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

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ABSTRACT

Previous studies have shown a dynamic karyotype evolution and the presence of complex sex chromosome systems in three cryptic *Leptidea* species from Western Palearctic. To further explore the chromosomal particularities of *Leptidea* butterflies, we examined the karyotype of an Eastern Palearctic species, *L. 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), together with the observed heterozygotes for a chromosomal fusion is 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 suggests that the multiple sex chromosome system, though different among species, is a common feature of the genus *Leptidea*. Furthermore, inter- and intraspecific variations in chromosome numbers along with complex meiotic pairing of these multiple sex chromosomes point to the role of chromosomal fissions, fusions, and translocations in the karyotype evolution of *Leptidea* butterflies.

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 (reviewed in Traut, Sahara & Marec, 2007). Furthermore, Lepidoptera are regarded as organisms with holokinetic chromosomes due to 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 (reviewed in Carpenter, Bloem & Marec, 2005). Lepidopteran chromosomes are usually small and uniform in shape, and the majority of species are reported to have haploid chromosome numbers ranging from n=28 to n=32 with the most common number of 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., 2015). Recent studies also suggest extensive conservation at the chromosomal level and evolutionary stability of whole lepidopteran genomic regions (Pringle et al., 2007; Sahara et al., 2007; 2013; Yasukochi et al., 2006; 2009; 2015; 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= ca224-226, where the latter comes from the recently reassessed number in the 
Atlas blue, *P. atlanticus*, and represents the highest chromosome number not only of 
Lepidoptera but 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, *Hypothena thea* 
(Nymphalidae; Brown, von Schultz & Suomalainen, 2004), and the Arizona giant-skipper, 
*Agathymus arxna* (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 due to chromosome fusion, e.g. *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 *P. brassicae* (n=15) and the small white 
*P. 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 *L. 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, *L. morsei* and *L. 
amurensis*, a high but constant number of chromosomes was reported, n=54 and n=61, 
respectively (Maeki, 1958). However, three recently recognized cryptic species from the 
Western Palearctic, *L. juvernica*, *L. reali*, and *L. 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*, 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 and 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 a frequent occurrence of multivalents during meiotic divisions. Hence, the uneven chromosome segregation of the multivalents is apparently being 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 $W_1W_2Z_1Z_2Z_3Z_4$ 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 several times independently 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 (reviewed in Gruetzner et al., 2006; Pokorná, Altmanová & Kratochvíl, 2014) and can also be found in a number of invertebrate species (e.g. del Cerro, Cuñado & Santos, 1998; Bardella et al., 2012; Palacios-Gimenez et al., 2013) including Lepidoptera (reviewed in 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 believed that they originated by non-disjunction 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 by 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 (2-4) 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). On the contrary, a neo-X1X2X3X4X5Y system in the spider Malthonica ferruginea probably evolved from an ancestral X1X2X30 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 by different chromosomal rearrangements including duplications, fissions, X-X and X-autosome fusions (Král et al., 2013). The evolutionary significance of the above mentioned complex multiple sex chromosome systems, including those recently described in three Leptidea butterfly species (Šichová 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 verify 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 analysed sex chromosome constitution using genomic in situ hybridization (GISH). Results obtained help us to better understand the karyotype and sex chromosome evolution in Leptidea butterflies.
MATERIALS 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, as 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 following the procedure described in 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%, 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 standard phenol-chloroform procedure (Blin & Stafford, 1976). We did not analyse all the larvae, since 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) that has been reported as reliable for the identification of Leptidea species (Dincă et al., 2011, 2013; Solovyev, Ilinsky & Kosterin, 2015; Šíchová et al., 2015). The COI marker was amplified using pairs of primers LepF1 (5’- ATTCAACCAATCATAAGATATTGG-3’) and LepR1 (5’-
TAAACTTCTGGATGTCCAAAAAATCA-3′) (Dincă et al., 2011). 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 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 by a final extension of 7 min at 72°C. PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced.

Sequences were edited and aligned using GENEIOUS PRO 4.7 created by Biomatters (http://www.geneious.com/). Our sequences were combined with Leptidea data available in GenBank, as follows: representatives of all unique COI haplotypes of Leptidea juvernica, L. reali, and L. sinapis identified by Dincă et al. (2013), as well as all COI sequences of these taxa published by Solovyev et al. (2015); all published COI sequences of other Leptidea taxa (L. morsei, L. amurensis, L. lactea, and L. duponcheli) that overlapped with our sequenced mtDNA fragment by at least 600 bp (Supplementary 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 2.4 (Guindon & Gascuel, 2003) implemented in GENEIOUS PRO 4.7. The substitution model used was GTR+I+G and was chosen according to AIC values obtained in jModeltest 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 according to the protocol of Sahara, Marec & Traut, (1999). The probes were labelled with Cy3-dUTP (GE Healthcare, Milwaukee, WI, USA) or Orange-dUTP (Abbott Molecular Inc., Des Plaines, IL, USA) using a Nick Translation Kit (Abbott Molecular Inc.) with 1 h incubation at 15°C. For chromosome counts we used fluorescence in
situ hybridization (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, St. Louis, MO, USA) in 10 μl of 50% formamide and 10% dextran sulphate in 2× SSC.

To identify sex chromosomes we used genomic in situ hybridization (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 standard phenol-chloroform procedure. A part of male gDNA was sonicated using a Sonopuls HD 2070 (Bandelin Electric, Berlin, Germany) and used as a competitor DNA (Šíchová et al., 2013). The extracted female gDNA was labelled with fluorescein-12-dUTP (Invitrogen, Carlsbad, CA, USA) or Green-dUTP (Abbott Molecular Inc.) using the Nick Translation Kit with 6 h 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 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 in Fuková, Nguyen & Marec (2005). The probe was labelled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) using the Nick Translation Kit (Abbott Molecular Inc.) with 1 h and 45 min incubation at 15°C. A biotin-labelled H3 histone probe was prepared from Leptidea sinapis gDNA using PCR according to the protocol described in Šíchová et al. (2013; 2015).

FISH experiments with 18S rDNA and H3 histone probes were performed as described in Fuková et al. (2005). Briefly, chromosome preparations were treated with 100 μg/mL 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 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.

**Microscopy and image processing**

Preparations from FISH experiments were observed under a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany) or under a DM 6000B microscope (Leica Microsystems Japan, Tokyo, Japan). Black-and-white images were recorded with a cooled F-View CCD camera using the AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany), installed on the Zeiss 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 Adobe Photoshop, version 7.0.

**RESULTS**

**Molecular identification of Leptidea specimens**

The ML tree based on mitochondrial COI sequences confirmed the taxonomical identity of the material used in this study. All analysed larvae clustered with the L. amurensis sequences downloaded from GenBank, but formed a well-supported and slightly differentiated clade with respect to all mainland specimens available (Figure 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 published as belonging to L. morsei (Hao et al., 2014), were clearly recovered by our analysis as L. amurensis (as indicated by the COI fragment analysed 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 (Figure 2A, B). Based on repeated counts, we found differences in chromosome numbers between sexes and among individual females. While seven examined male larvae had an identical diploid chromosome number of 2n=122 (Figure 2B), three out of six female larvae had 2n=118 and the other three female larvae had 2n=119 (Figure 2A). No intraindividual variability in chromosome number was found. In male meiotic metaphase I (MI) we observed 61 elements, most probably bivalents (Figure 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 (Figure 2C).

In female mitotic complements, four chromosomes stood out due to their size. Moreover, two of these large chromosomes were partially differentiated with heterochromatin (Figure 2A, 3B). Similarly, large chromosomes were observed in male mitotic nuclei (Figure 2B). However, in contrast with 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 (Figure 2D; asterisks). These DAPI-positive blocks were also found in other studied *Leptidea* species (Šichová *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 analysed meiotic complements from six female larvae. In all of them we observed a sex chromosome multivalent (see later; Figure 2D; arrow) and in four of them we also observed one extra trivalent (Figure 2D; arrowhead).

**Chromosomal location of major rDNA and H3 histone genes**

FISH with the 18S rDNA probe performed in three *Leptidea 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 (Figure 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 about two-fifths of the NOR-bivalent length (inset of Figure 2E).

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 (Figure 2F). Only the latter cluster was associated with a block of DAPI-positive heterochromatin (inset of Figure 2F). In accordance with this hybridization pattern, the H3 probe identified four mitotic chromosomes (not shown).

**Sex chromosome identification**

To identify the sex chromosomes we used GISH combined with tel-FISH on mitotic and meiotic chromosomes of *Leptidea amurensis* females. While 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 (Figure 3A, B; arrows). Two of these large chromosomes were preferentially labelled with the female gDNA probe (Figure 3A-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 W1 and
The W₁ chromosome was homogeneously labelled over most of its length except for a small terminal gap (Figure 3C). However, the W₂ chromosome was strongly highlighted with the probe only in a terminal segment (Figure 3C). The female-derived genomic probe also hybridized to heterochromatin blocks on autosomes (Figure 3C).

The analysis of female pachytene complements helped us to identify a complex sex chromosome multivalent with the following constitution: W₁W₂Z₁Z₂Z₃Z₄Z₅Z₆ (Figure 3D–H). In the multivalent, each W chromosome paired with 2-4 Z chromosomes (see a scheme in Figure 4). Three Z chromosomes paired each with two W chromosomes (Z₁ with W₁ and W₂; Z₂ and Z₃ with W₂ and W₃). On the contrary, three other Z chromosomes paired exclusively with one W chromosome each (Z₄ and Z₅ with W₁; Z₆ with W₂). These results were confirmed in four out of six female larvae. In two larvae we were not able to resolve the multivalent due to the lack of well-spread pachytene nuclei in chromosome preparations. In accordance with results from mitotic metaphases, the female gDNA probe highlighted two large W chromosomes, W₁ and W₂ (Figure 3D, F). These W chromosomes were also partially differentiated by DAPI-positive heterochromatin (Figure 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 (Figure 3E, the lower arrowhead). The fact that the W₃ sex chromosome was not largely differentiated by the female genomic probe suggests its recent origin (Figure 3F). Two largest Z chromosomes (Figure 3H) were designated Z₁ and Z₂. These Z chromosomes can represent two large bivalents in male meiotic metaphase I (Figure 2C) and also two large chromosomes that were not heterochromatinized in female mitotic nuclei (Figure 2A, 3A-B).

**DISCUSSION**

Previous studies on three cryptic *Leptidea* species from Western Palearctic (L. juvernica, L. reali, and L. sinapis) showed exceptional inter- and intra-specific variation in chromosome
numbers and location of cytogenetic markers (major rDNA and H3 histone genes) and a
curious sex determining system with 3–4 W and 3–4 Z chromosomes (Dincă et al., 2011;
2013; Lukhtanov et al., 2011; Šíchová et al., 2015). These results suggested a dynamic
karyotype evolution and stressed 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 due to incomplete faunistic data or cryptic diversity we used mitochondrial
COI sequences that accurately proved the taxonomical identity of the larvae used in this study.

Chromosome number variation

The first note about the chromosome number of L. amurensis dates back to 1958,
when Maeki analysed meiotic chromosomes in the stage of metaphase I (MI) spermatocytes.
He observed 61 elements in meiotic nuclei and, most importantly, described two elements that
stood out due to their size. 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 the haploid number of n=61 elements including two larger elements in metaphase I 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), while 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 the 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 shown 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 analysed larvae 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*, 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 intra-population variation in rDNA distribution is consistent with the evolutionary mobility of rDNA observed in Lepidoptera and other groups of organisms (Nguyen et al., 2010, and references therein; 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 when compared to other *Leptidea* species, point to the stability of the *L. amurensis* karyotype despite the high chromosome number. However, the 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 (Figures 3 and 4), which is even more complex than those found in Western Palearctic *Leptidea* species (cf. 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 by 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 (see Figure 3) indicating a high level of molecular differentiation (cf. 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 by 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 (this 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, though much less frequently (Traut et al., 2007). In addition, two types of multiple sex chromosome systems with three elements, $W_1W_2Z/ZZ$, and $WZ_2Z_1Z_2Z_2$, occur sporadically. They have been described only in seven genera from different lineages of the lepidopteran phylogenetic tree (reviewed in 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 in comparison with 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, *Cydia pomonella* (Tortricidae: Olethreutinae). 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 that gave 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 through 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 geographic subspecies of *S. cynthia*.

Conclusions

Based on our data, the emerging picture of chromosome evolution in *L. amurensis* is that: (i) the sex chromosome number is constant in both sexes, i.e. twelve sex chromosomes (*Z<sub>1</sub>-6/Z<sub>1</sub>-6*) in males and nine sex chromosomes (*W<sub>1</sub>-3/Z<sub>1</sub>-6*) in females; (ii) 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; (iii) 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 (Figure 5). The fact that we did not find heterozygous males with 2n=121 and homozygotes for the fusion in any sex can be due to the low number of individuals examined or recent origin of the fusion which is not yet widespread in the *L. amurensis* population.
Our 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 point to chromosome fission as the main force in the karyotype evolution of *L. amurensis*. In 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. *W₁⁻³Z₁⁻⁶* in females and *Z₁⁻⁶Z₁⁻⁶* in males. Meiotic configurations in females suggest that this system originated by complex chromosomal rearrangements between ancestral sex chromosomes and autosomes, including fusion, fission, and translocation events. The presence of sex chromosome multiples in the karyotypes of 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 contribute to the formation of reproductive barriers between the closely related *Leptidea* species.

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**Conflict of Interest**

Authors declare no conflict of interest.
Data archiving

Sequence data have been submitted to GenBank: accession numbers KR363156 - KR363165.
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FIGURE LEGENDS

Figure 1. Maximum likelihood tree inferred from mitochondrial COI sequences of Leptidea taxa. Specimens sequenced and analyzed in this study are indicated by an asterisk and were combined with available sequences of other Leptidea taxa from published studies. Samples with two asterisks correspond to COI sequences from mitochondrial genomes that were published as belonging to L. morsei, but were recovered as L. amurensis by our analysis. For L. juvernica, L. reali, and L. sinapis representatives of all haplotypes identified by Dincă et al. (2013) were used. For the origin of all specimens and GenBank accession numbers, see Supplementary 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 FISH 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 DAPI (blue). Asterisks indicate DAPI-positive blocks of heterochromatin. (A) Female mitotic metaphase (2n=119); arrows indicate four largest chromosomes in complement. (B) Male mitotic metaphase (2n=122); arrows indicate 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 in the upper right corner shows the pachytene NOR-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 in the upper right corner shows a block of DAPI-
positive heterochromatin in one H3 histone gene-bearing bivalent. Scale bar (A-C) = 5 µm; (D-F) = 10 µm.

Figure 3. Genomic in situ hybridization (GISH) combined with (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 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. Figures (A-C) show detailed analysis of female mitotic metaphase: (A) merged images of the female-derived genomic probe and DAPI staining; (B) DAPI image; (C) hybridization pattern of the female genomic probe. Figures (D-H) show detailed analysis of the sex chromosome multivalent W$_1$Z$_1$-6: (D) merged images of the female genomic probe, (TTAGG)$_n$ telomeric probe, and DAPI staining; (E) DAPI image; (F) hybridization pattern of the female genomic probe; (G) hybridization pattern of the (TTAGG)$_n$ telomeric probe; (H) schematic drawing of the sex chromosome multivalent; red dots indicate the ends of individual elements in the multivalent. Scale bar = 10 µm.

Figure 4. Simplified schematic drawing of the *Leptidea amurensis* sex chromosome multivalent in pachytene oocytes with the constitution W$_1$W$_2$W$_3$Z$_1$Z$_2$Z$_3$Z$_4$Z$_5$Z$_6$. 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 GISH 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.
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 9 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).

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*.