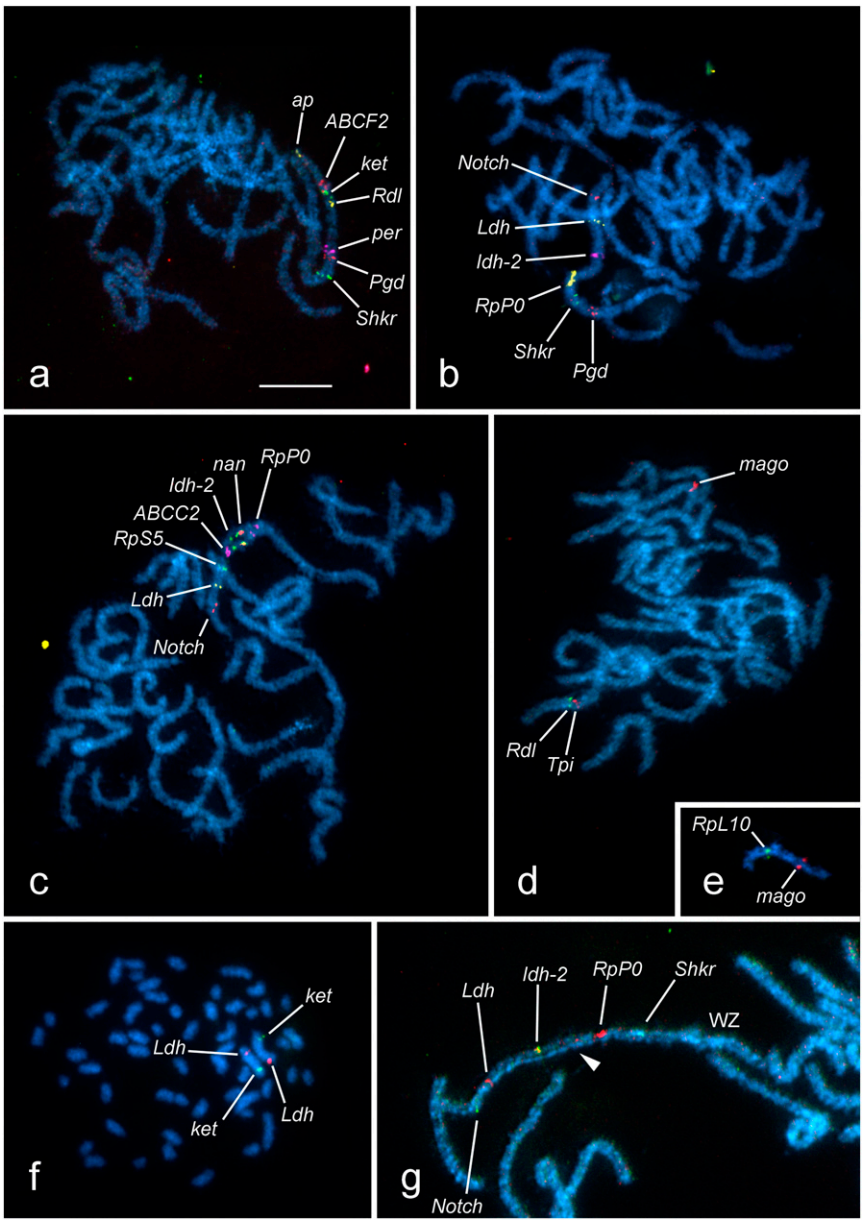

Fig. 1. BAC-FISH mapping of genes on chromosome preparations of the codling moth, *C. pomonella*. Chromosomes were counterstained with DAPI (light blue). Hybridization signals of BAC probes (yellow, green, red, and violet) indicate the physical positions of loci marked by abbreviated names. (*A–D*) Pachytene spermatocyte complements. (*A*) Three runs of BAC-FISH localized seven orthologs of *B. mori* Z-linked genes (*ap*, *ABCF2*, *ket*, *Rdl*, *per*, *Pgd*, and *Shkr*) to a single bivalent, the anticipated sex chromosome pair ZZ. (*B*) Two runs of BAC-FISH with orthologs of *B. mori* genes of the chromosomes Z (*Pgd*, *Shkr*, and *Ldh*) and 15 (*RpP0*, *Idh-2*, and *Notch*) revealed their positions on the same chromosome bivalent of *C. pomonella*. (*C*) Three runs of BAC-FISH localized six orthologs of *B. mori* chromosome 15 genes (*RpP0*, *nan*, *Idh-2*, *ABCC2*, *RpS5*, and *Notch*) and the Z-linked *Ldh* gene to the same bivalent. Note the position of *Ldh* between *RpS5* and *Notch* genes. (*D*) BAC-FISH localized two orthologs of *B. mori* Z-linked genes, *Rdl* and *Tpi*, to the anticipated Z chromosome bivalent, whereas *mago* (an ortholog of *B. mori* chromosome 15 gene) mapped to an autosome bivalent. (*E*) An autosome bivalent bearing hybridization signals of two orthologs of *B. mori* chromosome 15 genes, *RpL10* and *mago*. (*F*) Male mitotic metaphase consisting of $2n = 56$ chromosomes showing two BAC probes containing *ket* and *Ldh* genes, respectively, hybridized to two largest elements earlier identified as Z chromosomes. (*G*) A part of pachytene oocyte with the sex chromosome bivalent (WZ) easily discernible by DAPI-positive staining of a heterochromatic thread of the W chromosome (arrowhead) and characteristic twisting of paired chromosomes. Hybridization signals of BAC probes confined the *Shkr*, *RpP0*, *Idh-2*, *Ldh*, and *Notch* loci to the Z chromosome. (Scale bar: 10 μm.)

order of all but one marker (*Ldh*, see below) was conserved. However, a terminal position of the *apterous* gene and its distance from its closest neighbor *ABCF2* (Figs. 1*A* and 2) suggested a possible inversion in the subterminal chromosome region. The only exception to a strong colinearity was a BAC clone containing the *Ldh* gene, which hybridized to the other half of the same bivalent instead of its expected position between *Pgd* and *Shaker* (Fig. 1*B*). Six out of eight orthologs of the chromosome 15 genes of *B. mori* mapped to the same codling moth bivalent as the Z-linked markers. In this case, the genes retained the same gene order as their *B. mori* orthologs in chromosome 15, with *Ldh* inserted between *RpS5* and *Notch* (Fig. 1 *B* and *C*). The results of gene mapping indicate that a large chromosome rearrangement, probably a fusion event involving chromosome regions corresponding to the *B. mori* linkage groups (LG) Z and 15, differentiated karyotypes of the two species from a common ancestor. Two remaining orthologs of *B. mori* LG15 genes, namely *RpL10* and *mago*, mapped to another chromosome pair (Fig. 1 *D* and *E*), revealing a translocation corresponding to a 0.5- to 2.8-Mb segment of the *B. mori* chromosome 15. However, the distance between hybridization signals of the *RpL10* and *mago* genes on the codling moth autosome seems to greatly exceed the expected size of the translocated segment. A plausible explanation could be that the two originally closely linked genes were separated from each other by a subsequent inversion. All mapping data are integrated in Fig. 2.

BAC-FISH with selected probes on male mitotic chromosomes of the codling moth identified the rearranged chromosome as the largest element in the karyotype (Fig. 1*F*) reported earlier as the sex chromosome Z (18). Furthermore, in female preparations of pachytene oocytes, the BAC-derived probes hybridized to the WZ bivalent, which was easily discernible according to the DAPI-positive heterochromatic thread of the W chromosome. In this case, hybridization signals were confined only to the Z chromosome thread (Fig. 1*G*), which is in accordance with overall degeneration of the codling moth W chromosome (19). Taken together, we conclude that the codling moth Z chromosome is composed of two sets of genes, one originating from the ancestral Z chromosome and the other from an autosome referred to as chromosome 15 in the model species, *B. mori*.

Sex-Linkage Analysis of Selected Genes by qPCR. Because no BAC clone containing *Ace-1* was identified in the codling moth BAC library, quantitative real-time PCR (qPCR) using genomic DNA as template was used to determine a gene dose, i.e., copy number, of *Ace-1* in the codling moth males and females. The results clearly showed a twofold difference in the *Ace-1* gene dose between males and females, thus establishing its linkage to the Z sex chromosome (Fig. 3, *SI Text*, Fig. S1, Fig. S2, and Table S4).

Furthermore, two other tortricid species, the European grapevine moth *Lobesia botrana* (Olethreutinae) and the vine moth *Eupoecilia ambiguella* (Tortricinae), were studied to trace the evolutionary origin of the rearrangement between the sex chromosome Z and an autosome corresponding to *B. mori* chromosome 15. Partial sequences of *L. botrana* and *E. ambiguella* orthologs of the *Ace-1*, *EF-1α*, *mago*, and *Notch* genes were cloned and sequenced (see Table S2 for their accession nos.). Sex-linkage of *Ace-1*, *mago*, and *Notch* was then tested using qPCR with the *EF-1α* as a reference in all three tortricid species examined. The *Ace-1* and *Notch* gene doses differ significantly between males and females, suggesting their linkage to the Z chromosome (Fig. 3, Table S4). Therefore, Z chromosome–autosome fusion appears to be common to all species of subfamilies Olethreutinae and Tortricinae. Consistent with the results of BAC-FISH, the *C. pomonella mago* gene doses did not differ between males and females. Similar results were obtained by comparison of the *mago* to *EF-1α* gene dose ratios in *L. botrana*, suggesting that the *mago* gene is located on an autosome in both members of the subfamily Olethreutinae. However, different doses of the *mago* gene in males and females of *E. ambiguella*, a representative of the subfamily Tortricinae, indicate that this gene is located on the Z chromosome (Fig. 3, Table S4). Thus, the translocation of a chromosomal region containing the *mago* and *RpL10* genes to an autosome, identified in the codling moth by BAC-FISH (Figs. 1 *D* and *E* and 2), has no causal link with the Z chromosome–autosome fusion. The translocation event originated independently and much later, after the divergence of the subfamilies Olethreutinae and Tortricinae.

Discussion

We performed physical mapping of the Z sex chromosome in a major pest of pome fruit, the codling moth, *Cydia pomonella* (Tortricidae: Olethreutinae) (Figs. 1 *A*–*E* and 2). Although genome organization of the nontineoid Ditrysia (21) was shown to be highly conserved (22–24), our results revealed that a neo-Z chromosome formed following fusion between chromosomes corresponding to the linkage groups Z and 15 of the *Bombyx mori* reference genome, henceforth referred to as F(Z;15), thus supporting an earlier anecdotal prediction (15). Sex-linkage of the *Acetylcholinesterase 1* (*Ace-1*) and *Notch* orthologs of the *B. mori* chromosome 15 genes in two other tortricid pests (Fig. 3), *L. botrana* (Olethreutinae) and *E. ambiguella* (Tortricinae), strongly suggests that the F(Z;15) fusion occurred in a common ancestor of these lineages, which comprise about 97% of the tortricid species (25). The fate of the maternally inherited homolog of chromosome 15 cannot be conclusively resolved with current data sets. However, a previous molecular analysis of the codling moth W chromosome sequence library (19) along with the results of BAC-FISH (Fig. 1*G*) support the existence of extensive molecular degeneration of the codling moth W chromosome, ultimately leading to the loss of W-linked alleles.

Recently, resistance of the codling moth to a highly specific and virulent pathogen, *Cydia pomonella* granulovirus (CpGV) (Baculoviridae), has been reported. The CpGV resistance is mediated by a major gene with concentration-dependent dominance linked to the Z chromosome (26). Although other CpGV isolates were shown to overcome CpGV resistance (27, 28) caused by an early blockage of virus replication (29), its genetic basis remains elusive possibly due to false assumption of conserved gene content of the Z chromosome between *B. mori* and *C. pomonella*.

We found that three other targets for either chemical or biological insecticides, namely *Resistance to dieldrin* (*Rdl*), *Ace-1*, and *ABC transporter C2* (*ABCC2*), are linked to chromosome Z in the codling moth (Figs. 2 and 3), and presumably in all other species of the tortricid subfamilies Olethreutinae and Tortricinae, which comprise almost 700 economically important pests worldwide (30). Whereas *Rdl* orthologs conferring resistance to cyclodiene insecticides are also Z-linked in other Lepidoptera (31, 32), the *Ace-1* and *ABCC2* associated with insensitivity to organophosphates and carbamates, and resistance to *Bacillus thuringiensis* toxin Cry1Ab, respectively, are assignable to the autosomal linkage group corresponding to *B. mori* chromosome 15 in distantly related species (15, 33–35). By contrast, in most tortricids, the sex-linkage of these two genes is thus a direct consequence of F(Z;15). Theory predicts that recessive mutations conferring resistance spread faster in a pest population if they are Z-linked due to their hemizygosity in the females (36).

Although *ABCC2* mutations are reported to be recessive (33–35), the resistance conferred by insensitive Ace is in most cases semidominant. However, dominance levels of insensitive *Ace* alleles were shown to vary from recessivity to dominance and correlate with the activity of insensitive Ace forms in mosquito *Culex pipiens*. When activity of the resistant allele is low, heterozygotes, which possess only half the amount of insensitive Ace present in resistant homozygotes, display a lower tolerance to insecticide (37). This explanation seems to exclude the occurrence of recessive *Ace-1* conferred resistance in tortricids because there would be no difference in Ace-1 activity between heterozygous

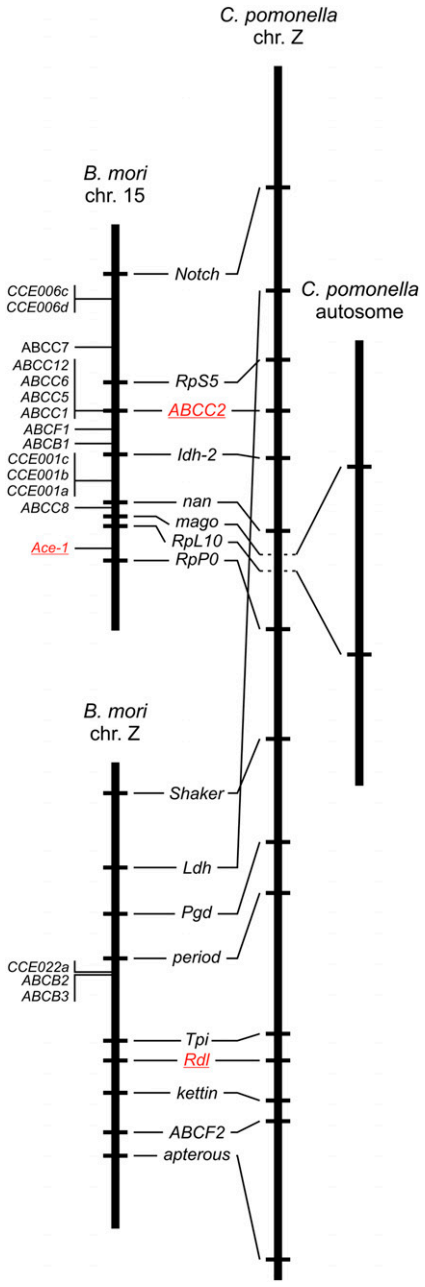

Fig. 2. A gene-based scheme of the Z chromosome of the codling moth, *C. pomonella*, integrating all BAC-FISH mapping data (Fig. 1) and its comparison with the *B. mori* chromosomes Z and 15. Locations of *B. mori* genes were retrieved from KAIKObase (Table S1). The mean relative positions of loci in the codling moth were calculated from data obtained by measuring physical distances between hybridization signals and the chromosome end in at least 10 ZZ bivalents; the distances were then related to the total length of the Z chromosome. Note the conserved synteny and conserved gene order between Z-linked genes of *B. mori* and the corresponding segment of the codling moth Z chromosome, except for *Ldh*, which moved to the part corresponding to *B. mori* chromosome 15. Carboxylesterases (CCE) and ABC transporters (ABC) with putative role in detoxification of synthetic and natural xenobiotics are annotated on the left of the *B. mori* chromosomes. Major genes conferring insecticide resistance are in red (for details, see *Discussion*).

males and hemizygous females due to absence of global dosage compensation in Lepidoptera (38). However, Kanga et al. (39) reported that Ace-1 insenstitivity, the major mechanism of carbamate resistance in a tortricid pest, *Grapholita molesta*, is both sex-linked and recessive. The recessivity of *G. molesta* Ace-1 insensitivity was probably facilitated by a female-specific modifier compensating for lower dosage of *Ace-1*, which evolved independently before resistance as suggested by Ace-1 activity ratios between sexes in both susceptible and resistant strains. Thus, the Z-linkage of both *ABCC2* and *Ace-1* is of importance to pest management programs attempting to delay the onset of insecticide resistance in tortricid pests.

It has recently been suggested that gene content might be relevant for maintenance of neo-sex chromosomes (40). The *Ace-1* and *ABCC2* genes belong to insect carboxylesterase and ATP-binding cassette (ABC) transporter gene families, whose members are involved in metabolism and regulated absorption of both insecticides and plant secondary metabolites, respectively (41–44). Along with glutathione *S*-transferases and cytochrome P450 monooxygenases (P450s), they represent the so-called performance genes affecting growth and survival of insect larvae on host plants (45). Recent analyses revealed an uneven distribution of performance gene clusters in the *B. mori* genome. In particular, chromosome 15 was shown to bear two clusters of Lepidoptera-specific esterases and a major cluster of ABC transporters (Fig. 2) (46, 47). Functions of these genes are largely unknown. However, sex-related response to organophosphates (48) correlating with sex-specific levels of general esterase activities (49) reported in *G. molesta* is consistent with sex-linkage and the absence of dosage compensation of involved genes. These findings suggest that the sex-linked esterases of tortricids play a role in detoxification of xenobiotics. Moreover, expansion of ABC transporters, including two genes located in *B. mori* chromosome 15, observed in the genome of the diamondback moth, *Plutella xylostella* (Yponomeutoidea), suggests their potential role in detoxification of plant secondary metabolites (50). Therefore, it is reasonable to assume that F(Z;15) physically linked a battery of performance genes to the tortricid Z chromosome.

Physical linkage between performance and either preference or host-independent isolation genes, shown to be disproportionately associated with the lepidopteran Z chromosome (51–54), is expected to generate genetic covariance between traits and thus facilitate ecological speciation under divergent selection (4, 55). Furthermore, performance genes are importantly associated with shifts in host plant utilization. Duplications and subsequent functional divergence of P450s have been reported to play a crucial role in dietary specialization of swallowtail butterflies of the genus *Papilio* (56). In general, duplications of performance genes are thought to be an adaptive response to environmental stress (57), a scenario well-supported by the role of gene amplification in metabolic resistance to insecticides (42, 58). Following this line of reasoning, we hereby hypothesize that duplicates of tortricid sex-linked performance genes, compensating for the loss of the W-linked alleles, were in all probability fixed as beneficial and acquired novel functions increasing the detoxification capacity of tortricid larvae. Therefore, F(Z;15) constitutes an evolutionary key innovation, potentially conferring physiological advantage in plant–herbivore interactions (59) and resulting in adaptive radiation of the species-rich tortricid subfamilies Tortricinae and Olethreutinae. Our findings thus not only contribute to management of tortricid pests but also allow a unique perspective concerning the role of neo-sex chromosomes in the adaptive radiation and ultimately speciation of phytophagous insects, a huge group of the class Insecta.

Materials and Methods

Insects. A laboratory strain (Krym-61) of the codling moth, *C. pomonella* (Olethreutinae) (for its origin and diet, see ref. 18) was used. Laboratory cultures of the European grapevine moth *L. botrana* (Olethreutinae) and the vine moth *E. ambiguella* (Tortricinae), both originating from field collections in wine-growing regions in Germany, were obtained from Annette Reineke (Research Center Geisenheim, Geisenheim, Germany) along with a rearing protocol and the composition of an artificial diet. The diet was prepared

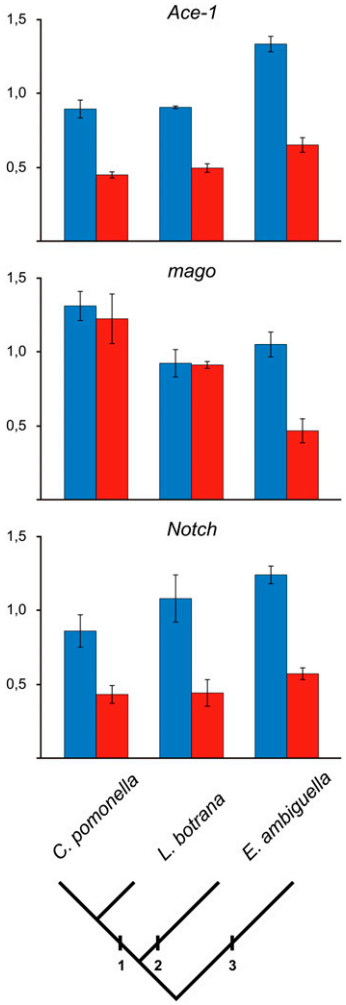

Fig. 3. Quantitative PCR comparison of male (blue columns) and female (red columns) doses of *Ace-1*, *mago*, and *Notch* genes normalized to the autosomal reference gene *EF-1α* in *C. pomonella* (Olethreutinae, Grapholitini), *L. botrana* (Olethreutinae, Olethreutini), and *E. ambiguella* (Tortricinae, Tortricini). Male and female genomic DNAs were used as templates. Error bars represent SDs calculated from three independent samples (Table S4). Twofold differences in both *Ace-1* and *Notch* gene doses between males and females suggest a Z-linkage of the genes in all three tortricids examined. However, *mago* gene doses did not differ significantly between males and females in both members of the subfamily Olethreutinae, *C. pomonella*, and *L. botrana*, thus indicating an autosomal location of the *mago* gene, in contrast with *E. ambiguella* where a two times higher dose of this gene in males compared with females suggests its Z-linkage. Phylogenetic relationships are based on ref. 20. 1, Olethreutinae; 2, Tortricinae; 3, Chlidanotinae.

according to the recipe of Christoph Hoffmann (Julius Kühn Institute, Siebeldingen, Germany). All three tortricid species were reared in a constant-temperature room under nondiapausing conditions (25 ± 1 °C; 16:8 light:dark).

Isolation of Genes for Comparative Mapping. Genes of interest were selected from a public genome database of the silkworm, *B. mori*, KAIKOBase (http://sgp.dna.affrc.go.jp/KAIKO) (Table S1). Degenerate primers were designed for regions of coding sequences conserved between the *B. mori* genes and other insect species and used for RT-PCR amplification of partial orthologous sequences in the tortricids examined (Table S2). The primer concentrations in RT-PCR were increased to 5 μM to compensate for their degeneration. First-strand cDNA synthesized from larval total RNA by oligo-dT primed SuperScript III Reverse Transcriptase (Invitrogen) was used as a template. Amplified fragments were cloned into pGEM-T Easy Vector (Promega) and confirmed by Sanger sequencing.

Identification of BAC Clones Containing Selected Genes. We used a copy of the codling moth BAC library constructed by GENEfinder Genomic Resource Laboratory (Texas A&M University, College Station, TX). Partial sequences of codling moth orthologs of selected *B. mori* genes were used as hybridization probes for screening of 18,432 *C. pomonella* BAC clones of average insert size 140 kbp, spotted as duplicates on high-density colony filters (obtained from GENEfinder Genomic Resources). Probes were labeled with alkali labile DIG-11-dUTP (Roche Diagnostics) using PCR and purified by gel filtration. Screening procedure followed a standard Southern hybridization protocol as described in ref. 19. Hybridization was carried out overnight at 42 °C. Positive BAC clones were confirmed by PCR with specific primers (Table S3). BAC-DNA was extracted using Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions.

BAC-FISH Mapping. Meiotic chromosomes were prepared from gonads of male and female larvae by the spreading technique as described in ref. 60. For FISH, BAC-DNA was labeled using a Nick Translation Kit (Abbott Molecular). Fifty microliters of labeling reaction mixture containing 1 μg of BAC-DNA and 25 μM dATP, dCTP, and dGTP each, 9 μM dTTP, and 16 μM fluorochrome-conjugated dUTP was incubated for 4 h at 15 °C. Two-color BAC-FISH with Cy3-dUTP (GE Healthcare) and ChromaTide Fluorescein-12-dUTP (Invitrogen)-labeled probes was performed following ref. 61, with some modifications. The same procedure was used for multicolor BAC-FISH, except that the probes that were labeled with Green-dUTP, Orange-dUTP, Red-dUTP (Abbott Molecular) and Cy5-dUTP (GE Healthcare). For BAC-FISH mapping, we used a reprobing protocol as described in ref. 62. Briefly, chromosome preparations were postfixed for 5 min in freshly prepared 4% formaldehyde in 2× SSC, washed twice in 2× SSC for 3 min, and incubated for 30 min in 5× Denhardt's solution in 2× SSC shortly before their denaturation in the first FISH round. The preparations were reprobed repeatedly with different probe mixtures. After each FISH round, the chromosomes were denatured during a stripping step, and the next probe mixture was applied directly to the dehydrated and air-dried slides.

Chromosome preparations were observed either in a Zeiss Axioplan 2 microscope (Carl Zeiss) or DM6000B fluorescence microscope (Leica Microsystems) equipped with appropriate fluorescence filter sets. Black-and-white images were captured with a cooled F-View CCD camera equipped with AnalySIS software, version 3.2 (Soft Imaging System), and a DFC350FX CCD camera with Leica LAS Image Analysis software (Leica Microsystems), respectively. The images were pseudocolored and superimposed with Adobe Photoshop CS3. Image analysis was carried out using freeware ImageJ (National Institutes of Health).

Quantitative Analysis of Gene Dose. qPCR using genomic DNA as a template was used to test sex-linkage of selected genes in the tortricid species studied. Gene doses of the target genes were compared with a single-copy autosomal (AA) reference gene, *elongation factor 1α* (*EF-1α*), in the male (AA, ZZ) and female (AA, WZ) genomes. If the target gene is autosomal, its copy number ratio to the autosomal reference gene is expected to be 1:1 in both sexes. In the case of Z-linkage, a target to autosomal reference gene dose ratio is expected to be 1:1 in males (ZZ) but 1:2 in females (WZ) (*SI Text*). W-linked genes should be missing completely in males.

Quantitative analysis was carried out in iQ 96-Well PCR Plates covered by Microseal "B" Adhesive Seals using the C1000 Thermal cycler CFX96 Real-Time System (Bio-Rad). Each qPCR reaction contained 1× SYBR Premix Ex Taq II (Perfect Real Time) (Takara), 0.4 μM forward and reverse primer (Table S5), and 100–150 ng of either male or female genomic DNA (gDNA) isolated from adult moths by a DNeasy Blood Tissue Kit (Qiagen). The target and reference genes were analyzed simultaneously in triplicates of three independent samples of both male and female gDNA. Default amplification efficiencies (*E*) of 1 were used to calculate target-to-reference gene dose ratio (*R*) using the formula $R = (1+E_{\text{target}})^{\text{CtTarget}}/(1+E_{\text{Ref}})^{\text{CtReference}}$. However, if *R* deviated considerably from the expected value of 1:1 in males, the PCR efficiencies were determined from the slope of the standard curve generated by plotting the threshold cycle (Ct) values against the log-concentrations of serial dilutions of male genomic DNA. The obtained data were processed using CFX Manager Software (Bio-Rad), and their significance was statistically assessed by unpaired two-tailed *t* test for unequal variances. The *t* test was used to test null hypothesis of no difference or a twofold difference in the means between males and females.

ACKNOWLEDGMENTS. We thank A. Reineke for cultures of *L. botrana* and *E. ambiguella*, D. G. Heckel for encouraging discussion at the early stage of this work, H. D. Loxdale for critical reading, and M. Korchová for technical assistance. This research was part of Entomology Institute Project Z50070508 and part of a Coordinated Research Project of the International Atomic Energy Agency, Vienna (Research Agreement 15838). Experiments were funded by Grant 523/09/2106 of the Czech Science Foundation and Grant IAA600960925 of the Grant Agency of the Academy of Sciences of the Czech

Republic. Additional support was provided from the Building up Modern Biotechnologies for Agriculture project (Registration 229518, European Union Program FP7-REGPOT-2008-1) and Grant 137/2010/P of the Grant Agency of the University of South Bohemia. K.S. was supported from Japan Society for the Promotion of Science Grant 23380030 and L.G.N. from US Department of Agriculture-Agricultural Research Service base funds.

1. Faria R, Navarro A (2010) Chromosomal speciation revisited: Rearranging theory with pieces of evidence. *Trends Ecol Evol* 25(11):660–669.
2. 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.
3. Butlin RK (2005) Recombination and speciation. *Mol Ecol* 14(9):2621–2635.
4. Hawthorne DJ, Via S (2001) Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature* 412(6850):904–907.
5. Servedio MR (2009) The role of linkage disequilibrium in the evolution of premating isolation. *Heredity (Edinb)* 102(1):51–56.
6. Wiley C, Shaw KL (2010) Multiple genetic linkages between female preference and male signal in rapidly speciating Hawaiian crickets. *Evolution* 64(8):2238–2245.
7. Merrill RM, Van Schooten B, Scott JA, Jiggins CD (2011) Pervasive genetic associations between traits causing reproductive isolation in *Heliconius* butterflies. *Proc Biol Sci* 278(1705):511–518.
8. Ellegren H (2011) Sex-chromosome evolution: Recent progress and the influence of male and female heterogamety. *Nat Rev Genet* 12(3):157–166.
9. Presgraves DC (2002) Patterns of postzygotic isolation in Lepidoptera. *Evolution* 56(6): 1168–1183.
10. Presgraves DC (2008) Sex chromosomes and speciation in *Drosophila*. *Trends Genet* 24(7):336–343.
11. Kitano J, Peichel CL (2012) Turnover of sex chromosomes and speciation in fishes. *Environ Biol Fishes* 94(3):549–558.
12. Traut W, Sahara K, Marec F (2007) Sex chromosomes and sex determination in Lepidoptera. *Sex Dev* 1(6):332–346.
13. Yoshido A, Marec F, Sahara K (2005) Resolution of sex chromosome constitution by genomic in situ hybridization and fluorescence in situ hybridization with $(TTAGG)_{(n)}$ telomeric probe in some species of Lepidoptera. *Chromosoma* 114(3):193–202.
14. Yoshido A, Sahara K, Marec F, Matsuda Y (2011) Step-by-step evolution of neo-sex chromosomes in geographical populations of wild silkmoths, *Samia cynthia* ssp. *Heredity (Edinb)* 106(4):614–624.
15. Heckel DG, Bryson PK, Brown TM (1998) Linkage analysis of insecticide-resistant acetylcholinesterase in *Heliothis virescens*. *J Hered* 89:71–78.
16. Cassanelli S, Reyes M, Rault M, Carlo Manicardi G, Sauphanor B (2006) Acetylcholinesterase mutation in an insecticide-resistant population of the codling moth *Cydia pomonella* (L.). *Insect Biochem Mol Biol* 36(8):642–653.
17. Fuková I, et al. (2009) Rapid assessment of the sex of codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) eggs and larvae. *J Appl Entomol* 133(4):249–261.
18. Fuková I, Nguyen P, Marec F (2005) Codling moth cytogenetics: Karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. *Genome* 48(6):1083–1092.
19. Fuková I, et al. (2007) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. *Chromosoma* 116(2):135–145.
20. Regier JC, et al. (2012) A molecular phylogeny for the leaf-roller moths (Lepidoptera: Tortricidae) and its implications for classification and life history evolution. *PLoS ONE* 7(4):e35574.
21. Mutanen M, Wahlberg N, Kaila L (2010) Comprehensive gene and taxon coverage elucidates radiation patterns in moths and butterflies. *Proc Biol Sci* 277(1695):2839–2848.
22. Baxter SW, et al. (2011) Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. *PLoS ONE* 6(4):e19315.
23. Heliconius Genome Consortium (2012) Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature* 487(7405):94–98.
24. Van't Hof AE, et al. (2012) Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): A model of industrial melanism. *Heredity (Edinb)* 110(3):283–295.
25. Gilligan TM, Baixeras J, Brown JW, Tuck KR (2012) *T@RTS: Online World Catalogue of the Tortricidae (Ver. 2.0)*. Available at www.tortricid.net/catalogue.asp. Accessed February 6, 2013.
26. Asser-Kaiser S, et al. (2007) Rapid emergence of baculovirus resistance in codling moth due to dominant, sex-linked inheritance. *Science* 317(5846):1916–1918.
27. Eberle KE, Asser-Kaiser S, Sayed SM, Nguyen HT, Jehle JA (2008) Overcoming the resistance of codling moth against conventional *Cydia pomonella* granulovirus (CpGV-M) by a new isolate CpGV-I12. *J Invertebr Pathol* 98(3):293–298.
28. Berling M, et al. (2009) *Cydia pomonella* granulovirus genotypes overcome virus resistance in the codling moth and improve virus efficiency by selection against resistant hosts. *Appl Environ Microbiol* 75(4):925–930.
29. Asser-Kaiser S, Radtke P, El-Salamouny S, Winstanley D, Jehle JA (2011) Baculovirus resistance in codling moth (*Cydia pomonella* L.) caused by early block of virus replication. *Virology* 410(2):360–367.
30. Zhang BC (1994) *Index of Economically Important Lepidoptera* (CAB International, Wallingford, UK).
31. Daly JC, Fisk JH (1998) Sex-linked inheritance of endosulphan resistance in *Helicoverpa armigera*. *Heredity (Edinb)* 81(1):55–62.
32. Yuan GR, Gao WY, Yang YH, Wu YD (2010) Molecular cloning, genomic structure, and genetic mapping of two *Rdl*-orthologous genes of GABA receptors in the diamondback moth, *Plutella xylostella*. *Arch Insect Biochem Physiol* 74(2):81–90.
33. Gahan LJ, Pauchet Y, Vogel H, Heckel DG (2010) An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLoS Genet* 6(12):e1001248.
34. Baxter SW, et al. (2011) Parallel evolution of *Bacillus thuringiensis* toxin resistance in Lepidoptera. *Genetics* 189(2):675–679.
35. Atsumi S, et al. (2012) Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. *Proc Natl Acad Sci USA* 109(25):E1591–E1598.
36. Orr HA (2010) The population genetics of beneficial mutations. *Philos Trans R Soc Lond B Biol Sci* 365(1544):1195–1201.
37. Bourguet D, et al. (1997) Variation of dominance of newly arisen adaptive genes. *Genetics* 147(3):1225–1234.
38. Harrison PW, Mank JE, Wedell N (2012) Incomplete sex chromosome dosage compensation in the Indian meal moth, *Plodia interpunctella*, based on de novo transcriptome assembly. *Genome Biol Evol* 4(11):1118–1126.
39. Kanga LHB, Pree DJ, Plapp FW, van Lier JL (2001) Sex-linked altered acetylcholinesterase resistance to carbamate insecticides in adults of the oriental fruit moth, *Grapholita molesta* (Lepidoptera:Tortricidae). *Pestic Biochem Physiol* 71(1):29–39.
40. Pala I, et al. (2012) Evidence of a neo-sex chromosome in birds. *Heredity (Edinb)* 108(3):264–272.
41. Sorensen JS, Dearing MD (2006) Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. *J Chem Ecol* 32(6):1181–1196.
42. Li XC, Schuler MA, Berenbaum MR (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* 52:231–253.
43. Buss DS, Callaghan A (2008) Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. *Pestic Biochem Physiol* 90(3):141–153.
44. Zangerl AR, Liao LH, Jogesh T, Berenbaum MR (2012) Aliphatic esters as targets of esterase activity in the parsnip webworm (*Depressaria pastinacella*). *J Chem Ecol* 38(2):188–194.
45. Berenbaum MR, Feeny PP (2008) *Specialization, Speciation and Radiation: The Evolutionary Biology of Herbivorous Insects*, ed Tilmon KJ (Univ California Press, Berkeley, CA), pp 3–19.
46. Yu QY, Lu C, Li WL, Xiang ZH, Zhang Z (2009) Annotation and expression of carboxylesterases in the silkworm, *Bombyx mori*. *BMC Genomics* 10:553.
47. Xie X, et al. (2012) Genome-wide analysis of the ATP-binding cassette (ABC) transporter gene family in the silkworm, *Bombyx mori*. *Mol Biol Rep* 39(7):7281–7291.
48. Shearer PW, Usmani KA (2001) Sex-related response to organophosphorus and carbamate insecticides in adult Oriental fruit moth, *Grapholita molesta*. *Pest Manag Sci* 57(9):822–826.
49. de Lame FM, Hong JJ, Shearer PW, Brattsten LB (2001) Sex-related differences in the tolerance of Oriental fruit moth (*Grapholita molesta*) to organophosphate insecticides. *Pest Manag Sci* 57(9):827–832.
50. You M, et al. (2013) A heterozygous moth genome provides insights into herbivory and detoxification. *Nat Genet* 45(2):220–225.
51. Thompson JN (1988) Evolutionary genetics of oviposition preference in swallowtail butterflies. *Evolution* 42(6):1223–1234.
52. Sperling FAH (1994) Sex-linked genes and species-differences in Lepidoptera. *Can Entomol* 126(3):807–818.
53. Prowell DP (1998) *Endless Forms: Species and Speciation*, eds Howard DJ, Berlocher SH (Oxford Univ Press, Oxford), pp 309–319.
54. Nygren GH, Nylin S, Stefanescu C (2006) Genetics of host plant use and life history in the comma butterfly across Europe: Varying modes of inheritance as a potential reproductive barrier. *J Evol Biol* 19(6):1882–1893.
55. Matsubayashi KW, Ohshima I, Nosil P (2010) Ecological speciation in phytophagous insects. *Entomol Exp Appl* 134(1):1–27.
56. Li WM, Schuler MA, Berenbaum MR (2003) Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: Specificity and substrate encounter rate. *Proc Natl Acad Sci USA* 100(Suppl 2):14593–14598.
57. Kondrashov FA, Rogozin IB, Wolf YI, Koonin EV (2002) Selection in the evolution of gene duplications. *Genome Biol* 3:0008.1.
58. Bass C, Field LM (2011) Gene amplification and insecticide resistance. *Pest Manag Sci* 67(8):886–890.
59. Ehrlich PR, Raven PH (1964) Butterflies and plants: A study in coevolution. *Evolution* 18(4):586–608.
60. 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(2):184–194.
61. Yoshido A, Bando H, Yasukochi Y, Sahara K (2005) The *Bombyx mori* karyotype and the assignment of linkage groups. *Genetics* 170(2):675–685.
62. Shibata F, Sahara K, Naito Y, Yasukochi Y (2009) Reprobing multicolor FISH preparations in lepidopteran chromosome. *Zoolog Sci* 26(3):187–190.

Supporting Information

Nguyen et al. 10.1073/pnas.1220372110

SI Text

A codling moth ortholog of *EF-1α* (LG5 in *Bombyx mori*), used as a reference gene for testing sex-linkage of selected codling moth genes by quantitative real-time PCR (qPCR), was cloned, sequenced (see Table S2 for its accession number) and used for BAC library screening (Table S3). Moreover, the codling moth BAC library was screened for an ortholog of the *Acetylcholinesterase 2* (*Ace-2*) gene (GenBank accession no. DQ267976), which is also autosomal in *B. mori* (Table S1). The *Ace-2* ortholog was used to evaluate the accuracy of qPCR for linkage testing. Subsequent FISH experiments with BAC clones containing either *Elongation factor 1 alpha* (*EF-1α*) or *Ace-2* and the Z-linked *Resistant to dieldrin* (*Rdl*) gene confirmed an autosomal location for both the *EF-1α* and *Ace-2* genes in the codling moth (Fig. S1 *A* and *B*).

Determination of sex-linkage by means of qPCR was first verified using the *kettin* and *Ace-2* genes that were already mapped to the codling moth Z chromosome and an autosome, respectively (Fig. 1 and Fig. S1*B*). The *kettin*-to-*EF-1α* gene dose ratio was about twice as high in males as in females, thus proving the Z-linkage of *kettin*. Conversely, results of quantitative analysis of the *Ace-2* gene did not differ significantly between males and females, which is consistent with the autosomal location of the codling moth *Ace-2* gene as confirmed by BAC-FISH. These results show that the qPCR is a useful and reliable tool for the gene dose based determination of sex-linked versus autosomal inheritance (Fig. 3, Fig. S2, and Table S4).

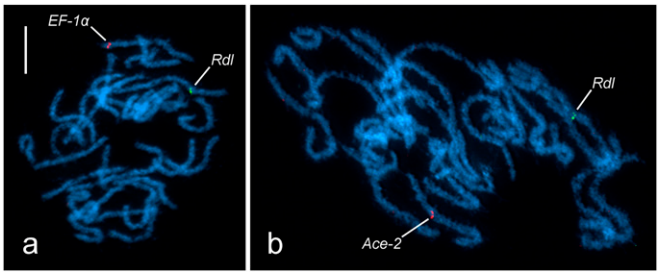

Fig. S1. BAC-FISH localization of two unmapped genes in comparison with a Z-linked gene on chromosome preparations of the codling moth, *Cydia pomonella*. Chromosomes were counterstained with DAPI (light blue). Hybridization signals of BAC probes (green and red) indicate the physical positions of loci marked by abbreviated names. (*A* and *B*) Pachytene spermatocyte complements. (*A*) Cohybridization of the BAC probe containing the Z-linked *Rdl* gene with BAC probe containing the *EF-1α* gene proved autosomal localization of *EF-1α*. (*B*) Sex-linkage of the *Ace-2* gene was excluded by cohybridization of BAC probe containing *Ace-2* with BAC probe in turn containing the Z-linked *Rdl* gene. (Scale bar: 10 μm.)

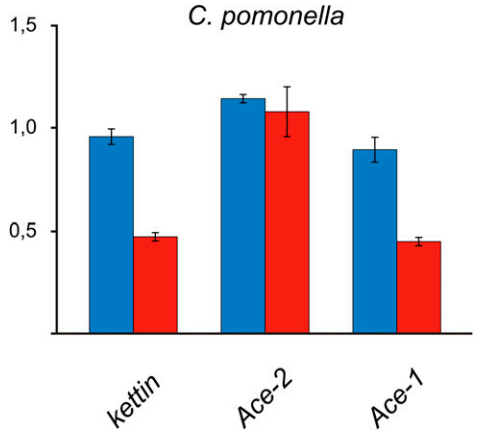

Fig. S2. qPCR determination of sex-linkage of the *Ace-1* gene in the codling moth, *Cydia pomonella*. Male (blue columns) and female (red columns) doses of *kettin, Ace-2*, and *Ace-1* genes normalized to the autosomal reference gene *EF-1α* are compared. A vertical bar at each column indicates the SD from three independent replicates. The *kettin* to *EF-1α* gene dose ratio was twice as high in males as in females due to the Z-linkage of *kettin* gene, whereas the relative dose of the autosomal *Ace-2* gene did not differ significantly between males and females. A twofold difference in the *Ace-1*-to-*EF-1a* gene dose ratios between males and females suggests Z-linkage of the *Ace-1* gene.

Table S1. *B. mori* orthologs of genes isolated in this study

Name	Symbol	Public ID*	Chromosome position*	Scaffold position*
Shaker	*Shkr*	BMgn003851	chr1: 20911282–20921258	Bm_scaf72: 1473937–1483913
lactate dehydrogenase	*Ldh*	BMgn012336	chr1: 17338625–17350610	Bm_scaf26: 2274069–2286054
Phosphogluconate dehydrogenase	*Pgd*	BMgn012298	chr1: 15112863–15127673	Bm_scaf26: 48307–63117
Period	*per*	BMgn000485	chr1: 12956618–13004501	Bm_scaf8: 7358664–7406547
Triosephosphate isomerase	*Tpi*	BMgn000559	chr1: 9023502–9027095	Bm_scaf8: 3425548–3429141
Resistant to dieldrin	*Rdl*	BMgn000568	chr1: 8060590–8089612	Bm_scaf8: 2462636–2491658
kettin	*ket*	BMgn000622	chr1: 6513219–6533895	Bm_scaf8: 915265–935941
ABC transporter family F protein ABCF2	*ABCF2*	BMgn002004	chr1: 4621452–4632826	Bm_scaf23: 4621452–4632826
apterous	*ap*	BMgn002127	chr1: 3487639–3516414	Bm_scaf23: 3487639–3516414
Ribosomal protein P0	*RpP0*	BMgn003309	chr15: 16146287–16150050	Bm_scaf42: 3154684–3158447
Acetylcholinesterase 1	*Ace-1*	BMgn003320	chr15: 15498774–15629164	Bm_scaf42: 2507171–2637561
Ribosomal protein L10	*RpL10*	BMgn003337	chr15: 14491197–14493497	Bm_scaf42: 1499594–1501894
mago nashi	*mago*	BMgn003398	chr15: 14026190–14028026	Bm_scaf42: 1034587–1036423
nanchung	*nan*	BMgn003369	chr15: 13344997–13360742	Bm_scaf42: 353394–369139
Isocitrate dehydrogenase 2	*Idh-2*	BMgn007586	chr15: 11046109–11053231	Bm_scaf66: 1438373–1445495
ABC transporter family C protein ABCC2	*ABCC2*	BMgn007793	chr15: 8949057–8952178	Bm_scaf3: 1002086–1005207
Ribosomal protein S5	*RpS5*	BMgn007710	chr15: 7586843–7587692	Bm_scaf3: 2366572–2367421
Notch	*N*	BMgn007929	chr15: 2349576–2409970	Bm_scaf3: 7544294–7604688
Elongation factor 1 alpha	*EF-1α*	BMgn003608	chr5: 17105811–17109595	Bm_scaf9: 615825–619609
Acetylcholinesterase 2	*Ace-2*	N/A	chr9	nscaf3045/nscaf3047

*KAIKObase version 3.2.2 (http://sgp.dna.affrc.go.jp/KAIKO; Accessed February 3, 2012).

Table S2. List of the partial sequences of tortricid genes obtained in this study

Species	Name*	GenBank acc. no.	Degenerate primer (forward)	Degenerate primer (reverse)
Cydia pomonella	*Shaker*	JQ771337	AAYGARTAYTTYTTYGAYAGRAA	ACRTGRTTRAARTTYTGNGAYTGCAT
	Ldh	JQ771341	GGNCARGTNGGNATGGC	CCDATNGCCCANGANGTRTA
	Pgd	JQ771338	GTNATGGGNCARAAYCTNAT	TGNCCNGTCCARTTNGTRTG
	Tpi	JQ771343	GGIAAYTGGAARATGAAYGG	CCICCIACYAARAAICCRTC
	Rdl	JQ771335	GAYTTYTAYTTYAGRCARTTYTGG	ATCCARTACATNAGRTTRAARCA
	kettin	JQ771344	AARGTIGAYACITTYGARTA	ATTGGGTATTATCGGAACG
	ABCF2	JQ771334	CARTGYGTNATGGARGTNGAYGA	GCRTCDATNGTYTCCATRTC
	apterous	JQ771339	GCNGTNGAYAGRCARTGGCA	CCAYTTNGCNCGNGCRTTYTGRAACC
	RpP0	JQ771358	ATGGGTAGGGAGGACAARGC	AGACCRAAGCCCATGTCGTC
	Ace-1	JQ771354	CGATACAAGGCATTCTGCCA	AAGTTTTGGTGCGCTAAGG
	RpL10	JQ771357	GACAAGCGTTTCWSYGGMAC	TTYCARATGAAGGTDYTGT
	mago	JQ771353	AAYTAYAARAAYGAYACNATGAT	TADATNGGYTTDATYTTRAARTG
	nanchung	JQ771346	CCNTTYGTNGTNATGATHTA	TANGTRTTNCCCATCATNGC
	Idh-2	JQ771360	GARATGGAYGGNGAYGARATG	RTGYTCRTACCADATYTTNGC
	ABCC2	JX258668	AARAGYCCNGTNTTYGGNATG	TTNRCNGTNGCYTCRTCCAT
	RpS5	JQ771355	GRTGGAGYTGYTAYGATGT	GAGTTWGATGARCCTTRGC
	Notch	JX307647	AAYAAYGCNGARTGYAAYTGGGA	ATYTGRAANACNCCCATNGCRTC
	EF-1α	JX258662	AARGARGCNCARGARATGGG	GCNACNGTYTGYCTCATRTC
Lobesia botrana	*Ace-1*	JQ771363	ACNGGNAARAARGTNGAYGCNTGG	GCRAARTTNGCCCARTAYCTCAT
	mago	JQ771369	AAYTAYAARAAYGAYACNATGAT	TADATNGGYTTDATYTTRAARTG
	Notch	JX258667	AAYAAYGCNGARTGYAAYTGGGA	ATYTGRAANACNCCCATNGCRTC
	EF-1α	JX258665	AARGARGCNCARGARATGGG	GCNACNGTYTGYCTCATRTC
Eupoecilia ambiguella	*Ace-1*	JQ771362	ACNGGNAARAARGTNGAYGCNTGG	GCRAARTTNGCCCARTAYCTCAT
	mago	JQ771368	AAYTAYAARAAYGAYACNATGAT	TADATNGGYTTDATYTTRAARTG
	Notch	JQ771361	AAYAAYGCNGARTGYAAYTGGGA	ATYTGRAANACNCCCATNGCRTC
	EF-1α	JX258666	AARGARGCNCARGARATGGG	GCNACNGTYTGYCTCATRTC

*For full gene names, see Table S1.

Table S3. List of the *C. pomonella* BAC clones mapped in this study

		Primers used for synthesis of hybridization probes		Primers used to confirm the presence of respective gene	
Gene*	BAC clone	Forward	Reverse	Forward	Reverse
Shaker	20G10	M13-26	M13-24	AGTCCAAGTTCTCGCATCGA	TACTCTGGCCACTGTGGTCG
Ldh	34N14	ATCGCCAGTAACCCCGTGG	CGCTGCTGTCTCCGTGTT	ATCGCCAGTAACCCCGTGG	CGCTGCTGTCTCCGTGTT
Pgd	03A23	M13-26	M13-24	TGCTAATGAAGCAAAAGGAACA	GCGCTGTGTGTCCATGTATT
period	23C16	ATAGACTTCGTCCACCCTTTG	CTGGATTTGCTGTCATTGTAGT	ACCTTCATACCCTTCCTGTTG	TAAAAGACGACCACTCCGTTT
Tpi	32P12	GGIAAYTGGAARATGAAYGG	ATIGCCCAIACIGGYTCRTA	CATTGGCGAGACCCTGGA	GTTCGTAGGCCAGCACCA
Rdl	23P13	M13-26	M13-24	AGGCAGTTCTGGACAGATCCACG	TGTATCGGATGTCCCGCATGGTG
kettin	33L16	GTCACAGGCAGACCTTACC	ATTGGGTATTATCGGAACG	GAAGCTGACGCGATTCGAT	TTAGGGGCTACCACTTGCT
ABCF2	25J19	M13-26	M13-24	CTCAAGACCAGCTAATGGACGTG	TCGTCCAGCAGTAGCAAGTGTGG
apterous	01K03	M13-26	M13-24	GCGGTGGACAGACAGTGGCA	GCCGGCAGTAGACCAGGTTG
RpP0	12O03	M13-26	M13-24	ATGGGTAGGGAGGACAAAGC	CCTTGATGAATTCCTTGATAG
RpL10	08A23	M13-26	M13-24	TTCTGGGAGACCAGCAGCAC	AACTTGATGGTGGCCTTGAC
mago	28B17	TGATCGGAGAGGAGCATATC	TAGATGGGCTTAATCTTGAAATG	TGATCGGAGAGGAGCATATC	TTTCAAATCCTGCACAAGGT
nanchung	40B18	CAGAATGGTGATGGGTGACTTGC	AGCTTCTATCTCGTGGTCGGTGC	CAGAATGGTGATGGGTGACTTGC	AGCTTCTATCTCGTGGTCGGTGC
Idh-2	12E19	M13-26	M13-24	CGCCTGATGAACAGAGAGTT	ATTTCCACCTTTCCAGGTTT
ABCC2	23H24	ACAATATCGGGCTTGTCCAC	TGTCCCACGGAGAAATTACC	ACAATATCGGGCTTGTCCAC	TGTCCCACGGAGAAATTACC
RpS5	32D15	GATGGAGCTGTTACGATGTC	TCGTCTGCGACGCACTCCGCG	GATGGAGCTGTTACGATGTC	TCGTCTGCGACGCACTCCGCG
Notch	19N22	M13-26	M13-24	CGGCCCGGACGGACAAGAGAT	ATGGACGCAGCAGCACCTTGA
EF-1α	09J15	M13-26	M13-24	TGATTACACTGTTTGGGGAGTC	TCCTTCATCTTGATTACTTCCG
Ace-2	11F21	AAGACAATGCGCGGGTATTTG	TCCTTCATCTTGATTACTTCCG	TGATTACACTGTTTGGGGAGTC	TCCTTCATCTTGATTACTTCCG

*For full gene names, see Table S1.

Table S4. Results of quantitative PCR

			Target-to-reference gene dose ratio, *R*							*P*	
Species	Target	Sex*	Sample I†	Sample II†	Sample III†	Mean ± SD	E_{target}‡	$E_{reference}$‡	Corrected mean ± SD‡	H_0 (1:1)§	H_0 (2:1)§
C. pomonella	*kettin*	M	2.05	1.80	1.96	1.94 ± 0.13	0.93	0.87	0.96 ± 0.04	0.001	0.798
		F	0.99	0.95	0.85	0.93 ± 0.07	0.93	0.87	0.47 ± 0.02	—	—
	Ace-2	M	2.15	2.24	2.21	2.20 ± 0.04	0.90	0.85	1.14 ± 0.02	0.523	—
		F	1.80	1.92	2.42	2.05 ± 0.33	0.90	0.85	1.08 ± 0.12	—	—
	Ace-1	M	2.23	1.92	2.24	2.13 ± 0.18	0.93	0.86	0.90 ± 0.06	0.004	0.841
		F	1.01	0.95	1.05	1.01 ± 0.05	0.93	0.86	0.45 ± 0.02	—	—
	mago	M	1.20	1.36	1.39	1.32 ± 0.10	—	—	—	0.464	—
		F	1.40	1.22	1.06	1.23 ± 0.17	—	—	—	—	—
	Notch	M	0.77	0.94	0.90	0.86 ± 0.11	—	—	—	0.005	0.757
		F	0.39	0.47	0.41	0.43 ± 0.06	—	—	—		
L. botrana	*Ace-1*	M	0.90	0.90	0.91	0.91 ± 0.01	—	—	—	0.001	0.145
		F	0.48	0.47	0.53	0.49 ± 0.03	—	—	—		
	mago	M	0.97	0.82	1.00	0.93 ± 0.10	—	—	—	0.788	—
		F	0.91	0.89	0.94	0.91 ± 0.02	—	—	—		
	Notch	M	1.27	0.97	1.00	1.08 ± 0.16	—	—	—	0.009	0.236
		F	0.49	0.34	0.50	0.44 ± 0.09	—	—	—		
E. ambiguella	*Ace-1*	M	1.33	1.40	1.31	1.34 ± 0.05	—	—	—	<0.001	0.514
		F	0.71	0.63	0.61	0.65 ± 0.05	—	—	—		
	mago	M	1.08	1.12	0.96	1.05 ± 0.08	—	—	—	0.001	0.309
		F	0.55	0.38	0.44	0.46 ± 0.08	—	—	—		
	Notch	M	2.11	2.35	2.27	2.24 ± 0.12	0.84	0.89	1.24 ± 0.06	<0.001	0.242
		F	1.05	0.94	0.88	0.96 ± 0.08	0.84	0.89	0.57 ± 0.04		

*M, male; F, female.
†Mean value ($n = 3$) in three independent samples (I–III).
‡If *R* was much higher than 1 in males, then it was corrected by the actual PCR efficiencies (*E*) calculated from the slope of the standard curve.
§Null hypothesis (H_0) of no difference (1:1) or a twofold difference (2:1) in the means between males and females was tested by unpaired two-tailed *t* test for unequal variances ($P > 0.05$ means no significant difference from the 1:1 and 2:1 ratios, respectively).

Table S5. List of primers used for qPCR

Species	Gene	Forward	Reverse
Cydia pomonella	*kettin*	ACCAGAAGGTACGTGGGCGA	CACGTTACCCGTGGCTTGGG
	Ace-1	CTGCCACATTCATGCGTTCA	ACCCAAAGCATAACAGCTGC
	mago	TGATCGGAGAGGAGCATATC	TTTCAAATCCTGCACAAGGT
	Notch	CAACGCCTTCCCCATCTTCAA	TTGTAACGGCGCAGAGGAAGC
	EF-1α	TACACTGTTTGGGGAGTCAGCT	TTCCCAATATCTTGAGCGCGT
	Ace- 2	CTGGTTCAAGGGATGGCAGA	ACCAATACCGCCGATTTTGT
Lobesia botrana	*Ace-1*	CCTGTTGAAAGTTGGGGAGACG	GGCCTGGGTCTAGGTGTGAC
	mago	CCCTTCTGGGTCGCGAGATTG	TGCACCCCTGCGTAATGGATG
	Notch	TCCAAGCATTCGCTATCGCC	GGGAACCATGTTATACCGG
	EF-1α	AGGTGCGAATACAACAATGG	GCAAGGCTGAAGGCAAGTG
Eupoecilia ambiguella	*Ace-1*	ACACTGCCTCATTCATGCGT	ACCCAGAGCATGACAGCTG
	mago	CGGAGACCTTCTGGATCACGGG	AGTTCACCAGAATTGCCCGTCT
	Notch	TCCAAGCATTCGCTATCGCC	GGGAACCATGTTATACCGG
	EF-1α	CGTTCCAATACCGCCGATTTTG	TTGGTTCAAGGGATGGAACGT

3.2. Paper II

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

Abstract

Moths of the family Tortricidae constitute one of the major microlepidopteran groups in terms of species richness and economic importance. Yet, despite their overall significance, our knowledge of their genome organization is very limited. In order to understand karyotype evolution in the family Tortricidae, we performed detailed cytogenetic analysis of *Grapholita molesta*, *G. funebrana*, *Lobesia botrana*, and *Eupoecilia ambiguella*, representatives of two main tortricid subfamilies, Olethreutinae and Tortricinae. Besides standard cytogenetic methods, we used fluorescence in situ hybridization for mapping of major rRNA and histone gene clusters and comparative genomic hybridization to determine the level of molecular differentiation of the W and Z sex chromosomes. Our results in combination with available data in the codling moth, *Cydia pomonella*, and other tortricids allow us a comprehensive reconstruction of chromosomal evolution across the family Tortricidae. The emerging picture is that the karyotype of a common ancestor of Tortricinae and Olethreutinae differentiated from the ancestral lepidopteran chromosome print of n = 31 by a sex chromosome-autosome fusion. This rearrangement resulted in a large neo-sex chromosome pair and a karyotype with n = 30 conserved in most Tortricinae species, which was further reduced to n = 28 observed in Olethreutinae. Comparison of the tortricid neo-W chromosomes showed differences in their structure and composition presumably reflecting stochasticity of molecular degeneration of the autosomal part of the neo-W chromosome. Our analysis also revealed conservative pattern of the histone distribution, which is in contrast with high rDNA mobility. Despite the dynamic evolution of rDNA, we can infer a single NOR-chromosome pair as an ancestral state not only in tortricids but probably in all Lepidoptera. The results greatly expand our knowledge of the genome architecture in tortricids, but also contribute to the understanding of chromosomal evolution in Lepidoptera in general.

OPEN ACCESS Freely available online

PLOS ONE

Chromosomal Evolution in Tortricid Moths: Conserved Karyotypes with Diverged Features

Jindra Šíchová[1,2], Petr Nguyen[1,2], Martina Dalíková[1,2], František Marec[1,2]*

1 Institute of Entomology, Biology Centre ASCR, České Budějovice, Czech Republic, **2** Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Abstract

Moths of the family Tortricidae constitute one of the major microlepidopteran groups in terms of species richness and economic importance. Yet, despite their overall significance, our knowledge of their genome organization is very limited. In order to understand karyotype evolution in the family Tortricidae, we performed detailed cytogenetic analysis of *Grapholita molesta*, *G. funebrana*, *Lobesia botrana*, and *Eupoecilia ambiguella*, representatives of two main tortricid subfamilies, Olethreutinae and Tortricinae. Besides standard cytogenetic methods, we used fluorescence *in situ* hybridization for mapping of major rRNA and histone gene clusters and comparative genomic hybridization to determine the level of molecular differentiation of the W and Z sex chromosomes. Our results in combination with available data in the codling moth, *Cydia pomonella*, and other tortricids allow us a comprehensive reconstruction of chromosomal evolution across the family Tortricidae. The emerging picture is that the karyotype of a common ancestor of Tortricinae and Olethreutinae differentiated from the ancestral lepidopteran chromosome print of n = 31 by a sex chromosome-autosome fusion. This rearrangement resulted in a large neo-sex chromosome pair and a karyotype with n = 30 conserved in most Tortricinae species, which was further reduced to n = 28 observed in Olethreutinae. Comparison of the tortricid neo-W chromosomes showed differences in their structure and composition presumably reflecting stochasticity of molecular degeneration of the autosomal part of the neo-W chromosome. Our analysis also revealed conservative pattern of the histone distribution, which is in contrast with high rDNA mobility. Despite the dynamic evolution of rDNA, we can infer a single NOR-chromosome pair as an ancestral state not only in tortricids but probably in all Lepidoptera. The results greatly expand our knowledge of the genome architecture in tortricids, but also contribute to the understanding of chromosomal evolution in Lepidoptera in general.

Citation: Šíchová J, Nguyen P, Dalíková M, Marec F (2013) Chromosomal Evolution in Tortricid Moths: Conserved Karyotypes with Diverged Features. PLoS ONE 8(5): e64520. doi:10.1371/journal.pone.0064520

Editor: Daniel Doucet, Natural Resources Canada, Canada

Received February 28, 2013; **Accepted** April 15, 2013; **Published** May 24, 2013

Funding: This research was part of a Coordinated Research Project of the IAEA, Vienna (Research Agreement No. 15838). Experiments were funded by GACR 523/09/2106 and GAAV IAA600960925 grants. Additional support was provided from GAJU 137/2010/P and 059/2010/P grants. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: marec@entu.cas.cz

Introduction

Moths and butterflies (Lepidoptera) constitute, with nearly 160,000 described species, one of the largest groups of animals [1]. Despite the species richness, the Lepidoptera are far more homogeneous, structurally and ecologically, than the other large insect orders such as Coleoptera, Diptera, and Hymenoptera [2]. This also applies to their cytogenetic characteristics. Holokinetic chromosomes of Lepidoptera possess very few differentiating features. They lack primary constrictions (centromeres *sensu stricto* [3]) and they are usually small, numerous, and uniform in shape. Although their holokinetic structure is expected to facilitate karyotype evolution via chromosome fusion and fission (see discussion in [4]), the architecture of lepidopteran genomes appears to be relatively stable. Most species have haploid chromosome numbers close to 30, and the modal number of n = 31 occurs from basal to advanced clades [5–8]. Recent comparative genomic studies revealed extensive conserved synteny of genes between the silkworm (*Bombyx mori*) and several other lepidopteran species (e.g. [4,9–13]), which suggests evolutionary stability of whole genomic regions. Additionally, these studies established the chromosome number of n = 31 as an ancestral karyotype of non-tineoid Ditrysia (*sensu* [14]). The high degree of conservation at the chromosomal level across the phylogenetic tree of Lepidoptera contrasts with exceptional diversity found in some taxa [15,16].

The family Tortricidae with about 10,300 described species of moths includes almost 700 potential pests of agricultural, forest, and ornamental plants [17–19], and it is thus among major lineages of basal Ditrysia in terms of species richness and economic importance. The family is comprised of three subfamilies, Chlidanotinae, Tortricinae, and Olethreutinae [20]. A recently published molecular analysis of phylogenetic relationships within tortricids confirmed Chlidanotinae as the earliest diverging lineage and supported Tortricinae and Olethreutinae as sister groups [21]. The overall significance of tortricids is demonstrated by numerous studies on various aspects of their taxonomy, biology, and pest control [22]. However, cytogenetics of tortricids is poorly explored. Nothing is known about chromosomes in Chlidanotinae, the smallest subfamily with about 240 species [20]. In the other two subfamilies, published cytogenetic data are available for 40 species, mostly reporting only chromosome numbers in males. In Tortricinae, 24 out of 25 species examined have the same haploid chromosome number of n = 30 [5,23–28]. These include, for

example, the spruce budworm *Choristoneura fumiferana* (Clemens), which is one of the most destructive pests of coniferous forests in North America. In Olethreutinae, eight out of 15 species examined have n = 28; other species have different, mostly reduced chromosome numbers [25,26,29–32].

The only tortricid in which detailed cytogenetic research was performed is the codling moth, *Cydia pomonella* (L.). This species belongs to the tribe Grapholitini of the subfamily Olethreutinae, and its larva is a well-known pest of pome fruits (apple, pear, and quince) and walnuts [33]. The codling moth has n = 28 and a WZ/ZZ (female/male) sex chromosome system [32]. In contrast to a typical lepidopteran karyotype, which shows a gradual decrease in chromosome size (e.g. [34]; reviewed in [7]), the codling moth karyotype consists of chromosomes of several size-groups and has two special features: (i) although it has two nucleolar organizer regions (NORs) as several other lepidopteran species, the NORs are located at the opposite ends of a single autosome [32,35]; (ii) both the W and Z sex chromosomes are remarkably larger than the autosomes [32], which is unusual in the Lepidoptera (cf. [4,34,36,37]). Nevertheless, basic characteristics of the codling moth sex chromosomes are similar to those found in other lepidopterans (reviewed in [38,39]). The W and Z chromosome, though similar in size, are highly differentiated from each other. The Z chromosome is composed of gene-rich euchromatin and resembles the autosomes. In contrast, the W chromosome is heterochromatic and composed mainly of repetitive DNA sequences [32,40].

Recently, we have physically mapped the large Z chromosome of the codling moth using fluorescence *in situ* hybridization (FISH) with bacterial artificial chromosome (BAC) probes, the so-called BAC-FISH, and showed that it is in fact a neo-Z chromosome that has arisen by fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 in the *Bombyx mori* reference genome. Further experiments, performed by quantitative PCR (qPCR) showed a Z-linkage of selected orthologs of *B. mori* chromosome 15 genes in two other tortricids, *Lobesia botrana* and *Eupoecilia ambiguella* (see below). The results suggest that the Z chromosome-autosome fusion originated in a common ancestor of the main tortricid subfamilies, Olethreutinae and Tortricinae [41].

In this study, we examined karyotype features in four other species of tortricids by standard cytogenetic techniques and by mapping multigene families (major rRNA genes and histone genes) using fluorescence *in situ* hybridization (FISH) with 18S rDNA and H3 histone probes. We also used comparative genomic hybridization (CGH) to determine the level of molecular differentiation of the W and Z sex chromosomes. Cytogenetic characteristics were compared with those of the codling moth [32] with the aim to understand karyotype and sex chromosome evolution in the family Tortricidae. Such complex comparisons have never been done across any lepidopteran family, except for the W chromosome divergence in the family Pyralidae [37]. For our research we chose two pests of pome and stone fruits, the Oriental fruit moth, *Grapholita molesta* (Busck) and the plum fruit moth, *Grapholita funebrana* (Treitschke), both close relatives of the codling moth (Olethreutinae: Grapholitini), and two pests of cultivated grapes, the European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) from the tribe Olethreutini and the vine moth, *Eupoecilia ambiguella* (Hübner) representing the tribe Cochylini of Tortricinae. Our choice was also motivated by the fact that *G. molesta* and possibly the two grape pests are candidate species for their control by sterile insect technique (SIT), which is currently used against the codling moth [42], and the acquired cytogenetic knowledge may facilitate transfer of the technology to these and other tortricid pests.

Materials and Methods

Insects

We used a laboratory wild-type strain of the codling moth, *Cydia pomonella*, referred to as Krym-61 (for its origin, diet and rearing conditions, see [32]). A laboratory culture of *Grapholita molesta* was obtained from Beatrice Christoffel and Silvia Dorn (Applied Entomology, Institute of Agricultural Sciences, ETH Zürich, Switzerland). The culture was established from a wild population collected in orchards in the province Emilia Romagna, Italy (see [43]). For *G. molesta*, we used the same diet and rearing conditions as for the codling moth. Laboratory cultures of *Lobesia botrana* and *Eupoecilia ambiguella*, both originating from field collections in wine-growing regions in Germany, along with a rearing protocol and composition of artificial diet were obtained from Annette Reineke (Department of Phytomedicine, Research Center Geisenheim, Germany). The diet was prepared according to the recipe of Christoph Hoffmann (Julius Kühn Institute, Siebeldingen, Germany). Briefly, agar (50 g) was boiled in 1.5 L of water, cooled down to about 60°C, and then supplemented with the following ingredients: wheat germ (187 g), casein (88 g), dried yeast (38 g), Wesson salt mixture (25 g), sugar (74 g), benzoic acid (4 g), cholesterol (2.5 g), methylparaben (2.5 g), ascorbic acid (40 g), Vanderzant vitamin mixture (15 g), chloramphenicol (1 g), formaldehyde (1 mL), and sunflower oil (5 mL). All four tortricid species were reared in a constant-temperature room under non-diapausing conditions (25±1°C; 16 h light : 8 h dark regime), without humidity control.

In *Grapholita funebrana*, it is difficult to establish a laboratory culture. Therefore, we used field-collected larvae from infested plum trees near České Budějovice, along the road between the villages of Zaliny and Ledenice.

This study was performed in strict accordance with the laws of the Czech Republic. Herewith we declare that all species used are agricultural pests not listed as endangered species (see the Decree of the Ministry of the Environment CR no. 395/1992 of the Legal Code, including updated versions), and no permissions are required for their collection and further use for research. The only field-collected species, *G. funebrana*, was sampled in a free-access state land, where no permission is needed.

Chromosome Preparations

In each species, two types of spread chromosome preparations were made. Meiotic chromosomes were obtained from gonads of the fifth instar male and female larvae as described in [34]. Briefly, after dissection in a physiological solution testes were pretreated for 10 min in a hypotonic solution (0.075 M KCl), fixed in Carnoy fixative (ethanol/chloroform/acetic acid, 6:3:1) for 15 minutes, dissociated with tungsten needles in a drop of 60% acetic acid and spread on the slide using a heating plate at 45°C. Ovaries were fixed without hypotonization to preserve the chromomere pattern of pachytene bivalents. Mitotic chromosomes were obtained from wing imaginal discs of the fifth instar larvae of both sexes. Wing discs were dissected out in a physiological solution, swollen for 20 min in 0.075 M KCl, and further processed as described above.

For visualization of the W chromosome, spread pachytene oocytes were stained in 2.5% lactic acetic orcein and inspected with phase-contrast optics. This technique is routinely used in Lepidoptera as it often allows identification of the sex chromosome bivalent by densely stained heterochromatin of the W chromosome, while autosome bivalents and the Z chromosome show a chromomere-interchromomere pattern [38]. For chromosome counts, preparations from wing discs were directly stained with

0.5 μg/mL DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) in antifade based on DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma-Aldrich). Preparations for FISH techniques were passed through a graded ethanol series (70%, 80%, and 100%, 1 min each) and stored at −80°C until further use.

Preparation of Polyploid Nuclei

Malpighian tubules from fifth instar male larvae, third and fifth instar female larvae, and adult females were dissected out in a physiological solution, briefly fixed in Carnoy fixative and stained in 1.5% lactic acetic orcein. Preparations were inspected in a light microscope for the presence of female specific sex chromatin (see [44]).

FISH with 18S rDNA and H3 Histone Probes

Unlabeled 1650 bp long 18S rDNA probe was generated by PCR from the codling moth genomic DNA (gDNA) extracted from adults by standard phenol-chloroform extraction as described in [32]. The probe was labeled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) by nick translation using Nick Translation Kit (Abbott Molecular Inc., Des Plaines, IL, USA).

To prepare a H3 histone probe specific to codling moth, we used two degenerate primers, forward (5′-ATGGCNCGTAC-NAARCARAC-3′) and reverse (5′-TANGCACGYTCNCG-GAT-3′). The primers were designed in conserved regions identified by multiple alignments of H3 amino acid sequences of several insect species. A codling moth orthologous sequence of the H3 histone gene was generated by PCR in an XP Thermal Cycler (Bioer Technology, Hangzhou, China). Reaction was carried out in 25-μL reaction volumes containing 1× Ex *Taq* buffer, 0.2 mM dNTP mix, 5 μmol each primer, 0.25 U TaKaRa Ex *Taq* Hot Start DNA polymerase (TaKaRa, Otsu, Japan), and about 100 ng of template cDNA prepared from total codling moth RNA as described below. An initial denaturation period of 5 min at 94°C was followed by 30 cycles of 30 s at 94°C, 1 min at 60°C, and 45 s at 72°C, and by a final extension step of 7 min at 72°C. The PCR product showed a single band of about 360 bp on a 1% agarose gel. The band was cut out from the gel, and the DNA was extracted using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The extracted DNA sequence was cloned into Promega pGEM T-Easy Vector (Promega), verified by sequencing, and the plasmid was used as a template for PCR amplification of the H3 histone probe. Labeling reaction was carried out in 15-μL volumes containing 1× Ex *Taq* buffer, 0.1 mM of each dATP, dGTP, and dCTP, 0.065 mM dTTP, 0.035 mM biotin-16-dUTP, 5 μmol each M-13 universal primers, 0.25 U TaKaRa Ex *Taq* Hot Start DNA polymerase, and about 5 ng of plasmid DNA. An initial denaturation period of 2 min at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, and by a final extension step of 2 min at 72°C.

A total RNA of the codling moth for preparation of the H3 histone probe was isolated from larvae of both sexes by RNA blue (Top-Bio, Prague, Czech Republic) following the supplier's protocol. RNAs were incubated with DNase I (USB Corporation, Cleveland, OH, USA) for 15 min at 37°C to remove potential contamination by DNAs. The first cDNA strand was synthesized by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol; then the reverse transcriptase was inactivated by heating for 15 min at 70°C. Samples were incubated with 5 U RNase H (TaKaRa) for 20 min at 37°C to remove template RNA. RNase H was inactivated by heating for 20 min at 65°C.

FISH with the 18S rDNA and H3 histone probes was carried out as described in [32]. Briefly, chromosome preparations were removed from freezer, dehydrated in the ethanol series, and digested with 100 μg/mL RNase A to remove an excessive amount of rRNAs. After denaturation the chromosomes were hybridized with a probe cocktail containing 15 ng of biotinylated probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich) per slide. Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA), amplified with biotinylated anti-streptavidin (Vector Labs. Inc., Burlingame, CA, USA) and again detected with Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 μg/mL DAPI and mounted in antifade based on DABCO.

Comparative Genomic Hybridization (CGH)

In each species, gDNA was extracted separately from adult males and females by DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany), except for *G. funebrana* where it was extracted from larvae. Labeling of gDNAs was done using Nick Translation Kit (Abbott Molecular Inc.). Male DNA was labeled with Cy3-dUTP (GE Healthcare, Milwaukee, WI, USA) and female DNA with fluorescein-12-dUTP (Invitrogen). Unlabeled male gDNAs, used as a species-specific competitor, were prepared as follows. In each species, the extracted gDNA was first amplified by GenomiPhi HY DNA Amplification Kit (GE Healthcare) and then sonicated using a Sonopuls HD 2070 (Bandelin Electric, Berlin, Germany), with two cycles of five pulses at 70% power.

CGH was performed according to [36] with several modifications. Briefly, after removal from the freezer, chromosome preparations were dehydrated in the ethanol series, treated with 100 μg/mL RNase A, and denatured. Then the preparations were hybridized with a denatured probe cocktail containing labeled female and male gDNAs (250 ng each), unlabeled sonicated male gDNA (2.5 μg), and sonicated salmon sperm DNA (25 μg) for 3 days at 37°C, washed for 5 min at 62°C in 0.1× SSC containing 1% Triton X-100, counterstained with 0.5 μg/mL DAPI and mounted in antifade based on DABCO.

Microscopy and Image Processing

Preparations were observed in a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Black-and-white images were recorded with a cooled F-View CCD camera and captured with AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany). In FISH preparations, images were captured separately for each fluorescent dye and pseudocolored (light blue for DAPI, green for fluorescein, and red for Cy3) and superimposed with Adobe Photoshop, version 7.0.

Results

Mitotic Karyotypes

Chromosome numbers were determined from mitotic metaphase chromosomes prepared from wing imaginal discs, which have a high mitotic index in the last (fifth) larval instar because of intensive proliferation of the cells. In each species, several tens of metaphase complements from several specimens of both sexes were examined.

Mitotic metaphase complements showed similar features in all tortricids examined (Figure 1a–h). They consisted of mostly rod-shaped chromosomes without any morphological landmarks such as the centromeres, as typical for holokinetic chromosomes in Lepidoptera. There was no difference in chromosome counts between sexes. Based on repeated counts we concluded that three

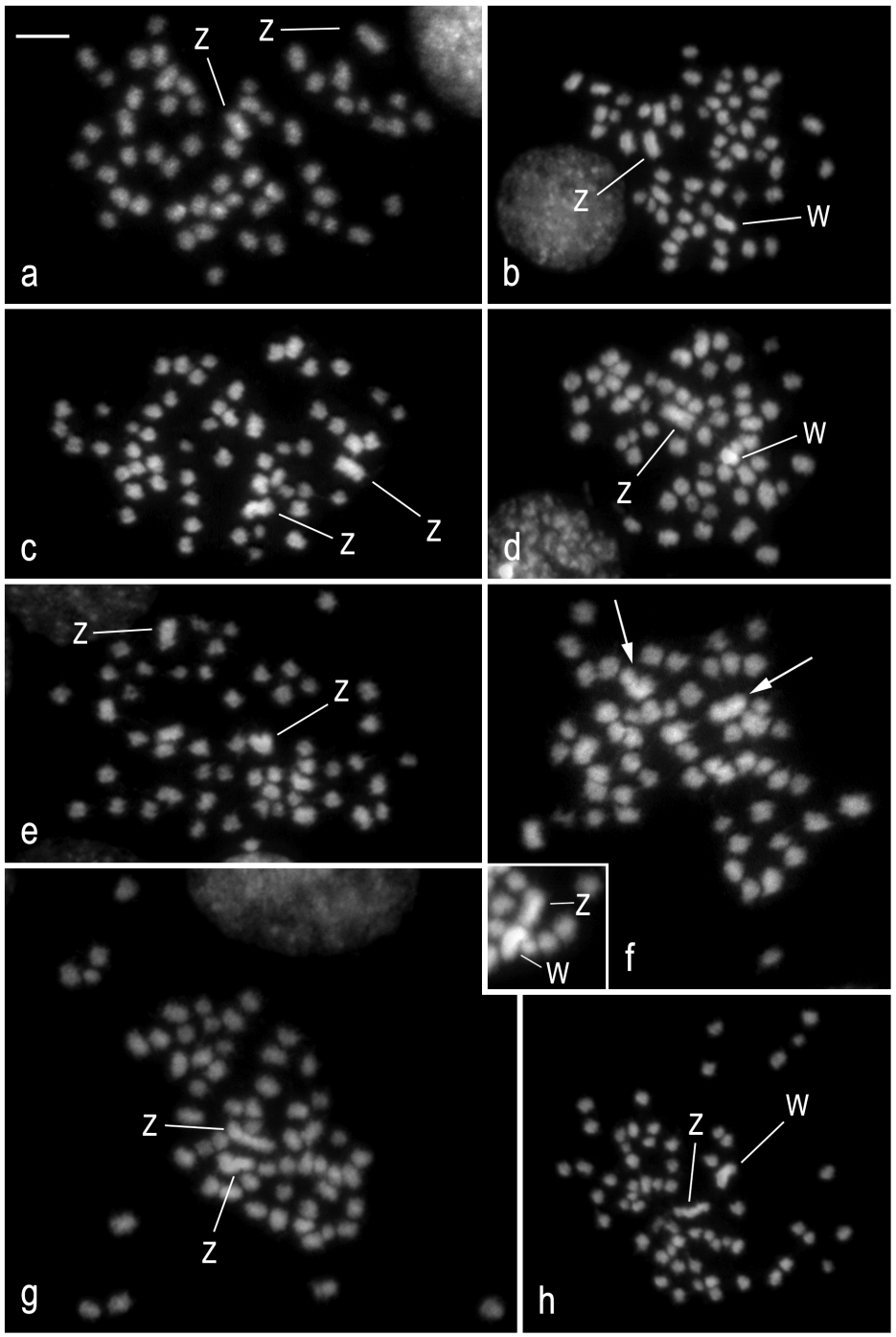

Figure 1. Chromosome preparations of wing discs in four members of the family Tortricidae. Spread mitotic chromosomes were stained with DAPI. White lines point to the largest chromosomes in the karyotype, the W and Z sex chromosomes. *Grapholita molesta* (**a**, **b**): **a** – male mitotic metaphase (2n = 56); **b** – female mitotic metaphase (2n = 56). *Grapholita funebrana* (**c**, **d**): **c** – male mitotic complement (2n = 56); **d** – female mitotic complement (2n = 56). *Lobesia botrana* (**e**, **f**): **e** – male mitotic nucleus (2n = 56); **f** – female mitotic nucleus (2n = 56) with indiscernible sex chromosome pair (arrows); the inset in the bottom left corner shows a detail of another mitotic nucleus with differentiated W and Z chromosomes. *Eupoecilia ambiguella* (**g**, **h**): **g** – spread male mitotic metaphase (2n = 60); **h** – spread female mitotic metaphase (2n = 60). Bar = 5 μm.
doi:10.1371/journal.pone.0064520.g001

species, *G. molesta* (Figure 1a, b), *G. funebrana* (Figure 1c, d), and *L. botrana* (Figure 1e, f), have identical numbers of 2n = 56 chromosomes like the codling moth, *C. pomonella* (see [32]), whereas the karyotype of *E. ambiguella* consists of a higher chromosome number of 2n = 60 (Figure 1g, h).

In male metaphases of each species, two chromosomes stood out by their large size (Figure 1a, c, e, g). Similarly, two large chromosomes were observed in female metaphases of *G. molesta*, *L. botrana*, and *E. ambiguella* (Figure 1b, f, h). However, in contrast to the largest chromosomes in males they differed from each other by size with the smaller (and/or more compact) chromosome more

intensely stained with DAPI. The exception was *G. funebrana*, which showed only one large and one DAPI-highlighted middle-sized chromosome in female metaphases (Figure 1d). Based on the comparison between male and female chromosome complements and also on the analysis of sex chromosome bivalents in pachytene oocytes (see later) we concluded that the two largest chromosomes represent a ZZ pair of the sex chromosomes in males and that the smaller DAPI-positive member of the WZ pair in females is the W chromosome composed of heterochromatin. Very large sex chromosomes including the DAPI-positive W chromosome were previously observed in *C. pomonella* (cf. [32]).

Chromosomal Location of Major rDNA

The tortricids examined did not differ in the number of rDNA sites. In each species, FISH with the 18S rDNA probe revealed a single cluster of rRNA genes, i.e. one NOR per haploid genome, associated with a small block of DAPI-positive heterochromatin (Figure 2a–h). However, the species differed in the location of rDNA. In pachytene nuclei of *G. molesta*, terminal hybridization signals localized an rDNA cluster to the end of an autosome bivalent (Figure 2a), whereas in the closely related *G. funebrana*, a large interstitial rDNA cluster was found in about one third of an autosome bivalent (Figure 2b, c). In *L. botrana*, an rDNA cluster was positioned near the end of a shorter autosome bivalent (Figure 2d). By contrast, pachytene spermatocytes of *E. ambiguella* showed an rDNA cluster at the end of the longest bivalent (Figure 2e), thus indicating its Z-linkage. Similarly, the probe localized an rDNA cluster to the end of the WZ bivalent in pachytene oocytes (Figure 2f). A comparison of hybridization signals in male and female mitotic metaphases confirmed the terminal location of rDNA in both the W and Z sex chromosomes in *E. ambiguella* (Figure 2g, h) and also confirmed the autosomal location of rDNA in the other species (not shown). Taken together with two rDNA clusters located at the opposite ends of a single autosome pair in the codling moth (see [32]), the results suggest dynamic repositioning of rDNA in tortricids.

Chromosomal Location of H3 Histone Genes

FISH with the H3 histone probe was also performed in the codling moth, *C. pomonella*, as it has not been done in this species yet. In pachytene spermatocytes of all five tortricid species, the probe localized a single cluster of H3 histone genes in a shorter autosome bivalent, and similar to rDNA the hybridization signals co-localized with a small block of DAPI-positive heterochromatin (Figure 3a-g). In four species (*C. pomonella*, Figure 3a; *G. funebrana*, Figure 3b; *G. molesta*, Figure 3c; *E. ambiguella*, Figure 3f, g), the hybridization signals positioned the H3 gene cluster near the midpoint of the bivalent, only in *L. botrana* near the end of the bivalent (Figure 3d, e). Similar sizes of the bivalent in all five species as well as similar positions in four of these species suggest a conserved chromosomal location of H3 histone gene cluster in the tortricids.

Differentiation of Sex Chromosomes

In each tortricid species, we first examined the status of sex chromatin, which is formed in polyploid somatic nuclei of lepidopteran females by multiple copies of the heterochromatic W chromosome. The sex chromatin is a suitable marker for determining the presence or absence of the W chromosome and also for possible interchromosomal rearrangements involving the W chromosome [38,44]. As expected, no heterochromatin was observed in somatic polyploid nuclei of males (Figure 4a, e, h, l). In young female larvae of all four species, oval nuclei of a lower ploidy level showed a single heterochromatin body (Figure 4b, g, i, m), indicating the presence of a single W chromosome in the female genomes. However, there were between-species differences in the sex chromatin status of older larvae and adult females. Like in the codling moth (see Figure 4 in [32]), a large single W-body was found in highly polyploid female nuclei of two species, *G. funebrana* (Figure 4f) and *E. ambiguella* (Figure 4n, o). In *G. molesta* larvae, the W-body did not grow proportionally with the nucleus growth (cf. Figure 4b and c). Moreover, the sex chromatin in branched nuclei of adult females was disintegrated into two or more smaller bodies (Figure 4d). Similarly in *L. botrana* females, highly polyploid nuclei of fifth instar larvae showed a very tiny (or none) W-body (Figure 4j), while branched nuclei of adult moths showed several smaller sex chromatin bodies (Figure 4k). The W-body fragmentation indicates that some parts of the W chromosomes in *G. molesta* and *L. botrana* are composed of transcriptionally active euchromatin, which might affect the W-body formation as shown in structural mutants of the W chromosome in the flour moth, *Ephestia kuehniella* [45,46].

To identify the WZ bivalent we first examined spread preparations of pachytene oocytes stained with lactic acetic orcein in three tortricid species, *G. molesta*, *L. botrana*, and *E. ambiguella*. This research was not done in *G. funebrana*, as we did not find an optimal stage of larvae in the field-collected samples of this species. In *G. molesta*, most pachytene bivalents showed an indistinctive pattern of chromomeres, and no bivalent was discernible from autosomal bivalents by W heterochromatin (Figure 5a). Whereas in *L. botrana*, a WZ bivalent was easily identified according to the deeply stained thread of the W chromosome (Figure 5b), clearly differentiated from the Z chromosome that was weakly stained except for several conspicuous chromomeres (Figure 5c). The WZ bivalent was also easily discernible in pachytene complements of *E. ambiguella*. One end of the bivalent was associated with the large nucleolus (Figure 5d). In some nuclei with not yet paired sex chromosomes, both the W and Z chromosome univalents were anchored in the nucleolus (Figure 5e). This finding is consistent with the results of rDNA-FISH, which localized rDNA clusters to the ends of both the W and Z chromosomes (see Figure 2h). The W chromosome of *E. ambiguella* was composed of a continuous thread of heterochromatin except for the end distal to the nucleolus, which showed a chromomere pattern similar to the Z chromosome (Figure 5d).

Molecular differentiation of the W and Z chromosomes was examined using CGH. In pachytene oocytes of each tortricid species (except *G. funebrana*; see above), CGH identified the WZ bivalent by strong binding of both the female-derived and male-derived genomic probes to the W chromosome, with slight preference for the female probe (Figures 6a–t). However, we found considerable between-species differences in the distribution of hybridization signals. In *C. pomonella*, which was used as a control, both probes strongly bound to the W thread of the WZ bivalent, except for short terminal segments at both ends that were less labeled with the female probe (Figure 6a, d, e). The W chromosome was also highlighted with DAPI (Figure 6c). A similar CGH pattern was reported by [32]. The W chromosome of *G. molesta* was also highlighted by both probes, but hybridization signals were much weaker and scattered along the entire W length, consistently with indistinctive staining pattern of DAPI (Figure 6f, h–j). In *L. botrana*, the W chromosome was decorated with strong but scattered hybridization signals of both probes in more than half of the WZ bivalent; however, in the remaining part the W chromosome was almost indistinguishable from the Z chromosome (Figure 6k, m–o). Similarly, the W chromosome of *E. ambiguella* showed a continuous pattern of strong hybridization

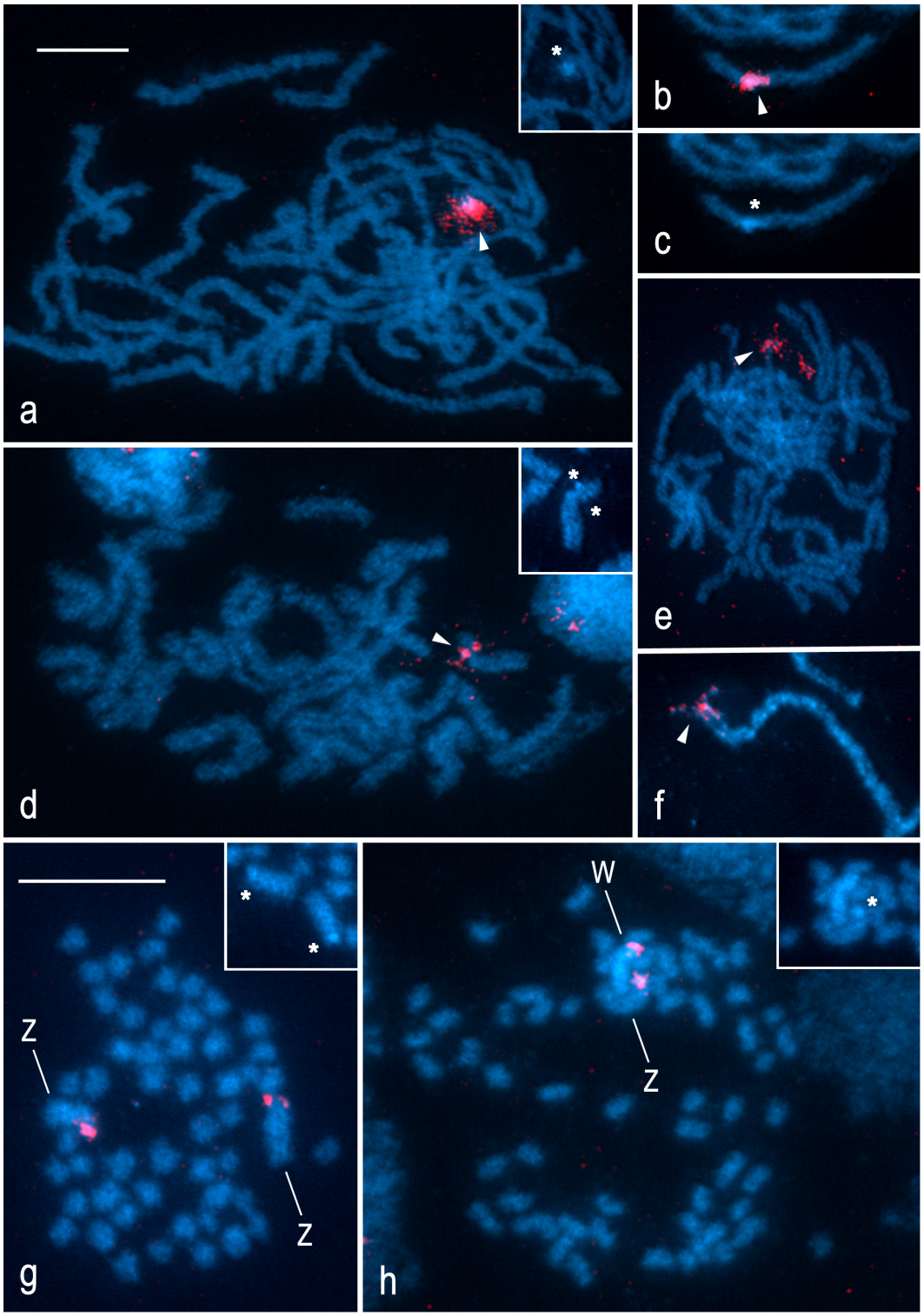

Figure 2. Localization of rDNA clusters in spread chromosome preparations of four species of the family Tortricidae by FISH with 18S rDNA probe. Chromosomes were counterstained with DAPI (blue). Asterisks show DAPI-positive blocks of heterochromatin in the NOR-regions; arrowheads indicate hybridization signals of the 18S rDNA probe (red). *Grapholita molesta*: **a** – male pachytene complement; the inset in the upper right corner shows DAPI image of the NOR-bivalent. *Grapholita funebrana* (**b**, **c**): **b** – composite FISH image of the NOR-bivalent (male pachytene); **c** – DAPI image of the same NOR-bivalent. *Lobesia botrana*: **d** – male pachytene nucleus; the inset in the upper right corner shows DAPI image of the NOR-bivalent. *Eupoecilia ambiguella* (**e**, **f**, **g**, **h**): **e** – male pachytene complement; **f** – a detail of the pachytene WZ bivalent (composite FISH image); **g** – spermatogonial metaphase; **h** – female mitotic metaphase (from wing disc); W and Z indicate sex chromosomes; the insets in the upper right corner of **f** and **g** show DAPI images of the NOR-sex-chromosomes. Bar = 10 μm; **a–f** and **g, h** have the same scale.
doi:10.1371/journal.pone.0064520.g002

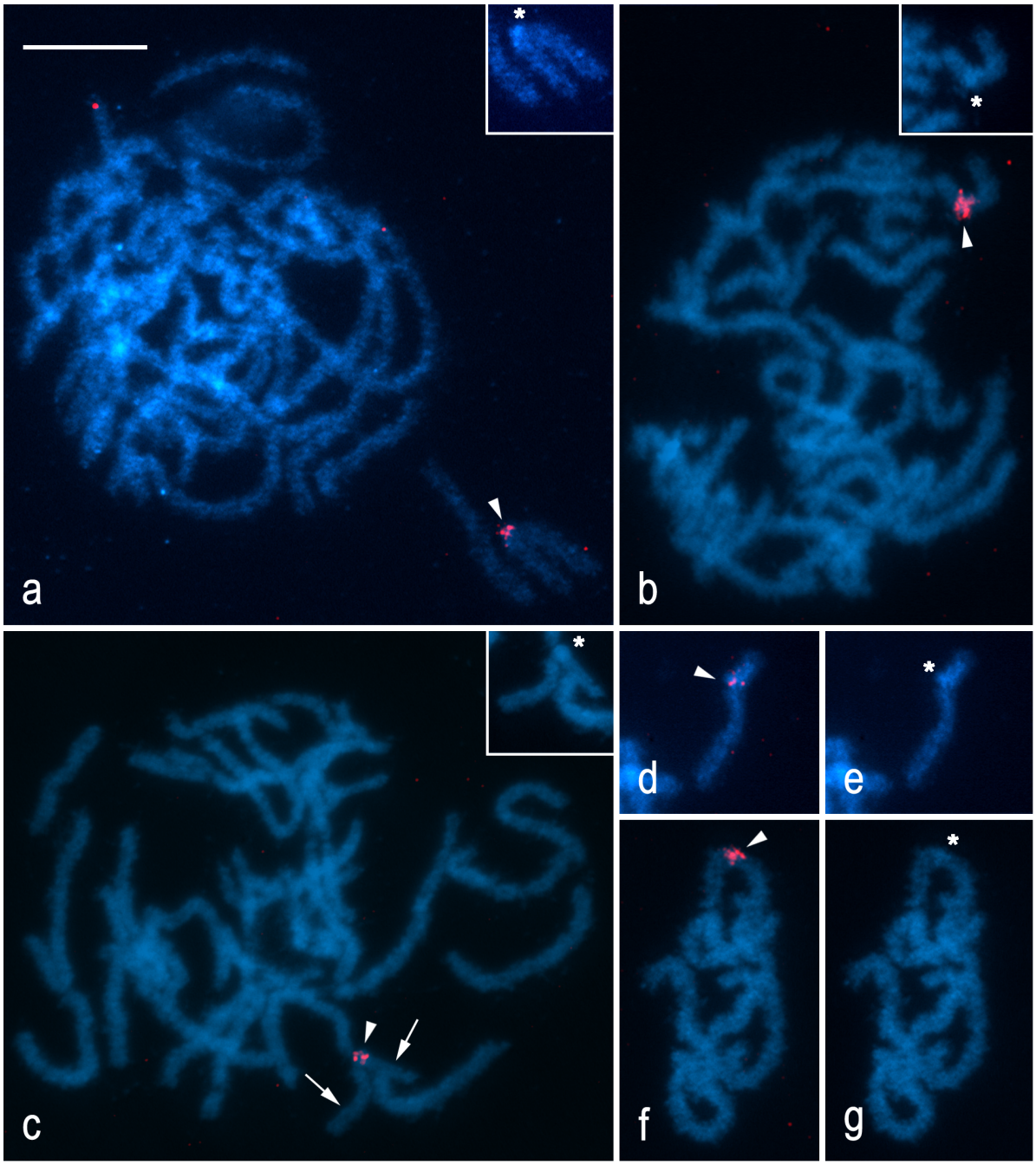

Figure 3. Localization of H3 histone gene clusters in pachytene spermatocytes of five tortricid species by FISH with H3 gene probe. Chromosomes were counterstained with DAPI (blue). Asterisks show DAPI-positive blocks of heterochromatin in the H3-region; arrowheads indicate hybridization signals of the H3 probe (red). *Cydia pomonella*: **a** – pachytene complement; the inset in the upper right corner shows DAPI image of the H3 cluster-carrying bivalent. *Grapholita funebrana*: **b** – pachytene complement; the inset in the upper right corner shows DAPI image of the H3 cluster-carrying bivalent. *Grapholita molesta*: **c** – pachytene complement; the inset in the upper right corner shows DAPI image of the H3 cluster-carrying bivalent. *Lobesia botrana* (**d**, **e**): **d** – composite FISH image of the H3 cluster-carrying bivalent; **e** – DAPI image of the same bivalent. *Eupoecilia ambiguella* (**f**, **g**): **f** – composite FISH image of a part of pachytene nucleus; **g** – DAPI image of the same part of pachytene nucleus. Bar = 10 μm.
doi:10.1371/journal.pone.0064520.g003

signals of both probes in more than half of the WZ bivalent, while the remaining part showed only a few spots evenly highlighted with both probes (Figure 6p, r–t). Interestingly, the pattern of hybridization signals was fully coincident with the heterochromatic segments highlighted with DAPI (Figure 6r).

Discussion

Our study confirmed highly conserved basic features of tortricid karyotypes. All three Olethreutinae species (*G. funebrana*, *G. molesta*, and *L. botrana*) have a haploid chromosome number of n = 28 like our reference species, the codling moth (*C. pomonella*). The n = 28 seems to be a modal number in this subfamily. So far, it has been

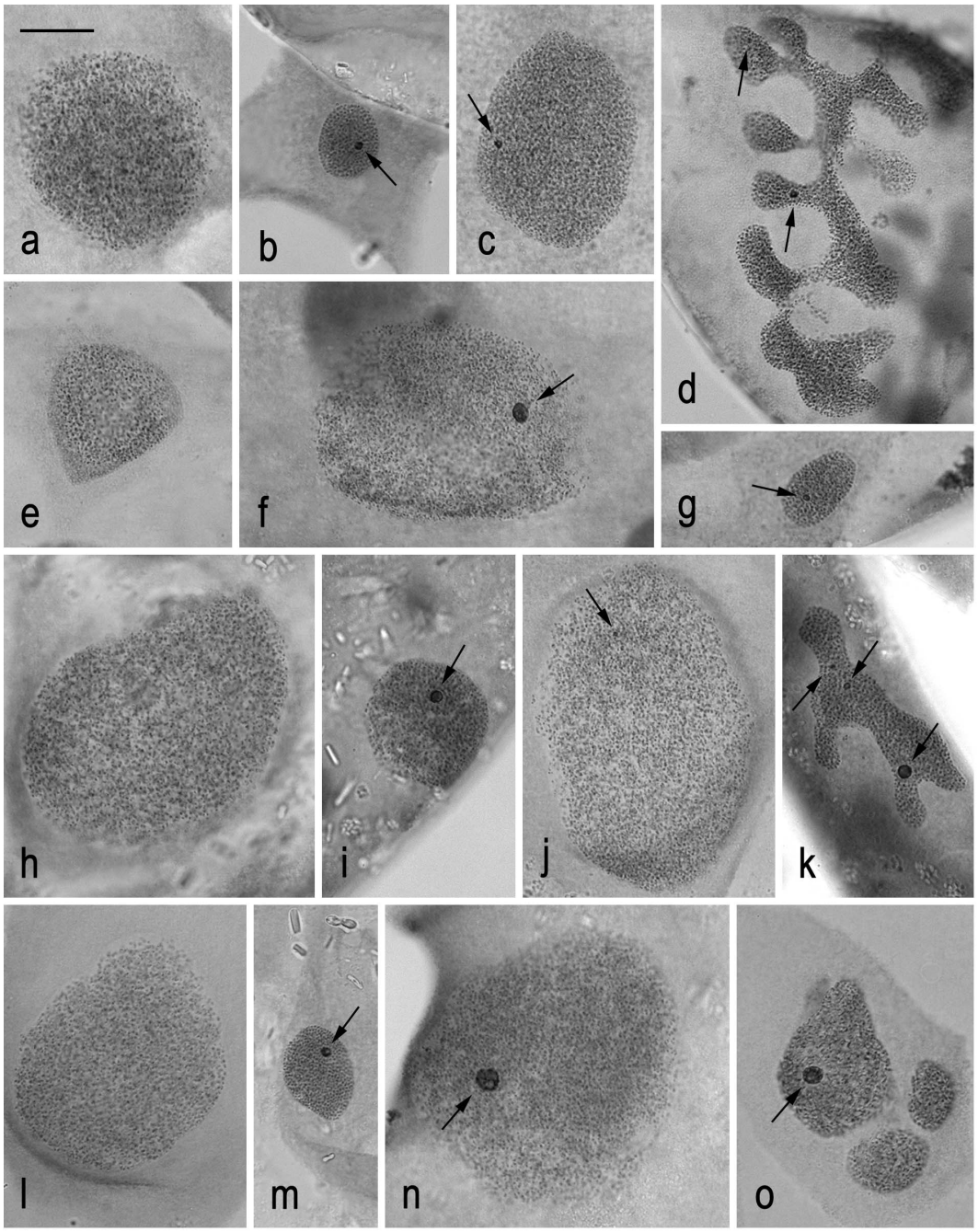

Figure 4. Sex chromatin status in polyploid somatic nuclei of Malpighian tubule cells in four tortricid species. The orcein-stained nuclei were prepared from fifth instar male larvae (**a**, **e**, **h**, **l**), third instar female larvae (**b**, **g**, **i**, **m**), fifth instar female larvae (**c**, **f**, **j**, **n**), and adult females (**d**, **k**, **o**). Arrows indicate deeply stained W chromatin body (-ies). *Grapholita molesta* (**a–d**): **a** – a male nucleus without W chromatin; **b** – a lower-ploidy female nucleus with a relatively large W-body; **c** – a highly polyploid female nucleus with a relatively small W-body; **d** – a branched, highly polyploid female nucleus with W chromatin disintegrated into several small bodies. *Grapholita funebrana* (**e–g**): **e** – a male nucleus without W chromatin; **f** – a highly polyploid female nucleus with a large W-body; **g** – a lower-ploidy female nucleus with a single W-body. *Lobesia botrana* (**h–k**): **h** – a male nucleus without W chromatin; **i** – a lower-ploidy female nucleus with a relatively large W-body; **j** – a highly polyploid female nucleus with a miniature W-body; **k** – a branched, highly polyploid female nucleus with W chromatin disintegrated into several bodies. *Eupoecilia ambiguella* (**l–o**): **l** – a male nucleus without W chromatin; **m** – a lower-ploidy female nucleus with a single W-body; **n** – a highly polyploid female nucleus with a conspicuous W-body; **o** – a fragmented, highly polyploid female nucleus with a conspicuous W-body. Bar = 10 μm.
doi:10.1371/journal.pone.0064520.g004

found in 11 out of 18 species examined, including all three species of the tribe Grapholitini, six out of nine species of Eucosmini, and two out of three species of Olethreutini but not in any of three Bactrini species (Table 1). However, the latter three species of the genus *Bactra* represent a special case with considerably reduced chromosome numbers, most likely due to chromosome fusions, and with multiple sex chromosomes in two of them [30]. Conversely, the subfamily Tortricinae exhibits much greater

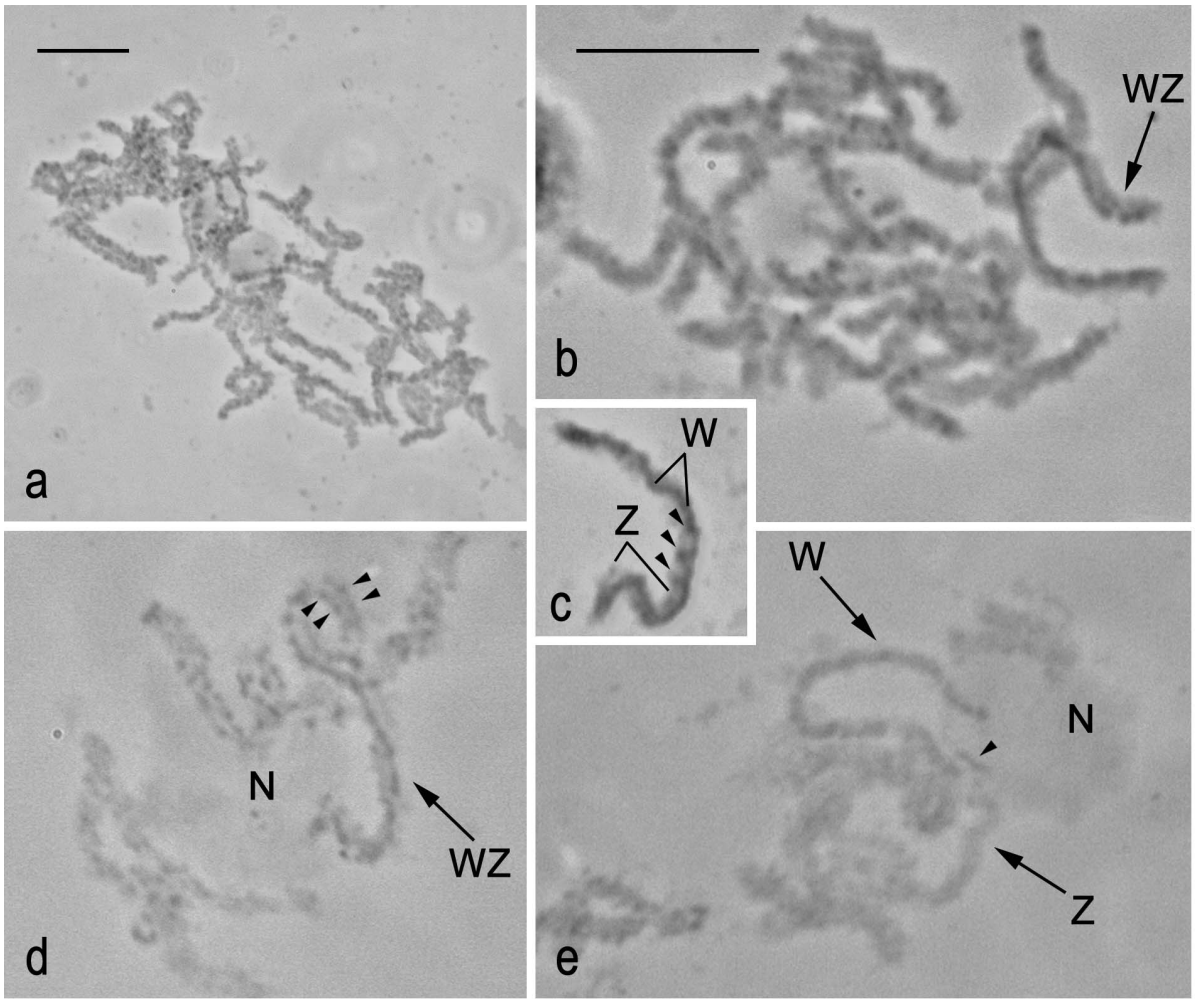

Figure 5. Identification of WZ bivalents in orcein-stained preparations of pachytene oocytes of three tortricid species. a – pachytene complement of *Grapholita molesta*; the WZ bivalent is indistinguishable. **b** – incomplete pachytene nucleus of *Lobesia botrana*; note a WZ bivalent (arrow) identified according to W-chromosome heterochromatin. **c** – a WZ bivalent of *L. botrana*; note the deeply stained W-chromosome thread while the Z-chromosome thread shows a chromomere pattern (see arrowheads pointing to deeply stained chromatin beads). **d** – a part of pachytene nucleus of *Eupoecilia ambiguella* with a WZ bivalent (arrow) anchored by one end in the nucleolus (N); note that most of the W chromosome is formed by a continuous thread except the end opposite to the nucleolar end, which shows a chromomere pattern similar to the Z chromosome (arrowheads). **e** – a part of zygotene/early pachytene nucleus of *E. ambiguella* with not yet paired sex chromosomes; note W and Z univalents (arrows) anchored by one end in the nucleolus (N); also note a deeply stained Z-end (arrowhead) inbuilt in the nucleolus. Bar = 10 μm; **b–e** have the same scale.
doi:10.1371/journal.pone.0064520.g005

karyotype stability with a clear modal chromosome number of n = 30 that has been reported for 25 out of 26 species from four tribes, including *E. ambiguella* (Cochylini) examined in this study, two species of Sparganothini, three species of Tortricini, and 19 out of 20 species of Archipini (Table 1). The only exception found in Totricinae is the rustic tortrix, *Clepsis senecionana*, with n = 29 [24].

Another conserved feature of the tortricid karyotype is a large pair of the sex chromosomes that was found in the codling moth [32,40], all four species examined in this study (except the middle-sized W chromosome in *G. funebrana*), and also found but not shown in the larch budmoth, *Zeiraphera griseana*, syn. *Z. diniana* (Guenée) (Olethreutinae; Eucosmini), with n = 28 ([31]; F. Marec, unpublished data on WZ bivalent). A similar large chromosome pair, supposed to be a pair of sex chromosomes, was also reported for almost each karyotyped tortricid species with modal or close to modal chromosome numbers [23–28]. Provided that the ancestral number in Lepidoptera is n = 31 and that a typical karyotype shows a gradual decrease in chromosome size [6,7], the invariable presence of large sex chromosomes suggests that a common ancestor of Tortricinae and Olethreutinae had a reduced karyotype with n = 30 as a result of a sex chromosome-autosome fusion. This chromosome number has been conserved in most Tortricinae species, but was further reduced to n = 28 in Olethreutinae, most probably by two fusion events involving autosomes. Then subsequent multiple chromosome fusions within Olethreutinae resulted in derived karyotypes with much lower chromosome numbers as found in the genus *Bactra* [30]. This scenario of karyotype evolution is consistent with recent evidence obtained in the codling moth, *L. botrana*, and *E. ambiguella*, showing that the tortricid Z chromosome is a neo-Z that has arisen by fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 in the silkworm, *B. mori*,

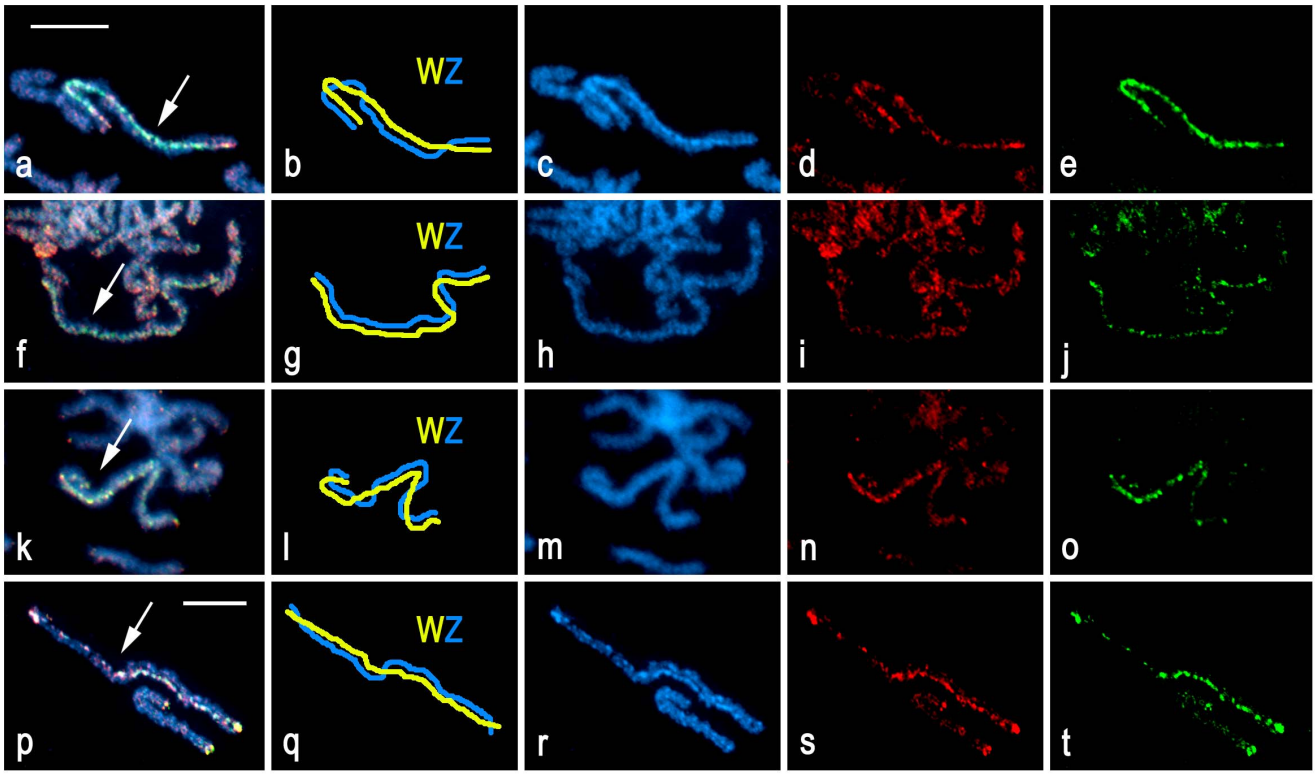

Figure 6. Comparative genomic hybridization (CGH) in pachytene oocytes of *Cydia pomonella* (a-e), *Grapholita molesta* (f-j), *Lobesia botrana* (k-o), and *Eupoecilia ambiguella* (p-t). Chromosomes were counterstained with DAPI (blue); female-derived genomic probes were labeled with fluorescein-12-dUTP (green), male-derived genomic probes with Cy3-dUTP (red). Figures **a–e**, **f–j**, **k–o**, and **p–t** show a detailed analysis of individual WZ bivalents: **a**, **f**, **k**, **p** – merged image of both probes including counterstaining; **b**, **g**, **l**, **q** – schematic interpretation of WZ bivalents; **c**, **h**, **m**, **r** – DAPI image; **d**, **i**, **n**, **s** – male genomic probe; **e**, **j**, **o**, **t** – female genomic probe. Arrows indicate WZ bivalents. Bar = 10 μm; **a–o** and **p–t** have the same scale.
doi:10.1371/journal.pone.0064520.g006

and that this happened in a common ancestor of the main tortricid subfamilies, Olethreutinae and Tortricinae [41].

To further explore physical characteristics of tortricid karyotypes we mapped chromosomal distribution of two multigene families, major rDNA (i.e. 18S, 5.8S and 28S rRNA gene clusters) and H3 histone genes. Interestingly, the tortricid species examined showed a single rDNA cluster (except codling moth) and a single cluster of H3 genes (including codling moth) per haploid genome. Each rDNA or histone gene site was associated with a small block of DAPI-positive heterochromatin, not seen in two rDNA sites of the codling moth [32]. However, distribution patterns of the two chromosome markers greatly differed. A similar, nearly central position of the H3 gene cluster in a middle-sized autosome with a slight exception for *L. botrana*, where the cluster was positioned more eccentrically, suggests a conservative pattern of the H3 histone gene location in tortricids. The finding is consistent with a highly conservative number (i.e. one per haploid genome) and chromosomal location of H3 gene clusters reported recently in other insects, such as Acrididae grasshoppers [47] and Scarabaeinae beetles [48]. On the contrary, major rDNAs showed a variable chromosome location in tortricids, irrespective of evolutionary relationships [20,21]. This is particularly obvious in closely related species, *G. molesta* and *G. funebrana* with a single rDNA cluster in a terminal position and an interstitial position, respectively, which contrasts with two terminal rDNA clusters at opposite ends of the NOR-chromosome in *C. pomonella* [32]. Moreover, a different rDNA location was also found in the other two species, specifically a subterminal rDNA cluster in an autosome of *L. botrana* and a terminal rDNA cluster in both the W and Z sex chromosomes of *E. ambiguella*. The high rDNA mobility in tortricids supports the concept of dynamic evolution of rDNA in Lepidoptera [35] and adds to growing evidence for the recognition of mobility as a common property of the major rDNAs, substantiated in extensive surveys of different organisms including insects [48–50].

There are two interesting features in the distribution of the major rDNAs in tortricids. One of them is the NOR-autosome bearing two terminal rDNA clusters in *C. pomonella*. Fuková et al. [32] hypothesized that the curious NOR-autosome might have arisen through fusion of two ancestral NOR-chromosomes by their non-rDNA ends. However, the hypothesis lacks a support in our results. On the contrary, the single rDNA cluster in the other tortricids examined suggests that a common ancestor of Olethreutinae and Tortricinae had a single NOR-chromosome pair. Hence the two terminal rDNA sites in *C. pomonella* are more likely result of rDNA expansion, for example, by ectopic recombination that has been proposed as a primary motive force of rDNA dynamics in Lepidoptera [35]. Since the family Tortricidae is the most basal lineage examined for NOR distribution so far (for Lepidoptera phylogeny, see [14,51]), we can hypothesize that a single NOR was present also in the ancestral lepidopteran karyotype with n = 31. The other interesting feature is the W- and Z-location of rDNA in *E. ambiguella*, which has so far been only reported in the butterfly *Bicyclus anynana* [52] and the tussock moth *Orgyia thyellina*, though in the latter species the NOR was

Table 1. Karyotype numbers (2n) in Tortricidae.

Tribe	Species[a]	2n	Reference(s)
Subfamily Tortricinae			
Archipini	*Adoxophyles orana*	60	[5,23]
	Aphelia paleana	60	[24]
	Archips breviplicanus	60	[5,23]
	Archips cerasivorana[b]	60	[5,23,25]
	Archips crataegana	60	[27]
	Archips fervidana	60	[25]
	Archips fuscocupreanus	60	[5,23]
	Choristoneura biennis	60	[25,28]
	Choristoneura conflictana	60	[25]
	Choristoneura fumiferana	60	[5,25,28]
	Choristoneura lambertiana	60	[28]
	Choristoneura occidentalis	60	[25,28]
	Choristoneura orae	60	[28]
	Choristoneura pinus	60	[5,25,28]
	Choristoneura retiniana	60	[28]
	Clepsis senecionana	58	[24]
	Homona coffearia[c]	60	[5]
	Homona magnanima	60	[5,23]
	Lozotaenia forsterana	60	[24]
	Pandemis heparana	60	[5,23]
Cochylini	*Eupoecilia ambiguella*	60	this study
Sparganothini	*Cenopis penitana*	60	[25]
	Sparganothis directana	60	[25]
Tortricini	*Acleris forsskaleana*	60	[24]
	Acleris variana	60	[25]
	Tortrix viridana	60	[26]
Subfamily Olethreutinae			
Bactrini	*Bactra furfurana*	33/32[i]	[30]
	Bactra lacteana	31/30[i]	[30]
	Bactra robustana	46	[30]
Eucosmini	*Blastesthia tessulatana*[d]	56	[26]
	Epinotia radicana[e]	58	[25]
	Epinotia solandriana	56	[25]
	Gypsonoma haimbachiana	50	[25]
	Retinia albicapitana[f]	54	[25]
	Rhyacionia buoliana	56	[25]
	Zeiraphera canadensis	56	[25]
	Zeiraphera fortunana	56	[25]
	Zeiraphera griseana[g]	56	[31]
Grapholitini	*Cydia pomonella*	56	[26,32]
	Grapholita funebrana	56	this study
	Grapholita molesta	56	this study
Olethreutini	*Lobesia botrana*	56	this study
	Phiaris mori	44	[29]
	Pseudosciaphila duplex[h]	56	[25]

[a]Species names are used according to [19];
[b]syn. *Choristoneura cerasivorana;*
[c]syn. *Homona menciana;*
[d]syn. *Pseudococcyx tessulatana;*
[e]syn. *Epinotia (Griselda) radicana;*
[f]syn. *Petrova albicapitana;*
[g]*Zeiraphera diniana;*
[h]*Sciaphila duplex;*
[i]species with multiple sex chromosomes W_1W_2Z/ZZ (female/male).
doi:10.1371/journal.pone.0064520.t001

located in the originally autosomal part of the neo-sex chromosomes [53]. However, the sex-chromosome location of rDNA in other insects seems to be rather common as shown, for example, in tiger beetles of the genus Cicindela [54], bushcrickets of the genus *Odontura* [55], and Triatominae bugs [56]. In dipteran insects, the association of rDNA with sex chromosomes even seems to be an ancestral character for the whole order [57–60]. The rare occurrence on the sex chromosomes in Lepidoptera suggests that the sex-linkage of rDNA is not favorable, possibly due to the inactivation of the W chromosome in somatic nuclei of females [44].

The sex chromosomes of tortricids examined here showed some common features in addition to their large size. Similar to codling moth, the W chromosomes were largely composed of heterochromatin, and in CGH experiments they were differentiated by both the female and male genomic DNA probes, with slight preference for the female probes. These results suggest preponderance of common repetitive sequences and transposons and a low amount of W-specific sequences on the W chromosome (cf. [32,37,40]). However, a detailed analysis carried out in this study revealed considerable between-species differences in the formation of W chromatin bodies in the highly polyploid somatic nuclei of females (see Results), in the level of W-chromosome heterochromatinization, and in the pattern of molecular differentiation of the W and Z chromosomes. Only the codling moth W chromosome showed a conspicuous heterochromatinization and uniform CGH pattern along the entire W thread of the pachytene WZ bivalents ([32]; this study), unlike the indistinctive and scattered pattern of the W chromosome in the closely related *G. molesta*.

On the contrary, the CGH patterns in *L. botrana* and *E. ambiguella* suggest that their W chromosomes are composed of two parts, the highly differentiated and poorly differentiated parts. The latter finding strongly suggests that not only the Z chromosome (see above) but also the tortricid W chromosome had originated by fusion between an ancestral W chromosome (the highly differentiated part) and an autosome (the weakly differentiated part), most probably also corresponding to the *B. mori* chromosome 15 (see [41]). Following the W chromosome-autosome fusion event, the complete absence of meiotic recombination in lepidopteran females resulted in independent molecular degeneration of the autosomal part of the neo-W

chromosome in different lineages of tortricids. The resulting molecular divergence could be then responsible for the observed between-species differences in the structure and composition of the tortricid W chromosomes.

In conclusion, our study confirmed conserved karyotypes of tortricids in terms of chromosome numbers, n = 30 in Tortricinae and n = 28 in Olethreutinae, and the large pair of the WZ sex chromosomes. However, differences in the molecular differentiation of the W chromosomes and in the pattern of rDNA distribution suggest a divergence in the internal architecture of tortricid karyotypes. In the codling moth, there is an interest to develop genetic sexing strains with the aim to increase the efficiency of the pest control using SIT [42]. For the creation of genetic sexing strains it has been proposed to insert a dominant conditional lethal mutation into the maternally inherited W chromosome [61]. A similarity between tortricid karyotypes along with the intimate knowledge of their sex chromosomes ([41]; this study) supports the application of technologies developed for the codling moth in other tortricid pests.

Acknowledgments

Our thanks go to Beatrice Christoffel and Silvia Dorn (ETH Zürich, Switzerland) for *Grapholita molesta* culture, and Annette Reineke (Research Center Geisenheim, Germany) for cultures of *Lobesia botrana* and *Eupoecilia ambiguella*. We also wish to thank Marie Korchová for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: JŠ FM. Performed the experiments: JŠ PN MD. Analyzed the data: JŠ FM. Contributed reagents/materials/analysis tools: PN MD. Wrote the paper: FM JŠ PN.

References

1. van Nieukerken EJ, Kaila L, Kitching IJ, Kristensen NP, Lees DC, et al. (2011) Order Lepidoptera Linnaeus, 1758. In: Zhang ZQ, editor. Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa 3148: 212–221.
2. Kristensen NP, Scoble MJ, Karsholt O (2007) Lepidoptera phylogeny and systematics: the state of inventorying moth and butterfly diversity. Zootaxa 1668: 699–747.
3. Wolf KW (1996) The structure of condensed chromosomes in mitosis and meiosis of insects. Int J Insect Morphol Embryol 25: 37–62.
4. Yasukochi Y, Tanaka-Okuyama M, Shibata F, Yoshido A, Marec F, et al. (2009) Extensive conserved synteny of genes between the karyotypes of *Manduca sexta* and *Bombyx mori* revealed by BAC-FISH mapping. PLoS ONE 4: e7465.
5. Robinson R (1971) Lepidoptera genetics. Oxford: Pergamon Press. 687 p.
6. Lukhtanov VA (2000) Sex chromatin and sex chromosome systems in nonditrysian Lepidoptera (Insecta). J Zoolog Syst Evol Res 38: 73–79.
7. De Prins J, Saitoh K (2003) Karyology and sex determination. In: Kristensen NP, editor. Lepidoptera, moths and butterflies: Morphology, physiology, and development. Berlin: Walter de Gruyter. 449–468 pp.
8. Brown KS Jr, Freitas AVL, Wahlberg N, von Schoultz B, Saura AO, et al. (2007) Chromosomal evolution in the South American Nymphalidae. Hereditas 144: 137–148.
9. 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.
10. d'Alençon E, Sezutsu H, Legeai F, Permal E, Bernard-Samain S, et al. (2010) Extensive synteny conservation of holocentric chromosomes in Lepidoptera despite high rates of local genome rearrangements. Proc Natl Acad Sci USA 107: 7680–7685.
11. Baxter SW, Davey JW, Johnston JS, Shelton AM, Heckel DG, et al. (2011) Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. PLoS ONE 6: e19315.
12. The *Heliconius* Genome Consortium (2012) Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. Nature 487: 94–98.
13. Van't Hof AE, Nguyen P, Dalíková M, Edmonds N, Marec F, et al. (2013) Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism. Heredity 110: 283–295.
14. Mutanen M, Wahlberg N, Kaila L (2010) Comprehensive gene and taxon coverage elucidates radiation patterns in moths and butterflies. Proc Biol Sci 277: 2839–2848.
15. Lukhtanov VA, Kandul NP, Plotkin JB, Dantchenko AV, Haig D, et al. (2005) Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus* butterflies. Nature 436: 385–389.
16. 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.
17. van der Geest LPS, Evenhuis HH, editors (1991) Tortricid pests: their biology, natural enemies and control. Amsterdam: Elsevier. 808 p.
18. Zhang BC, editor (1994) Index of economically important Lepidoptera. Wallingford: CAB International. 600 p.
19. Gilligan TM, Baixeras J, Brown JW, Tuck KR (2012) T@RTS: Online world catalogue of the Tortricidae.Version 2.0. Available: http://www.tortricid.net/catalogue.asp. Accessed 2013 Feb 25.
20. Horak M (1998) The Tortricoidea. In: Kristensen NP, editor. Lepidoptera: moths and butterflies. Vol. 1: Evolution, systematics, and biogeography. Handbook of Zoology, Vol. 4, Part 35, Arthropoda: Insecta. Berlin & New York: Walter de Gruyter. 199–215 pp.
21. Regier JC, Brown JW, Mitter C, Baixeras J, Cho S, et al. (2012) A molecular phylogeny for the leaf-roller moths (Lepidoptera: Tortricidae) and its implications for classification and life history evolution. PLoS ONE 7: e35574.
22. Brown JW, Byun BK, Gilligan TM (2010) Tortricid literature library. Version 1.0. Available: http://www.tortricidae.com/library.asp. Accessed 2013 Feb 25.
23. Saitoh K (1966) The chromosomes of two tortricid species (Lepidoptera) with notes on the chromosomes of two other allied species. Jap J Genet 41: 275–278.
24. Suomalainen E (1971) Unequal sex chromosomes in a moth *Lozotaenia forsterana* F. (Lepidoptera: Tortricidae). Hereditas 68: 313–316.
25. Ennis TJ (1976) Sex chromatin and chromosome numbers in Lepidoptera. Can J Genet Cytol 18: 119–130.
26. Ortiz E, Templado J (1976) Los cromosomas de tres especies de tortrícidos (Lep. Tortricidae). EOS-Rev Esp Entomol 51: 77–84.
27. Lukhtanov VA, Kuznetsova VG (1989) Karyotype structure in higher Lepidoptera (Papilionomorpha). Entomological Review 68: 12–31.
28. Harvey GT (1997) Interspecific crosses and fertile hybrids among the coniferophagous *Choristoneura* (Lepidoptera: Tortricidae). Can Entomol 129: 519–536.
29. Saitoh K, Kudoh K (1968) Notes on chromosomes of *Exartema mori* (Lepidoptera: Olethreutidae). Kontyu 36: 403–405.
30. Suomalainen E (1969) On the sex chromosome trivalent in some Lepidoptera females. Chromosoma 28: 298–308.
31. Emelianov I, Marec F, Mallet J (2004) Genomic evidence for divergence with gene flow in host races of the larch budmoth. Proc Biol Sci 271: 97–105.
32. 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.
33. Barnes MM (1991) Codling moth occurrence, host race formation, and damage. In: van der Geest LPS, Evenhuis HH, editors. Tortricid pests: their biology, natural enemies and control. Amsterdam: Elsevier. 313–327 pp.
34. Mediouni J, Fuková I, Frydrychová R, Dhoubi MH, Marec F (2004) Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). Caryologia 57: 184–194.
35. 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.
36. Traut W, Sahara K, Otto TD, Marec F (1999) Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. Chromosoma 108: 173–180.
37. Vítková M, Fuková I, Kubíčková S, Marec F (2007) Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera). Chromosome Res 15: 917–930.
38. Traut W, Sahara K, Marec F (2007) Sex chromosomes and sex determination in Lepidoptera. Sex Dev 1: 332–346.
39. Marec F, Sahara K, Traut W (2010) Rise and fall of the W chromosome in Lepidoptera. In: Goldsmith MR, Marec F, editors. Molecular biology and genetics of the Lepidoptera. Boca Raton: CRC Press. 49–63 pp.
40. Fuková I, Traut W, Vítková M, Nguyen P, Kubíčková S, et al. (2007) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. Chromosoma 116: 135–145.
41. Nguyen P, Sýkorová M, Šíchová J, Kůta V, Dalíková M, et al. (2013) Neo-sex chromosomes and adaptive potential in tortricid pests. Proc Natl Acad Sci USA 110: 6931–6936.
42. Vreysen MJB, Carpenter JE, Marec F (2010) Improvement of the sterile insect technique for codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera, Tortricidae) to facilitate expansion of field application. J Appl Entomol 134: 165–181.
43. Notter-Hausmann C, Dorn S (2010) Relationship between behavior and physiology in an invasive pest species: oviposition site selection and temperature-

dependent development of the Oriental fruit moth (Lepidoptera: Tortricidae). Environ Entomol 39: 561–569.
44. Traut W, Marec F (1996) Sex chromatin in Lepidoptera. Q Rev Biol 71: 239–256.
45. Traut W, Weith A, Traut G (1986) Structural mutants of the W chromosome in *Ephestia* (Insecta, Lepidoptera). Genetica 70: 69–79.
46. Marec F, Traut W (1994) Sex chromosome pairing and sex chromatin bodies in W-Z translocation strains of *Ephestia kuehniella* (Lepidoptera). Genome 37: 426–435.
47. 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.
48. 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.
49. Roy V, Monti-Dedieu L, Chaminade N, Siljak-Yakovlev S, Aulard S, et al. (2005) Evolution of the chromosomal location of rDNA genes in two *Drosophila* species subgroups: *ananassae* and *melanogaster*. Heredity 94: 388–395.
50. Cabrero J, Camacho JPM (2008) Location and expression of ribosomal RNA genes in grasshoppers: abundance of silent and cryptic loci. Chromosome Res 16: 595–607.
51. Regier JC, Mitter C, Zwick A, Bazinet AL, Cummings MP, et al. (2013) A large-scale, higher-level, molecular phylogenetic study of the insect order Lepidoptera (moths and butterflies). PLoS ONE 8: e58568.
52. Van't Hof AE, Marec F, Saccheri IJ, Brakefield PM, Zwaan BJ (2008) Cytogenetic characterization and AFLP-based genetic linkage mapping for the butterfly *Bicyclus anynana*, covering all 28 karyotyped chromosomes. PLoS ONE 3: e3882.
53. Yoshido A, Marec F, Sahara K (2005) Resolution of sex chromosome constitution by genomic in situ hybridization and fluorescence in situ hybridization with $(TTAGG)_n$ telomeric probe in some species of Lepidoptera. Chromosoma 114: 193–202.
54. Galián J, Proença SJR, Vogler AP (2007) Evolutionary dynamics of autosomal-heterosomal rearrangements in a multiple-X chromosome system of tiger beetles (Cicindelidae). BMC Evol Biol 7: 158.
55. Warchalowska-Sliwa E, Maryanska-Nadachowska A, Grzywacz B, Karamysheva T, Lehmann AW, et al. (2011) Changes in the numbers of chromosomes and sex determination system in bushcrickets of the genus *Odontura* (Orthoptera: Tettigoniidae: Phaneropterinae). Eur J Entomol 108: 183–195.
56. Panzera Y, Pita S, Ferreiro MJ, Ferrandis I, Lages C, et al. (2012) High dynamics of rDNA cluster location in kissing bug holocentric chromosomes (Triatominae, Heteroptera). Cytogenet Genome Res 138: 56–67.
57. Marchi A, Pili E (1994) Ribosomal RNA genes in mosquitoes: localization by fluorescence *in situ* hybridization (FISH). Heredity 72: 599–605.
58. Rafael MS, Santos IP Jr, Tadei WP, Carvalho KA, Recco-Pimente SM, et al. (2006) Cytogenetic study of *Anopheles albitarsis* (Diptera: Culicidae) by C-banding and in situ hybridization. Hereditas 143: 62–67.
59. Brianti MT, Ananina G, Recco-Pimentel SM, Klaczko LB (2009) Comparative analysis of the chromosomal positions of rDNA genes in species of the *tripunctata* radiation of *Drosophila*. Cytogenet Genome Res 125: 149–157.
60. Drosopoulou E, Nakou I, Šíchová J, Kubíčková S, Marec F, et al. (2012) Sex chromosomes and associated rDNA form a heterochromatic network in the polytene nuclei of *Bactrocera oleae* (Diptera: Tephritidae). Genetica 140: 169–180.
61. Marec F, Neven LG, Robinson AS, Vreysen M, Goldsmith MR, et al. (2005) Development of genetic sexing strains in Lepidoptera: From traditional to transgenic approaches. J Econ Entomol 98: 248–259.

3.3. Paper III

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.

Abstract

Background

Chromosomal rearrangements have the potential to limit the rate and pattern of gene flow within and between species and thus play a direct role in promoting and maintaining speciation. Wood white butterflies of the genus *Leptidea* are excellent models to study the role of chromosome rearrangements in speciation because they show karyotype variability not only among but also within species. In this work, we investigated genome architecture of three cryptic *Leptidea* species (*L. juvernica*, *L. sinapis* and *L. reali*) by standard and molecular cytogenetic techniques in order to reveal causes of the karyotype variability.

Results

Chromosome numbers ranged from 2n = 85 to 91 in *L. juvernica* and 2n = 69 to 73 in *L. sinapis* (both from Czech populations) to 2n = 51 to 55 in *L. reali* (Spanish population). We observed significant differences in chromosome numbers and localization of cytogenetic markers (rDNA and H3 histone genes) within the offspring of individual females. Using FISH with the $(TTAGG)_n$ telomeric probe we also documented the presence of multiple chromosome fusions and/or fissions and other complex rearrangements. Thus, the intraspecific karyotype variability is likely due to irregular chromosome segregation of multivalent meiotic configurations. The analysis of female meiotic chromosomes by GISH and CGH revealed multiple sex chromosomes: $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica*, $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis* and $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ in *L. reali*.

Conclusions

Our results suggest a dynamic karyotype evolution and point to the role of chromosomal rearrangements in the speciation of *Leptidea* butterflies. Moreover, our study revealed a curious sex determination system with 3–4 W and 3–4 Z chromosomes, which is unique in the Lepidoptera and which could also have played a role in the speciation process of the three *Leptidea* species.

Šíchová et al. BMC Evolutionary Biology (2015) 15:89
DOI 10.1186/s12862-015-0375-4

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Dynamic karyotype evolution and unique sex determination systems in *Leptidea* wood white butterflies

Jindra Šíchová[1,2], Anna Voleníková[2], Vlad Dincă[3,4], Petr Nguyen[1,2], Roger Vila[4], Ken Sahara[5] and František Marec[1,2*]

Abstract

Background: Chromosomal rearrangements have the potential to limit the rate and pattern of gene flow within and between species and thus play a direct role in promoting and maintaining speciation. Wood white butterflies of the genus *Leptidea* are excellent models to study the role of chromosome rearrangements in speciation because they show karyotype variability not only among but also within species. In this work, we investigated genome architecture of three cryptic *Leptidea* species (*L. juvernica*, *L. sinapis* and *L. reali*) by standard and molecular cytogenetic techniques in order to reveal causes of the karyotype variability.

Results: Chromosome numbers ranged from 2n = 85 to 91 in *L. juvernica* and 2n = 69 to 73 in *L. sinapis* (both from Czech populations) to 2n = 51 to 55 in *L. reali* (Spanish population). We observed significant differences in chromosome numbers and localization of cytogenetic markers (rDNA and H3 histone genes) within the offspring of individual females. Using FISH with the $(TTAGG)_n$ telomeric probe we also documented the presence of multiple chromosome fusions and/or fissions and other complex rearrangements. Thus, the intraspecific karyotype variability is likely due to irregular chromosome segregation of multivalent meiotic configurations. The analysis of female meiotic chromosomes by GISH and CGH revealed multiple sex chromosomes: $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica*, $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis* and $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ in *L. reali*.

Conclusions: Our results suggest a dynamic karyotype evolution and point to the role of chromosomal rearrangements in the speciation of *Leptidea* butterflies. Moreover, our study revealed a curious sex determination system with 3–4 W and 3–4 Z chromosomes, which is unique in the Lepidoptera and which could also have played a role in the speciation process of the three *Leptidea* species.

Keywords: Lepidoptera, Wood white butterflies, Karyotype variability, Fluorescence *in situ* hybridization Chromosome fusion and fission, Multiple sex chromosomes, Speciation

Background

Speciation, *i.e.* the origin of new species, is a complex evolutionary process which leads to the formation of barriers preventing gene flow between emerging species. Defining the factors that generate such barriers is a central goal for evolutionary biologists. Among animals, moths and butterflies (insect order Lepidoptera) represent an ideal model group for the study of various aspects of speciation. This is mainly due to the immense diversity of Lepidoptera, which include nearly 160,000 species and belong to the most speciose groups of animals [1]. Moreover, the study of moths and butterflies provides a number of practical advantages. Many species can be easily collected in the field, reared and hybridized in laboratory conditions and experiments can be replicated fairly often due to the relatively short generation time of many species.

Among traditional models, the *Heliconius* butterflies have been the subject of a high number of evolutionary studies showing that various wing patterns, resulting from predator-induced selection through Müllerian mimicry, ultimately lead to divergence and speciation

* Correspondence: marec@entu.cas.cz
[1]Institute of Entomology, Biology Centre CAS, 370 05 České Budějovice, Czech Republic
[2]Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic
Full list of author information is available at the end of the article

(*e.g.* [2–6]). The shift in colour pattern mimicry also played a key role in generating pre-mating isolation as male mate preferences often led to strong assortative mating between individuals with similar wing pattern phenotypes [7]. Swallowtail butterflies of the genus *Papilio* are another diverse group of Lepidoptera where the evolution of mimicry greatly contributed to their spectacular radiation [8–10]. Lepidoptera also include models for research of sex pheromone communication and its role as a pre-zygotic barrier [11, 12]. A well-known example is the European corn borer, *Ostrinia nubilalis*, which comprises two sympatric races that are prevented from mating by utilizing opposite sex pheromone isomers of the same compound [13, 14]. The butterfly subgenus *Agrodiaetus* is one of the few taxa where reinforcement of pre-zygotic isolation has been demonstrated [15].

It is generally accepted that chromosomal rearrangements have the potential to limit introgression and thus facilitate the development and maintenance of reproductive isolation by means of suppressed recombination [16–18]. Reduced recombination enables the accumulation of genetic incompatibilities and leads to divergence and speciation. Suppression of recombination is an intrinsic feature of sex chromosomes which were suggested to play a disproportionate role in lepidopteran speciation [19, 20]. Recent studies in geographic subspecies of wild silkworms, *Samia cynthia* ssp. (Saturniidae), suggest that chromosomal rearrangements resulting in multiple sex chromosomes may also contribute to the formation of reproductive barriers and thus promote divergence and eventually speciation [21, 22]. Moreover, the holokinetic nature of lepidopteran chromosomes, *i.e.* the lack of a distinct primary constriction (the centromere), is expected to facilitate karyotype evolution mainly via chromosomal fusion and fission by reducing the risk of formation of dicentric and acentric chromosomes [23]. However, results of comparative genomics revealed a high degree of conserved synteny of genes between the silkworm *Bombyx mori* (Bombycoidea) and several other lepidopteran species [24–30]. The extensive conservation of chromosome print across Lepidoptera suggests evolutionary stability of lepidopteran karyotypes, with most of haploid chromosome numbers ranging n = 28–32 [31] and the most common and probably also ancestral number of n = 31 [30, 32].

The remarkably stable chromosome numbers and highly conserved synteny of genes between chromosomes of distant species contrast with the exceptional diversity of karyotypes found in some lepidopteran taxa. Probably the greatest interspecific karyotype variation in the animal kingdom was found in blue butterflies (Lycaenidae: Polyommatinae) of the genus *Polyommatus* with haploid chromosome numbers ranging from n = 10 to n = 223 [15, 33–35]. The latter, observed in the Atlas blue, *Polyommatus atlantica*, represents the highest chromosome number not only of Lepidoptera but of all animals [36]. In addition, blue butterflies of the subgenus *Agrodiaetus* represent the group with the largest difference in the number of chromosomes between sister species. Karyotypes of *Polyommatus* (*Agrodiaetus*) *biruni* and *P.* (*A.*) *posthumus* consist of n = 10 and n = 90 elements, respectively, with no intermediate karyomorphs. The similarity in genome size of these closely related species suggests that the karyotype variation is not caused by polyploidy but arose through chromosomal rearrangements such as fusion and fission [37]. However, recent comparative phylogenetic studies found little evidence supporting the role of chromosomal rearrangements in the speciation of *Agrodiaetus* blues and rather stressed the importance of reinforcement of their pre-zygotic isolation [15].

Exceptional intraspecific variability of karyotypes was also found in wood white butterflies of the genus *Leptidea* comprising several Eurasian species [38, 39]. In this genus, chromosome numbers vary greatly between and within species. While two species with predominantly Eastern Palaearctic distribution, *L. morsei* and *L. amurensis*, probably have a constant number of chromosomes (n = 54 and n = 61, respectively; [40]), three cryptic species mainly from the Western Palaearctic have a variable number of chromosomes [38, 41]. The most striking case is *L. sinapis*, which shows a gradual decrease in the diploid chromosome number from 2n = 106 in Spain to 2n = 56 in eastern Kazakhstan, resulting in a 6000 km wide chromosomal cline of recent origin [39]. Excluding polyploidy, this is the widest known within-species chromosome number range for any animal or plant. Moreover, a variable number of chromosomes was described in the other two cryptic species, *L. reali* (2n = 52–54) and *L. juvernica* (2n = 80–84) [38].

Although the nature of dynamic evolution of *Leptidea* karyotypes and its role in speciation is not yet known, the chromosomal cline found in *L. sinapis* provided strong evidence for rapid and extensive within-species accumulation of numerous chromosomal rearrangements [39]. While such clinal speciation is theoretically possible, it is difficult to document without further research. In this study, we integrated standard cytogenetic techniques and FISH (fluorescence *in situ* hybridization) mapping of major ribosomal RNA (rRNA) and H3 histone genes to study among- and within-species variability in the karyotypes of three cryptic *Leptidea* species (*L. juvernica*, *L. sinapis* and *L. reali*). We also determined the sex chromosome constitution using genomic *in situ* hybridization (GISH) and examined molecular differentiation of the sex chromosomes through comparative genomic hybridization (CGH). Cytogenetic

characteristics were compared with the aim of understanding karyotype and sex chromosome evolution in *Leptidea* butterflies.

Results

Molecular identification of *Leptidea* specimens

Morphometric analysis of genitalia allowed us to identify only two groups, *L. sinapis* and the group consisting of *L. reali* and *L. juvernica*, whose genitalia cannot be reliably distinguished [38]. Phylogenetic analyses based on two DNA markers, the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene and the nuclear internal transcribed spacer 2 (*ITS2*) sequence, revealed three supported major clades corresponding to *L. juvernica*, *L. sinapis* and *L. reali* (Fig. 1 and Additional file 1: Figure S1). Relationships among these clades coincide with previous results [38, 41] with *L. juvernica* being sister to the species pair *L. sinapis* and *L. reali*.

Karyotype differences in chromosome number and structure

Chromosome numbers of all three *Leptidea* species were counted from mitotic metaphase complements prepared from wing imaginal discs of the last instar larvae and stained by means of FISH with $(TTAGG)_n$ telomeric probes (tel-FISH) to facilitate identification of individual chromosome elements. In each *Leptidea* species, several tens of mitotic metaphases were analysed in the progeny of individual females.

Based on repeated counts we found that chromosome numbers differ considerably in all three species studied. Moreover, we observed differences in the number of chromosomes even among the offspring of individual females. We established that, in the population studied, the chromosome number is not fixed and ranges from 2n = 85 to 91 in *L. juvernica* and 2n = 69 to 73 in *L. sinapis* (both from Czech populations) to 2n = 51 to 55 in *L. reali* (Spanish population). Mitotic complements of *L. juvernica* and *L. sinapis* also displayed a higher variability in chromosome size, having mostly middle- or small-sized chromosomes (*L. juvernica*, Fig. 2a) or a mixture of large- and small-sized chromosomes (*L. sinapis*, Fig. 3c, d, f), while in *L. reali* we observed larger chromosomes of a similar size (Fig. 2b).

In male meiotic metaphase I (MI) and pachytene complements of all studied species we observed complex chromosomal rearrangements (Fig. 2c, d) and conspicuous heterochromatin blocks highlighted with DAPI (Fig. 2d). However, in female pachytene complements these DAPI-positive blocks did not allow the identification of a sex chromosome bivalent according to the W chromosome, which is usually the only largely heterochromatic element present in lepidopteran karyotypes [42, 43].

Chromosomal location of major rDNA

FISH with the biotin-labelled 18S ribosomal DNA (rDNA) probe combined with the digoxigenin-labelled $(TTAGG)_n$ telomeric probe did not reveal any difference in the number of major rDNA clusters in offspring of three *L. juvernica* and three *L. reali* females. In both species, the rDNA probe mapped to two mitotic metaphase chromosomes of a similar size (Fig. 3a, b; for simplification, hybridization signals of the telomeric probe are not shown) and to a single bivalent in the pachytene stage (insets of Fig. 3a, b). This clearly indicates the presence of a single pair of chromosomes, each carrying a cluster of rRNA genes forming a nucleolar organizer region (NOR). However, we found a substantial interspecific difference in the location of rDNA. While the NOR-bivalent in pachytene nuclei of *L. juvernica* showed the cluster of rRNA genes associated with a large interstitial block of DAPI-positive heterochromatin (inset of Fig. 3a), rDNA occupied a large terminal segment of the NOR-bivalent in *L. reali*. In the latter species, rDNA was not associated with heterochromatin, which was observed at the opposite end of the NOR-bivalent (inset of Fig. 3b).

In *L. sinapis*, we found intraspecific variability in the number and position of rDNA clusters both within and among the offspring of individual females (Fig. 3c-g; hybridization signals of the telomeric probe are not shown). In mitotic metaphase complements from the offspring of one female the 18S rDNA probe localized four rDNA sites at the ends of four middle-sized chromosomes (Fig. 3c), thus indicating two pairs of NOR-chromosomes. However, in mitotic metaphases from the offspring of another female we found either two terminal and one interstitial signal (Fig. 3d) or three terminal hybridization signals (Fig. 3f). The difference among siblings was confirmed in pachytene nuclei where we observed two hybridization signals in a trivalent and one in an element of a bivalent that was heterozygous for rDNA (Fig. 3e) or a pair of signals in a small bivalent and one signal in a larger element of another bivalent (Fig. 3g), respectively.

Chromosomal location of H3 histone genes

FISH with the H3 histone probe combined with tel-FISH showed constant results only in *L. reali*. In all examined larvae from progenies of three different females we found one interstitial cluster of H3 histone genes in a large pachytene bivalent and two clusters in mitotic metaphase complements (Fig. 4a; hybridization signals of the telomeric probe are not shown). In the pachytene bivalent, the H3 cluster was localized next to a small block of DAPI-positive heterochromatin (inset of Fig. 4a).

In *L. sinapis* and *L. juvernica*, we observed intraspecific variability in the number and location of H3 histone gene clusters both within and among offspring of

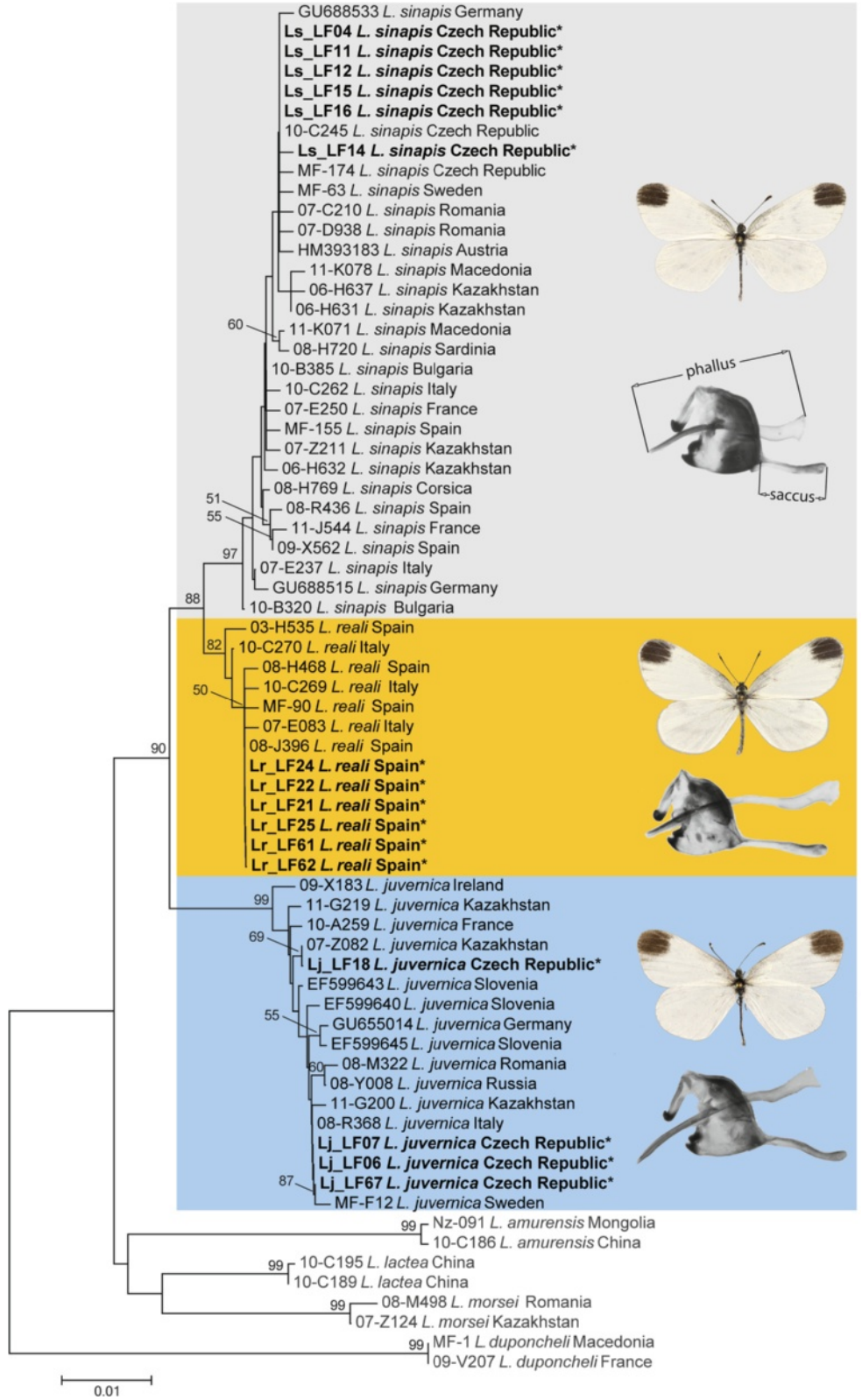

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Neighbor-joining tree of mitochondrial *COI* haplotypes of *L. sinapis* (grey background), *L. reali* (orange background) and *L. juvernica* (blue background). Specimens sequenced and analysed in this study are indicated by an asterisk and were combined with representatives of all available haplotypes of *L. sinapis, L. reali* and *L. juvernica* identified in a previous study [41]. *Leptidea amurensis*, *L. lactea*, *L. morsei* and *L. duponcheli* were used as outgroup. For the origin of all specimens and GenBank accession numbers, see Additional file 5: Table S1. The scale represents 0.01 substitutions per site. Bootstrap supports (100 replicates) are shown next to the recovered nodes. Representative male specimens and genitalia (drawn to scale, with *phallus* and *saccus* indicated) are shown

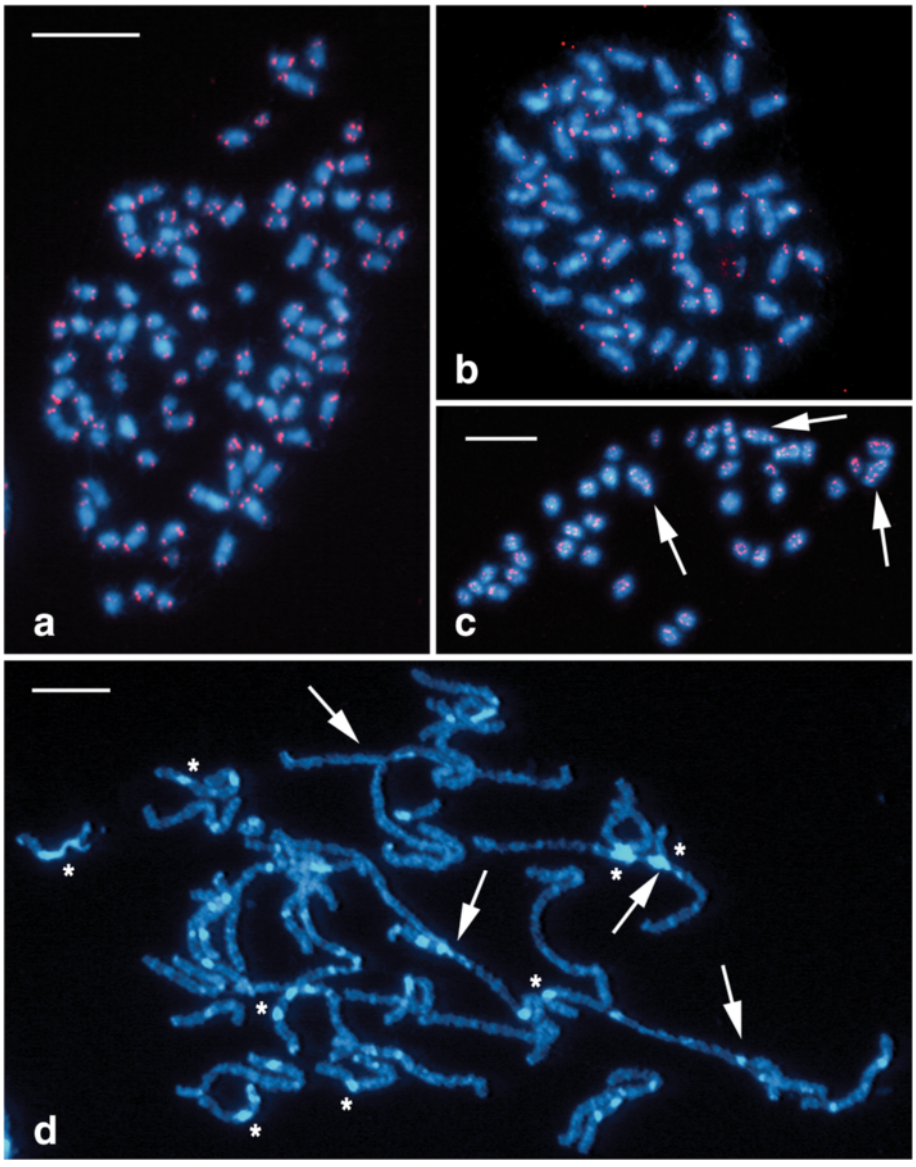

Fig. 2 Karyotype analysis of mitotic and meiotic chromosomes of *Leptidea* species by FISH with the $(TTAGG)_n$ telomeric probe. Hybridization signals of the Cy3-dUTP-labelled telomeric probe (red) indicate chromosome ends in (**a**–**c**). Chromosomes were counterstained with DAPI (blue). White arrows indicate chromosome multivalents and asterisks show heterochromatic blocks. **a** Mitotic metaphase of *L. juvernica* female with numerous middle- or small-sized chromosomes (2n = 85). **b** Mitotic metaphase of *L. reali* female with large chromosomes of a similar size (2n = 55). **c** Meiotic metaphase I of *L. juvernica* male showing several chromosome multivalents. **d** Meiotic pachytene complement of *L. juvernica* female showing several chromosome multivalents and numerous blocks of DAPI-highlighted heterochromatin. Scale bars = 10 μm; (**a**) and (**b**) have the same scale

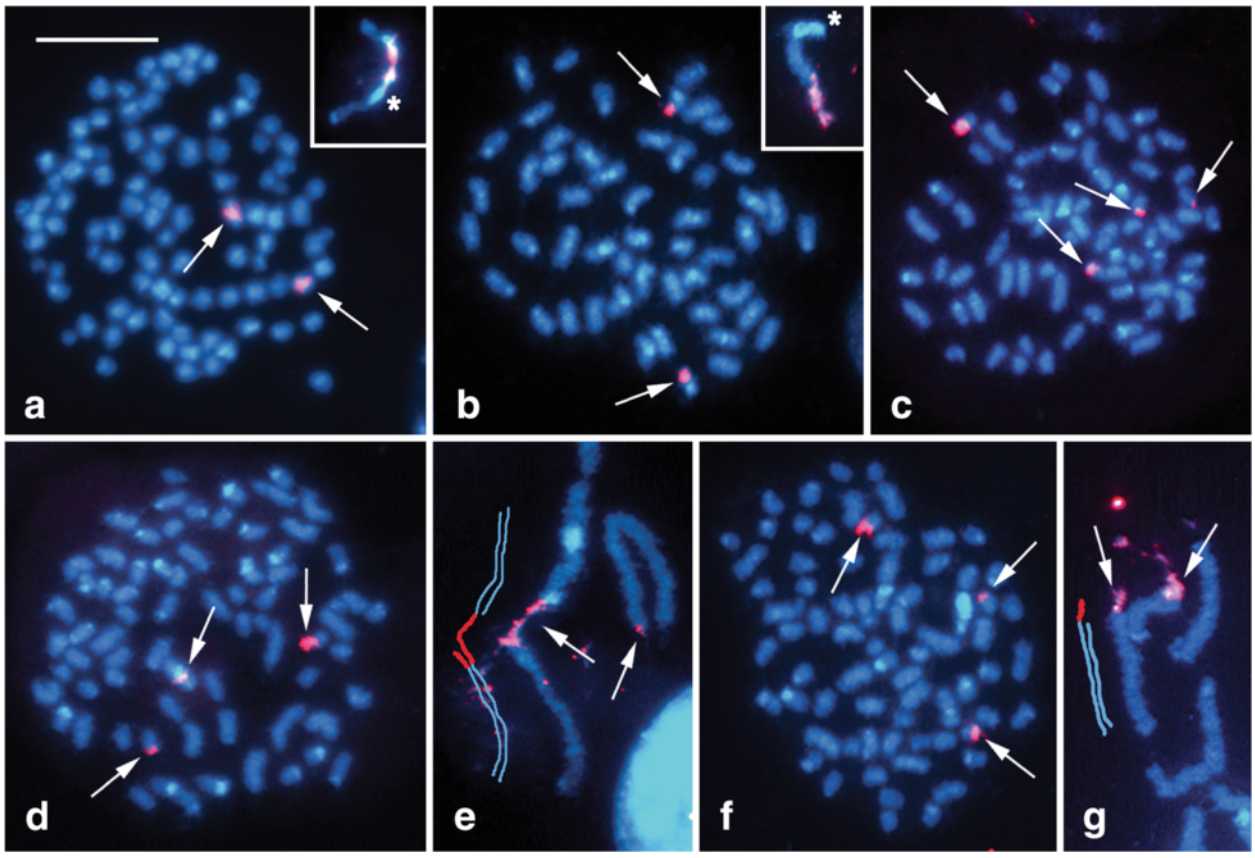

Fig. 3 Localization of rDNA clusters in spread chromosome preparations of three *Leptidea* species by FISH with 18S rDNA probe. Chromosomes were counterstained with DAPI (blue). Arrows show hybridization signals of the 18S rDNA probe (red); asterisks indicate DAPI-positive blocks of heterochromatin. **a** Mitotic metaphase of *L. juvernica* male (2n = 90); the inset in the upper right corner shows the pachytene NOR-bivalent with a large interstitial rDNA cluster. **b** Mitotic metaphase of *L. reali* female (2n = 52); the inset in the upper right corner shows the pachytene NOR-bivalent with a large terminal rDNA cluster. **c** Male mitotic metaphase (2n = 69) with a typical hybridization pattern found in the offspring of one *L. sinapis* female. Figures (**d**–**g**) show a variable pattern in the offspring of another *L. sinapis* female: (**d**) mitotic metaphase of male offspring (2n = 71); (**e**) hybridization signals on pachytene chromosomes of the same male offspring (schematic drawing shows the structure of a trivalent carrying two out of three rDNA clusters); (**f**) mitotic metaphase of female offspring (2n = 73); (**g**) hybridization signals on pachytene chromosomes of the same female offspring (schematic drawing shows the structure of a bivalent heterozygous for a terminal rDNA cluster). Scale bar = 10 μm

individual females (Fig. 4b-i; hybridization signals of the telomeric probe are not shown). In some offspring of one *L. sinapis* female we observed an interstitial cluster of H3 histone genes in a long pachytene bivalent (inset of Fig. 4b) corresponding to two hybridization signals in a pair of mitotic chromosomes (Fig. 4b) like in *L. reali*. However, in other offspring of the same female, a single H3 histone gene array mapped to a subterminal region of a short pachytene bivalent (inset of Fig. 4c), corresponding to terminal hybridization signals in a pair of small mitotic chromosomes (Fig. 4c). In the offspring of another *L. sinapis* female, hybridization signals positioned two H3 gene clusters to a pachytene trivalent, one terminal in a short chromosome and the other interstitial in a long chromosome (Fig. 4d). In accordance with this hybridization pattern, the H3 probe identified two mitotic chromosomes, one small and one large (Fig. 4e). In pachytene nuclei of *L. juvernica*, the H3 probe hybridized most often to a tetravalent. We found three clusters of hybridization signals, one terminal in a short element and two interstitial in two long elements of the tetravalent (Fig. 4g). The hybridization pattern was confirmed in mitotic nuclei, where the probe mapped H3 gene arrays to the end of a small chromosome and to the middle of two larger chromosomes (Fig. 4f). The number and location of H3 gene clusters was characteristic for the offspring of four *L. juvernica* females. However, in the offspring of another female we found an additional (fourth) H3 gene cluster located at the end of one element of a pachytene bivalent (Fig. 4i), corresponding to a total number of four hybridization signals in mitotic nuclei (Fig. 4h).

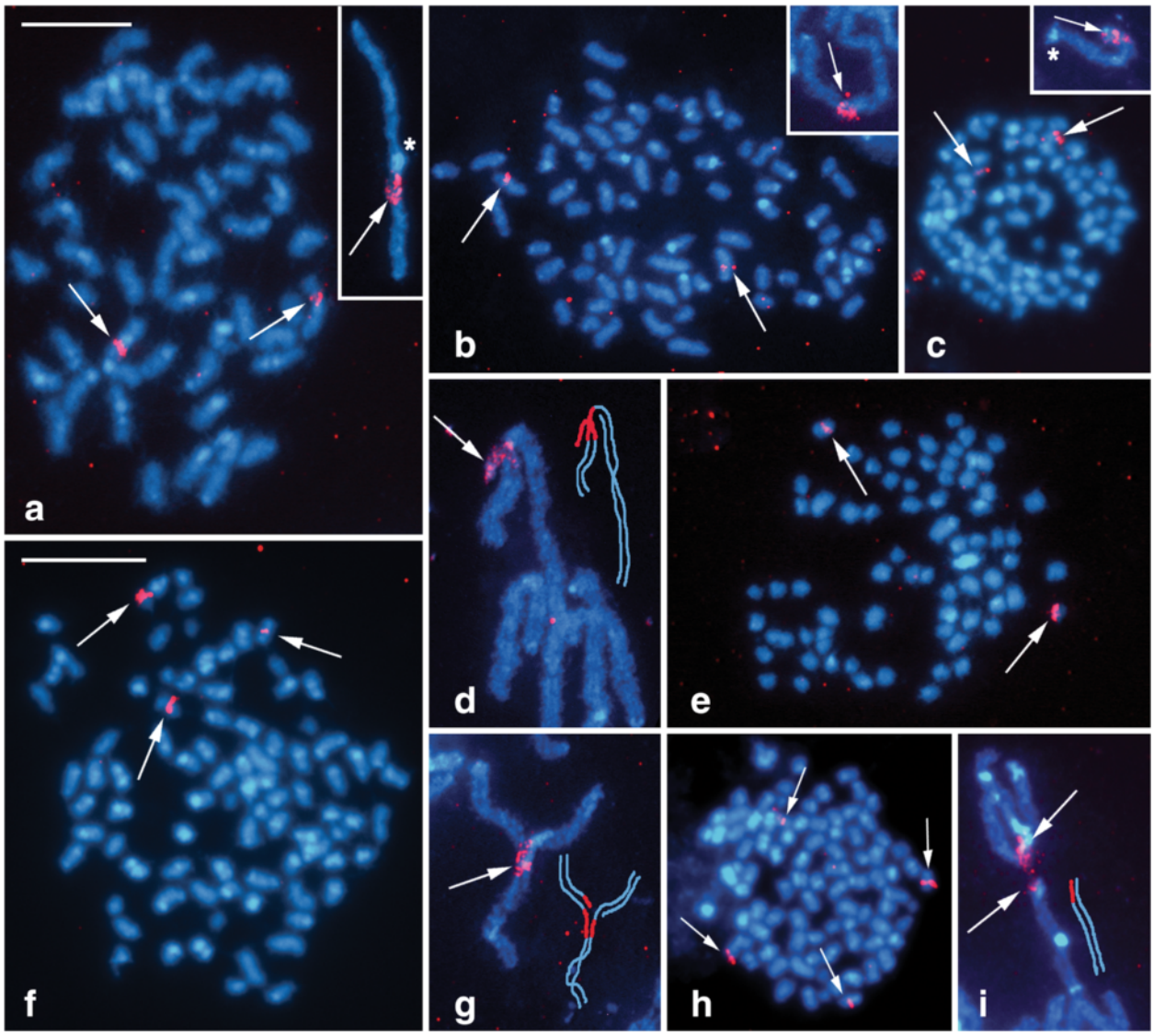

Fig. 4 Localization of H3 histone gene clusters in spread chromosome preparations of three *Leptidea* species by FISH with H3 gene probe. Chromosomes were counterstained with DAPI (blue). Arrows indicate hybridization signals of the H3 probe (red); asterisks show DAPI-positive blocks of heterochromatin. **a** Mitotic metaphase of *L. reali* female; the inset in the upper right corner shows the pachytene H3 cluster-carrying bivalent. Figures (**b**–**e**) show intraspecific variability in the location of H3 histone gene clusters in *L. sinapis*: (**b**) mitotic metaphase of male larva; the inset shows the pachytene bivalent carrying a cluster of H3 genes; (**c**) mitotic metaphase of another male from the same offspring; the inset shows the pachytene bivalent carrying a cluster of H3 genes; (**d**) pachytene trivalent observed in the female offspring of another female (schematic drawing shows the structure of the trivalent and positions of two H3 clusters); (**e**) female mitotic metaphase of the same individual. Figures (**f**–**i**) show intraspecific variability in the location of H3 histone gene clusters in *L. juvernica*: (**f**) male mitotic metaphase with three hybridization signals, observed in the vast majority of *L. juvernica* larvae; (**g**) pachytene tetravalent of the same individual (schematic drawing shows the structure of the tetravalent and positions of three H3 clusters); (**h**) mitotic metaphase with four hybridization signals found in one male offspring of another female; (**i**) pachytene tetravalent with three hybridization signals (see schematic drawing in **g**) and bivalent with the fourth hybridization signal located at the end of one homologue. Scale bars = 10 μm; except for (**f**) all images have the same scale

Sex chromosome constitution

We first examined the presence or absence of female specific sex chromatin in polyploid somatic nuclei of all three *Leptidea* species. The sex chromatin consists of multiple copies of the W chromosome, which usually form one conspicuous heterochromatin body in somatic interphase nuclei of lepidopteran females [44]. In the majority of female larvae of all three species, we observed one larger, more intensely stained heterochromatin body and two tiny indistinct bodies (Additional file 2: Figure S2a). Yet the larger body was much smaller in comparison to the sex chromatin typically observed in

females of other lepidopteran species (*cf.* [45, 46]). In other females we found a variable number of tiny heterochromatin bodies ranging from none to four. Similar findings were made in branched nuclei of adult females with a higher level of ploidy (Additional file 2: Figure S2b). In the majority of *Leptidea* males, no sex chromatin was observed in polyploid cells (Additional file 2: Figure S2c). However, in a few male specimens we found a tiny heterochromatin body of uncertain origin (Additional file 2: Figure S2d). The small size and fragmentation of sex chromatin in *Leptidea* females indicate the presence of interchromosomal rearrangements involving the W chromosome (see [42, 47]).

To identify the W chromosome we examined spread preparations of pachytene oocytes using a combination of GISH and tel-FISH. While GISH differentiated the W chromosome thread in female pachytene nuclei, the telomeric probe helped us to determine chromosomal ends. The female-derived genomic probe also hybridized to heterochromatin blocks on autosomes, which made the identification of the W chromosome more difficult in pachytene nuclei and impossible in mitotic metaphases. Nevertheless, the analysis revealed multiple sex chromosomes in all three *Leptidea* species with 3–4 W and 3–4 Z chromosomes (Fig. 5a–l; hybridization signals of the telomeric probe are shown in Additional file 3: Figure S3a–l). In *L. juvernica*, we observed a $W_1W_2W_3Z_1Z_2Z_3Z_4$ sex chromosome constitution (Fig. 5a–d). While the female genomic DNA (gDNA) probe strongly bound to two W chromosomes, the third one was highlighted only partially (Fig. 5c). Moreover, two of the three W chromosomes were partially differentiated by DAPI-positive heterochromatin (Fig. 5b, two upper arrows). We found only a small heterochromatin block at the very end of the third W chromosome (Fig. 5b, the lower arrow). In *L. sinapis*, we found a $W_1W_2W_3Z_1Z_2Z_3$ sex chromosome system (Fig. 5e–h) with an intensely stained block of heterochromatin on one of the W chromosomes (Fig. 5f, the middle arrow). We also found a small heterochromatin block at the very end of the smallest W chromosome (Fig. 5f, the upper arrow) while the third W chromosome was discernible only due to hybridization signals of the female gDNA probe (Fig. 5g). In the third species, *L. reali*, the sex chromosome constitution was $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ (Fig. 5i–l). Except for the smallest W, the W chromosomes were highlighted with the female gDNA probe (Fig. 5k), but the staining pattern of DAPI was indistinctive with only few small heterochromatin blocks of higher intensity (Fig. 5j, arrows).

The level of molecular differentiation of the W and Z chromosomes was examined using CGH. In pachytene oocytes of the three *Leptidea* species, the WZ multivalent was discernible from autosomes due to stronger binding of both female- and male-derived probes to the W chromosomes (Fig. 6a–o). A detailed analysis of the WZ multivalent at the pachytene stage of *L. juvernica* revealed a similar labelling pattern of both probes (Fig. 6a–e). In *L. sinapis*, the W chromosomes were decorated with strong but scattered hybridization signals of both genomic probes (Fig. 6f, h, i) with a slight preference for the female probe (Fig. 6h). The highest level of molecular differentiation of the W and Z chromosomes was observed in *L. reali* (Fig. 6k–o), where three out of four W chromosomes were preferentially labelled by the female-derived probe (Fig. 6m). However, the smallest W chromosome was almost indistinguishable from the Z chromosome (Fig. 6m–o). Hybridization signals of the male-derived genomic probe were considerably weaker, except for a few intense heterochromatin blocks located on one W chromosome (Fig. 6n).

Discussion

We performed a detailed karyotype analysis of three cryptic *Leptidea* species (*L. juvernica*, *L. sinapis* and *L. reali*) by means of standard and molecular cytogenetic techniques. Previous studies showed both inter- and intraspecific variation in chromosome numbers in all three studied species. However, the results were based on chromosome counts from squash preparations of metaphase I spermatocytes [38, 39], which did not allow the analysis of complex meiotic figures such as multivalents. Using FISH with $(TTAGG)_n$ telomeric probes, we confirmed the presence of numerous multivalents in female pachytene nuclei as well as in male pachytene and metaphase I complements in all three species. Detailed analysis of male and female mitotic metaphases prepared from wing imaginal discs allowed us to determine more accurately the range of diploid chromosome numbers that were 2n = 51–55 in *L. reali* (Spanish population), 2n = 69–73 in *L. sinapis* and 2n = 85–91 in *L. juvernica* (both Czech populations). These numbers are broadly in line with previous findings [38, 39]. Nevertheless, we further extended the range of chromosome numbers observed in *L. juvernica* and *L. reali* and provided data from new localities for *L. juvernica* and *L. sinapis*.

Besides inter- and intraspecific karyotype variability, the analysis of mitotic chromosomes allowed us to identify differences in chromosome numbers even within offspring of individual females of all three species. Although such intrapopulation variability could be caused by the presence of supernumerary chromosomes, *i.e.* B-chromosomes, this scenario was deemed unlikely in the case of *Leptidea* species [39]. Yet, we cannot totally exclude the involvement of B-chromosomes, especially as they were observed in related white butterflies from the family Pieridae [34]. In wild silkworms (*Samia cynthia*), chromosomal polymorphism among geographical populations/subspecies was ascribed to repeated autosome-sex chromosome fusions resulting in

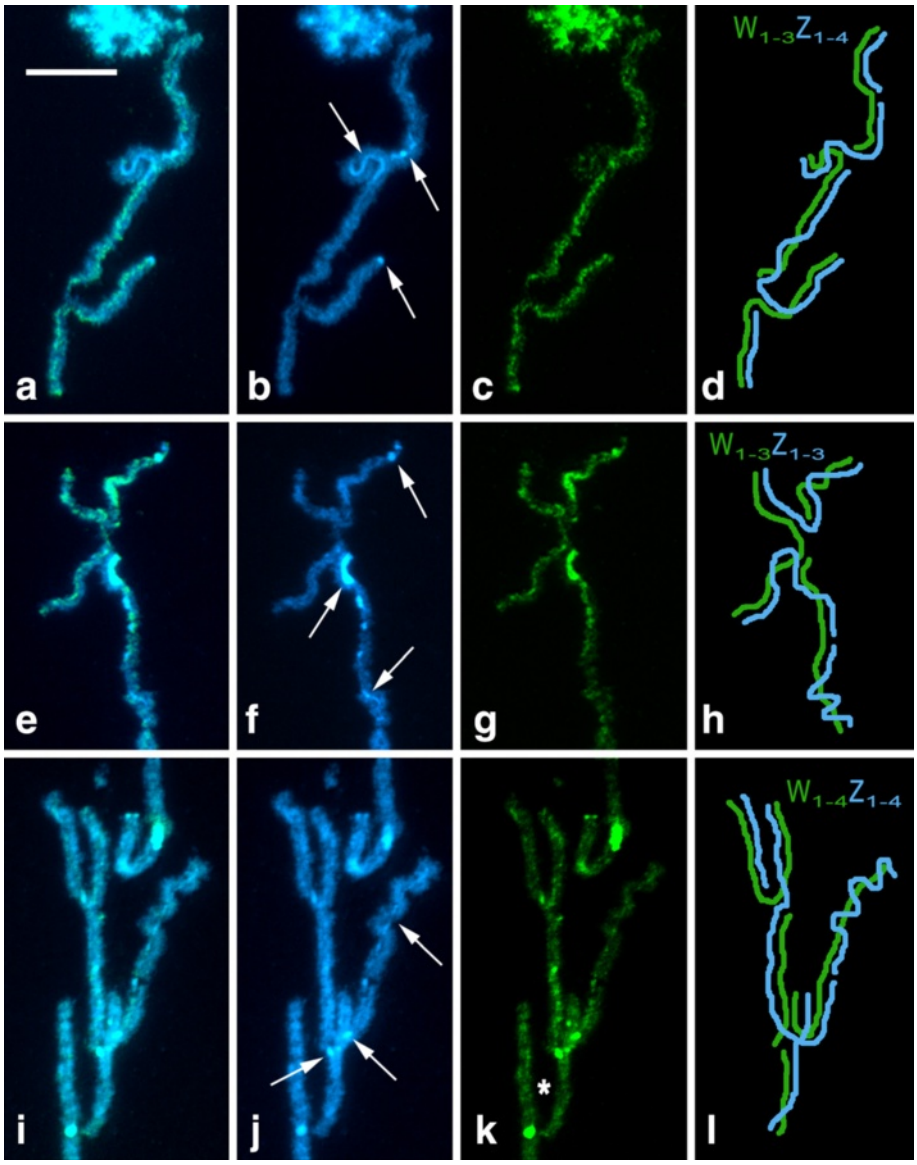

Fig. 5 Genomic *in situ* hybridization (GISH) in pachytene oocytes of *Leptidea juvernica* (**a–d**), *L. sinapis* (**e–h**) and *L. reali* (**i–l**). Female-derived genomic probes were labelled with fluorescein-12-dUTP (green) and chromosomes were counterstained with DAPI (blue). Figures (**a**–**d**), (**e**–**h**) and (**i**–**l**) show detailed analyses of sex chromosome multivalents $W_{1-n}Z_{1-n}$: (**a**, **e**, **i**) merged images of female genomic probes and DAPI staining; (**b**, **f**, **j**) DAPI images; arrows indicate DAPI-positive W-chromosome segments and heterochromatic blocks at the end of the W chromosomes; (**c**, **g**, **k**) hybridization pattern of the female genomic probes; the asterisk indicates an undifferentiated segment of one of the W chromosomes; (**d**, **h**, **l**) schematic drawings of the sex chromosome multivalents. Scale bar = 10 μm

neo-sex chromosomes and reduced chromosome numbers [21]. Similar intraspecific variation resulting in reduced chromosome numbers was also reported in grasshoppers (*e.g.* [48]) and mammals (*e.g.* [49, 50]), mainly as a result of Robertsonian translocations. However, karyotype variation observed in the three *Leptidea* species surpasses previous reports. Our findings, namely the relatively low number of large chromosomes in *L. reali*, variability in chromosome size in the other two species and the occurrence of multivalents in meiotic nuclei of all three species, suggest that the *Leptidea* karyotypes are differentiated by multiple chromosome fusions and fissions. In addition, our preliminary data showing a similar size of interphase nuclei in the *Leptidea* species studied (Additional file 4: Figure S4) suggest that their karyotypes did not differentiate through polyploidy. Uneven chromosome segregation of multivalents during meiotic division is thus the most plausible explanation for the intraspecific and intrapopulation karyotype variations.

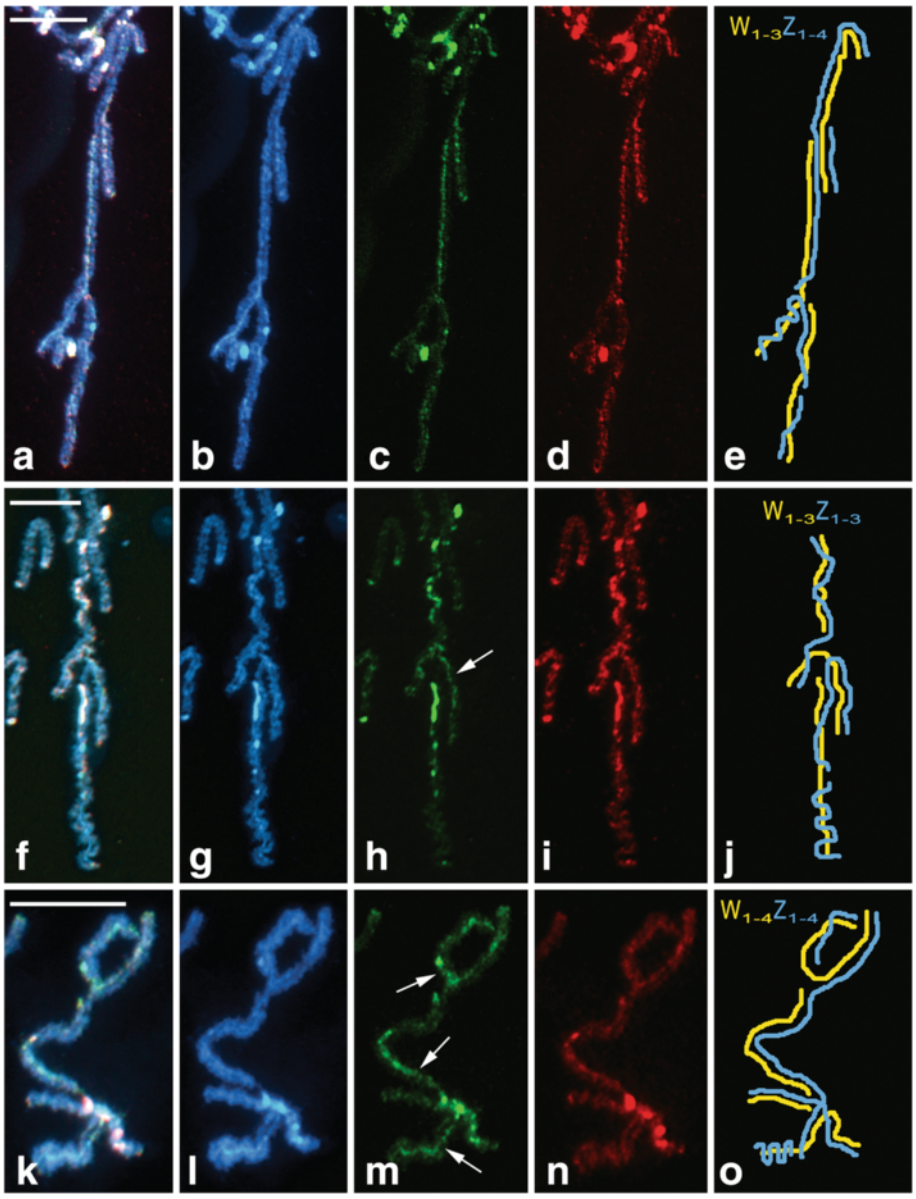

Fig. 6 Comparative genomic hybridization (CGH) in pachytene oocytes of *Leptidea juvernica* (**a–e**), *L. sinapis* (**f–j**) and *L. reali* (**k–o**). Female-derived genomic probes were labelled with fluorescein-12-dUTP (green), male-derived genomic probes were labelled with Cy3-dUTP (red) and chromosomes were counterstained with DAPI (blue). Figures (**a**–**e**), (**f**–**j**) and (**k**–**o**) show detailed analyses of sex chromosome multivalents $W_{1-n}Z_{1-n}$: (**a**, **f**, **k**) merged images of both genomic probes and DAPI staining; (**b**, **g**, **l**) DAPI images; (**c**, **h**, **m**) female genomic probes; arrows indicate W-chromosome segments with female-specific hybridization pattern; (**d**, **i**, **n**) male genomic probes; (**e**, **j**, **o**) schematic drawings of the sex chromosome multivalents. Scale bars = 10 μm

The karyotypes of *Leptidea* species also differed in the number and location of two cytogenetic markers used in this study, clusters of rRNA genes (major rDNA) and H3 histone genes. In *L. reali*, the species with the lowest chromosome number, all larvae showed consistent results with a single terminal rDNA cluster and an interstitial cluster of H3 genes per haploid genome. The highest variability in the number and position of both cytogenetic markers was observed within and among the offspring of individual *L. sinapis* females. In *L. juvernica*, the number and location of H3 histone genes differed in the progeny of individual females, while one rDNA cluster was always located in the middle of one pachytene bivalent. Except for *L. reali*, both cytogenetic markers often marked multivalents. Interspecific differences as well as intrapopulation variability in rDNA distribution

in *L. sinapis* are in agreement with the hypothesis on dynamic evolution of genes for major RNAs in Lepidoptera [51]. However, the differences in the number and location of H3 histone genes in *L. sinapis* and especially *L. juvernica* are rather surprising, since this marker shows a highly conserved pattern in the lepidopteran family Tortricidae [52] and other insect groups, such as the Acrididae grasshoppers [53] and Scarabaeinae beetles [54]. Our results thus support the previously reported intraspecific variability in the karyotype of *L. sinapis* [39] and highlight the ongoing explosive karyotype evolution in all three *Leptidea* species.

To further explore *Leptidea* karyotypes and to identify their sex chromosomes we performed GISH combined with tel-FISH. In pachytene nuclei of lepidopteran females, the WZ bivalent is usually easily discernible with GISH by deep staining of the W chromosome thread with the fluorescently labelled female gDNA probe [43, 45, 55]. In all three *Leptidea* species, the analysis revealed unique sex chromosome systems with the following constitutions: $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica*, $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis* and $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ in *L. reali*. The constitution seemed to be stable in the progenies of individual females. Multiple sex chromosome systems have been documented in mammals [56, 57], fish [58, 59] and spiders [60, 61]. However, the majority of moths and butterflies show a WZ/ZZ sex chromosome system. Multiple sex chromosomes have so far been found only in seven genera and only in two different constitutions, either with W_1W_2Z or WZ_1Z_2 trivalents in females [46]. Thus, the sex chromosome constitutions observed in *Leptidea* stand out for its complexity and the number of chromosomes involved in the multivalent in meiosis. In addition, this is the first case of multiple sex chromosomes in butterflies (Papilionoidea). Besides sex chromosomes, the gDNA probes also highlighted heterochromatin blocks abundantly present in the karyotypes of all three studied species. In Lepidoptera, heterochromatin is usually confined to the NOR containing rDNA repeats [51, 52] and to the W chromosome [44, 62]. So far, similar heterochromatin blocks have been found only in subtelomeric regions of the white butterfly *Pieris brassicae* (Pieridae) [51] and on chromosome 24 of *Bombyx mori* [63]. In *Leptidea*, however, heterochromatin was evenly distributed throughout the whole genome. Such distribution suggests the preponderance of tandemly arranged repetitive sequences in *Leptidea* genomes in comparison with other lepidopteran species, which could ultimately contribute to the remarkable karyotype diversity in this group.

In addition to the different sex chromosome constitution, the three *Leptidea* species also differed in their overall genomic hybridization pattern. In *L. sinapis* and *L. juvernica*, all W chromosomes were at least partially differentiated by strong binding of fluorescently labelled gDNA probes with GISH and CGH, indicating the accumulation of repetitive sequences and transposable elements in the W chromosomes (*cf.* [64]). In *L. reali*, one of the W chromosomes was not highlighted by any gDNA probe. This W chromosome probably represents an evolutionarily young element, which did not have sufficient time to differentiate. Individual Z chromosomes involved in multivalents thus probably correspond to the so-called evolutionary strata, which were also reported in mammals, birds and plants [65–69]. Moreover, similar hybridization patterns of male and female genomic probes in CGH experiments suggest a predominance of common repetitive sequences and transposons and a low amount of W-specific sequences on the W chromosomes of *L. sinapis* and *L. juvernica*. On the contrary, the preferential binding of the female-derived genomic probe to three of the four W chromosomes in *L. reali* suggest a relatively high proportion of W-specific sequences.

Observed differences in chromosome numbers and location of the major rDNA and H3 histone gene clusters as well as the existence of complex sex chromosome systems corroborate the role of chromosomal rearrangements in the speciation of the closely related *Leptidea* species examined in this study. It has been shown that chromosomal rearrangements have a potential to limit gene flow and thus facilitate the development and maintenance of reproductive isolation by means of suppressed recombination [16–18]. The majority of studies on the effects of chromosome fusion and fission on speciation have been done in organisms with monocentric chromosomes that exhibit Robertsonian translocations, *i.e.* centric fusions [70–72]. These studies confirmed the role of chromosomal fusions in reducing the frequency of recombination. The variation in chromosome size and number is explained as a result of frequent fusion and fission events also in taxa with holokinetic chromosomes [39, 73], in which kinetochores are distributed along most of the poleward facing chromosome surface [74]. In this case, fusion is likely to behave as a stable centric fusion and fission leads to viable chromosomal fragments that are normally inherited during meiosis [75, 76]. A recent study stressed the effect of chromosome fusion on the recombination rate in holokinetics [77]. Moreover, studies in sedges (*Carex*, Cyperaceae) proved that fusion and fission of holokinetic chromosomes also have the potential to restrict gene flow and lead to divergence and eventually speciation [78].

The complex sex chromosome constitution revealed in this study is likely another factor involved in the speciation of *Leptidea* butterflies. It has been proposed that the Z sex chromosome could play a disproportionately larger role in adaptive evolution compared to autosomes [79–81]. This so-called 'large-Z effect' was reported in both birds [79, 81–83] and Lepidoptera ([5, 84], the two

largest taxa with female heterogamety. Furthermore, detailed studies on the neo-sex chromosome evolution in geographic populations of *S. cynthia* and leaf-rollers of the family Tortricidae suggest that sex chromosomal rearrangements play a major role in the formation of reproductive barriers between populations and contribute to radiation in some lepidopteran taxa, respectively [21, 85]. In *Leptidea*, the multiple sex chromosome system most likely originated by complex translocations between the ancestral WZ pair and several autosomes, which increased the number of sex-linked genes and thus accelerated the accumulation of genetic incompatibilities among populations. This is supported by the intraspecific stability of their multiple sex chromosomes systems, which is in stark contrast to the evolutionary dynamics of their autosomes. Another signal of reinforcement could be the fact that the most recently diverged sister species, *L. sinapis* and *L. reali*, display not only the largest differences in chromosome numbers in sympatry [38, 39] but also the most different sex determination system (as shown in this study).

Conclusions

To conclude, we confirmed significant differences in the number and structure of chromosomes within and among closely related wood white butterflies. We showed that the distribution of cytogenetic markers differs remarkably even in the offspring of individual females, probably due to irregular segregation of multivalents in meiosis. Our results suggest rapid karyotype evolution in the examined *Leptidea* species and stress the role of chromosomal rearrangements, especially multiple chromosome fusions and fissions, in their speciation. Remarkably, all three *Leptidea* species have complex sex chromosome systems with 3–4 W and 3–4 Z chromosomes. Such sex chromosome constitutions are unique among Lepidoptera and should be counted as an additional factor potentially contributing to the speciation process in *Leptidea* butterflies. Taken together, these findings add to accumulating evidence on the important role of chromosomal rearrangements in speciation and also point to the relevance of multiple sex chromosomes in species divergence and the formation of reproductive barriers.

Methods

Sample collecting

Fresh adult specimens of *Leptidea juvernica* and *L. sinapis* were collected in the Czech Republic, namely in the surroundings of České Budějovice and near Havraníky village in the Podyjí National Park in South Moravia, respectively. The third species, *L. reali*, was collected in the Montseny area near Barcelona, Spain. In the laboratory, fertilized females were kept in plastic containers to lay eggs. The bodies of all collected individuals were then placed into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C until DNA extraction, except for their genitalia which were immediately used for morphometric analysis. Hatched larvae were reared on corresponding host plants, *Lathyrus pratensis* for *L. juvernica* and *L. reali* and *Securigera varia* for *L. sinapis*, at room temperature and normal day/night regime.

Genitalia preparation and morphometric analysis

Male and female genitalia were dissected in a physiological solution and inspected under a stereomicroscope. Lengths of two elements of the male genitalia, *phallus* and *saccus* and one element of the female genitalia, *ductus bursae*, were measured. These diagnostic characters discriminate *L. sinapis* from the other two species, *L. juvernica* and *L. reali*, which cannot be reliably distinguished from each other based on morphological features (Fig. 1; [38]).

Specimen sequencing

Genomic DNA (gDNA) was extracted from legs of every female that gave progeny used in cytogenetic studies, *i.e.* from 6 *L. sinapis*, 6 *L. reali* and 4 *L. juvernica* females, using the NucleoSpin Tissue XS kit (Macherey-Nagel, Düren, Germany) according to the supplier's protocol. To confirm the taxonomic determination of the examined specimens, molecular phylogenetic trees were constructed using one mitochondrial gene, cytochrome *c* oxidase subunit 1 (*COI*) and one nuclear marker, the internal transcribed spacer 2 (*ITS2*). For each individual, a partial sequence of both markers was amplified by polymerase chain reaction (PCR) using two pairs of primers: for *COI* (658 bp) LepF1 (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LepR1 (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′); for *ITS2* (684 bp) ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [38].

PCR was carried out in 25-μL reaction volumes containing 1× Ex *Taq* buffer (TaKaRa, Otsu, Japan), 0.2 mM dNTP mix, 5 μmol of each primer, 0.25 U Ex *Taq* Hot Start DNA polymerase (TaKaRa) and about 100 ng of template gDNA. The typical thermal cycling profile for *COI* consisted of an initial denaturation period of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min at 44 °C and 1 min at 72 °C and by a final extension step of 7 min at 72 °C. The profile was similar for the nuclear marker *ITS2* except for the annealing temperature, which was 50 °C. PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Sequences were edited and aligned using GENEIOUS PRO 4.7.5 created by Biomatters (http://www.geneious.com/). Our sequences were combined with all available *COI* and *ITS2* haplotypes of *Leptidea sinapis, L. reali* and *L. juvernica* identified in a previous study [41] and with sequences of *L. morsei*, *L. amurensis*, *L. lactea* and *L. duponcheli* that were used as outgroup (Additional file 5: Table S1). Thus, the final *COI* alignment contained 69 nucleotide sequences and was 658 bp long, while the *ITS2* alignment involved 28 sequences and consisted of 684 positions.

To confirm the identification of the examined specimens, neighbor-joining trees [86] were built for *COI* and *ITS2*. Both trees were based on *p*-distance [87] and pairwise deletion. Node supports were assessed through 100 bootstrap replicates [88]. The trees were inferred in MEGA6 [89].

Chromosome preparation

In each *Leptidea* species, two types of spread chromosome preparations were made from fifth instar male and female larvae. Mitotic chromosomes were obtained from wing imaginal discs characterized by a high mitotic index [52], while meiotic chromosomes were obtained from ovaries and testes. In both cases we used the procedure described in [45]. All preparations were passed through a graded ethanol series (70 %, 80 % and 100 %, 1 min each) and stored at −80 °C until further use.

Preparation of polyploid nuclei

Malpighian tubules were dissected out from fifth instar larvae of both sexes and adult females in a physiological solution. Removed tubules were fixed in ethanol/chloroform/acetic acid (6:3:1) for 1 minute and stained in 1.5 % lactic acetic orcein. Preparations were inspected under a light microscope for the presence of female specific sex chromatin [44].

FISH with fluorochrome-labelled probes

For the chromosome counts we used spread chromosome preparations from wing imaginal discs stained by FISH with $(TTAGG)_n$ telomeric probes (tel-FISH), which helped us to identify the chromosome ends. The telomeric probes were generated by non-template PCR as described in [90] and labelled by Cy3-dUTP (GE Healthcare, Milwaukee, WI, USA) using a Nick Translation Kit (Abbott Molecular Inc., Des Plaines, IL, USA) with 1 hour incubation at 15 °C. For tel-FISH we followed the procedure described in [55]. The probe cocktail contained 100 ng of Cy3-labelled telomeric probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA) in 10 μl of 50 % formamide and 10 % dextran sulfate in 2× SSC.

GISH and CGH were used to identify the sex chromosomes and examine their molecular differentiation [43, 91]. GISH was combined with tel-FISH for better resolution of the sex chromosome constitution [55]. Genomic DNAs for both GISH and CGH experiments were extracted separately from adult *Leptidea* males and females by standard phenol-chloroform procedure. Male gDNA was also amplified by GenomiPhi HY DNA Amplification Kit (GE Healthcare), thereafter sonicated using a Sonopuls HD 2070 (Bandelin Electric, Berlin, Germany) and used as a competitor DNA [52]. The extracted male gDNA was labelled with Cy3-dUTP (GE Healthcare) and female gDNA with fluorescein-12-dUTP (Invitrogen, Carlsbad, CA, USA) using the Nick Translation Kit with 8 hours incubation at 15 °C.

For GISH combined with tel-FISH the probe cocktail contained fluorescein-labelled female gDNA (300 ng), Cy3-labelled telomeric probe (100 ng), unlabelled sonicated male gDNA (3 μg) and sonicated salmon sperm DNA (25 μg). The probe cocktail for CGH was similar to GISH, except that it contained Cy3-labelled male gDNA (300 ng) instead of the telomeric probe. The preparations were counterstained with 0.5 mg/mL DAPI and mounted in antifade based on DABCO (Sigma-Aldrich).

FISH with biotin- and digoxigenin-labelled probes

Unlabelled 18S rDNA probe was generated by PCR from the codling moth (*Cydia pomonella*) gDNA extracted from adults by standard phenol-chloroform procedure as described in [43]. The probe was labelled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) by nick translation using the Nick Translation Kit with 1 hour and 45 minutes incubation at 15 °C.

Unlabelled H3 histone probe was obtained by PCR from *L. sinapis* gDNA. PCR was carried out using degenerate forward (5′-ATGGCNCGTACNAARCARAC-3′) and reverse (5′-TANGCACGYTCNCGGAT-3′) primers and the final PCR product was cloned as described in [52]. The probe was labelled in 25-μL PCR reaction containing 1× Ex *Taq* buffer, 0.1 mM dATP, dGTP and dCTP, 0.065 mM dTTP, 0.035 mM biotin-16-dUTP, 5 μmol of each M-13 universal primers, 0.25 U TaKaRa Ex *Taq* Hot Start DNA polymerase and about 5 ng of plasmid DNA. The thermal cycle profile consisted of an initial denaturation period of 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C and a final extension step of 2 min at 72 °C.

In FISH experiments, 18S rDNA and H3 histone probes were combined with telomeric probes. Unlabelled telomeric probe generated by non-template PCR (see above) was labelled with digoxigenin (Roche Diagnostics GmbH) using the Nick Translation Kit. The detection of biotin was carried out as described in [43]: the signals were detected with Cy3-conjugated streptavidin (Jackson

ImmunoRes. Labs. Inc., West Grove, PA, USA), amplified with biotinylated anti-streptavidin (Vector Labs. Inc., Burlingame, CA, USA) and again detected with Cy3-conjugated streptavidin. The detection of digoxigenin was carried out by Fluorescent Antibody Enhancer Set for DIG Detection (Roche Diagnostics GmbH). Like in the above-mentioned FISH experiments, the preparations were counterstained with 0.5 μg/mL DAPI and mounted in the DABCO-based antifade.

Microscopy and image processing

Preparations from FISH experiments were observed under a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Black-and-white images were recorded with a cooled F-View CCD camera using AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany). In all preparations, images were captured separately for each fluorescent dye, pseudocoloured (light blue for DAPI, green for fluorescein and red for Cy3) and superimposed with Adobe Photoshop, version 7.0.

Additional files

Additional file 1: Figure S1. Neighbor-joining tree of nuclear *ITS2* haplotypes of *L. sinapis* (grey background), *L. reali* (orange background) and *L. juvernica* (blue background). Specimens sequenced and analysed in this study are indicated by an asterisk. *Leptidea amurensis*, *L. lactea*, *L. morsei* and *L. duponcheli* were used as outgroup. For the origin of all specimens and GenBank accession numbers, see Additional file 5: Table S1. The scale represents 0.01 substitutions per site. Bootstrap supports (100 replicates) are shown next to the recovered nodes.

Additional file 2: Figure S2. The status of sex chromatin in polyploid nuclei of three *Leptidea* species. The orcein-stained preparations were made from Malpighian tubule cells of the fifth instar larvae (**a**, **c**, **d**) and adult females (**b**). Black arrows indicate a larger deeply stained heterochromatin body, while arrowheads show smaller bodies. (**a**) A lower-ploidy female nucleus of *L. sinapis* with one larger and two smaller bodies. (**b**) A highly polyploid female nucleus of *L. sinapis* with two bodies, one larger and one smaller. (**c**) A male nucleus of *L. reali* without distinguishable heterochromatin bodies. (**d**) A male nucleus of *L. reali* with one smaller body. Scale bar = 10 μm.

Additional file 3: Figure S3. Analysis of sex chromosome multivalents of pachytene oocytes in *Leptidea juvernica* (a–d), *L. sinapis* (e–h) and *L. reali* (i–l) using FISH with the $(TTAGG)_n$ telomeric probe. Hybridization signals of the Cy3-dUTP-labelled telomeric probe (red) indicate chromosome ends. Chromosomes were counterstained with DAPI (blue). Figures (**a**–**d**), (**e**–**h**) and (**i**–**l**) show sex chromosome multivalents $W_{1-n}Z_{1-n}$: (**a**, **e**, **i**) merged images of the $(TTAGG)_n$ telomeric probe and DAPI staining; (**b**, **f**, **j**) DAPI images; note DAPI-highlighted heterochromatic segments of the W chromosomes; (**c**, **g**, **k**) hybridization pattern of the $(TTAGG)_n$ telomeric probe; (**d**, **h**, **l**) schematic drawings of the sex chromosome multivalents; yellow dots indicate the ends of individual chromosomes involved in the multivalents. Scale bar = 10 μm.

Additional file 4: Figure S4. Comparison of interphase nuclei sizes in three *Leptidea* species. The y-axis shows the number of pixels. Micrographs of interphase nuclei were taken from DAPI-stained spread preparations of wing discs from three different larvae of each *Leptidea* species, using the same resolution. In these micrographs, we measured the area of 144 nuclei of *L. juvernica*, 154 nuclei of *L. reali* and 130 nuclei of *L. sinapis*. The measurements were carried out using the software JMicroVision v1.2.7 [Roduit N: JMicroVision: Image analysis toolbox for measuring and quantifying components of high-definition images. Version 1.2.7. http://www.jmicrovision.com (accessed 27 March 2015)]. Calibration was performed using an image resolution so that the area of each nucleus was measured in pixels. The average size of nuclei was calculated for each species independently and then compared between species by one-way ANOVA using the software Statistica for Windows, version 8.0 (StatSoft, Inc., Tulsa, OK, USA). The comparison of interphase nuclei revealed no statistically significant between-species differences in their size ($F_{(2, 9)} = 0.6782$; $P = 0.5425$). The mean (± S.E.) area of interphase nuclei was 22434 ± 2296 pixels for *L. juvernica*, 19781 ± 1965 pixels for *L. reali* and 19835 ± 1021 pixels for *L. sinapis*.

Additional file 5: Table S1. List of specimens included in phylogenetic analyses. Sequences obtained in this study are in blue, the other sequences were downloaded from GenBank and are representative for all the *COI* and *ITS2* haplotypes of *Leptidea sinapis*, *L. reali* and *L. juvernica* identified in a previous study [41]. The haplotype numbers correspond to those in [41].

Abbreviations

CGH: Comparative genomic hybridization; *COI*: Cytochrome *c* oxidase subunit 1; FISH: Fluorescence *in situ* hybridization; gDNA: Genomic DNA; GISH: Genomic *in situ* hybridization; H3: Histone H3; *ITS2*: Internal transcribed spacer 2; MI: Meiotic metaphase I; NOR: Nucleolar organizer region; PCR: Polymerase chain reaction; rDNA: Ribosomal DNA; rRNA: Ribosomal RNA; tel-FISH: Fluorescence *in situ* hybridization with $(TTAGG)_n$ telomeric probe.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KS performed first cytogenetic analyses and obtained preliminary data in *Leptidea juvernica* and *L. sinapis*. JŠ and FM conceived and designed the experiments and collected population samples of *L. juvernica* and *L. sinapis*. RV collected and supplied samples of the Spanish population of *L. reali*. JŠ performed most experiments and analysed the data. AV and PN participated in the preparation of FISH probes, some FISH experiments and isolation of genes for molecular analyses. VD and RV performed phylogenetic analyses. JŠ, PN and FM interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Our thanks go to Marie Korchová for excellent technical assistance and Leonela Carabajal Paladino for help with statistical analysis. This research was funded by Grant 14-22765S of the Czech Science Foundation, Grant IAA600960925 of the Grant Agency of The Czech Academy of Sciences, Grant 063/2012/P of the Grant Agency of the University of South Bohemia and Grant CGL2013-48277-P from the Spanish Ministerio de Economía y Competitividad. JŠ and AV were supported by Grant 052/2013/P of the Grant Agency of the University of South Bohemia, VD by a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme (project no. 625997), PN by Grant 14-35819P of the Czech Science Foundation and KS by JSPS 23380030 grant and JSPS Excellent Young Researchers Overseas Visit Program (21–7147).

Author details

[1]Institute of Entomology, Biology Centre CAS, 370 05 České Budějovice, Czech Republic. [2]Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic. [3]Biodiversity Institute of Ontario, University of Guelph, N1G 2W1 Guelph, ON, Canada. [4]Institut de Biologia Evolutiva (CSIC-Universitat Pompeu-Fabra), 08003 Barcelona, Spain. [5]Laboratory of Applied Entomology, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan.

Received: 29 December 2014 Accepted: 7 May 2015
Published online: 19 May 2015

References

1. van Nieukerken EJ, Kaila L, Kitching IJ, Kristensen NP, Lees DC, Minet J, et al. Order Lepidoptera Linnaeus, 1758. In Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness. Edited by Zhang ZQ. Zootaxa. 2011;3148:212–21.
2. Brown KS. The biology *of Heliconius* and related genera. Annu Rev Entomol. 1981;26:427–56.

3. Sheppard PM, Turner JRG, Brown KS, Benson WW, Singer MC. Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. Philos Trans R Soc Lond B. 1985;308:433–610.
4. Mallet J, Joron M. Evolution of diversity in warning color and mimicry: polymorphism, shifting balance and speciation. Annu Rev Ecol Syst. 1999;30:201–33.
5. Jiggins CD, Linares M, Naisbit RE, Salazar C, Yang ZH, Mallet J. Sex-linked hybrid sterility in a butterfly. Evolution. 2001;55:1631–8.
6. Kronforst MR, Kapan DD, Gilbert LE. Parallel genetic architecture of parallel adaptive radiations in mimetic *Heliconius* butterflies. Genetics. 2006;174:535–9.
7. Jiggins CD, Naisbit RE, Coe RL, Mallet J. Reproductive isolation caused by colour pattern mimicry. Nature. 2001;411:302–5.
8. Scriber JM, Hagen RH, Lederhouse RC. Genetics of mimicry in the tiger swallowtail butterflies, *Papilio glaucus* and *P. canadensis* (Lepidoptera: Papilionidae). Evolution. 1996;50:222–36.
9. Kunte K, Shea C, Aardema ML, Scriber JM, Juenger TE, Gilbert LE, et al. Sex chromosome mosaicism and hybrid speciation among tiger swallowtail butterflies. PLoS Genet. 2011;7, e1002274.
10. Zhang W, Kunte K, Kronforst MR. Genome-wide characterization of adaptation and speciation in tiger swallowtail butterflies using de novo transcriptome assemblies. Genome Biol Evol. 2013;5:1233–45.
11. Ando T, Inomata SI, Yamamoto M. Lepidopteran sex pheromones. Top Curr Chem. 2004;239:51–96.
12. Dopman EB, Robbins PS, Seaman A. Components of reproductive isolation between North American pheromone strains of the European corn borer. Evolution. 2010;64:881–902.
13. Olsson SB, Kesevan S, Groot AT, Dekker T, Heckel DG, Hansson BS. *Ostrinia* revisited: Evidence for sex linkage in European corn borer *Ostrinia nubilalis* (Hübner) pheromone reception. BMC Evol Biol. 2010;10:285.
14. Leary GP, Allen JE, Bunger PL, Luginbill JB, Linn Jr CE, Macallister IE, et al. Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. Proc Natl Acad Sci U S A. 2012;109:14081–6.
15. Lukhtanov VA, Kandul NP, Plotkin JB, Dantchenko AV, Haig D, Pierce NE. Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus* butterflies. Nature. 2005;436:385–9.
16. Noor MA, Grams KL, Bertucci LA, Reiland J. Chromosomal inversions and the reproductive isolation of species. Proc Natl Acad Sci U S A. 2001;98:12084–8.
17. Rieseberg LH. Chromosomal rearrangements and speciation. Trends Ecol Evol. 2001;16:351–8.
18. Faria R, Navarro A. Chromosomal speciation revisited: rearranging theory with pieces of evidence. Trends Ecol Evol. 2010;25:660–9.
19. Sperling FAH. Sex-linked genes and species-differences in Lepidoptera. Can Entomol. 1994;126:807–18.
20. Prowell DP. Sex Linkage and Speciation in Lepidoptera. In: Howard DJ, Berlocher SH, editors. Endless Forms: Species and Speciation. New York: Oxford University Press; 1998. p. 309–19.
21. Yoshido A, Sahara K, Marec F, Matsuda Y. Step-by-step evolution of neo-sex chromosomes in geographical populations of wild silkmoths, *Samia cynthia* ssp. Heredity. 2011;106:614–24.
22. Yoshido A, Šíchová J, Kubíčková S, Marec F, Sahara K. Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp. Chromosome Res. 2013;21:149–64.
23. Carpenter JE, Bloem S, Marec F. Inherited Sterility in Insects. In: Dyck VA, Hendrichs J, Robinson AS, editors. Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management. Dordrecht: Springer; 2005. p. 115–46.
24. Pringle EG, Baxter SW, Webster CL, Papanicolaou A, Lee SF, Jiggins CD. Synteny and chromosome evolution in the Lepidoptera: Evidence from mapping in *Heliconius melpomene*. Genetics. 2007;177:417–26.
25. Beldade P, Saenko SV, Pul N, Long AD. A gene-based linkage map for *Bicyclus anynana* butterflies allows for a comprehensive analysis of synteny with the lepidopteran reference genome. PLoS Genet. 2009;5, e1000366.
26. Yasukochi Y, Tanaka-Okuyama M, Shibata F, Yoshido A, Marec F, Wu C, et al. Extensive conserved synteny of genes between the karyotypes of *Manduca sexta* and *Bombyx mori* revealed by BAC-FISH mapping. PLoS ONE. 2009;4, e7465.
27. d'Alençon E, Sezutsu H, Legeai F, Permal E, Bernard-Samain S, Gimenez S, et al. Extensive synteny conservation of holocentric chromosomes in Lepidoptera despite high rates of local genome rearrangements. Proc Natl Acad Sci U S A. 2010;107:7680–5.
28. Baxter SW, Davey JW, Johnston JS, Shelton AM, Heckel DG, Jiggins CD, et al. Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. PLoS ONE. 2011;6, e19315.
29. Sahara K, Yoshido A, Shibata F, Fujikawa-Kojima N, Okabe T, Tanaka-Okuyama M, et al. FISH identification of *Helicoverpa armigera* and *Mamestra brassicae* chromosomes by BAC and fosmid probes. Insect Biochem Mol Biol. 2013;43:644–53.
30. Van't Hof AE, Nguyen P, Dalíková M, Edmonds N, Marec F, Saccheri IJ. Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism. Heredity. 2013;110:283–95.
31. Suomalainen E. Chromosome evolution in the Lepidoptera. Chromosomes Today. 1969;2:132–8.
32. Ahola V, Lehtonen R, Somervuo P, Salmela L, Koskinen P, Rastas P, et al. The Glanville fritillary genome retains an ancient karyotype and reveals selective chromosomal fusions in Lepidoptera. Nat Commun. 2014;5:4737.
33. Robinson R. Lepidoptera Genetics. Oxford: Pergamon Press; 1971.
34. De Prins J, Saitoh K. Karyology and Sex Determination. In: Kristensen NP, editor. Lepidoptera, Moths and Butterflies: Morphology, Physiology and Development. Berlin: Walter de Gruyter; 2003. p. 449–68.
35. Kandul NP, Lukhtanov VA, Dantchenko DA, Coleman JWS, Sekercioglu C, Haig D, et al. Phylogeny of *Agrodiaetus* Hübner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sequences of *COI* and *COII* and nuclear sequences of *EF1-* α: karyotype diversification and species radiation. Syst Biol. 2004;53:278–98.
36. de Lesse H. Les nombres de chromosomes dans le groupe de *Lysandra argester* et leur incidence sur la taxonomie. Bull Soc Entomol Fr. 1970;75:64–8.
37. Lukhtanov VA, Danchenko AD. Principles of the highly ordered arrangement of metaphase I bivalents in spermatocytes of *Agrodiaetus* (Insecta, Lepidoptera). Chromosome Res. 2002;10:5–20.
38. Dincă V, Lukhtanov VA, Talavera G, Vila R. Unexpected layers of cryptic diversity in wood white *Leptidea* butterflies. Nat Commun. 2011;2:324.
39. Lukhtanov VA, Dincă V, Talavera G, Vila R. Unprecedented within-species chromosome number cline in the Wood White butterfly *Leptidea sinapis* and its significance for karyotype evolution and speciation. BMC Evol Biol. 2011;11:109.
40. Maeki K. On the cytotaxonomical relationship in *Leptidea* (Lepidoptera-Rhopalocera). Jpn J Genet. 1958;33:283–5.
41. Dincă V, Wiklund C, Lukhtanov VA, Kodandaramaiah U, Norén K, Dapporto L, et al. Reproductive isolation and patterns of genetic differentiation in a cryptic butterfly species complex. J Evol Biol. 2013;26:2095–106.
42. Marec F, Traut W. Sex chromosome pairing and sex chromatin bodies in W-Z translocation strains of *Ephestia kuehniella* (Lepidoptera). Genome. 1994;37:426–35.
43. Fuková I, Nguyen P, Marec F. Codling moth cytogenetics: karyotype, chromosomal location of rDNA and molecular differentiation of sex chromosomes. Genome. 2005;48:1083–92.
44. Traut W, Marec F. Sex chromatin in Lepidoptera. Q Rev Biol. 1996;71:239–56.
45. Mediouni J, Fuková I, Frydrychová R, Dhouibi MH, Marec F. Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). Caryologia. 2004;57:184–94.
46. Traut W, Sahara K, Marec F. Sex chromosomes and sex determination in Lepidoptera. Sex Dev. 2007;1:332–46.
47. Traut W, Weith A, Traut G. Structural mutants of the W chromosome in *Ephestia* (Insecta, Lepidoptera). Genetica. 1986;70:69–79.
48. Colombo PC. Cytogeography of three parallel Robertsonian polymorphisms in the water-hyacinth grasshopper, *Cornops aquaticum* (Orthoptera: Acrididae). Eur J Entomol. 2008;105:59–64.
49. Castiglia R, Annesi F, Capanna E. Geographical pattern of genetic variation in the Robertsonian system of *Mus musculus domesticus* in central Italy. Biol J Linn Soc Lond. 2005;84:395–405.
50. White TA, Bordewich M, Searle JB. A network approach to study karyotypic evolution: the chromosomal races of the common shrew (*Sorex araneus*) and house mouse (*Mus musculus*) as model systems. Syst Biol. 2010;59:262–76.
51. Nguyen P, Sahara K, Yoshido A, Marec F. Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica. 2010;138:343–54.
52. Šíchová J, Nguyen P, Dalíková M, Marec F. Chromosomal evolution in tortricid moths: conserved karyotypes with diverged features. PLoS ONE. 2013;8, e64520.
53. Cabrero J, López-León MD, Teruel M, Camacho JPM. Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. Chromosome Res. 2009;17:397–404.

54. Cabral-de-Mello DC, Oliveira SG, de Moura RC, Martins C. 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. 2011;12:88.
55. Yoshido A, Marec F, Sahara K. Resolution of sex chromosome constitution by genomic *in situ* hybridization and fluorescence *in situ* hybridization with $(TTAGG)_n$ telomeric probe in some species of Lepidoptera. Chromosoma. 2005;114:193–202.
56. Rens W, Grützner F, O'Brien PCM, Fairclough HF, Graves JAM, Ferguson-Smith MA. 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. 2004;101:16257–61.
57. Veyrunes F, Waters PD, Miethke P, Rens W, McMillan D, Alsop AE, et al. Bird-like sex chromosomes of platypus imply recent origin of mammal sex chromosomes. Genome Res. 2008;18:965–73.
58. Cioffi MB, Sánchez A, Marchal JA, Kosyakova N, Liehr T, Trifonov V, et al. Cross-species chromosome painting tracks the independent origin of multiple sex chromosomes in two cofamiliar Erythrinidae fishes. BMC Evol Biol. 2011;11:186.
59. Blanco DR, Vicari MR, Lui RL, Artoni RF, de Almeida MC, Traldi JB, et al. Origin of the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system of *Harttia punctata* (Siluriformes, Loricariidae) inferred from chromosome painting and FISH with ribosomal DNA markers. Genetica. 2014;142:119–26.
60. Král J. Evolution of multiple sex chromosomes in the spider genus *Malthonica* (Araneae: Agelenidae) indicates unique structure of the spider sex chromosome systems. Chromosome Res. 2007;15:863–79.
61. Král J, Kořínková T, Forman M, Krkavcová L. Insights into the meiotic behavior and evolution of multiple sex chromosome systems in spiders. Cytogenet Genome Res. 2011;133:43–66.
62. Traut W, Marec F. Sex chromosome differentiation in some species of Lepidoptera (Insecta). Chromosome Res. 1997;5:283–91.
63. Yoshido A, Bando H, Yasukochi Y, Sahara K. The *Bombyx mori* karyotype and the assignment of linkage groups. Genetics. 2005;170:675–85.
64. Sahara K, Marec F, Eickhoff U, Traut W. Moth sex chromatin probed by comparative genomic hybridization (CGH). Genome. 2003;46:339–42.
65. Lahn BT, Page DC. Four evolutionary strata on the human X chromosome. Science. 1999;286:964–7.
66. Bergero R, Forrest A, Kamau E, Charlesworth D. Evolutionary strata on the X chromosomes of the dioecious plant *Silene latifolia:* evidence from new sex-linked genes. Genetics. 2007;75:1945–54.
67. Nam K, Ellegren H. The chicken (*Gallus gallus*) Z chromosome contains at least three nonlinear evolutionary strata. Genetics. 2008;180:1131–6.
68. Mácha J, Teichmanová R, Sater AK, Wells DE, Tlapáková T, Zimmerman LB, et al. Deep ancestry of mammalian X chromosome revealed by comparison with the basal tetrapod *Xenopus tropicalis*. BMC Genomics. 2012;13:315.
69. Wang J, Na J-K, Yu Q, Gschwend AR, Han J, Zeng F, et al. Sequencing papaya X and Y^h chromosomes reveals molecular basis of incipient sex chromosome evolution. Proc Natl Acad Sci U S A. 2012;109:13710–5.
70. Baker RJ, Bickham JW. Speciation by monobrachial centric fusions. Proc Natl Acad Sci U S A. 1986;83:8245–8.
71. Bidau CJ, Giménez MD, Palmer CL, Searle JB. The effects of Robertsonian fusions on chiasma frequency and distribution in the house mouse (*Mus musculus domesticus*) from a hybrid zone in northern Scotland. Heredity. 2001;87:305–13.
72. Basset P, Yannic G, Brünner H, Hausser J. Restricted gene flow at specific parts of the shrew genome in chromosomal hybrid zones. Evolution. 2006;60:1718–30.
73. Hipp AL, Rothrock PE, Roalson EH. The evolution of chromosome arrangements in *Carex* (Cyperaceae). Bot Rev. 2009;75:96–109.
74. Melters DP, Paliulis LV, Korf IF, Chan SWL. Holocentric chromosomes: convergent evolution, meiotic adaptations and genomic analysis. Chromosome Res. 2012;20:579–93.
75. Wrensch DL, Kethley JB, Norton RA. Cytogenetics of Holokinetic Chromosomes and Inverted Meiosis: Keys to the Evolutionary Success of Mites, with Generalization on Eukaryotes. In: Houck MA, editor. Mites: Ecological and Evolutionary Analyses of Life-History Patterns. New York: Chapman & Hall; 1994. p. 282–343.
76. Marec F, Tothová A, Sahara K, Traut W. Meiotic pairing of sex chromosome fragments and its relation to atypical transmission of a sex-linked marker in *Ephestia kuehniella* (Insecta: Lepidoptera). Heredity. 2001;87:659–71.
77. Bureš P, Zedek F. Holokinetic drive: centromere drive in chromosomes without centromeres. Evolution. 2014;68:2412–20.
78. Hipp AL, Rothrock PE, Whitkus R, Weber JA. Chromosomes tell half of the story: the correlation between karyotype rearrangements and genetic diversity in sedges, a group with holocentric chromosomes. Mol Ecol. 2010;19:3124–38.
79. Sætre GP, Borge T, Lindroos K, Haavie J, Sheldon BC, Primmer C, et al. Sex chromosome evolution and speciation in *Ficedula* flycatchers. Proc Biol Sci. 2003;270:53–9.
80. Ellegren H. Genomic evidence for large-Z effect. Proc Biol Sci. 2009;276:361–6.
81. Štorchová R, Reif J, Nachman MW. Female heterogamety and speciation: reduced introgression of the Z chromosome between two species of nightingales. Evolution. 2010;64:456–71.
82. Carling MD, Brumfield RT. Haldane's rule in an avian system: using cline theory and divergence population genetics to test for differential introgression of mitochondrial, autosomal and sex-linked loci across the *Passerina* bunting hybrid zone. Evolution. 2008;62:2600–15.
83. Carling MD, Brumfield RT. Speciation in *Passerina* buntings: introgression patterns of sex- linked loci identify a candidate gene region for reproductive isolation. Mol Ecol. 2009;18:834–47.
84. Naisbit RE, Jiggins CD, Linares M, Salazar C, Mallet J. Hybrid sterility. Haldane's rule and speciation in *Heliconius cydno* and *H. melpomene*. Genetics. 2002;161:1517–26.
85. Nguyen P, Sýkorová M, Šíchová J, Kůta V, Dalíková M, Čapková Frydrychová R, et al. Neo-sex chromosomes and adaptive potential in tortricid pests. Proc Natl Acad Sci U S A. 2013;110:6931–6.
86. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406–25.
87. Nei M, Kumar S. Molecular Evolution and Phylogenetics. New York: Oxford University Press; 2000.
88. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39:783–91.
89. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.
90. Sahara K, Marec F, Traut W. TTAGG telomeric repeats in chromosomes of some insects and other arthropods. Chromosome Res. 1999;7:449–60.
91. Traut W, Sahara K, Otto TD, Marec F. Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. Chromosoma. 1999;108:173–80.

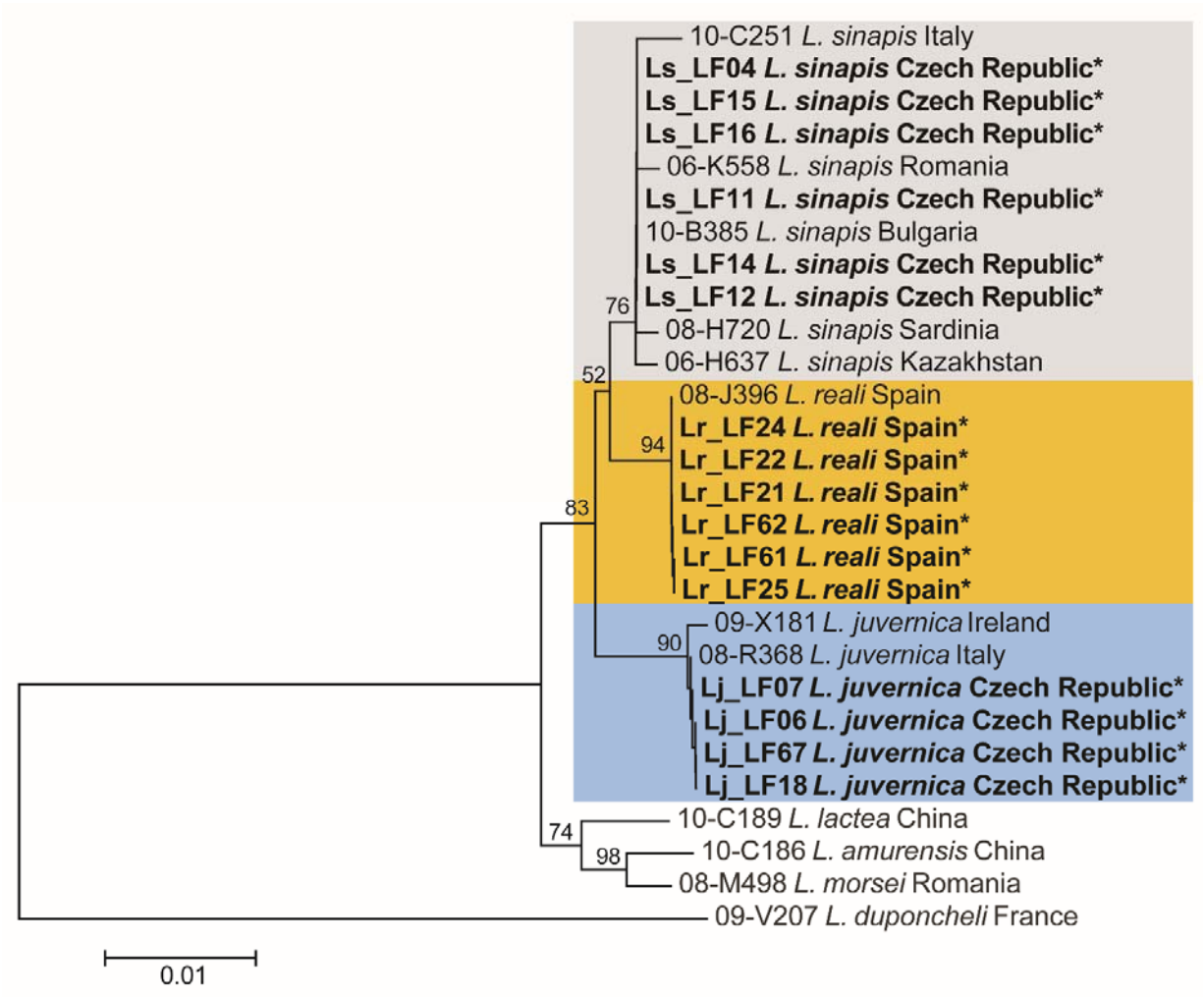

Figure S1 Neighbor-joining tree of nuclear *ITS2* haplotypes of *L. sinapis* (grey background), *L. reali* (orange background), and *L. juvernica* (blue background). Specimens sequenced and analysed in this study are indicated by an asterisk. *Leptidea amurensis*, *L. lactea*, *L. morsei*, and *L. duponcheli* were used as outgroup. For the origin of all specimens and GenBank accession numbers, see Additional file 5: Table S1. The scale represents 0.01 substitutions per site. Bootstrap supports (100 replicates) are shown next to the recovered nodes.

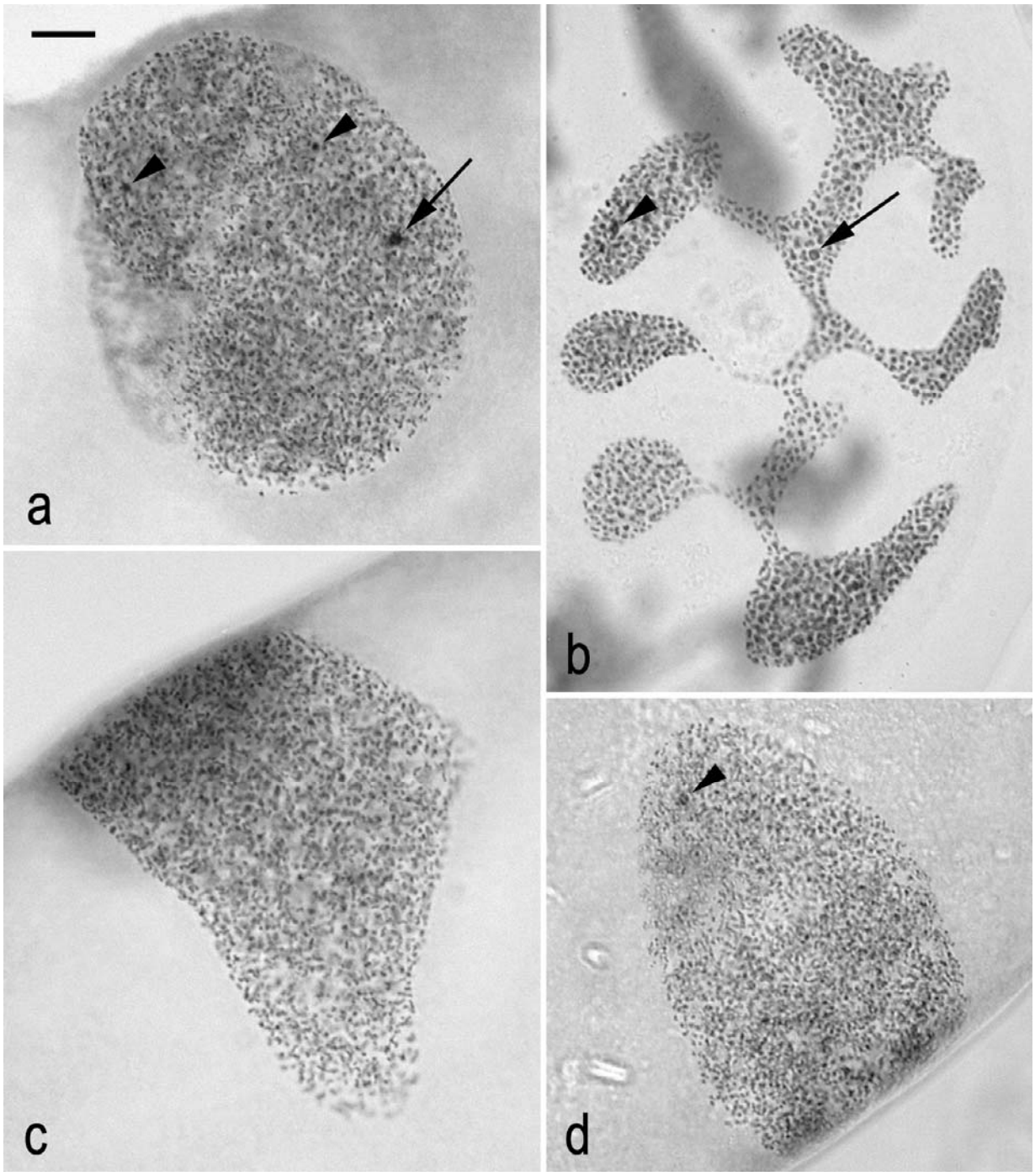

Figure S2 The status of sex chromatin in polyploid nuclei of three *Leptidea* species. The orcein-stained preparations were made from Malpighian tubule cells of the fifth instar larvae (**a**, **c**, **d**) and adult females (**b**). Black arrows indicate a larger deeply stained heterochromatin body, while arrowheads show smaller bodies. (**a**) A lower-ploidy female nucleus of *L. sinapis* with one larger and two smaller bodies. (**b**) A highly polyploid female nucleus of *L. sinapis* with two bodies, one larger and one smaller. (**c**) A male nucleus of *L. reali* without distinguishable heterochromatin bodies. (**d**) A male nucleus of *L. reali* with one smaller body. Scale bar = 10 µm.

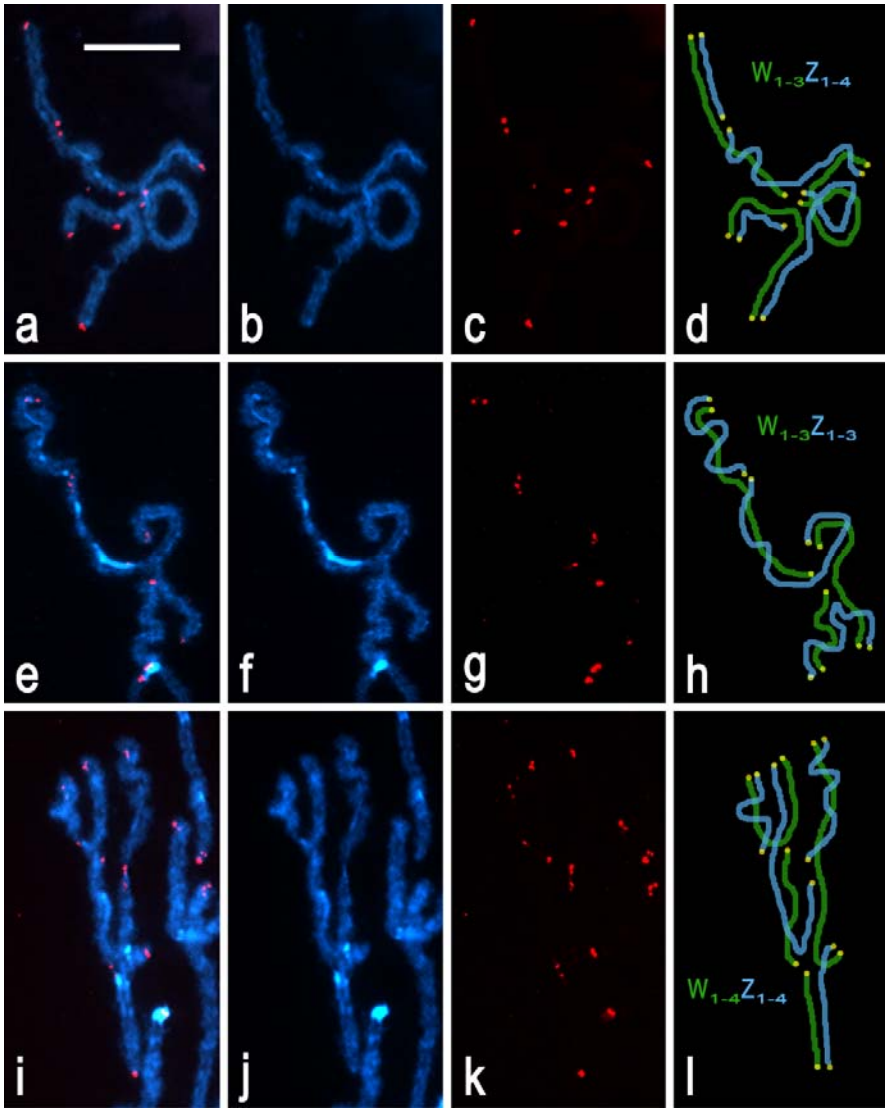

Figure S3 Analysis of sex chromosome multivalents of pachytene oocytes in *Leptidea juvernica* (a-d), *L. sinapis* (e-h), and *L. reali* (i-l) using FISH with the $(TTAGG)_n$ telomeric probe. Hybridization signals of the Cy3-dUTP-labelled telomeric probe (red) indicate chromosome ends. Chromosomes were counterstained with DAPI (blue). Figures (**a**-**d**), (**e**-**h**), and (**i**-**l**) show sex chromosome multivalents $W_{1-n}Z_{1-n}$: (**a**, **e**, **i**) merged images of the $(TTAGG)_n$ telomeric probe and DAPI staining; (**b**, **f**, **j**) DAPI images; note DAPI-highlighted heterochromatic segments of the W chromosomes; (**c**, **g**, **k**) hybridization pattern of the $(TTAGG)_n$ telomeric probe; (**d**, **h**, **l**) schematic drawings of the sex chromosome multivalents; yellow dots indicate the ends of individual chromosomes involved in the multivalents. Scale bar = 10 µm.

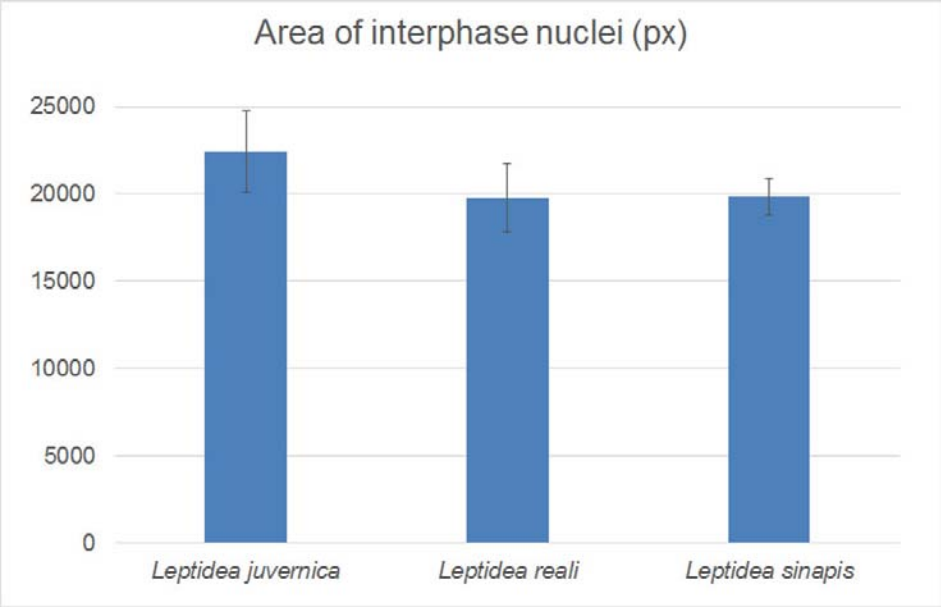

Figure S4 Comparison of interphase nuclei sizes in three *Leptidea* species. The y-axis shows the number of pixels. Micrographs of interphase nuclei were taken from DAPI-stained spread preparations of wing discs from three different larvae of each *Leptidea* species, using the same resolution. In these micrographs, we measured the area of 144 nuclei of *L. juvernica*, 154 nuclei of *L. reali*, and 130 nuclei of *L. sinapis*. The measurements were carried out using the software JMicroVision v1.2.7 [Roduit N: JMicroVision: Image analysis toolbox for measuring and quantifying components of high-definition images. Version 1.2.7. http://www.jmicrovision.com (accessed 27 March 2015)]. Calibration was performed using an image resolution so that the area of each nucleus was measured in pixels. The average size of nuclei was calculated for each species independently and then compared between species by one-way ANOVA using the software Statistica for Windows, version 8.0 (StatSoft, Inc., Tulsa, OK, USA). The comparison of interphase nuclei revealed no statistically significant between-species differences in their size ($F_{(2, 9)} = 0.6782$; $P = 0.5425$). The mean (± S.E.) area of interphase nuclei was 22434 ± 2296 pixels for *L. juvernica*, 19781 ± 1965 pixels for *L. reali*, and 19835 ± 1021 pixels for *L. sinapis*.

Table S1 List of specimens included in phylogenetic analyses. Sequences obtained in this study are in blue, the other sequences were downloaded from GenBank and are representative for all the *COI* and *ITS2* haplotypes of *Leptidea sinapis* , *L. reali* , and *L. juvernica* identified in a previous study [41]. The haplotype numbers correspond to those in [41].

Sample ID	Species	*COI* haplotype[1)]	*COI* accession number	*ITS2* haplotype[1)]	*ITS2* accession number	Locality	Country
RVcoll.08-R368	*L. juvernica*	**hj1**	KC865949	**hj1**	KC865858	Val di Tovo- Laghi	Italy
RVcoll.08-M322	*L. juvernica*	**hj2**	HQ004596	**hj1**	JF512789	Valea Belchia, Harghita	Romania
RVcoll.10-A259	*L. juvernica*	**hj6**	JF512651	**hj1**	JF512783	Gresse-en-Vercors, Isère	France
RVcoll.07-Z082	*L. juvernica*	**hj7**	JF512648	**hj1**	JF512768	South Altai, Uspenka	Kazakhstan
RVcoll.09-X181	*L. juvernica*	**hj9**	JF512715	**hj2**	JF512788	Kilternan, Dublin	Ireland
RVcoll.08-J396	*L. reali*	**hr1**	JF512603	**hr1**	JF512797	Viladrau, Barcelona	Spain
RVcoll.03-H535	*L. reali*	**hr6**	JF512617	**hr1**	JF512790	Saldes, Barcelona	Spain
RVcoll.07-E083	*L. reali*	**hr7**	JF512712	**hr1**	JF512795	Roccaraso, L'Aquila	Italy
RVcoll.10-B385	*L. sinapis*	**hs1**	JF512693	**hs1**	JF512817	Paril Village, Khadzhidimovo	Bulgaria
MF-63	*L. sinapis*	**hs14**	KC866102	**hs1**	KC865928	Riala	Sweden
MF-174	*L. sinapis*	**hs16**	KC866101	**hs1**	KC865919	Hodonín, South Moravia	Czech Republic
RVcoll.08-R436	*L. sinapis*	**hs24**	GU675857	**hs1**	KC865933	Ames, Novais	Spain
RVcoll.10-C245	*L. sinapis*	**hs5**	JF512697	**hs1**	JF512847	Příbram, Central Bohemia	Czech Republic
RVcoll.07-E237	*L. sinapis*	**hs8**	JF512597	**hs1**	JF512810	Novalesa-Moncenisio, Torino	Italy
RVcoll.08-H720	*L. sinapis*	**hs23**	KC865994	**hs2**	KC865883	Gairo	Sardinia
RVcoll.08-H769	*L. sinapis*	**hs3**	KC866005	**hs2**	KC865886	Fozzaninco	Corsica
RVcoll.06-H637	*L. sinapis*	**hs2**	JF513027	**hs3**	KC865931	Landman, Zyryanovsk	Kazakhstan
RVcoll.06-K558	*L. sinapis*	**hs1**	JF513036	**hs4**	KC865939	Bădeni, Cluj	Romania
RVcoll.10-C251	*L. sinapis*	**hs1**	JF512699	**hs5**	JF512832	Monte di Malo, Veneto	Italy
MF-F12	*L. juvernica*	**hj10**	KC866126			Riala	Sweden
GenBank	*L. juvernica*	**hj11**	EF599645			Barje	Slovenia

GenBank	*L. juvernica*	**hj12**	EF599643			Barje	Slovenia
GenBank	*L. juvernica*	**hj13**	EF599640			Vrhnika	Slovenia
RVcoll.08-Y008	*L. juvernica*	**hj3**	JF512578			Peterhof, St. Petersburg	Russia
RVcoll.11-G200	*L. juvernica*	**hj4**	KC865980			Narymski Mts.	Kazakhstan
RVcoll.11-G219	*L. juvernica*	**hj5**	KC865982			Balgyn	Kazakhstan
GenBank	*L. juvernica*	**hj8**	GU655014			Neustadt/Donau, Plattenberg, Bavaria	Germany
RVcoll.09-X183	*L. juvernica*	**hj9**	JF512716			Gortmore Point, Lough Derg, Tipperary	Ireland
RVcoll.10-C269	*L. reali*	**hr2**	JF512616			Cascia, Perugia	Italy
RVcoll.10-C270	*L. reali*	**hr3**	JF512704			Sibillini Mountains	Italy
RVcoll.08-H468	*L. reali*	**hr4**	GU676645			Hormiguera, Cantabria	Spain
MF-90	*L. reali*	**hr5**	KC866117			Pla de la Calma, Montseny	Spain
RVcoll.07-C210	*L. sinapis*	**hs10**	JF512592			Schitul Pahomie, Vâlcea	Romania
RVcoll.06-H631	*L. sinapis*	**hs11**	JF513025			Landman, Zyryanovsk	Kazakhstan
RVcoll.06-H632	*L. sinapis*	**hs12**	JF513047			Landman, Zyryanovsk	Kazakhstan
GenBank	*L. sinapis*	**hs13**	HM393183			Zahmer Kaiser, Aschinger Alm, Tyrol	Austria
RVcoll.10-C262	*L. sinapis*	**hs15**	KC866089			Corciano, Perugia	Italy
MF-155	*L. sinapis*	**hs17**	KC866100			Sant Celoni, Barcelona	Spain
RVcoll.11-K071	*L. sinapis*	**hs18**	KC866098			Mala Reka, Mavrovo	Macedonia
GenBank	*L. sinapis*	**hs19**	GU688515			Ruhpolding, Bavaria	Germany
RVcoll.10-B320	*L. sinapis*	**hs20**	KC866088			Studen Kladenets, Krumovgrad	Bulgaria
GenBank	*L. sinapis*	**hs21**	GU688533			Lenggries Isarauen, Bavaria	Germany
RVcoll.11-K078	*L. sinapis*	**hs22**	KC866104			Mala Reka, Mavrovo	Macedonia
RVcoll.11-J544	*L. sinapis*	**hs25**	KC866097			Manosque, Alpes-de-Haute-Provence	France
RVcoll.07-Z211	*L. sinapis*	**hs4**	JF513046			Saur Mts., Malyi Zhemeney	Kazakhstan
RVcoll.07-E250	*L. sinapis*	**hs6**	JF513034			NE Bézaudun-sur-Bine, Drôme	France
RVcoll.09-X562	*L. sinapis*	**hs7**	KC866082			Sorauren, Navarra	Spain
RVcoll.07-D938	*L. sinapis*	**hs9**	JF513026			Ciupercenii de Olteț, Gorj	Romania

Nz091	*L. amurensis*		JF512621			Bulgan	Mongolia
RVcoll.10-C186	*L. amurensis*		JF512622		JF512841	Jiexiu county, Shanxi	China
RVcoll.09-V207	*L. duponcheli*		JF512569		JF512852	Oraison, Alpes de Haute Provence	France
MF-1	*L. duponcheli*		KC866120			Skopje	Macedonia
RVcoll.10-C189	*L. lactea*		JF512717		JF512849	Qin Ling Shan, Madao, Liuba County, Shaanxi	China
RVcoll.10-C195	*L. lactea*		JF512718			Qin Ling Shan, Zhouzhi (Erqu) County, Shaanxi	China
RVcoll.07-Z124	*L. morsei*		JF512618			South Altai, Markakol	Kazakhstan
RVcoll.08-M498	*L. morsei*		HQ004591		JF512840	Bădeni, Cluj	Romania
Ls_LF04	*L. sinapis*		KM488575		KM488591	NP Podyjí, South Moravia	Czech Republic
Ls_LF11	*L. sinapis*		KM488578		KM488594	NP Podyjí, South Moravia	Czech Republic
Ls_LF12	*L. sinapis*		KM488579		KM488595	NP Podyjí, South Moravia	Czech Republic
Ls_LF14	*L. sinapis*		KM488580		KM488596	NP Podyjí, South Moravia	Czech Republic
Ls_LF15	*L. sinapis*		KM488581		KM488597	NP Podyjí, South Moravia	Czech Republic
Ls_LF16	*L. sinapis*		KM488582		KM488598	NP Podyjí, South Moravia	Czech Republic
Lr_LF21	*L. reali*		KM488584		KM488600	NP Montseny, Barcelona	Spain
Lr_LF22	*L. reali*		KM488585		KM488601	NP Montseny, Barcelona	Spain
Lr_LF24	*L. reali*		KM488586		KM488602	NP Montseny, Barcelona	Spain
Lr_LF25	*L. reali*		KM488587		KM488603	NP Montseny, Barcelona	Spain
Lr_LF61	*L. reali*		KM488588		KM488604	NP Montseny, Barcelona	Spain
Lr_LF62	*L. reali*		KM488589		KM488605	NP Montseny, Barcelona	Spain
Lj_LF06	*L. juvernica*		KM488576		KM488592	České Budějovice, South Bohemia	Czech Republic
Lj_LF07	*L. juvernica*		KM488577		KM488593	České Budějovice, South Bohemia	Czech Republic
Lj_LF18	*L. juvernica*		KM488583		KM488599	České Budějovice, South Bohemia	Czech Republic
Lj_LF67	*L. juvernica*		KM488590		KM488606	České Budějovice, South Bohemia	Czech Republic

3.4. Paper IV

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 of the northeast-Asian wood white butterfly, *Leptidea amurensis*. *Biol J Linnean Soc* (published online 21 January 2016). DOI: 10.1111/bij.12756.

Abstract

Previous studies have shown a dynamic karyotype evolution and the presence of complex sex chromosome systems in three cryptic *Leptidea* species from the Western Palearctic. To further explore the chromosomal particularities of Leptidea butterflies, we examined the karyotype of an Eastern Palearctic species, *Leptidea amurensis*. We found a high number of chromosomes that differed between the sexes and slightly varied in females (i.e. 2n = 118–119 in females and 2n = 122 in males). The analysis of female meiotic chromosomes revealed multiple sex chromosomes with three W and six Z chromosomes. The curious sex chromosome constitution [i.e. $W_{1–3}/Z_{1–6}$ (females) and $Z_{1–6}/Z_{1–6}$ (males)] and the observed heterozygotes for a chromosomal fusion are together responsible for the sex-specific and intraspecific variability in chromosome numbers. However, in contrast to the Western Palearctic *Leptidea* species, the single chromosomal fusion and static distribution of cytogenetic markers (18S rDNA and H3 histone genes) suggest that the karyotype of *L. amurensis* is stable. The data obtained for four *Leptidea* species suggest that the multiple sex chromosome system, although different among species, is a common feature of the genus *Leptidea*. Furthermore, inter- and intraspecific variations in chromosome numbers and the complex meiotic pairing of these multiple sex chromosomes indicate the role of chromosomal fissions, fusions, and translocations in the karyotype evolution of *Leptidea* butterflies.

The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2016, ••, ••–••. With 5 figures.

Fissions, fusions, and translocations shaped the karyotype and multiple sex chromosome constitution of the northeast-Asian wood white butterfly, *Leptidea amurensis*

JINDRA ŠÍCHOVÁ[1,2], MIZUKI OHNO[3], VLAD DINCĂ[4,5], MICHIHITO WATANABE[6], KEN SAHARA[3] and FRANTIŠEK MAREC[1,2]*

[1]*Institute of Entomology, Biology Centre CAS, 370 05, České Budějovice, Czech Republic*
[2]*Faculty of Science, University of South Bohemia, 370 05, České Budějovice, Czech Republic*
[3]*Laboratory of Applied Entomology, Faculty of Agriculture, Iwate University, Morioka, 020-8550, Japan*
[4]*Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, N1G 2W1, Canada*
[5]*Institut de Biologia Evolutiva, (CSIC-Universitat Pompeu-Fabra), 08003, Barcelona, Spain*
[6]*NPO Mt. Fuji Nature Conservation Center, 6603 Funatsu, Fujikawaguchiko-machi, Yamanashi, 401-0301, Japan*

Received 25 September 2015; revised 11 November 2015; accepted for publication 12 November 2015

Previous studies have shown a dynamic karyotype evolution and the presence of complex sex chromosome systems in three cryptic *Leptidea* species from the Western Palearctic. To further explore the chromosomal particularities of *Leptidea* butterflies, we examined the karyotype of an Eastern Palearctic species, *Leptidea amurensis*. We found a high number of chromosomes that differed between the sexes and slightly varied in females (i.e. $2n$ = 118–119 in females and $2n$ = 122 in males). The analysis of female meiotic chromosomes revealed multiple sex chromosomes with three W and six Z chromosomes. The curious sex chromosome constitution [i.e. $W_{1–3}/Z_{1–6}$ (females) and $Z_{1–6}/Z_{1–6}$ (males)] and the observed heterozygotes for a chromosomal fusion are together responsible for the sex-specific and intraspecific variability in chromosome numbers. However, in contrast to the Western Palearctic *Leptidea* species, the single chromosomal fusion and static distribution of cytogenetic markers (18S rDNA and H3 histone genes) suggest that the karyotype of *L. amurensis* is stable. The data obtained for four *Leptidea* species suggest that the multiple sex chromosome system, although different among species, is a common feature of the genus *Leptidea*. Furthermore, inter- and intraspecific variations in chromosome numbers and the complex meiotic pairing of these multiple sex chromosomes indicate the role of chromosomal fissions, fusions, and translocations in the karyotype evolution of *Leptidea* butterflies. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000–000.

KEYWORDS: chromosome fusion – chromosome number variation – fluorescence *in situ* hybridization – genomic *in situ* hybridization – Pieridae.

INTRODUCTION

Moths and butterflies (Lepidoptera) exhibit several peculiar cytogenetic features by which they differ greatly from other groups of insects, except for their sister group, caddisflies (Trichoptera). The most striking feature is the chromosomal mechanism of sex determination, with female heterogamety represented by Z0/ZZ and WZ/ZZ (female/male) sex chromosome systems or their numerical variations. Female heterogamety is associated with achiasmatic meiosis in females (i.e. the absence of crossing over and chiasmata in meiotic prophase I oocytes) (Traut, Sahara & Marec, 2007). Furthermore, Lepidoptera

*Corresponding author. E-mail: marec@entu.cas.cz

are regarded as organisms with holokinetic chromosomes as a result of the lack of a distinct primary constriction (the centromere) and parallel disjunction of sister chromatids during mitotic metaphase. During cell division, the spindle microtubules attach to a large kinetochore plate covering most of the chromosome surface (Carpenter, Bloem & Marec, 2005). Lepidopteran chromosomes are usually small and uniform in shape, and most species are reported to have haploid chromosome numbers ranging from $n = 28$ to $n = 32$, with the most common number being $n = 31$ (Suomalainen, 1969; Robinson, 1971; De Prins & Saitoh, 2003; Brown *et al.*, 2007). Based on the occurrence across the lepidopteran phylogenetic tree, the modal chromosome number of $n = 31$ has been proposed as the ancestral number for Lepidoptera (Suomalainen, 1969; Lukhtanov, 2000). The putative ancestral karyotype is strongly supported by recent results of comparative chromosome mapping (Baxter *et al.*, 2011; Sahara *et al.*, 2013; Van't Hof *et al.*, 2013; Ahola *et al.*, 2014; Yasukochi *et al.*, 2016). Recent studies also suggest extensive conservation at the chromosomal level and evolutionary stability of whole lepidopteran genomic regions (Yasukochi *et al.*, 2006, 2009, 2016; Pringle *et al.*, 2007; Sahara *et al.*, 2007, 2013; Van't Hof *et al.*, 2013; Ahola *et al.*, 2014).

The ancestral chromosome number is also common in butterflies (i.e. species of the superfamily Papilionoidea) (Robinson, 1971; Brown *et al.*, 2007). However, some groups of butterflies greatly deviate from this general pattern of karyotype stability. This applies especially to butterflies of the genus *Polyommatus* (Lycaenidae), which display the greatest interspecific variation in chromosome numbers known in the animal kingdom, ranging from $n = 10$ to $n = 224$–226, where the latter comes from the recently reassessed number in the Atlas blue, *Polyommatus atlanticus*, and represents the highest chromosome number not only of Lepidoptera, but also of all non-polyploid eukaryotes (Kandul *et al.*, 2004; Lukhtanov, 2015). The karyotype variation is most likely caused by chromosomal rearrangements involving fusions and fissions (Lukhtanov *et al.*, 2005). In Lepidoptera, the smallest chromosome number of $n = 5$ was also found in Papilionoidea, namely in a neotropical butterfly, *Hypothyris thea* (Nymphalidae; Brown, von Schoultz & Suomalainen, 2004), and the Arizona giant-skipper, *Agathymus aryxna* (Hesperiidae; De Prins & Saitoh, 2003). Butterflies of the family Pieridae represent another group with dynamic karyotype evolution. Many species of this family have reduced chromosome numbers, apparently as a result of chromosome fusion; for example, *Eurema brigitta* ($n = 12$) from the subfamily Coliadinae, *Leptosia alcesta* ($n = 12$), *Pinacopteryx eriphia* ($n = 13$), and two well-known agricultural pests, the large white *Pieris brassicae* ($n = 15$) and the small white *Pieris rapae* ($n = 25$), all from Pierinae (Robinson, 1971; Lukhtanov, 1991). The highest chromosome number in Pieridae was described in wood white butterflies of the genus *Leptidea* (Dismorphiinae), namely in *Leptidea duponcheli* with $n = 102$–104 (Lorković, 1941; de Lesse, 1960). Moreover, exceptional variation in chromosome numbers not only between but also within species was found in this genus (Dincă *et al.*, 2011; Lukhtanov *et al.*, 2011; Šíchová *et al.*, 2015). For two species with predominantly Eastern Palearctic distribution, *Leptidea morsei* and *Leptidea amurensis*, a high but constant number of chromosomes was reported ($n = 54$ and 61, respectively) (Maeki, 1958). However, three recently recognized cryptic species from the Western Palearctic, *Leptidea juvernica*, *Leptidea reali*, and *Leptidea sinapis*, have a variable number of chromosomes (Dincă *et al.*, 2011, 2013). Their diploid chromosome numbers range from $2n = 51$–55 in *L. reali* and $2n = 80$–91 in *L. juvernica* to $2n = 56$–106 in *L. sinapis*, with the latter representing the widest known intraspecific chromosome number variability, excluding cases of polyploidy (Dincă *et al.*, 2011; Lukhtanov *et al.*, 2011; Šíchová *et al.*, 2015). Interestingly, detailed analyses of their karyotypes revealed different chromosome numbers even in the progenies of individual females, as well as a variable number and location of two cytogenetic markers, major rDNA and H3 histone genes. The results obtained suggested a dynamic karyotype evolution through multiple chromosome fusions and fissions resulting in the frequent occurrence of multivalents during meiotic divisions. Hence, the uneven chromosome segregation of the multivalents is apparently responsible for the intraspecific karyotype variation in the *Leptidea* butterflies (Šíchová *et al.*, 2015). In addition to the variable number of chromosomes, each of the three cryptic species has a unique set of multiple sex chromosomes with $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica*, $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ in *L. reali*, and $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis* (Šíchová *et al.*, 2015).

Chromosomal rearrangements that give rise to complex multiple sex chromosomes can result in unbalanced segregation, which could have serious consequences for the fertility and/or viability of individuals. Yet, multiple sex chromosomes, composed of more than four elements, evolved independently several times in different plant and animal lineages. In plants, the presence of well-established sex chromosomes is generally rare (Vyskot & Hobza, 2004), and meiotic sex chromosome multivalents were reported only in a few cases, such as a translocation chain composed of four X and five Y in *Viscum fischeri*

(Wiens & Barlow, 1975). In animals, sex chromosome trivalents or quadrivalents are common in vertebrates except for birds (Gruetzner *et al.*, 2006; Pokorná, Altmanová & Kratochvíl, 2014) and can also be found in a number of invertebrate species (del Cerro, Cuñado & Santos, 1998; Bardella *et al.*, 2012; Palacios-Gimenez *et al.*, 2013) including Lepidoptera (Marec, Sahara & Traut, 2010). Neo-sex chromosomes of this type usually arise by sex chromosome–autosome fusion or translocation (Bertollo *et al.*, 1997; Bertolotto, Rodrigues & Yonenaga-Yassuda, 2001; Yoshido *et al.*, 2011). However, two-four multiple X chromosomes of spiders represent a special case. They form univalents that associate with each other during meiotic prophase and it is considered that they originated as a result of nondisjunction of an ancestral X chromosome (Král *et al.*, 2011). Similarly, three X chromosomes were recently reported in a heteropteran insect but, in this case, they probably originated as a result of fragmentation (Kaur & Gaba, 2015). Nevertheless, known meiotic multiples of more than four sex chromosomes are confined to invertebrates and monotremes. In the latter, the duck-billed platypus is an extraordinary case with a chain of ten sex chromosomes that arose by sex chromosome–autosome translocations (Grützner *et al.*, 2004; Rens *et al.*, 2004). In invertebrates, the most complicated sex chromosome systems were described for some termites, in which males are permanent translocation heterozygotes and form sex-linked chains or rings of up to 19 chromosomes in meiosis (Syren & Luykx, 1981). A variable number of X chromosomes (two to four) plus a single Y chromosome are the hallmark of the North American tiger beetles (Cicindelidae). However, changes in the number of X chromosomes are probably caused by fusion or fission of the X chromosomes (i.e. without the participation of autosomes) (Galián, Proença & Vogler, 2007). By contrast, a neo-$X_1X_2X_3X_4X_5Y$ system in the spider *Malthonica ferruginea* probably evolved from an ancestral $X_1X_2X_30$ system, which included an additional pair of homomorphic proto-sex chromosomes, by Robertsonian fusion between the proto-Y chromosome and an autosome (Král, 2007). Moreover, some mygalomorph spiders exhibit up to 13 X chromosomes in males; the X-multiples originated as a result of different chromosomal rearrangements, including duplications, fissions, and X–X and X–autosome fusions (Král *et al.*, 2013). The evolutionary significance of these types of complex multiple sex chromosome systems, including those recently described in three *Leptidea* butterfly species (Šíchová *et al.*, 2015), is poorly understood and deserves our full attention.

All three *Leptidea* species (*L.* juvernica, *L. reali*, and *L. sinapis*), showing an exceptional karyotype variation and unique system of multiple sex chromosomes, are mainly distributed in the Western Palearctic. To extend our knowledge and to confirm the results of the earlier study of Maeki (1958), we performed a detailed karyotype analysis in one species from the Eastern Palearctic, the northeast-Asian wood white *L. amurensis*. A comparison of male and female mitotic chromosomes allowed us to determine more accurately the range of diploid chromosome numbers in this species. We also mapped major rDNA and H3 histone genes by fluorescence *in situ* hybridization (FISH) and analyzed sex chromosome constitution using genomic *in situ* hybridization (GISH). The results obtained help us to better understand the karyotype and sex chromosome evolution in *Leptidea* butterflies.

MATERIAL AND METHODS

Insect collecting and dissection

Leptidea amurensis larvae and adults were collected in one area around Mt Takazasu, Yamanashi (Honshu island), Japan. In this locality, the confusion of *L. amurensis* with other species is unlikely because the closely-related *L. morsei* occurs on the island of Hokkaido (Maeki, 1958). In the laboratory, adult females were kept in plastic containers to lay eggs and all collected newly-hatched larvae were reared on the host plant, *Vicia amoena*. Once the larvae reached the fifth instar, two types of spread chromosome preparations were made in accordance with the procedure described by Mediouni *et al.* (2004) and Yoshido, Sahara & Yasukochi (2014). Briefly, both gonads for meiotic chromosomes and wing imaginal discs for mitotic chromosomes were dissected, swollen for 15 min in a hypotonic solution (0.075 M KCl), fixed in Carnoy fixative (ethanol/chloroform/acetic acid, 6 : 3 : 1) for 15 min, macerated with tungsten needles in a drop of 60% acetic acid, and spread on the slide using a heating plate at 45 °C. Ovaries were directly fixed without hypotonic treatment to preserve the pattern of W-chromosome heterochromatin. The bodies of all dissected larvae were frozen in liquid nitrogen and stored at −20 °C until DNA extraction. The preparations were passed through graded ethanol series (70%, 80%, and 100% for 1 min each) and stored at −20°C until further use.

Specimen sequencing and sequence analysis

To confirm species determination, DNA was extracted from two male and eight female larvae using a standard phenol–chloroform procedure (Blin & Stafford, 1976). We did not analyze all the larvae because the probability of confusion with another *Leptidea* species was negligible. The species level identification was

confirmed based on 658-bp sequences of the mitochondrial gene cytochrome *c* oxidase subunit 1 (*COI*), which has been reported as reliable for the identification of *Leptidea* species (Dincă *et al.*, 2011, 2013; Šíchová *et al.*, 2015; Solovyev, Ilinsky & Kosterin, 2015). The *COI* marker was amplified using pairs of primers LepF1 (5′-ATTCAACCAATCATAAAGATA TTGG-3′) and LepR1 (5′-TAAACTTCTGGATGTC CAAAAAATCA-3′) (Dincă *et al.*, 2011). A polymerase chain reaction (PCR) was carried out in 25-μL reaction volumes containing 1 × Ex *Taq* buffer (Takara), 0.2 mM dNTP mix, 5 μmol of each primer, 0.25 U of Ex *Taq* Hot Start DNA polymerase (Takara), and approximately 100 ng of template DNA. The PCR profile for *COI* consisted of denaturation for 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min at 44 °C, and 1 min at 72 °C, and then a final extension of 7 min at 72 °C. PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced.

Sequences were edited and aligned using GENEIOUS PRO, version 4.7 created by Biomatters (http://www.geneious.com). Our sequences were combined with *Leptidea* data available in GenBank as representatives of all unique *COI* haplotypes of *L. juvernica*, *L. reali*, and *L. sinapis* identified by Dincă *et al.* (2013), as well as all *COI* sequences of these taxa reported by Solovyev *et al.* (2015); all published *COI* sequences of other *Leptidea* taxa (*L. morsei*, *L. amurensis*, *Leptidea lactea*, and *L. duponcheli*) that overlapped with our sequenced mtDNA fragment by at least 600 bp (see Supporting information, Table S1). Thus, the final *COI* alignment included nucleotide sequences of 114 specimens and was 658 bp long.

To confirm the identification of the examined specimens and to place them into a broader phylogenetic context, a maximum likelihood (ML) analysis was performed using PHYML, version 2.4 (Guindon & Gascuel, 2003) implemented in GENEIOUS PRO, version 4.7. The substitution model used was GTR + I + G and was chosen according to Akaike information criterion values obtained in JMODELTEST, version 2 (Darriba *et al.*, 2012). Node supports were assessed through 100 bootstrap replicates (Felsenstein, 1985).

FISH WITH FLUOROCHROME-LABELLED PROBES

Unlabelled $(TTAGG)_n$ telomeric probes, used for the identification of chromosome ends, were prepared by non-template PCR *sensu* Sahara, Marec & Traut (1999). The probes were labelled with Cy3-dUTP (GE Healthcare) or Orange-dUTP (Abbott Molecular Inc.) using a Nick Translation Kit (Abbott Molecular Inc.) with 1 h of incubation at 15 °C. For chromosome counts, we used FISH with $(TTAGG)_n$ telomeric probes (tel-FISH) on spread chromosome preparations from wing imaginal discs (Šíchová *et al.*, 2015). The hybridization mixture contained 100 ng of telomeric probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich) in 10 μL of 50% formamide and 10% dextran sulphate in 2 × SSC.

To identify sex chromosomes, we used GISH combined with tel-FISH as described in Yoshido, Marec & Sahara (2005) and Šíchová *et al.* (2015). Male and female gDNA was extracted from larvae by a standard phenol–chloroform procedure. A part of the male gDNA was sonicated using a Sonopuls HD 2070 (Bandelin Electric) and used as competitor DNA (Šíchová *et al.*, 2013). The extracted female gDNA was labelled with fluorescein-12-dUTP (Invitrogen) or Green-dUTP (Abbott Molecular Inc.) using the Nick Translation Kit with 6 h of incubation at 15 °C. The hybridization mixture contained female gDNA (300 ng), Cy3-labelled telomeric probe (100 ng), unlabelled sonicated male gDNA (3 μg), and sonicated salmon sperm DNA (25 μg). The preparations were counterstained with 0.5 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) and mounted in antifade based on DABCO (Sigma-Aldrich).

FISH WITH BIOTIN-LABELLED PROBES

Fragments of 18S rDNA were generated from the codling moth (*Cydia pomonella*) gDNA using PCR as described by Fuková, Nguyen & Marec (2005). The probe was labelled with biotin-16-dUTP (Roche Diagnostics GmbH) using the Nick Translation Kit (Abbott Molecular Inc.) with 1 h and 45 min of incubation at 15 °C. A biotin-labelled H3 histone probe was prepared from *L. sinapis* gDNA using PCR in accordance with the protocol described by Šíchová *et al.* (2013, 2015).

FISH experiments with 18S rDNA and H3 histone probes were performed as described by Fuková *et al.* (2005). Briefly, chromosome preparations were treated with 100 μg mL^{-1} RNase A for 1 h to remove the excess of rRNAs, and were denatured and hybridized with 15 ng of biotinylated probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich) per slide. Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoResearch Labs. Inc.), amplified with biotinylated anti-streptavidin (Vector Laboratories Inc.), and detected again with Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 μg mL^{-1} DAPI and mounted in antifade based on DABCO.

MICROSCOPY AND IMAGE PROCESSING

Preparations from FISH experiments were observed under an Axioplan 2 microscope (Carl Zeiss) or under

a DM 6000B microscope (Leica Microsystems Japan). Black-and-white images were recorded with a cooled F-View CCD camera using the ANALYSIS, version 3.2 (Soft Imaging System GmbH), installed on the Axioplan 2 microscope or with a DFC350FX CCD camera installed on the DM 6000B microscope. In all preparations, images were captured separately for each fluorescent dye, pseudocoloured (light blue for DAPI, green for fluorescein and Green, red for Cy3, and yellow for Orange), and superimposed with PHOTOSHOP, version 7.0 (Adobe Systems Inc.).

RESULTS

Molecular identification of *Leptidea* specimens

The ML tree based on mitochondrial *COI* sequences confirmed the taxonomical identity of the material used in the present study. All analyzed larvae clustered with the *L. amurensis* sequences downloaded from GenBank but formed a well-supported and slightly differentiated clade with respect to all of the mainland specimens available (Fig. 1), likely reflecting the geographical isolation of the Japanese population of *L. amurensis* (minimum *p*-distance to the nearest mainland conspecific was 0.5%). Moreover, two samples, NC_022686 and JX274648, corresponding to mitochondrial genomes reported as belonging to *L. morsei* (Hao *et al.*, 2014), were clearly recovered by our analysis as *L. amurensis* (as indicated by the *COI* fragment analyzed here).

Chromosome number and structure

Chromosome numbers of *L. amurensis* were determined from repeated counts of mitotic metaphase chromosomes prepared from wing imaginal discs. To facilitate the identification of individual chromosomes, we used FISH with $(TTAGG)_n$ telomeric probes. Mitotic metaphase complements showed similar characteristics in all studied larvae. The karyotypes consisted of a high number of various-sized chromosomes with DAPI-positive heterochromatin blocks evenly distributed throughout the whole genome (Fig. 2A, B). Based on repeated counts, we found differences in chromosome numbers between sexes and among individual females. Although seven examined male larvae had an identical diploid chromosome number of $2n = 122$ (Fig. 2B), three out of six female larvae had $2n = 118$ and the other three female larvae had $2n = 119$ (Fig. 2A). No intra-individual variability in chromosome number was found. In male meiotic metaphase I (MI), we observed 61 elements, most probably bivalents (Fig. 2C), which corresponded to the diploid chromosome number found in male mitotic complements. We also observed two MI bivalents that were slightly larger than the other bivalents (Fig. 2C).

In female mitotic complements, four chromosomes stood out because of their size. Moreover, two of these large chromosomes were partially differentiated with heterochromatin (Figs 2A, 3B). Similarly, large chromosomes were observed in male mitotic nuclei (Fig. 2B). However, in contrast to the two largest chromosomes in females, they were not heterochromatinized and the size difference was not as pronounced. Thus, based on the comparison between male and female mitotic complements, we concluded that the two largest heterochromatinized chromosomes in females represent two W chromosomes: W_1 and W_2.

In male and female meiotic pachytene complements, we observed conspicuous heterochromatin blocks highlighted with DAPI that were predominantly present at the chromosomal ends in the majority of bivalents (Fig. 2D, asterisks). These DAPI-positive blocks were also found in other studied *Leptidea* species (Šíchová *et al.*, 2015). However, they were distributed throughout the whole chromosomes. Compared to other studied *Leptidea* species, we found a low number of chromosomal rearrangements in female pachytene nuclei. In total, we analyzed meiotic complements from six female larvae. In all of them, we observed a sex chromosome multivalent (see below) (Fig. 2D, arrow) and, in four of them, we also observed one extra trivalent (Fig. 2D, arrowhead).

Chromosomal location of major rDNA and H3 histone genes

FISH with the 18S rDNA probe performed in three *L. amurensis* larvae did not reveal any differences in the number and location of rDNA sites. The probe localized one rDNA cluster at the end of one small-sized pachytene bivalent (Fig. 2E). In mitotic complements, the probe mapped correspondingly to two small mitotic metaphase chromosomes (not shown). These results clearly indicate the presence of a single bivalent bearing the nucleolar organizer region (NOR). The NOR was associated with a large block of DAPI-positive heterochromatin, covering approximately two-fifths of the NOR-bivalent length (Fig. 2E, inset).

FISH with the H3 histone probe was performed in three *L. amurensis* larvae. In all examined larvae, we found two clusters in middle-sized pachytene bivalents: one terminal and one interstitial (Fig. 2F). Only the latter cluster was associated with a block of DAPI-positive heterochromatin (Fig. 2F, inset). In accordance with this hybridization pattern, the H3 probe identified four mitotic chromosomes (not shown).

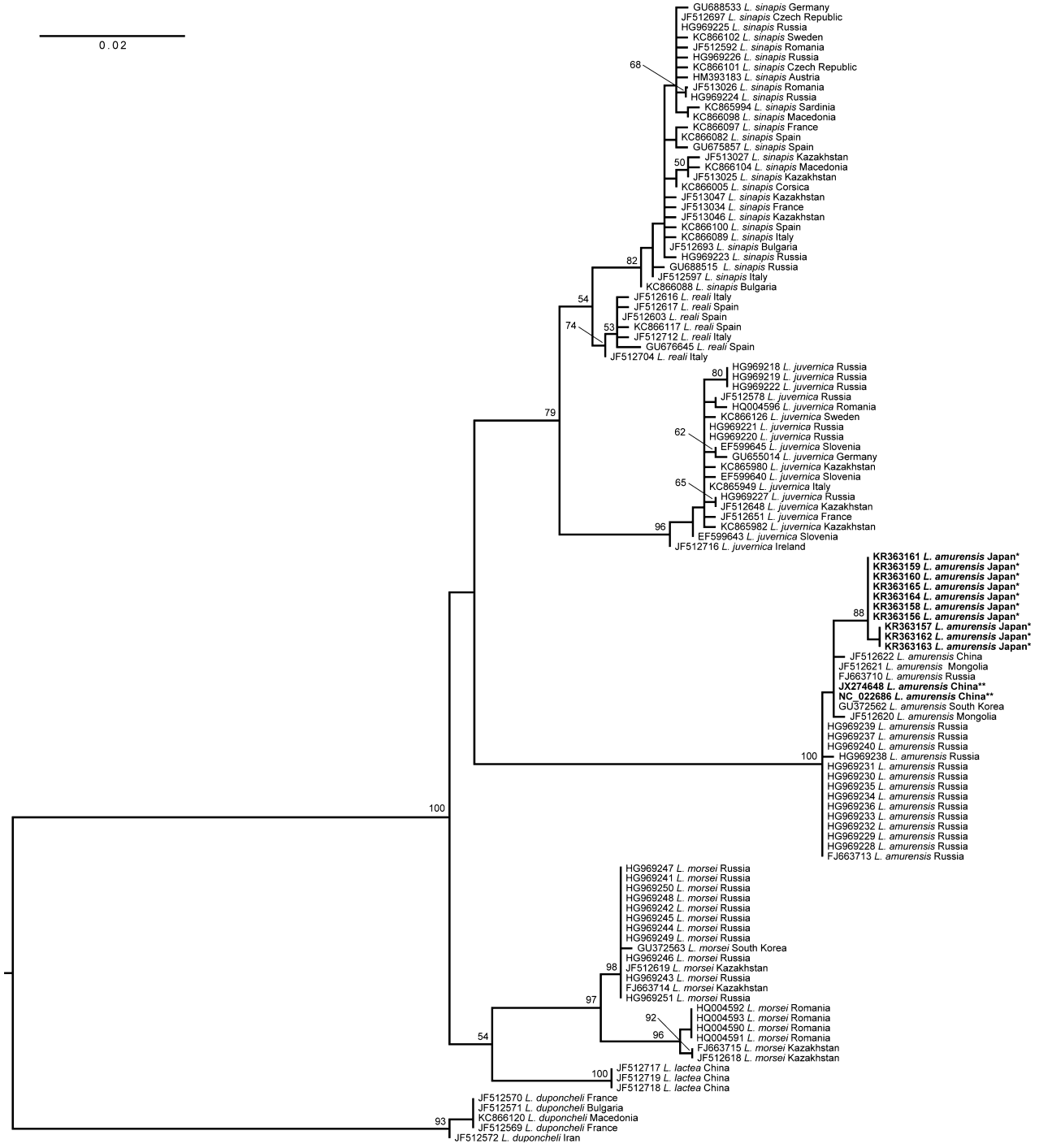

Figure 1. Maximum likelihood tree inferred from mitochondrial gene cytochrome *c* oxidase subunit 1 (*COI*) sequences of *Leptidea* taxa. Specimens sequenced and analyzed in the present study are indicated by an asterisk and were combined with the available sequences of other *Leptidea* taxa from published studies. Samples with two asterisks correspond to *COI* sequences from mitochondrial genomes that were reported as belonging to *Leptidea morsei* but were recovered as *Leptidea amurensis* by our analysis. For *Leptidea juvernica*, *Leptidea reali*, and *Leptidea sinapis*, representatives of all haplotypes identified by Dincă *et al.* (2013) were used. For the origin of all specimens and GenBank accession numbers, see the Supporting information (Table S1). The scale represents 0.02 substitutions per site. Bootstrap supports (≥ 50; 100 replicates) are shown next to the recovered nodes.

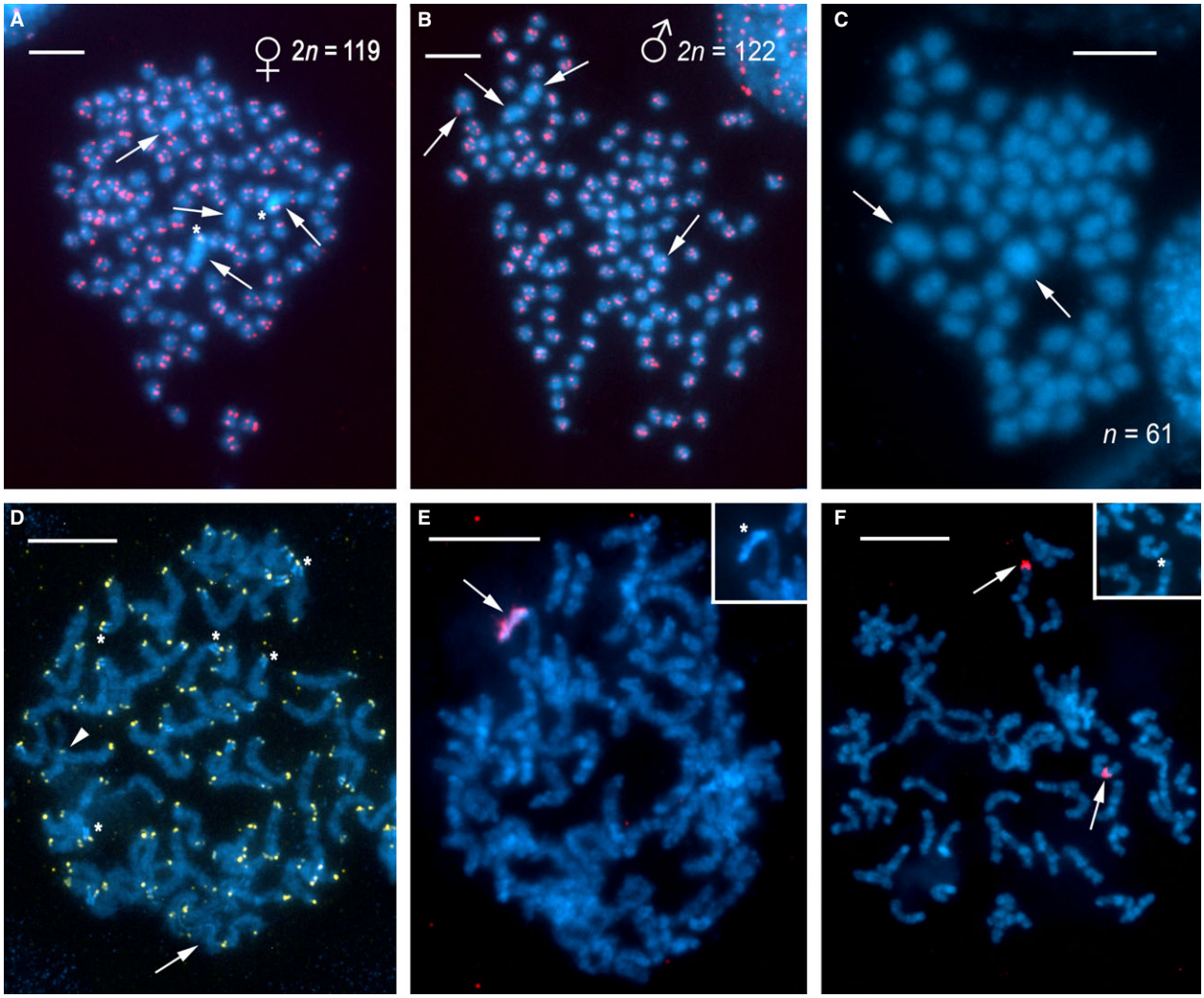

Figure 2. Karyotype analysis of mitotic and meiotic chromosomes (A–D) and fluorescence *in situ* hybridization localization of rDNA (E) and H3 histone gene (F) clusters in spread chromosome preparations of *Leptidea amurensis* larvae. Hybridization signals of the Cy3-dUTP- (red) and orange-labelled (yellow) $(TTAGG)_n$ telomeric probe indicate chromosome ends in (A, B, D), and hybridization signals of the 18S rDNA and H3 histone probe (red) are marked with arrows in (E, F). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Asterisks indicate DAPI-positive blocks of heterochromatin. A, female mitotic metaphase ($2n$ = 119); arrows indicate the four largest chromosomes in complement. B, male mitotic metaphase ($2n$ = 122); arrows indicate the four large chromosomes. C, male metaphase I (MI) complement (n = 61); arrows indicate two large chromosomal elements. D, female pachytene complement; the arrow indicates a sex chromosome multivalent; the arrowhead indicates a trivalent. E, female pachytene complement with a large terminal rDNA cluster (arrow); the inset to the upper right shows the pachytene nucleolar organizer region-bivalent with a large terminal DAPI-positive block of heterochromatin. F, female pachytene complement with one terminal and one interstitial H3 histone gene cluster (arrows); the inset to the upper right shows a block of DAPI-positive heterochromatin in one H3 histone gene-bearing bivalent. Scale bar (A–C) = 5 μm; (D–F) = 10 μm.

Sex chromosome identification

To identify the sex chromosomes, we used GISH combined with tel-FISH on mitotic and meiotic chromosomes of *L. amurensis* females. Although GISH was expected to differentiate the W chromosome(s) by strong binding of the female genomic probe, the telomeric probe helped us to determine the ends of individual chromosomes. In total, the analysis was performed in chromosome preparations from six female larvae. In all female mitotic metaphase complements, we observed four large chromosomes (Fig. 3A, B, arrows). Two of these large chromosomes

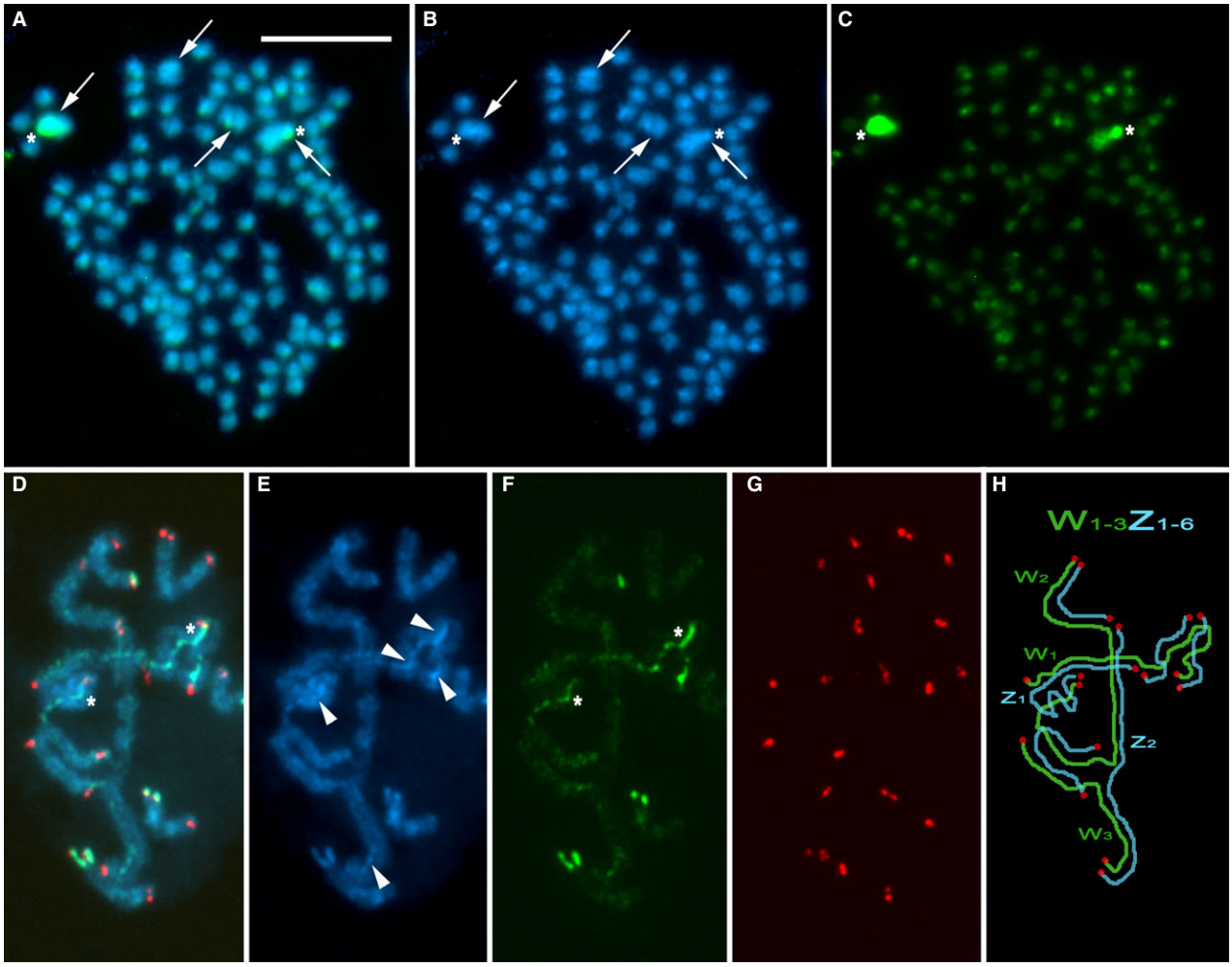

Figure 3. Genomic *in situ* hybridization combined with the $(TTAGG)_n$ telomeric probe in pachytene oocytes of *Leptidea amurensis* females. Female-derived genomic probes were labelled with fluorescein-12-dUTP or green-dUTP (green), and the telomeric probe with Cy3-dUTP (red). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). The arrows indicate four large chromosomes from the complement. Asterisks show two W chromosomes, W_1 and W_2, which are more intensely highlighted with the female genomic probe. Arrowheads indicate DAPI-positive heterochromatin segments of the W sex chromosomes. A–C, detailed analysis of female mitotic metaphase: merged images of the female-derived genomic probe and DAPI staining (A); DAPI image (B); hybridization pattern of the female genomic probe (C). D–H, detailed analysis of the sex chromosome multivalent $W_{1–3}Z_{1–6}$: merged images of the female genomic probe, $(TTAGG)_n$ telomeric probe, and DAPI staining (D); DAPI image (E); hybridization pattern of the female genomic probe (F); hybridization pattern of the $(TTAGG)_n$ telomeric probe (G); schematic drawing of the sex chromosome multivalent (H); red dots indicate the ends of individual elements in the multivalent. Scale bar = 10 μm.

were preferentially labelled with the female gDNA probe (Fig. 3A, B, C, asterisks), indicating that these are the W sex chromosomes. They were the largest chromosomes in the female karyotype of *L. amurensis* and were designated W_1 and W_2. The W_1 chromosome was homogeneously labelled over most of its length, except for a small terminal gap (Fig. 3C). However, the W_2 chromosome was strongly highlighted with the probe only in a terminal segment (Fig. 3C). The female-derived genomic probe also hybridized to heterochromatin blocks on autosomes (Fig. 3C).

The analysis of female pachytene complements helped us to identify a complex sex chromosome multivalent with the following constitution: $W_1W_2W_3Z_1Z_2Z_3Z_4Z_5Z_6$ (Fig. 3D, E, F, G, H). In the multivalent, each W chromosome paired with two to four Z chromosomes (Fig. 4, scheme). Three Z chromosomes paired each with two W chromosomes (Z_1 with W_1 and W_2; Z_2 and Z_3 with W_2 and W_3). By

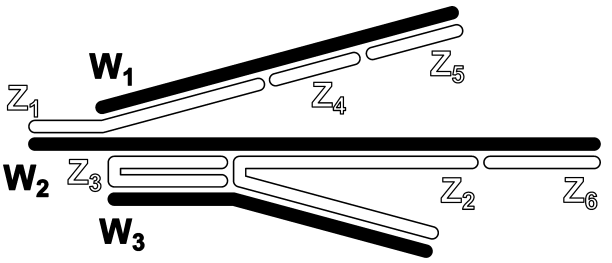

Figure 4. Simplified schematic drawing of the *Leptidea amurensis* sex chromosome multivalent in pachytene oocytes with the constitution $W_1W_2W_3Z_1Z_2Z_3Z_4Z_5Z_6$. In the multivalent, the W_1, W_2, W_3, Z_1 and Z_2 chromosomes are designated according to their morphological and labelling characteristics (i.e. with genomic *in situ* hybridization, W_1 was homogeneously stained over most of its length, W_2 was preferentially labelled at one chromosomal end, and W_3 was poorly differentiated; Z_1 and Z_2 were the largest Z chromosomes from the multivalent). The Z_3, Z_4, Z_5, and Z_6 chromosomes are named arbitrarily.

contrast, three other Z chromosomes paired exclusively with one W chromosome each (Z_4 and Z_5 with W_1; Z_6 with W_2). These results were confirmed in four out of six female larvae. In two larvae, we were unable to resolve the multivalent because of the lack of well-spread pachytene nuclei in chromosome preparations. In accordance with the results from mitotic metaphases, the female gDNA probe highlighted two large W chromosomes, W_1 and W_2 (Fig. 3D, F). These W chromosomes were also partially differentiated by DAPI-positive heterochromatin (Fig. 3E). Along with the strong binding of the gDNA probe, this pattern indicated the accumulation of repetitive sequences and transposable elements in both W chromosomes (Sahara *et al.*, 2003). However, we found only a small heterochromatin block at the terminal part of the third W chromosome (Fig. 3E, lower arrowhead). The fact that the W_3 sex chromosome was not largely differentiated by the female genomic probe suggests its recent origin (Fig. 3F). Two largest Z chromosomes (Fig. 3H) were designated Z_1 and Z_2. These Z chromosomes can represent two large bivalents in male meiotic MI (Fig. 2C) and also two large chromosomes that were not heterochromatinized in female mitotic nuclei (Figs 2A, 3A, B).

DISCUSSION

Previous studies on three cryptic *Leptidea* species from the Western Palearctic (*L. juvernica*, *L. reali*, and *L. sinapis*) showed exceptional inter- and intraspecific variation in chromosome numbers and location of cytogenetic markers (major rDNA and H3 histone genes) and a curious sex determining system with three or four W and three or four Z chromosomes (Dincă *et al.*, 2011, 2013; Lukhtanov *et al.*, 2011; Šíchová *et al.*, 2015). These results suggested a dynamic karyotype evolution and emphasized the role of chromosomal rearrangements in the speciation of *Leptidea* butterflies. The present study enabled us to extend the research and examine the karyotype of *L. amurensis*, a wood white butterfly with Eastern Palearctic distribution, previously reported as a species with a constant haploid chromosome number of $n = 61$ (Maeki, 1958).

Wood white butterflies are generally similar to each other in external morphology and, especially for some of the species in this genus, it is often very difficult to reliably assign specimens to species. In such cases, it is necessary to use several additional characters, including molecular (mitochondrial and/or nuclear DNA markers), cytological (chromosome number), and morphological (genitalia morphometry) data, for the precise identification of closely-related species (Dincă *et al.*, 2011; Lukhtanov *et al.*, 2011). In the Takazasu region of Japan, the probability of misidentifying specimens of *L. amurensis* is small because no other *Leptidea* species has been reported from this area. Nevertheless, to eliminate any potential misidentification as a result of incomplete faunistic data or cryptic diversity, we used mitochondrial *COI* sequences that accurately confirmed the taxonomical identity of the larvae used in the present study.

Chromosome number variation

The first note about the chromosome number of *L. amurensis* dates back to 1958, when Maeki (1958) analyzed meiotic chromosomes in the stage of MI spermatocytes. Sixty-one elements were observed in meiotic nuclei and, most importantly, two elements that stood out as a result of their size were described. Unfortunately, meiotic spermatocytes do not allow the analysis of complex meiotic figures such as multivalents. Taking into account the complicated structures of meiotic chromosomes found in three closely-related *Leptidea* species (Šíchová *et al.*, 2015) and the absence of data from *L. amurensis* females, we carried out a comparative analysis of male and female mitotic metaphase complements, which allowed us to determine more accurately the range of diploid chromosome numbers in *L. amurensis*. Our results in males confirmed the findings of Maeki (1958). All studied males showed a diploid chromosome number of $2n = 122$ in mitotic metaphase nuclei and a haploid number of $n = 61$

elements, including two larger elements in MI of meiotic spermatocytes. However, females showed a lower number of chromosomes in mitotic metaphase, either $2n = 118$ or $2n = 119$. The sex-specific difference in chromosome numbers between *L. amurensis* males and females resulted from the unique constitution of multiple sex chromosomes: females had a total of nine sex chromosomes (three W and six Z) and males had twelve sex chromosomes (six Z-chromosome pairs), whereas the difference in *L. amurensis* females depended on the presence or absence of a trivalent in pachytene oocytes. The trivalent indicated the occurrence of chromosome fusion that reduced the chromosome number by one in heterozygous females. No male heterozygotes and no homozygotes of either sex were found for the fusion.

Previous studies showed that fusion polymorphism involving autosomes and/or sex chromosomes leads almost exclusively to variation in chromosome numbers (Papeschi, 1994; Poggio *et al.*, 2013; Yoshido *et al.*, 2013). In species with holokinetic chromosomes, where the kinetic activity is distributed along most of the chromosome surface, a fusion does not dramatically alter meiotic segregation, as in the case of a monocentric chromosome that may become dicentric after fusion. Especially in lepidopteran females, which have the achiasmatic meiosis, meiotic trivalents often exhibit regular segregation of chromosomes and generate genetically balanced gametes (Marec *et al.*, 2001; Melters *et al.*, 2012). Moreover, it was reported that chromosomal fusions affect the frequency of recombination in both monocentric and holokinetic chromosomes (Basset *et al.*, 2006; Hipp, Rothrock & Roalson, 2009; Bureš & Zedek, 2014). The reduced recombination enables the accumulation of genetic incompatibilities and can ultimately lead to divergence and speciation (Noor *et al.*, 2001; Rieseberg, 2001; Faria & Navarro, 2010).

To further explore the variability of *L. amurensis* karyotypes, we mapped the chromosomal location of two cytogenetic markers, clusters of major rDNA and H3 histone genes that were also mapped by FISH in three Western Palearctic species, *L. juvernica*, *L. reali*, and *L. sinapis*. In *L. reali*, the species with the lowest chromosome number of the three ($2n = 51$–55), all larvae analyzed showed consistent results with a single terminal rDNA cluster and an interstitial cluster of H3 genes per haploid genome. In *L. juvernica* and *L. sinapis*, which are species with higher chromosome numbers ($2n = 80$–91 in *L. juvernica* and $2n = 56$–106 in *L. sinapis*), significant differences in the number and location of both cytogenetic markers were observed, even among the offspring of individual females (Šíchová *et al.*, 2015). Such inter- and intrapopulation variation in rDNA distribution is consistent with the evolutionary mobility of rDNA observed in Lepidoptera and other groups of organisms (Nguyen *et al.*, 2010; Pucci *et al.*, 2014). However, the variability in otherwise conserved H3 histone gene clusters is rather surprising and highlights the ongoing explosive karyotype evolution in *Leptidea* species (Šíchová *et al.*, 2015). Interestingly, all examined larvae of *L. amurensis* exhibited a single terminal rDNA cluster and two clusters of H3 genes, one terminal and one interstitial, per haploid genome. These findings, together with the low number of chromosomal multivalents compared to other *Leptidea* species, indicate the stability of the *L. amurensis* karyotype despite the high chromosome number. However, analysis of other *L. amurensis* populations should be conducted to confirm these results.

Multiple sex chromosomes

In pachytene oocytes of *L. amurensis* females, the multiple sex chromosomes formed a complex $W_1W_2W_3Z_1Z_2Z_3Z_4Z_5Z_6$ multivalent (Figs 3, 4), which is even more complex than those found in Western Palearctic *Leptidea* species (Šíchová *et al.*, 2015). Three Z chromosomes of the multivalent paired each with two W chromosomes, suggesting that the Z chromosomes and corresponding segments of the W chromosomes originated as a result of autosome sex chromosome translocations. However, three other Z chromosomes paired only with one W chromosome, which indicates that these components of the multivalent evolved either via chromosome fission (Z chromosomes) or fusion (corresponding parts of the W chromosomes). We assume that the W_1 chromosome, which was the only W almost entirely highlighted by GISH (Fig. 3), indicating a high level of molecular differentiation (Fuková *et al.*, 2005), is largely composed of an ancestral W chromosome. This would favour the origin of Z_4 and Z_5 by fission of an ancestral Z chromosome. A very weak hybridization pattern of the W_3 chromosome suggests a recent autosomal origin of this element, which has not yet accumulated a sufficient amount of repetitive sequences to be differentiated by GISH. Similarly, the multiple sex chromosome systems in three Western Palearctic *Leptidea* species most likely originated as a result of complex translocations between the ancestral WZ pair and several autosomes (Šíchová *et al.*, 2015). These complex changes could be facilitated by the presence of transposable elements, as in the case of gene insertions in *Drosophila melanogaster* (Jakubczak, Xiong & Eickbush, 1990), *Apis mellifera*, and other Hymenoptera (Bigot *et al.*, 1992), or the preponderance of other repetitive sequences, which is supported by the presence of evenly

distributed heterochromatin blocks in the three previously studied *Leptidea* species (Šíchová *et al.*, 2015) and also in the genome of *L. amurensis* (present study).

By contrast to the highly complex constitution of *L. amurensis* multiple sex chromosomes, the majority of moths and butterflies have a WZ/ZZ (female/male) chromosome system of sex determination. Systems without the W chromosome (Z0/ZZ) also occur, although much less frequently (Traut *et al.*, 2007). In addition, two types of multiple sex chromosome systems with three elements, W_1W_2Z/ZZ, and $WZ_1Z_2/Z_1Z_1Z_2Z_2$, occur sporadically. They have been described only in seven genera from different lineages of the lepidopteran phylogenetic tree (Marec *et al.*, 2010). Their origin can be ascribed either to sex chromosome fission or to sex chromosome–autosome fusion, where the remaining autosome becomes a W_2 or Z_2 chromosome (Marec *et al.*, 2010). However, only the latter mechanism has been clearly demonstrated in Lepidoptera (Yoshido *et al.*, 2011; Sahara, Yoshido & Traut, 2012). The so-called neo-sex chromosomes, resulting from the fusion with an autosome, can evolve in both the Z and W chromosomes of a particular species, if each member of an autosome pair fuses with one sex chromosome. These neo-sex chromosomes can be exceptionally large compared to the other chromosomes of the respective genome and may play an important role in the evolution of large groups of Lepidoptera. Such a neo-Z chromosome originating through a fusion of the ancestral Z chromosome with an autosome has been recently demonstrated in the codling moth, *C. pomonella* (Tortricidae: Olethreutinae). The available data suggest that this fusion happened in a common ancestor of the main tortricid subfamilies, Olethreutinae and Tortricinae, and that it increased the adaptive potential of tortricids contributing to their spectacular radiation (Nguyen *et al.*, 2013). Furthermore, studies on the neo-sex chromosomes in populations of *Samia cynthia* suggest that repeated autosome-sex chromosome fusions giving rise to neo-sex chromosomes may accelerate the accumulation of genetically based incompatibilities and ultimately contribute to the formation of reproductive barriers between populations (Yoshido *et al.*, 2011, 2013). Similarly, sex chromosome multiples of *L. amurensis* represent a highly derived neo-sex chromosome system that originated as a result of complex chromosomal rearrangements. These rearrangements increased the number of sex-linked genes and thus could have played a major role in the divergence and speciation of *Leptidea* butterflies as in the case of above-mentioned leaf-rollers of the family Tortricidae and geographical subspecies of *S. cynthia*.

CONCLUSIONS

Based on our data, the emerging picture of chromosome evolution in *L. amurensis* is that: (1) the sex chromosome number is constant in both sexes [i.e. twelve sex chromosomes ($Z_{1–6}/Z_{1–6}$) in males and nine sex chromosomes ($W_{1–3}/Z_{1–6}$) in females]; (2) based on the most frequent diploid chromosome number of $2n = 122$ found in males, the modal autosome number is 110, which means a total diploid chromosome number of $2n = 119$ in females; and (3) the autosomal fusion in heterozygotes reduces the number of autosomes to 109, resulting in a total diploid chromosome number of $2n = 121$ in males and $2n = 118$ in females (Fig. 5). The fact that we did not find heterozygous males with $2n = 121$ and homozygotes for the fusion in any sex may be a result of the low number of individuals examined or the recent origin of the fusion, which is not yet widespread in the *L. amurensis* population.

The present study confirmed the high number of small chromosomes in the karyotype of *L. amurensis*, one of the highest in *Leptidea* species and twice the ancestral number of $n = 31$ in Lepidoptera. These findings indicate that chromosome fission is the main force in the karyotype evolution of *L. amurensis*. By contrast to the previously studied Western Palearctic species (Šíchová *et al.*, 2015), the karyotype of *L. amurensis* is relatively stable but shows a striking difference in chromosome numbers between sexes. We clearly showed that this difference results from the unique constitution of multiple sex chromosomes (i.e. $W_{1–3}Z_{1–6}$ in females and $Z_{1–6}Z_{1–6}$ in males). Meiotic configurations in females suggest that this system originated as a result of complex chromosomal

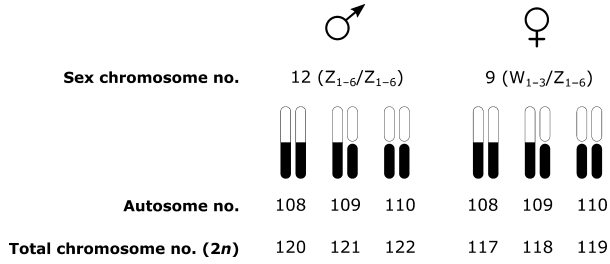

Figure 5. Schematic drawing of a chromosomal fusion reducing the number of autosomes in *Leptidea amurensis*. The sex chromosome number appears to be constant in each sex and consists of 12 sex chromosomes (Z_{1-6}/Z_{1-6}) in males and nine sex chromosomes (W_{1-3}/Z_{1-6}) in females. The original autosome number is 110, resulting in a total diploid chromosome number of $2n = 122$ in males and $2n = 119$ in females. In fusion heterozygotes, the autosome number is reduced to 109, resulting in a total diploid chromosome number of $2n = 121$ in males and $2n = 118$ in females. Individuals that are homozygous for the fusion with 108 autosomes should also occur in wild populations (although not found in the present study).

rearrangements between ancestral sex chromosomes and autosomes, including fusion, fission, and translocation events. The presence of sex chromosome multiples in the karyotypes of the four *Leptidea* species examined so far suggests that a multiple sex chromosome system is an ancestral trait for all *Leptidea*. Our findings also support a hypothesis according to which the complex rearrangements of sex chromosomes contributes to the formation of reproductive barriers between the closely-related *Leptidea* species.

ACKNOWLEDGEMENTS

We thank V. A. Lukhtanov and two anonymous reviewers for their helpful comments that greatly improved the manuscript. This research was funded by Grant 14-22765S of the Czech Science Foundation (JŠ and FM) and by Grant 23380030 of the Japan Society for the Promotion of Science (KS). JŠ acknowledges the additional support by Grant 052/2013/P of the Grant Agency of the University of South Bohemia (USB) and from the Development Project of SBU for Internationalization. VD was supported by a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme (project no. 625997). The authors declare that there are no conflicts of interest. Sequence data have been submitted to GenBank: accession numbers KR363156–KR363165.

REFERENCES

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. *Nature Communications* **5:** 4737.

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

Basset P, Yannic G, Brünner H, Hausser J. 2006. Restricted gene flow at specific parts of the shrew genome in chromosomal hybrid zones. *Evolution* **60:** 1718–1730.

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.

Bertollo LAC, Fontes MS, Fenocchio AS, Cano J. 1997. The X_1X_2Y sex chromosome system in the fish *Hoplias malabaricus*. I. G-, C- and chromosome replication banding. *Chromosome Research* **5:** 493–499.

Bertolotto CEV, Rodrigues MT, Yonenaga-Yassuda Y. 2001. Banding patterns, multiple sex chromosome system and localization of telomeric $(TTAGGG)_n$ sequences by FISH on two species of *Polychrus* (Squamata, Polychrotidae). *Caryologia* **54:** 217–226.

Bigot Y, Lutcher F, Hamelin MH, Périquet G. 1992. The 28S ribosomal RNA-encoding gene of Hymenoptera - Inserted sequences in the retrotransposon-rich regions. *Gene* **121:** 347–352.

Blin N, Stafford DW. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Research* **3:** 2303–2308.

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

Brown KS Jr, Freitas AVL, Wahlberg N, von Schoultz B, Saura AO, Saura A. 2007. Chromosomal evolution in the South American Nymphalidae. *Hereditas* **144:** 137–148.

Bureš P, Zedek F. 2014. Holokinetic drive: centromere drive in chromosomes without centromeres. *Evolution* **68:** 2412–2420.

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*. Dordrecht: Springer, 115–146.

del Cerro AL, Cuñado N, Santos JL. 1998. Synaptonemal complex analysis of the X_1X_2Y trivalent in *Mantis religiosa* L. males: inferences on the origin and maintenance of the sex-determining mechanism. *Chromosome Research* **6:** 5–11.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:** 772.

De Prins J, Saitoh K. 2003. Karyology and sex determination. In: Kristensen NP, ed. *Lepidoptera, moths and butterflies: morphology, physiology, and development*. Berlin: Walter de Gruyter, 449–468.

Dincă V, Lukhtanov VA, Talavera G, Vila R. 2011. Unexpected layers of cryptic diversity in wood white *Leptidea* butterflies. *Nature Communications* **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. *Journal of Evolutionary Biology* **26:** 2095–2106.

Faria R, Navarro A. 2010. Chromosomal speciation revisited: rearranging theory with pieces of evidence. *Trends in Ecology & Evolution* **25:** 660–669.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

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.

Galián J, Proença SJR, Vogler AP. 2007. Evolutionary dynamics of autosomal-heterosomal rearrangements in a multiple-X chromosome system of tiger beetles (Cicindelidae). *BMC Evolutionary Biology* **7:** 158.

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 PCM, 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.

Guindon S, Gascuel O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum-likelihood. *Systematic Biology* **52:** 696–704.

Hao JJ, Hao JS, Sun XY, Zhang LL, Yang Q. 2014. The complete mitochondrial genomes of the Fenton's wood white, *Leptidea morsei*, and the lemon emigrant, *Catopsilia pomona*. *Journal of Insect Science* **14:** 130.

Hipp AL, Rothrock PE, Roalson EH. 2009. The evolution of chromosome arrangements in *Carex* (Cyperaceae). *Botanical Review* **75:** 96–109.

Jakubczak JL, Xiong Y, Eickbush TH. 1990. Type-I (R1) and type-II (R2) ribosomal DNA insertions of *Drosophila melanogaster* are retrotransposable elements closely related to those of *Bombyx mori*. *Journal of Molecular Biology* **212:** 37–52.

Kandul NP, Lukhtanov VA, Dantchenko AV, Coleman JWS, Sekercioglu CH, Haig D, Pierce NE. 2004. Phylogeny of *Agrodiaetus* Hübner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sequences of *COI* and *COII* and nuclear sequences of *EF1-α*: karyotype diversification and species radiation. *Systematic Biology* **53:** 278–298.

Kaur H, Gaba K. 2015. First report on a multiple sex chromosome system ($X_1X_2X_30$) and population variations in the frequency of ring bivalents in Pyrrhocoridae (Hemiptera: Heteroptera). *European Journal of Entomology* **112:** 419–425.

Král J. 2007. Evolution of multiple sex chromosomes in the spider genus *Malthonica* (Araneae: Agelenidae) indicates unique structure of the spider sex chromosome systems. *Chromosome Research* **15:** 863–879.

Král J, Kořínková T, Forman M, Krkavcová L. 2011. Insights into the meiotic behavior and evolution of multiple sex chromosome systems in spiders. *Cytogenetic and Genome Research* **133:** 43–66.

Král J, Kořínková T, Krkavcová L, Musilová J, Forman M, Herrera IMÁ, Haddad CR, Vítková M, Henriques S, Vargas JGP, Hedin M. 2013. Evolution of karyotype, sex chromosomes, and meiosis in mygalomorph spiders (Araneae: Mygalomorphae). *Biological Journal of Linnean Society* **109:** 377–408.

de Lesse H. 1960. Spéciation et variation chromosomique chez les Lépidoptères Rhopalocères. *Annales des sciences naturelles - Zoologie et biologie animale* **12:** 1–223.

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

Lukhtanov VA. 1991. Evolution of the karyotype and system of higher taxa of the Pieridae (Lepidoptera) of the world fauna. *Entomologicheskoe Obozrenie* **70:** 619–641.

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

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. *Comparative Cytogenetics* **9:** 683–690.

Lukhtanov VA, Kandul NP, Plotkin JB, Dantchenko AV, Haig D, Pierce NE. 2005. Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus* butterflies. *Nature* **436:** 385–389.

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 Evolutionary Biology* **11:** 109.

Maeki K. 1958. On the cytotaxonomical relationship in *Leptidea* (Lepidoptera-Rhopalocera). *Japanese Journal of Genetics* **33:** 283–285.

Marec F, Tothová A, Sahara K, Traut W. 2001. Meiotic pairing of sex chromosome fragments and its relation to atypical transmission of a sex-linked marker in *Ephestia kuehniella* (Insecta: Lepidoptera). *Heredity* **87:** 659–671.

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*. Boca Raton, FL: CRC Press, 49–63.

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.

Melters DP, Paliulis LV, Korf IF, Chan SWL. 2012. Holocentric chromosomes: convergent evolution, meiotic adaptations and genomic analysis. *Chromosome Research* **20:** 579–593.

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. *Proceedings of the National Academy of Sciences of the United States of America* **110:** 6931–6936.

Noor MAF, Grams KL, Bertucci LA, Reiland J. 2001. Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 12084–12088.

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 Evolutionary Biology* **13:** 167.

Papeschi AG. 1994. Chromosome rearrangements in *Belostoma plebejum* (Stål) (Belostomatidae, Heteroptera). *Caryologia* **47:** 223–231.

Poggio MG, Gaspe MS, Papeschi AG, Bressa MJ. 2013. Cytogenetic study in a mutant of *Triatoma infestans* (Hemiptera: Reduviidae) carrying a spontaneous autosomal fusion and an extra chromosome. *Cytogenetic and Genome Research* **139:** 44–51.

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

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.

Pucci MB, Barbosa P, Nogaroto V, Almeida MC, Artoni RF, Pansonato-Alves JC, Foresti F, Moreira-Filho O, Vicari MR. 2014. Population differentiation and speciation in the genus *Characidium* (Characiformes: Crenuchidae): effects of reproductive and chromosomal barriers. *Biological Journal of the Linnean Society* **111:** 541–553.

Rens W, Grützner F, O'Brien PCM, Fairclough H, Graves JAM, Ferguson-Smith MA. 2004. Resolution and evolution of the duck-billed platypus karyotype with an $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ male sex chromosome constitution. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 16257–16261.

Rieseberg LH. 2001. Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution* **16:** 351–358.

Robinson R. 1971. *Lepidoptera genetics*. Oxford: Pergamon Press.

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

Sahara K, Marec F, Eickhoff U, Traut W. 2003. Moth sex chromatin probed by comparative genomic hybridization (CGH). *Genome* **46:** 339–342.

Sahara K, Yoshido A, Marec F, Fuková I, Zhang H-B, Wu C-C, 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, Traut W. 2012. Sex chromosome evolution in moths and butterflies. *Chromosome Research* **20:** 83–94.

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 Biochemistry and Molecular Biology* **43:** 644–653.

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, 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 Evolutionary Biology* **15:** 89.

Solovyev VI, Ilinsky Y, Kosterin OE. 2015. Genetic integrity of four species of *Leptidea* (Pieridae, Lepidoptera) as sampled in sympatry in West Siberia. *Comparative Cytogenetics* **9:** 299–324.

Suomalainen E. 1969. Chromosome evolution in the Lepidoptera. *Chromosomes Today* **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.

Traut W, Sahara K, Marec F. 2007. Sex chromosomes and sex determination in Lepidoptera. *Sexual Development* **1:** 332–346.

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.

Vyskot B, Hobza R. 2004. Gender in plants: sex chromosomes are emerging from the fog. *Trends in Genetics* **20:** 432–438.

Wiens D, Barlow BA. 1975. Permanent translocation heterozygosity and sex determination in East African mistletoes. *Science* **187:** 1208–1209.

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, 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.

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.

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

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

Yoshido A, Šíchová J, Kubíčková S, Marec F, Sahara K. 2013. Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp. *Chromosome Research* **21:** 149–164.

Yoshido A, Sahara K, Yasukochi Y. 2014. Silk moths (Lepidoptera). In: Sharakhov IV, ed. *Protocols for cytogenetic mapping of arthropod genomes*. Boca Raton, FL: CRC Press, 219–256.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. List of *Leptidea* specimens included in the DNA analyses. Sequences obtained in the present study are indicated in blue, whereas the other sequences were downloaded from GenBank. Because a large number of sequences of *Leptidea juvernica*, *Leptidea reali*, and *Leptidea sinapis* are available in GenBank, we used only representatives for all unique mitochondrial gene cytochrome *c* oxidase subunit 1 (*COI*) haplotypes of these three species identified by Dincă *et al.* (2013), as well as the specimens from Solovyev *et al.* (2015). The haplotype numbers for these species correspond to those in Dincă *et al.* (2013). Samples NC_022686 and JX274648 appear as mitogenomes of *Leptidea morsei* in GenBank, although our *COI* analyses recovered them as *Leptidea amurensis*.

Supplementary Table S1 List of *Leptidea* specimens included in the DNA analyses. Sequences obtained in this study are in blue, while the other sequences were downloaded from GenBank. Because a large number of sequences of *L. juvernica* , *L. reali* , and *L. sinapis* are available in GenBank, we used only representatives for all unique *COI* haplotypes of these three species identified by Dincă *et al.* (2013), as well as the specimens from Solovyev *et al.* (2015). The haplotype numbers for these species correspond to those in Dincă *et al.* (2013). Samples NC_022686 and JX274648 appear as mitogenomes of *L. morsei* in GenBank, but our *COI* analyses recovered them as *L. amurensis* .

GenBank accession number	Species	*COI* haplotype	Locality	Country
JF512622	*L. amurensis*		Jiexiu county, Shanxi	China
NC_022686	*L. amurensis*		Shanxi or Hainan province	China
JX274648	*L. amurensis*		Shanxi or Hainan province	China
KR363156	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363157	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363158	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363159	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363160	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363161	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363162	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363163	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363164	*L. amurensis*		Mt. Takazasu, Honshu	Japan
KR363165	*L. amurensis*		Mt. Takazasu, Honshu	Japan
JF512620	*L. amurensis*		10 km NE Hishig-Ondor	Mongolia
JF512621	*L. amurensis*		Bulgan	Mongolia
FJ663713	*L. amurensis*		Altai Krai, Myuta	Russia
FJ663710	*L. amurensis*		Buryatia, Selenduma distr., Sosnovka	Russia
HG969228	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969229	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969230	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969231	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969232	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969233	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969234	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969235	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969236	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969237	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969238	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969239	*L. amurensis*		Novosibirsk Academy Town	Russia
HG969240	*L. amurensis*		Novosibirsk Academy Town	Russia
GU372562	*L. amurensis*		Yeongwol, Gangwon	South Korea
JF512571	*L. duponcheli*		Paril village, Khadzhidimovo	Bulgaria
JF512570	*L. duponcheli*		Digne les Bains, Alpes-de-Haute, Provence	France
JF512569	*L. duponcheli*		Oraison, Alpes de Haute Provence	France
JF512572	*L. duponcheli*		Elborz, 10km W Ghachsar	Iran
KC866120	*L. duponcheli*		Skopje	Macedonia
JF512651	*L. juvernica*	hj6	Gresse-en-Vercors, Isère	France
GU655014	*L. juvernica*	hj8	Neustadt/Donau, Plattenberg, Bavaria	Germany
JF512716	*L. juvernica*	hj9	Gortmore Point, Lough Derg, Tipperary	Ireland
KC865949	*L. juvernica*	hj1	Val di Tovo- Laghi	Italy
KC865982	*L. juvernica*	hj5	Balgyn	Kazakhstan
KC865980	*L. juvernica*	hj4	Narymski Mts.	Kazakhstan
JF512648	*L. juvernica*	hj7	South Altai, Uspenka	Kazakhstan
HQ004596	*L. juvernica*	hj2	Valea Belchia, Harghita	Romania
HG969218	*L. juvernica*		Novosibirsk Academy Town	Russia
HG969219	*L. juvernica*		Novosibirsk Academy Town	Russia
HG969220	*L. juvernica*		Novosibirsk Academy Town	Russia
HG969221	*L. juvernica*		Novosibirsk Academy Town	Russia
HG969222	*L. juvernica*		Novosibirsk Academy Town	Russia
HG969227	*L. juvernica*		Novosibirsk Academy Town	Russia
JF512578	*L. juvernica*	hj3	Peterhof, St. Petersburg	Russia

Supplementary Table S1 Continued.

GenBank accession number	Species	*COI* haplotype	Locality	Country
EF599645	*L. juvernica*	hj11	Barje	Slovenia
EF599643	*L. juvernica*	hj12	Barje	Slovenia
EF599640	*L. juvernica*	hj13	Vrhnika	Slovenia
KC866126	*L. juvernica*	hj10	Riala, Norrtälje municipality	Sweden
JF512719	*L. lactea*		Fengxian (Shuangshipu) county, Hekou vic., Shaanxi	China
JF512717	*L. lactea*		Qin Ling Shan, Madao, Liuba county, Shaanxi	China
JF512718	*L. lactea*		Qin Ling Shan, Zhouzhi (Erqu) county, Shaanxi	China
JF512618	*L. morsei*		South Altai, Markakol	Kazakhstan
JF512619	*L. morsei*		South Altai, Uspenka, Vostochno-Kazakhstanskaya obl.	Kazakhstan
FJ663714	*L. morsei*		Ust-Kamenogorsk region, Alekseevka distr., Mramorny Pass, Azytau Mts., Uspenka	Kazakhstan
FJ663715	*L. morsei*		Ust-Kamenogorsk region, Alekseevka distr., Mramorny Pass, Azytau Mts., Uspenka	Kazakhstan
HQ004591	*L. morsei*		Bădeni, Cluj	Romania
HQ004593	*L. morsei*		Badeni, Cluj county	Romania
HQ004590	*L. morsei*		Badeni, Cluj county	Romania
HQ004592	*L. morsei*		Hoia forest (Cluj-Napoca), Cluj county	Romania
HG969241	*L. morsei*		Novosibirsk Academy Town	Russia
HG969247	*L. morsei*		Novosibirsk Academy Town	Russia
HG969242	*L. morsei*		Novosibirsk Academy Town	Russia
HG969243	*L. morsei*		Novosibirsk Academy Town	Russia
HG969244	*L. morsei*		Novosibirsk Academy Town	Russia
HG969245	*L. morsei*		Novosibirsk Academy Town	Russia
HG969246	*L. morsei*		Novosibirsk Academy Town	Russia
HG969248	*L. morsei*		Novosibirsk Academy Town	Russia
HG969249	*L. morsei*		Novosibirsk Academy Town	Russia
HG969250	*L. morsei*		Novosibirsk Academy Town	Russia
HG969251	*L. morsei*		Novosibirsk Academy Town	Russia
GU372563	*L. morsei*		Haesan-ryeong, Gangwon	South Korea
JF512616	*L. reali*	hr2	Cascia, Perugia	Italy
JF512712	*L. reali*	hr7	Roccaraso, L'Aquila	Italy
JF512704	*L. reali*	hr3	Sibillini Mountains	Italy
GU676645	*L. reali*	hr4	Hormiguera, Cantabria	Spain
KC866117	*L. reali*	hr5	Pla de la Calma, Montseny	Spain
JF512617	*L. reali*	hr6	Saldes, Barcelona	Spain
JF512603	*L. reali*	hr1	Viladrau, Barcelona	Spain
HM393183	*L. sinapis*	hs13	Zahmer Kaiser, Aschinger Alm, Tyrol	Austria
JF512693	*L. sinapis*	hs1	Paril Village, Khadzhidimovo	Bulgaria
KC866088	*L. sinapis*	hs20	Studen Kladenets, Krumovgrad	Bulgaria
KC866101	*L. sinapis*	hs16	Hodonín, South Moravia	Czech Republic
JF512697	*L. sinapis*	hs5	Příbram, Central Bohemia	Czech Republic
KC866097	*L. sinapis*	hs25	Manosque, Alpes-de-Haute-Provence	France
JF513034	*L. sinapis*	hs6	NE Bézaudun-sur-Bine, Drôme	France
KC866005	*L. sinapis*	hs3	Fozzaninco	Corsica (France)
GU688533	*L. sinapis*	hs21	Lenggries Isarauen, Bavaria	Germany
GU688515	*L. sinapis*	hs19	Ruhpolding, Bavaria	Germany
KC866089	*L. sinapis*	hs15	Corciano, Perugia	Italy
JF512597	*L. sinapis*	hs8	Novalesa-Moncenisio, Torino	Italy
KC865994	*L. sinapis*	hs23	Gairo, Ogliastra	Sardinia (Italy)
JF513025	*L. sinapis*	hs11	Landman, Zyryanovsk	Kazakhstan
JF513047	*L. sinapis*	hs12	Landman, Zyryanovsk	Kazakhstan
JF513027	*L. sinapis*	hs2	Landman, Zyryanovsk	Kazakhstan
JF513046	*L. sinapis*	hs4	Saur Mts., Malyi Zhemeney	Kazakhstan
KC866098	*L. sinapis*	hs18	Mala Reka, Mavrovo	Macedonia
KC866104	*L. sinapis*	hs22	Mala Reka, Mavrovo	Macedonia

Supplementary Table S1 Continued.

GenBank accession number	Species	*COI* haplotype	Locality	Country
JF513026	*L. sinapis*	hs9	Ciupercenii de Olteț, Gorj	Romania
JF512592	*L. sinapis*	hs10	Schitul Pahomie, Vâlcea	Romania
HG969223	*L. sinapis*		Novosibirsk Academy Town	Russia
HG969224	*L. sinapis*		Novosibirsk Academy Town	Russia
HG969225	*L. sinapis*		Novosibirsk Academy Town	Russia
HG969226	*L. sinapis*		Novosibirsk Academy Town	Russia
GU675857	*L. sinapis*	hs24	Ames, Novais	Spain
KC866100	*L. sinapis*	hs17	Sant Celoni, Barcelona	Spain
KC866082	*L. sinapis*	hs7	Sorauren, Navarra	Spain
KC866102	*L. sinapis*	hs14	Riala, Norrtälje municipality	Sweden

4. Synthesis and perspectives

4.1. Karyotype and sex chromosome evolution in tortricid moths

Moths of the family Tortricidae are among one of the major microlepidopteran groups in terms of species richness and economic importance. The family includes almost 700 potential pests of forest, agricultural, and ornamental plants. The overall significance of tortricids is demonstrated by numerous studies on their taxonomy, ecology, and pest control (Brown *et al*. 2010). However, the genome architecture of tortricid moths is poorly understood. In order to reconstruct the karyotype evolution in the family Tortricidae we performed detailed cytogenetic analysis of two pests of pome and stone fruit, *Grapholita molesta* and *G. funebrana* (Olethreutinae; Grapholitini), and two pests of cultivated grapes, *Lobesia botrana* (Olethreutinae; Olethreutini) and *Eupoecilia ambiguella* (Tortricinae; Cochylini), together with comparative mapping of the Z chromosome in codling moth, *Cydia pomonella* (Olethreutinae; Grapholitini).

Results of our cytogenetic analyses together with chromosome data available in the codling moth, *C. pomonella* (Fuková *et al.* 2005), and other members of the family Tortricidae were comprehensively reviewed, suggesting conserved basic features of tortricid karyotypes. All three analysed species of the subfamily Olethreutinae (*G. molesta*, G*. funebrana*, and *L. botrana*) have a haploid chromosome number of n=28, similar to our reference genome of *C. pomonella (*Fuková *et al.* 2005*)*. Moreover, this chromosome number seems to be a modal number in this subfamily, as it has been found in 11 out of 18 of species examined (see Table 1 in section 3.2.). However, much greater chromosome number stability with n=30 has been found in 25 out of 26 species of the subfamily Tortricinae, including *E. ambiguella* examined in our study. The only exception found in Tortricinae is the rustic tortrix, *Clepsis senecionana*, with the haploid chromosome number of n=29 (Suomalainen 1971).

Another stable feature of tortricid karyotypes is the presence of a large pair of sex chromosomes that was described in *C. pomonella* (Fuková *et al.* 2005) and all four species from our study (*G. molesta*, *G. funebrana*, *L. botrana*, and *E. ambiguella*). Moreover, a similar large pair of chromosomes, most probably a pair of sex chromosomes, was reported for almost each karyotyped tortricid species (Saitoh 1966, Suomalainen 1971, Ennis 1976, Ortiz and Templado 1976, Lukhtanov and Kuznetsova 1989, Harvey 1997). Giving the fact the chromosome number of n=31 represents an ancestral karyotype in Lepidoptera, the presence of large sex chromosomes suggests that the very first chromosome rearrangement, which differentiated the common ancestor of Olethreutinae and Tortricinae, is a sex chromosome-autosome fusion. This rearrangement gave rise to large neo-sex chromosomes and a karyotype with haploid chromosome number of n=30 which has been described in the majority of Tortricinae species, but was further reduced to n=28 in the subfamily

Olethreutinae, most probably by other two fusion events involving autosomes. This scenario of karyotype and sex chromosome evolution is consistent with the results presented in section 3.1., where we show that the Z sex chromosome of *C. pomonella* is a neo-Z chromosome that originated by fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 in the silkworm *B. mori* reference genome. Furthermore, two other tortricids, *L. botrana* (Olethreutinae) and *E. ambiguella* (Tortricinae), were tested to trace the evolutionary origin of this F(Z;15) fusion. The results of sex-linkage analysis of *Acetylcholinesterase 1* (*Ace-1*) and *Notch* orthologous genes of *B. mori* chromosome 15 in these tortricid pests clearly suggest that the chromosomal rearrangement occurred in a common ancestor of these subfamilies.

The chromosome fusion leading to the neo-Z chromosome in tortricid moths poses an intriguing question about its role in the divergence and radiation of this species-rich family. Both chromosomes that are involved in the rearrangement, the Z chromosome and chromosome 15, are well known for their genetic content. As mentioned in section 1.1., the Z sex chromosome plays a disproportionately larger role in adaptive evolution than the autosomes and thus can be involved in postzygotic reproductive isolation (Presgraves 2008, Ellegren 2009, Štorchová *et al*. 2010). This so-called 'large-Z effect' was reported in both birds (Ellegren 2009, Štorchová *et al*. 2010) and Lepidoptera (Sperling 1994, Prowell 1998), the two largest taxa with female heterogamety. A possible explanation for the 'large-Z effect' is rapid divergence of Z-linked coding sequences compared with autosomal sequences, the so-called 'fast-Z effect' (Presgraves 2008). New recessive mutations are not masked by standard alleles in the heterozygous state and thus immediately fixed by selection. Moreover, it has been shown that the Z chromosome of moths and butterflies contains female preference genes (Sperling 1994, Iyengar *et al*. 2002) and genes that are responsible for the differences between closely related species (Sperling 1994).

The fusion of the Z sex chromosome with chromosome 15 brought under sex-linked inheritance two major genes that are assigned to the autosomal linkage group corresponding to *B. mori* chromosome 15, namely *Ace-1* and *ABCC2* genes. Both *Ace-1* and *ABCC2* genes are members of insect carboxylesterase and ATP-binding cassette (ABC) transporter gene families, which are involved in insecticide resistance and detoxification of plant secondary metabolites, respectively (Sorensen and Dearing 2006, Li *et al*. 2007, Buss and Callaghan 2008, Zangerl *et al*. 2012). Together with other genes they represent the so-called 'performance' genes, which affect growth and survival of larvae on their host plants (Berenbaum and Feeny 2008). Theory predicts that the recessive mutation conferring metabolism and regulated absorption of both insecticides and plant secondary metabolites spread faster in a pest population once is Z-linked due to its hemizygosity in the females (see paragraph above). Thus, we can assume that the F(Z;15) fusion represents an important

innovation conferring evolutionary advantage in plant-herbivores interactions and resulting in adaptive radiation of the tortricid subfamilies Tortricinae and Olethreutinae.

And what happened with the female-limited homolog of chromosome 15 in codling moth and other tortricid pests? Unfortunately, it is not possible to draw any conclusion with current data. As mentioned in section 1.2., the W sex chromosome is heterochromatic and consists mainly from interspersed repetitive elements (Traut and Marec 1997, Fuková *et al.* 2005, Abe *et al.* 2005, Vítková *et al.* 2007). Repetitive sequences are known to undergo rapid evolution and the absence of crossing-over in lepidopteran females may accelerate molecular differentiation and degeneration of the W chromosomes (Marec 1996). Previous molecular analysis of the codling moth W chromosome (Fuková *et al*. 2007) along with the results from four representatives of the family Pyralidae (Vítková *et al.* 2007) supports the existence of extensive molecular degeneration of lepidopteran W chromosome. However, the W chromosomes of other tortricids examined in section 3.2. showed some interesting features. Detailed cytogenetic analysis revealed significant interspecific differences in the level of heterochromatinization of the W chromosomes and in the pattern of molecular differentiation of the W and Z chromosomes. Only the codling moth W chromosome showed a continuous heterochromatinization and uniform hybridization pattern along the entire W chromosome. On the other hand, results in *L. botrana* and *E. ambiguella* suggest that their W chromosomes consist of highly and poorly differentiated parts. These findings support a hypothesis that not only the Z chromosome but also the tortricid W chromosome had originated by fusion between an ancestral W chromosome (the highly differentiated part) and an autosome (the weakly differentiated part), probably also corresponding to the *B. mori* chromosome 15. Whether this scenario applies also to other lepidopteran species remains uncertain as we cannot exclude the possibility that the W chromosome arose independently in different lepidopteran lineages.

4.2. Karyotype and sex chromosome evolution in *Leptidea* butterflies

Wood white butterflies of the genus *Leptidea* represent an emerging 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). Over the past 30 years, originally a single wood white species, *L. sinapis*, was split into three closely related cryptic species (*L. sinapis*, *L. reali*, and *L. juvernica*) that are virtually indistinguishable externally from each other and two of them (*L. reali* and *L. juvernica*) also have extremely similar genitalia (Dincă *et al*. 2011). In addition, the three species show remarkable inter- and intraspecific variation in chromosome numbers (Dincă *et al*. 2011, Lukhtanov *et al.* 2011). This particularly regards to *L. sinapis* with an exceptional chromosomal cline ranging from 2n = 56 in Kazakhstan to 2n = 106 in Spain (Lukhtanov *et al.* 2011). In order to confirm the results of previous studies and to understand karyotype evolution

in this genus, we performed a detailed molecular cytogenetic analysis of three cryptic *Leptidea* species (*L. sinapis*, *L. reali*, and *L. juvernica*) from the Western Palearctic, and one species, the northeast-Asian wood white (*L. amurensis*), from the Eastern Palearctic.

Previous studies on chromosomal counts in *Leptidea* species were done on metaphase I spermatocytes (Dincă *et al*. 2011, Lukhtanov *et al.* 2011), which did not allow the identification of complex meiotic figures such as multivalents. Our detailed cytogenetic analysis performed on mitotic and meiotic chromosomes revealed an exceptional inter- and intraspecific variation in chromosome numbers, as well as the presence of chromosome multiples in all analysed *Leptidea* species. The chromosome numbers ranged from 2n = 85 to 91 in *L. juvernica* and 2n = 69 to 73 in *L. sinapis* to 2n = 51 to 55 in *L. reali*. By contrast, the chromosome number of *L. amurensis* was relatively stable with remarkable differences in chromosome numbers between sexes, i.e. 2n = 118–119 in females and 2n = 122 in males, with no intermediate karyomorphs. These results are in concordance with previous findings (Maeki 1958, Dincă *et al*. 2011, Lukhtanov *et al.* 2011). Nevertheless, we extended the range of chromosome numbers observed in *L. juvernica* and *L. reali* and confirmed chromosome number variation in *L. amurensis*, previously reported as a species with constant chromosome number of 2n = 122 (Maeki 1958). Similar differences in chromosome numbers, especially in plants, can be caused by polyploidy (Coghlan 2005). However, our preliminary data showing a similar size of interphase nuclei in three cryptic *Leptidea* species (*L. sinapis*, *L. reali*, and *L. juvernica*) suggest that their karyotypes did not differentiate through polyploidy. Providing that the chromosome print of n=31 represents an ancestral karyotype of non-tineoid Ditrysia, our findings clearly indicate that chromosome fission is the main force in the karyotype evolution of *Leptidea* butterflies.

The analysis of mitotic chromosomes helped us to identify the unprecedented variability in chromosome numbers even within the offspring of individual females of all studied *Leptidea* species. It is known that in some systems, the population variability may be caused by the presence of supernumerary chromosomes, i.e. B-chromosomes (Camacho *et al.* 2000). These additional chromosomes are dispensable but they can accumulate through processes of mitotic or meiotic drive (Jones *et al*. 2008). However, this scenario was deemed rather unlikely in *Leptidea* butterflies (Lukhtanov *et al.* 2011). Our findings, namely the higher the chromosome numbers in a population, the smaller the size of chromosomes and the occurrence of multivalents in meiotic nuclei of all studied species suggest that the *Leptidea* karyotypes are differentiated by multiple chromosome fusion and fission. Uneven chromosome segregation of multivalents during meiotic division is thus the most plausible explanation for the extraordinary intraspecific karyotype variation.

In the karyotypes of all analysed *Leptidea* butterflies we also found significant differences in the number and distribution of two cytogenetic markers, i.e. clusters of major rDNA and H3 histone genes. In *L. reali* and *L. amurensis*, the species with the lowest and highest chromosome number

respectively, the results were consistent within a species. In *L. juvernica* and *L. sinapis*, which are species with higher chromosome numbers, significant differences in the number and location of both cytogenetic markers were observed, even among the offspring of individual females. Such inter- and intrapopulation variation in rDNA distribution is consistent with the evolutionary mobility of rDNA observed in Lepidoptera (Nguyen *et al*. 2010, section 3.2.). On the other hand, the differences in the number and location of H3 histone gene clusters are unexpected, since this marker is highly conserved in the lepidopteran family Tortricidae (section 3.2.) as well as other insect groups, i.e. grasshoppers and beetles (Cabrero *et al*. 2009, Cabral-de-Mello 2011). These results together with observed differences in chromosome numbers highlight the ongoing explosive karyotype evolution in *Leptidea.*

The exceptional variability in chromosome numbers and localization of cytogenetic markers (rDNA and H3 histone genes), even among the offspring of individual females, contrasts with intraspecific stability of sex chromosome constitution. In all analysed *Leptidea* species, our results revealed unique but species-specific sex chromosome systems with the following constitutions: $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica*, $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis*, $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ in *L. reali*, and $W_1W_2W_3Z_1Z_2Z_3Z_4Z_5Z_6$ in *L. amurensis* (Figure 1). In general, sex chromosome trivalents or quadrivalents are common in vertebrates except for birds (Gruetzner *et al.* 2006, Pokorná *et al*. 2014) and can also be found in a number of invertebrate species (Marec *et al*. 2010, Bardella *et al.* 2012, Palacios-Gimenez *et al.* 2013). On the other hand, multiple sex chromosomes with more than four sex chromosomes are rare and have been documented only in some invertebrates and monotreme mammals. In the latter, the duck-billed platypus represents an extraordinary ten sex chromosome system that arose by sex chromosome–autosome translocations (Grützner *et al.* 2004, Rens *et al.* 2004). In invertebrates, the most complicated sex chromosome systems were observed in some termites, in which males are permanent translocation heterozygotes and form sex-linked chains or rings of up to 19 chromosomes in meiosis (Syren and Luykx 1981). In Lepidoptera, the majority of moth and butterflies have a WZ/ZZ sex chromosome system (section 1.2.). Multiple sex chromosomes are rare and so far have been found only in seven genera in two different constitutions, either with W_1W_2Z or WZ_1Z_2 trivalents in females. Their origin can be ascribed either to sex chromosome fission or to sex chromosome–autosome fusion, where the remaining autosome becomes a W_2 or Z_2 chromosome (Marec *et al*. 2010). Thus, the unique sex chromosome constitutions observed in all analysed *Leptidea* butterflies stand out for its complexity and the number of elements involved in the multivalent in meiosis.

Sex chromosome multiples of *Leptidea* butterflies represent highly derived neo-sex chromosome systems that originated as a result of complex chromosomal rearrangements. It is known that complex chromosomal rearrangements that give rise to sex chromosome multiples can

result in unbalanced segregation during meiotic division, which could have fatal consequences for the fertility and/or viability of individuals. Yet, multiple sex chromosomes have been found in all four *Leptidea* species suggesting that the presence of multiple sex chromosomes is an ancestral trait for the genus *Leptidea*. What is then the evolutionary advantage of this complex system? Recent studies have shown that neo-sex chromosomes contribute to reproductive isolation between closely related species of stickleback fish, which supports the idea that the turnover of sex chromosomes may actually promote speciation (Kitano and Peichel 2012). Moreover, in Lepidoptera, detailed studies on the neo-sex chromosome evolution in geographic subpopulations of wild silkmoths *Samia cynthia* ssp. (Yoshido *et al*. 2011) and leaf-rollers of the family Tortricidae (section 3.1. and 4.1.) point to the fact that the chromosome rearrangements are important forces in the formation of reproductive barriers between populations and contribute to radiation in some lepidopteran taxa, respectively. Similarly, chromosomal rearrangements that give rise to sex chromosome multiples in *Leptidea* butterflies increased the number of sex-linked genes and thus could have played a major role in the 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 perform detailed comparative analysis of multiple sex chromosomes in *Leptidea* species using genomic tools, recently established for *L. juvernica*. These are sequenced transcriptome to facilitate the search for orthologous genes, array-CGH for the identification of sex-linked genes, and a newly constructed library of bacterial artificial chromosomes (BAC) from genomic DNA of *L. juvernica* females. The BAC library will be used for physical mapping of identified sex-linked genes on *Leptidea* chromosomes by BAC-FISH in order to clarify the structure and origin of multiple sex chromosomes based on their genetic content, identify major chromosomal mechanisms underlying the formation of multiple sex chromosomes, and reconstruct the evolution of multiple sex chromosomes in the genus *Leptidea*. In addition, we propose to examine the karyotypes and sex chromosomes also in two basal species, *L. morsei* and *L. duponcheli*. Knowledge of sex chromosomes in these two species is essential to elucidate a step-by-step evolution of multiple sex chromosome systems in the genus *Leptidea*.

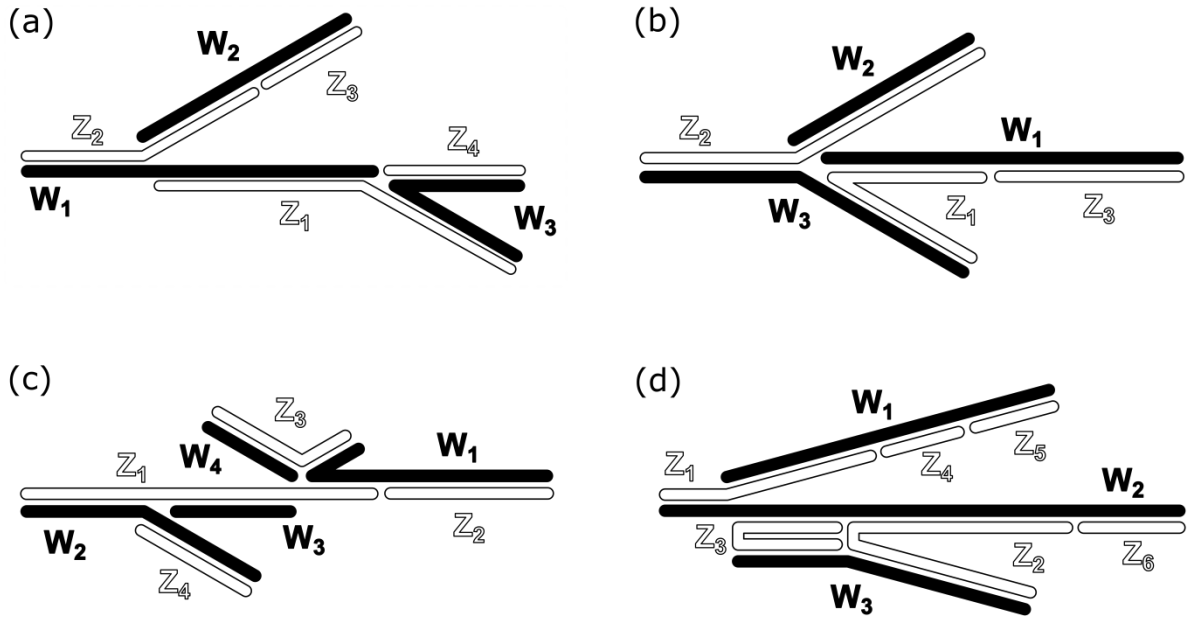

Figure 1 Simplified schematic drawing of *Leptidea* sex chromosome systems with the following constitutions: $W_1W_2W_3Z_1Z_2Z_3Z_4$ in *L. juvernica* (**a**), $W_1W_2W_3Z_1Z_2Z_3$ in *L. sinapis* (**b**), $W_1W_2W_3W_4Z_1Z_2Z_3Z_4$ in *L. reali* (**c**), and $W_1W_2W_3Z_1Z_2Z_3Z_4Z_5Z_6$ in *L. amurensis* (**d**). Individual elements in the sex-chromosome multivalents are designated arbitrarily. Their origin will be identified using recently established genomic tools.

5. 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.

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

Bardella VB, Dias A.L., 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.

Basset P, Yannic G, Brunner H, Hausser J (2006) Restricted gene flow at specific parts of the shrew genome in chromosomal hybrid zones. *Evolution* **60:** 1718-1730.

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.

BerenbaumMR, Feeny PP (2008) Specialization, Speciation and Radiation: The Evolutionary Biology of Herbivorous Insects, ed Tilmon KJ (Univ California Press, Berkeley, CA), pp 3-19.

Bidau CJ, Giménez MD, Palmer CL, Searle JB (2001) The effects of Robertsonian fusions on chiasma frequency and distribution in the house mouse (*Mus musculus domesticus*) from a hybrid zone in northern Scotland. *Heredity* **87:** 305-13.

Brown JW, Byun BK, Gilligan TM (2010) Tortricid literature library. Version 1.0. Available: http://www.tortricidae.com/library.asp. Accessed 2013 Feb 25.

Brown KM, Burk LM, Henagan LM, Noor MA (2004) A test of the chromosomal rearrangement model of speciation in *Drosophila pseudoobscura*. *Evolution* **58:** 1856-60.

Bureš P, Zedek F (2014) Holokinetic drive: centromere drive in chromosomes without centromeres. *Evolution* **68:** 2412-2420.

Buss DS, Callaghan A (2008) Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. *Pestic Biochem Physiol* 90: 141-153.

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.

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.* Dordrecht: Springer, 115-146.

Charlesworth B (1991) The evolution of sex chromosomes. *Science* **251:** 1030-1033.

Charlesworth B, Coyne JA, Barton NH (1987) The relative rates of evolution of sex chromosomes and autosomes. *Am Nat* **130:** 113-146

Coghlan A, Eichler EE, Oliver SG, Paterson AH, Stein L (2005) Chromosome evolution in eukaryotes: a multi-kingdom perspective. *Trends Genet* **21:** 673-682.

Coluzzi M (1982) Spatial distribution of chromosomal inversions and speciation in Anopheline mosquitoes. In Mechanisms of Speciation (Barigozzi, C., ed.), pp. 143–153, Alan R. Liss

Coyne JA, Orr HA (1998) The evolutionary genetics of speciation. *Philos Trans R Soc London Ser B* **353:** 287-305.

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.

Dernburg AF (2001) Here, there, and everywhere: kinetochore function on holocentric chromosomes. *J Cell Biol* **153:** F33-8.

Dincă V, Lukhtanov VA, Talavera G, Vila R (2011) Unexpected layers of cryptic diversity in wood white *Leptidea* butterflies. *Nat Commun* **2:** 324.

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

Ellegren H (2011) Sex-chromosome evolution: Recent progress and the influence of male and female heterogamety. *Nat Rev Genet* **12:** 157-166.

Ennis TJ (1976) Sex chromatin and chromosome numbers in Lepidoptera. *Can J Genet Cytol* **18:** 119-130.

Faria R, Navarro A (2010) Chromosomal speciation revisited: rearranging theory with pieces of evidence. *Trends Ecol Evol* **25:** 660-669.

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.

Futuyama DJ, Mayer GC (1980) Nonallopatric speciation in animals. *Syst Zool* **19:** 254-271.

Gavrilets S (2003) Perspective: models of speciation: what have we learned in 40 years? *Evolution* **57:** 2197-215.

Goldsmith M.R., Marec F., eds. (2010) *Molecular Biology and Genetics of the Lepidoptera*. CRC Press, Boca Raton, FL, USA.: Pp. 362.

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.

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.

Harvey GT (1997) Interspecific crosses and fertile hybrids among the coniferophagous *Choristoneura* (Lepidoptera: Tortricidae). *Can Entomol* **129:** 519-536.

Henikoff S, Ahmad K, Malik HS (2001) The centromere paradox: stable inheritance with rapidly evolving DNA. *Science* **293:** 1098-1102.

Hipp AL (2007) Nonuniform processes of chromosome evolution in sedges (Carex: Cyperaceae). *Evolution* **61:** 2175-94.

Hipp AL, Rothrock PE, Whitkus R, Weber JA (2010) Chromosomes tell half of the story: the correlation between karyotype rearrangements and genetic diversity in sedges, a group with holocentric chromosomes. *Mol Ecol* **19**: 3124-3138.

Imai HT, Maruyama T, Gojobori T, Inoue Y, Crozier RH (1986) Theoretical bases for karyotype evolution. 1. The minimum-interaction hypothesis. *Am Nat* **128:** 900-920.

Iyengar VK, Reeve HK, Eisner T (2002) Paternal inheritance of a female moth's mating preference. *Nature* **419:** 830-832.

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.

Kandul NP, Lukhtanov VA, Dantchenko AV, Coleman JW, Sekercioglu CH, Haig D, Pierce NE (2004) Phylogeny of *Agrodiaetus* Hübner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sequences of COI and COII and nuclear sequences of *EF1-α*: karyotype diversification and species radiation. *Syst Biol* **53:** 278-298.

Kawakami T, Butlin RK, Cooper SJB (2011) Chromosomal speciation revisited: Modes of diversification in Australian morabine grasshoppers (*Vandiemenella*, *viatica* species group). *Insects* **2:** 49-61.

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

Lande R (1985) The fixation of chromosomal rearrangements in a subdivided population with local extinction and recolonisation. *Heredity* **54:** 323-332.

Li XC, Schuler MA, Berenbaum MR (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52:** 231-253.

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

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, Kuznetsova VG (1989) Karyotype structure in higher Lepidoptera (Papilionomorpha). *Entomological Review* **68:** 12-31.

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, Kandul NP, Plotkin JB, Dantchenko AV, Haig D, Pierce NE (2005) Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus* butterflies. *Nature* **436:** 385-389.

Machado CA, Kliman RM, Markert JA, Hey J (2002) Inferring the history of speciation from multilocus DNA sequence data: the case of *Drosophila pseudoobscura* and close relatives. *Mol Biol Evol* **19:** 472-488.

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

Mank JE, Axelsson E, Ellegren H (2007) Fast-X on the Z: rapid evolution of sex-linked genes in birds. *Genome Res* **17**: 618-624.

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

Marec F (1996) Synaptonemal complexes in insects. *Int J Insect Morphol Embryol* **25:** 205-233.

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*. Boca Raton, FL: CRC Press, 49-63.

Marec F, Tothová A, Sahara K, Traut W (2001) Meiotic pairing of sex chromosome fragments and its relation to atypical transmission of a sex-linked marker in *Ephestia kuehniella* (Insecta: Lepidoptera). *Heredity* **87:** 659-671.

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.

Melters DP, Paliulis LV, Korf IF, Chan SWL (2012) Holocentric chromosomes: convergent evolution, meiotic adaptations and genomic analysis. *Chromosome Res* **20:** 579-593.

Nachman MW, Myers P (1989) Exceptional chromosomal mutations in a rodent population are not strongly underdominant. *Proc Natl Acad Sci U S A* **86:** 6666–6670.

Navarro A, Barton NH (2003) Chromosomal speciation and molecular divergence-accelerated evolution in rearranged chromosomes. *Science* **300:** 321-324.

Navarro A, Betran E, Barbadilla A, Ruiz A (1997) Recombination and gene flux caused by gene conversion and crossing over in inversion heterokaryotypes. *Genetic*s **146:** 695-709.

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.

Noor MA, Grams KL, Bertucci LA, Reiland J (2001) Chromosomal inversions and the reproductive isolation of species. *Proc Natl Acad Sci USA* **98:** 12084-12088.

Normark BB (1999) Evolution in a putatively ancient asexual aphid lineage: recombination and rapid karyotype change. *Evolution* **53:** 1458-1469.

Orr HA, Presgraves DC (2000) Speciation by postzygotic isolation: forces, genes and molecules. *BioEssays* **22:** 1085-94.

Ortiz E, Templado J (1976) Los cromosomas de tres especies de tortrı´cidos (Lep. Tortricidae). *EOS-Rev Esp Entomol* **51:** 77-84.

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.

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.

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.

Prowell DP (1998) Sex linkage and speciation in Lepidoptera. *In:* Howard DJ, Berlocher SH (eds) *Endless Forms: Species and Speciation*, pp. 309-319. Oxford University Press, New York.

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.

Rieseberg LH (2001) Chromosomal rearrangements and speciation. *Trends Ecol Evol* **16:** 351-358.

Robinson R (1971) *Lepidoptera Genetics*. Oxford: Pergamon Press.

Sahara K, Marec F, Eickhoff U, Traut W (2003) Moth sex chromatin probed by comparative genomic hybridization (CGH). *Genome* **46**: 339-342.

Sahara K, Yoshido A, Marec F, Fuková 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 Molec Biol* **43:** 644-653.

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

Saitoh K (1966) The chromosomes of two tortricid species (Lepidoptera) with notes on the chromosomes of two other allied species. Jap J Genet **41:** 275-278.

Sorensen JS, Dearing MD (2006) Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. *J Chem Ecol* **32:** 1181-1196.

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

Suomalainen E (1969) Chromosome evolution in the Lepidoptera. *Chromosomes Today* **2:** 132-138.

Suomalainen E (1971) Unequal sex chromosomes in a moth *Lozotaenia forsterana* F. (Lepidoptera: Tortricidae). *Hereditas* **68:** 313-316.

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

Štorchová 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.

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

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. *Trends Ecol Evol* **16:** 330-343.

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.

van Nieukerken EJ, Kaila L, Kitching IJ, Kristensen NP, Lees DC, et al. (2011) Order Lepidoptera Linnaeus, 1758. In: Zhang ZQ, editor. Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. *Zootaxa* **3148**: 212-221.

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

White MJD (1978) Modes of speciation. W.H. Freeman: San Francisco, CA, USA. pp. 1-455.

White BJ, Cheng C, Simard F, Costantini C, Besansky NJ (2010) Genetic association of physically unlinked islands of genomic divergence in incipient species of *Anopheles gambiae*. *Mol Ecol* **19:** 925-39.

Wrensch DL, Kethley JB, Norton RA (1994) Cytogenetics of holokinetic chromosomes and inverted meiosis: keys to the evolutionary success of mites, with generalization on eukaryotes. *In:* Houck MA (ed) *Mites: Ecological and Evolutionary Analyses of Life-History Patterns*, pp. 282-343. Chapman & Hall, New York.

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, 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, Marec F, Sahara K (2005) Resolution of sex chromosome constitution by genomic *in situ* hybridization and fluorescence *in situ* hybridization with $(TTAGG)_n$ telomeric probe in some species of Lepidoptera. *Chromosoma* **114:** 193-202.

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

Yoshido A, Šíchová J, Kubíčková S, Marec F, Sahara K (2013) Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp. *Chromosome Res* **21**: 149-164.

Zangerl AR, Liao LH, Jogesh T, Berenbaum MR (2012) Aliphatic esters as targets of esterase activity in the parsnip webworm (*Depressaria pastinacella*). *J Chem Ecol* **38:** 188-194.

6. Curriculum vitae

Jindra Šíchová

Birth Date: April 26, 1986
Place of Birth: Hradec Králové, Czech Republic
E-mail: sichjindra@seznam.cz

Research Interests:
Karyotype evolution, evolution of sex chromosomes, comparative genomics of Lepidoptera

University Education:

since 2011 Doctoral study in Molecular and Cell Biology and Genetics at the Faculty of Science, University of South Bohemia, České Budějovice. Thesis: The role of sex chromosomes in speciation of butterflies using the genus *Leptidea* as a model.

2011 Master's degree 'with distinctions' in Genetics and Gene Engineering at the Faculty of Science, University of South Bohemia, České Budějovice. Thesis: Molecular divergence of sex chromosomes compared to autosomes in related species of tortricids.

2009 Bachelor's degree in Biology at the Faculty of Science, University of South Bohemia, České Budějovice. Thesis: Analysis of the codling moth (*Cydia pomonella*) Z chromosome by means of laser microdissection.

Professional and Research Experience:

2014 Karyotype and multiple sex chromosome analysis of the northeast-Asian wood white, *Leptidea amurensis*; Iwate University, Faculty of Agriculture, Morioka, Japan. (c/o Ken Sahara, 44 days)

Publications:

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 of the northeast-Asian wood white butterfly, *Leptidea amurensis*. *Biol J Linnean Soc* (published online 21 January 2016) DOI: 10.1111/bij.12756.

Carabajal Paladino LZ, Ferrari ME, Lauría JP, Cagnotti C, **Šíchová J**, López S (2016) The effect of X-rays on cytological traits of *Tuta absoluta* (Lepidoptera: Gelechiidae). *Fla Entomol*: (in press).

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 Evolutionary Biology* 15: 89.

Carabajal Paladino LZ, Nguyen P, **Šíchová J**, Marec F (2014) Mapping of single-copy genes by TSA-FISH in the codling moth, *Cydia pomonella. BMC Genetics* 15: S15.

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

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: 6931-6936.

Yoshido A, **Šíchová J**, Kubíčková S, Marec F, Sahara K (2013) Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp. *Chromosome Res* 21: 149-164.

Drosopoulou E, Nakou I, **Šíchová J**, Kubíčková S, Marec F, Mavragani-Tsipidou P (2012) Sex chromosomes and associated rDNA form a heterochromatic network in the polytene nuclei of *Bactrocera oleae* (Diptera: Tephritidae). *Genetica* 140: 169-180.

Other Contributions (Selected):

Šíchová J, Voleníková A, Dincă V, Nguyen P, Vila R, Marec F (2014) Dynamic karyotype evolution and multiple sex chromosomes in wood white butterflies. In: The 9th International Workshop on the Molecular Biology and Genetics of the Lepidoptera, 17-23 August 2014, Orthodox Academy of Crete, Kolympari, Crete. Greece, p. 31. (poster).

Šíchová J, Voleníková A, Nguyen P, Vila R, Dinca V, Marec F (2013) Dynamic karyotype evolution and multiple sex chromosomes in wood white butterflies. In: 19th International Chromosome Conference, 2-6 September 2013, Bologna, Italy, p. 161. (poster)

Nguyen P, Sýkorová M, **Šíchová J**, Kůta V, Dalíková M, Čapková Frydrychová R, Neven LG, Sahara K, Marec F (2013) The role of neo-sex chromosomes in the evolution of tortricid moths (Lepidoptera: Tortricidae). in: Conference Jacques Monod, Recent Advances on the Evolution of Sex and Genetic Systems, 22 - 26 May 2013, Roscoff, France: p. 59. (poster)

Drosopoulou E, Nakou I, **Šíchová J**, Kubíčková S, Marec F, Mavragani-Tsipidou P (2012) What is the fate of sex chromosomes in polytene nuclei of *Bactrocera oleae* (Diptera: Tephritidae)? in: Abstracts, 2nd International Symposium of TEAM, 3-6 July 2012. Kolymbari. Crete, Greece, p. 78. (poster)

Šíchová J, Nguyen P, Dalíková M, Sýkorová M, Marec F (2011) Lepidoptera: Do they have fast-Z evolution? in: Jackson D., Griffin D. (eds.) Delegate Book, 18th International Chromosome Conference, 29 August - 2 September 2011, Manchester, UK, poster no. 170. (poster)

Šíchová J, Vítková M, Marec F (2009) Laser microdissection of codling moth Z chromosome for Zoo-FISH and sequence library. In: Kostas I., Gordon, K. (eds) Program, Abstracts, Participants, Authors, The 8th International Workshop on the Molecular Biology and Genetics of the Lepidoptera, 23-29 August 2009, Orthodox Academy of Crete, Kolympari, Crete. Greece, p. 86. (poster)

Grants:

Grant 052/2013/P "Struktura, funkce a evoluce genomů hmyzu a jeho patogenů" of the Grant Agency of the University of South Bohemia in České Budějovice. Role: member of research team.

Grant 063/2012/P "The role of sex chromosomes in speciation of butterflies using the genus *Leptidea* as a model" of the Grant Agency of the University of South Bohemia in České Budějovice. Role: PI.

Grant 059/2010/P "Mechanism of rDNA mobility in lepidopteran genomes" of the Grant Agency of the University of South Bohemia in České Budějovice. Role: member of research team.

Grant SGA2008/001 "Evolution of lepidopteran Z chromosome" of the Student grant Agency of the University of South Bohemia in České Budějovice. Role: member of research team.

sichjindra@seznam.cz

Contrasting patterns of karyotype and sex chromosome evolution in Lepidoptera
Ph.D. Thesis

University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 31
370 05 České Budějovice, Czech Republic

Phone: +420 387 772 244
www.prf.jcu.cz, e-mail: sekret@prf.jcu.cz