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Comparative mapping of sex-linked genes in Lepidoptera

Ph.D. Thesis

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Annotation

Sequenced genome of the silkmoth, *Bombyx mori*, allowed comparative genomic studies in Lepidoptera. The presented thesis is concerned with synteny of sex-linked genes in moths and butterflies. I took advantage of available BAC libraries and by means of fluorescence in situ hybridization I constructed physical maps of the Z chromosome of the peppered moth, *Biston betularia*, and the codling moth, *Cydia pomonella*. The results revealed unexpected dynamics of the lepidopteran Z chromosome, which was found to be involved in both inter- and intrachromosomal rearrangements.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval 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, 19. 9. 2013 .....

Petr Nguyen

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List of papers and author's contribution

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

I. 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 (IF = 4,110).

As a corresponding author I hereby confirm that Petr Nguyen performed BAC-FISH mapping of Z-linked genes, constructed a physical map of the B. betularia Z chromosome, and contributed to the draft of the manuscript.

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Ilik J. Saccheri

II. 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 = 9,737).

Hereby I declare that Petr Nguyen conceived the study, designed experiments, isolated most of the studied genes, performed physical mapping of the genes, supervised qPCR analysis, wrote the first draft of the manuscript and decisively contributed to the revision of the manuscript.

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František Marec

Contents

1. General introduction

There is no doubt that sex chromosomes belong to the most intriguing topics of contemporary evolutionary genetics, as they are involved in processes such as ecological adaptation, speciation or genomic conflict (e.g. Meiklejohn and Tao 2010, Ellegren 2011, Ezaz and Graves 2012, Bachtrog 2013, Nguyen *et al.* 2013). Recent research has brought new twist to our understanding of evolution and differentiation of sex chromosomes (Guioli *et al.* 2012, Mank 2013, Vicoso and Bachtrog 2013). And although newly developed sequencing technologies have played an enormous role in the recent progress; it has been data from non-model organisms that lead to reexamining the classical model of sex chromosome differentiation (Ezaz and Graves 2012, Mank 2013).

This thesis deals with sex chromosomes of moths and butterflies (Lepidoptera), which represent, with some 160 000 described species, the second most diverse animal group to beetles (Coleoptera) (Kristensen *et al.* 2007) and the largest taxon with female heterogamety, i.e. with the WZ/ZZ sex chromosome system and its variations (Ellegren 2011). Despite their importance and diversity, or may be due to them, there are still considerable gaps in our knowledge of lepidopteran genome architecture and evolution. The thesis aims at some of these gaps and if there is any common thread to it, it is definitely a notion that Z chromosomes of moths and butterflies are highly dynamic entities, which is in stark contrast to the current view (reviewed by Sahara *et al.* 2012).

1.1. Sex chromosome evolution and differentiation

According to a classical model of sex chromosome differentiation, sex chromosomes evolve from a pair of autosomes, which acquired a sex determining factor. In theory, the process is initiated by separation of male and female functions by two sterility mutations that arise in co-sexual, i.e. hermaphroditic or monoecious, population, which is followed by selection for linkage and suppression of recombination between the genes involved (Charlesworth and Charlesworth 1978, Charlesworth 2013).

Although the dioecy is relatively rare in plants (Charlesworth 2013), this early stage of sex chromosomes evolution has been recently reported in a wild strawberry, *Fragaria virginiana*, where females co-exist with pollen-bearing hermaphrodites and males. In *F. virginiana*, it was shown that sex is determined by at least two loci linked together, which still recombine at a very low frequency (Spigler *et al.* 2008). However, no such incipient sex chromosomes are known in animals, which probably stems from the fact that sexual dimorphism is common to all coelomate bilaterians (Haag and Doty 2005). Thus, the majority of animal sex-determining systems have probably not evolved from hermaphroditism but rather through transition from environmental to genetic sex

determination (Pokorná and Kratochvíl 2009) or acquisition of a new upstream master gene, which took control of sex development (van Doorn and Kirkpatrick 2007, Hasselmann *et al.* 2008).

Once the separate sexes are established, sexually antagonistic alleles, i.e. alleles beneficial to one sex but detrimental to the other, are expected to accumulate in the vicinity of the sex determining region. To preserve their linkage disequilibrium, recruited sexually antagonistic loci favor a suppression of recombination between homomorphic proto-sex chromosomes (Charlesworth *et al.* 2005, but see Ironside 2010 for alternative hypotheses). Two hypotheses have been invoked to explain how restriction of recombination spreads along sex chromosomes. Stepwise model highlights the role of inversions in suppressing of recombination, which is supported by evolutionary strata of different age, i.e. sex chromosome regions of different levels of divergence, observed in mammals (Kohn *et al.* 2004, Mácha *et al.* 2012), birds (Nam and Ellegren 2008), snakes (Vicoso *et al.* 2013a), papaya (Wang *et al.* 2012), and white campion *Silene latifolia* (Bergero *et al.* 2007, 2013, Hobza *et al.* 2007). However, it was shown that the multiple inversions on the Y chromosome are a consequence rather than a cause of suppressed recombination in *S. latifolia* (Bergero *et al.* 2008). Thus, other mechanisms such as heterochiasmy, i.e. sex-specific differences in recombination, are probably involved in a gradually proceeding cessation of recombination in the early stage of sex chromosome differentiation (Perrin 2009, Bachtrog 2013, Natri *et al.* 2013).

The lack of recombination triggers differentiation of homomorphic sex chromosomes. Non-recombining loci are locked in a complete linkage disequilibrium with selection acting on the entire chromosome. Furthermore, the effective population size of the sex-limited chromosome drops considerably (Mank 2012, Bachtrog 2013). This reduces a net efficacy of natural selection and leads to the loss of genetic diversity and the accumulation of genetic load via background selection and selective sweeps (Charlesworth and Charlesworth 2000, Bachtrog 2013). In addition, chromosome variants bearing the fewest deleterious mutations are irreversibly lost in a stochastic process of Muller's ratchet (Charlesworth and Charlesworth 2000, Kaiser and Charlesworth 2010, Bachtrog 2013). All these processes ultimately result in accumulation of repetitive sequences and loss of gene function accompanied by structural changes and heterochromatinization of the sex-limited chromosome (Charlesworth *et al.* 2005, Bachtrog 2013).

Numerous cytogenetic and molecular analyses of ancient sex chromosome systems document this final step of sex chromosome differentiation (e.g. Skaletsky *et al.* 2003, Abe *et al.* 2005, Fuková *et al.* 2007, Vítková *et al.* 2007, Hughes *et al.* 2010). However, the key to understanding the forces driving sex chromosome degeneration is held by evolutionary young sex chromosomes, which are formed either *de novo* or in sex chromosome-autosome rearrangements, i.e. neo-sex chromosomes. Among other model systems, fruit flies of genus *Drosophila* stand out. In *Drosophila* spp. karyotype evolution, independent reshuffling of chromosomal arms, so-called Muller's elements (Bhutkar *et al.*

2008), gave rise to neo-sex chromosomes of different age and level of differentiation (Bachtrog *et al.* 2008, Zhou and Bachtrog 2012b, Zhou *et al.* 2012). Genomic studies of neo-sex chromosomes of fruit flies *D. albomicans*, *D. miranda*, and *D. pseudoobscura*, which are 0.1 My, 1 My, and 15 My old, respectively, offer us a unique insight into molecular changes of sex-limited loci over evolutionary time (reviewed by Bachtrog 2013). The *Drosophila* neo-Y chromosome stops to recombine immediately upon neo-sex chromosome formation due to male achiasmatic meiosis. In *D. albomicans*, the process of neo-Y chromosome differentiation began with chromosome-wide downregulation of neo-Y linked genes caused either by epigenetic changes in the chromatin structure or mutations in regulatory sequences (Zhou and Bachtrog 2012a). In the next step observed in *D. miranda*, gene function was lost and repetitive sequences accumulated on the neo-Y chromosome. Only male-beneficial genes were retained, which led to masculinization of sex-limited gene content and increased its rates of evolution (Zhou and Bachtrog 2012b). In less than 15 My, *D. pseudoobscura* neo-Y chromosome reached the final stage of sex chromosome evolution as it is formed solely by heterochromatin and harbors only handful of fertility factors (Bachtrog 2013, but see Carvalho 2002 for alternative origin of the *D. pseudoobscura* Y chromosome). Recently evolved sex chromosomes of *S. latifolia* show genetic degeneration, which is much alike the pattern observed in *D. albomicans*. In comparison with their X-linked counterparts, *S. latifolia* Y-linked alleles were reported to have lower expression and accumulate presumably deleterious mutations. However, *S. latifolia* sex chromosomes are estimated to have evolved about 10 Mya, which suggests much lower rate of molecular decay of non-recombining loci in plants (Bergero and Charlesworth 2011). The difference is probably due to high transcriptional activity of Y-linked genes in male haploid gametophyte, i.e. pollen, inducing stronger purifying selection against deleterious recessive mutations (Chibalina and Filatov 2011).

Molecular differentiation of sex chromosomes has far-reaching consequences. In the heterogametic sex, genetic degeneration of sex-limited loci results in sex chromosome monosomy. Since gene dose, i.e. copy number, is often correlated with an expression level, selection for sex chromosome dosage compensation was predicted by Ohno (1967, cited in Mank 2013). Dosage compensation, though conferred by different mechanisms, was indeed confirmed in placental mammals, fruit fly *D. melanogaster* and worm *Caenorhabditis elegans* (Straub and Becker 2007) as well as in several other species (reviewed by Mank 2013). In *S. latifolia*, it was shown that decrease in expression of Y-linked alleles is compensated by increased expression of X-linked alleles. Thus *de novo* evolution of dosage compensation accompanies the process of sex chromosome differentiation (Muyle *et al.* 2012). However, recent studies suggest a lack of global, i.e. chromosome-wide, dosage compensation in all taxa with female heterogamety studied so far, namely birds (Ellegren *et al.* 2007), trematode worm *Schistosoma mansoni* (Vicoso and Bachtrog 2011), moths (Koike *et al.* 2003,

Zha *et al.* 2009, Harrison *et al.* 2012), and snakes (Vicoso *et al.* 2013a). Why do male and female heterogametic taxa differ in dosage compensation still remains unknown. Whatever the reason is, it is clear that dosage compensation is not general feature of sex chromosomes (Mank 2013).

Yet another consequence of molecular differentiation is meiotic sex chromosome inactivation caused by structural differences between heteromorphic sex chromosomes. Fully differentiated sex chromosomes lack any homology outside their pseudoautosomal regions, where crossing-overs occur to secure proper segregation of sex chromosomes during meiosis (Otto *et al.* 2011). Unpaired loci trigger the so-called meiotic silencing of unsynapsed chromatin, an epigenetic mechanism which ensures transcriptional inactivation of unpaired sequences during first meiotic prophase (Turner 2007). Meiotic sex chromosome inactivation is presumably a manifestation of this general mechanism reported from various organisms (Bean *et al.* 2004, Turner *et al.* 2006, Hense *et al.* 2007, Hornecker *et al.* 2007) including chicken (Schoenmakers *et al.* 2009), which lead to a conclusion that it constitutes an universal characteristic of heteromorphic sex chromosomes (Namekawa and Lee 2009). However, existence of meiotic sex chromosome inactivation has been recently questioned in *D. melanogaster* (Meiklejohn *et al.* 2011, Mikhaylova and Nurminsky 2011, 2012), though it was previously suggested as a driving force behind relocation of testis-expressed genes out of the X chromosome in this species (Vibranovski *et al.* 2009). Furthermore, Guioli *et al.* (2012) found no sign of meiotic sex chromosome inactivation in chicken germ line.

Restriction of recombination obviously represents a milestone in the evolution of sex chromosomes, which dooms sex-limited chromosomes to decay and possibly to an ultimate loss (Graves 2006, Steinemann and Steinemann 2005, Marec *et al.* 2010). But some sex chromosomes can escape their grim fate. There are lineages such as birds or snakes where differentiation of sex chromosomes progressed in some taxa but, for reasons unknown, was halted in others (Mank and Ellegren 2007, Vicoso *et al.* 2013a,b). Extant birds split into two clades, the Paleognathae comprising ratites and tinamous, and the Neognathae comprising all other birds. In neognathous birds such as chicken, highly heteromorphic WZ sex chromosomes evolved by a differentiation process described above (Mank and Ellegren 2007). Despite their well-established homeology with the gonosomes of neognathous birds, paleognathous sex chromosomes differ in the level of their molecular degeneration (Shetty *et al.* 1999, Vicoso *et al.* 2013b). Tinamou sex chromosomes reached an intermediate stage of differentiation (Mank and Ellegren 2007, Tsuda *et al.* 2007), whereas in ratites, e.g. ostriches and emus, sex chromosomes are largely homomorphic. How do ratite sex chromosomes defy the evolutionary forces driving suppression of recombination is not clear. Adolfsson and Ellegren (2013) suggested that inability to develop a dosage compensation mechanism can be responsible for arrested differentiation of ostrich sex chromosomes. Vicoso *et al.* (2013b) analyzed the genome and transcriptome of emu and found that sex-linked loci accumulated genes

with male-biased expression. This expression pattern is consistent with transcription down-regulation of sexually antagonistic alleles in the sex to which they are detrimental and was interpreted as another resolution of sexual conflict caused by accumulation of sexually antagonistic mutations. It was suggested that sex-biased expression can diminish selection for restriction of recombination between sex chromosomes and thus contribute to maintenance of homomorphic sex chromosomes.

Similarly, the representatives of the snake families Boidae and Pythonidae have homomorphic WZ sex chromosomes, which contrast with heteromorphic sex chromosomes observed in other snake lineages such as Viperidae, Elapidae, and Colubridae (Vicoso *et al.* 2013a). In general, homomorphic sex chromosomes seem to be a rule rather than exception in cold-blooded vertebrates (Perrin 2009). Recently, several hypotheses were proposed to explain the lack of sex chromosome differentiation, which occurs even under suppressed recombination. A centerpiece of one group of explanations is rapid turnover of sex chromosomes caused either by translocation of a sex-determining gene or recruitment of an upstream sex-determining factor, which replaces existing sex chromosomes before their degeneration proceeds. The turnover can be driven by sex-ratio selection either optimizing or restoring sex ratios (Grossen *et al.* 2011), sexually antagonistic selection (van Doorn and Kirkpatrick 2007), or deleterious mutation load (Blaser *et al.* 2013).

However, homeologous chromosomes were shown to be sex-linked in three diverged frog genera, namely *Bufo*, *Hyla*, and *Rana*, which suggests that either turnovers are preferentially biased towards some chromosomes (Brelsford *et al.* 2013) or other mechanisms preserves the homomorphic sex chromosomes observed in these frogs (Stöck *et al.* 2011, Rodrigues *et al.* 2013, Stöck *et al.* 2013). Perrin (2009) hypothesized that homomorphic sex chromosomes could be maintained by recombination in XY sex-reversed females. This so-called "fountain of youth" hypothesis is supported by genealogies of sex-linked genes in *Hyla* spp. and *Bufo viridis* subgroup (Stöck *et al.* 2011, Stöck *et al.* 2013).

Finally, even though sex chromosomes pass through the valley of molecular decay and evolutionary death, they are not beyond salvation. Vicoso and Bachtrog (2013) showed that the *Drosophila* heterochromatic dot chromosome matching Muller's element F corresponds to the well differentiated X chromosome common to the dipteran suborder Brachycera. This ancestral sex chromosome reverted back to autosome, while sex determination was taken over by Muller's element A in the brachyceran family Drosophilidae. And the *Drosophila* dot chromosome needs not to be the only case of sex chromosome reversal. In newts *Triturus cristatus* and *T. marmoratus*, a balanced lethal system causing 50 percent embryonic mortality was observed. All adult individuals of both sexes are heteromorphic for the chromosome 1, which harbors recessive deleterious alleles in its non-recombining region. Grossen *et al.* (2012) suggested that chromosome 1 used to be an

ancestral Y chromosome. According to their hypothesis, the balanced lethal system is a result of sex chromosome turnover, which was induced by environmental changes with feminizing effects and subsequently fixed by sex ratio selection.

1.2. Lepidopteran cytogenetics

Moths and butterflies represent a prominent lineage of exclusively herbivorous insects, which plays a crucial role in terrestrial ecosystems and which is of great importance to human society (Grimaldi and Engel 2005, Goldsmith and Marec 2010). As for their chromosomes, they make up a fascinating study object, since they display several cytogenetic peculiarities.

Lepidopteran mitotic complement usually consists of numerous dot-like elements, which are uniform in both shape and size (De Prins and Saitoh 2003, Mediouni *et al.* 2004). Modal haploid chromosome number is n=31 in Lepidoptera and almost identical, n=30, in their sister clade Trichoptera (Suomalainen 1966, De Prins and Saitoh 2003). Based on this comparison, ancestral lepidopteran karyotype was assumed to comprise n=31 chromosomes, which is in agreement with karyological data obtained in basal lepidopteran lineages (Lukhtanov 2000). One of the most profound features of lepidopteran chromosomes is a lack of primary constriction, i.e. centromere (Wolf *et al.* 1997). Microtubules attach to a kinetochore plate covering a relatively large portion, though not all, of the chromosome surface (Gasner and Klemetson 1974, Wolf *et al.* 1992) and sister chromatids separate by parallel disjunction in anaphase (Murakami and Imai 1974). Thus chromosomes of Lepidoptera can be referred to as functionally, yet not fully, holokinetic (Carpenter *et al.* 2005).

Lepidopteran chromosomes lack any morphological landmarks (Mediouni *et al.* 2004, Fuková *et al.* 2005), which disable identification of individual chromosomes. Also banding techniques failed to produce any pattern (Bedo 1984, De Prins and Saitoh 2003), and thus for a long time cytogenetic research was limited to mere chromosome counting in Lepidoptera. Karyotypic analysis was later facilitated by the use of prolonged pachytene chromosomes paired in bivalents, which showed distinct chromomere patterns allowing the identification of some chromosomes including the sex chromosome pair in female oocytes (Traut 1976, Traut and Clarke 1997, Traut and Marec 1997). The use of pachytene chromosomes also enabled silver staining of active nucleoli (Fuková *et al.* 2005).

Introduction of fluorescence *in situ* hybridization (FISH) and its modifications represented an important milestone in lepidopteran cytogenetics. Traut *et al.* (1999) adapted comparative genomic hybridization (CGH) used in diagnostics of tumors for identification of differentiated sex chromosomes (Sahara *et al.* 2003a, Mediouni *et al.* 2004, Fuková *et al.* 2005, Vítková *et al.* 2007, Šíchová *et al.* 2013). Simplification of this method gave rise to genomic *in situ* hybridization (GISH) (Sahara *et al.* 2003b). This method was successfully combined with W-specific probes derived from

bacterial artificial chromosomes (BACs) or insect telomeric probe $(TTAGG)_n$, to study W chromosome aberrations and multiple sex chromosomes, respectively (Sahara *et al.* 2003b, Yoshido *et al.* 2005b). Recently, clusters of major ribosomal RNAs and histone genes have been used to study evolutionary dynamics of lepidopteran karyotypes (Nguyen *et al.* 2010, Šíchová *et al.* 2013).

In 2004, Japanese and Chinese groups made a major breakthrough as they independently released two draft sequences of *Bombyx mori* genome (Mita *et al.* 2004, Xia *et al.* 2004). A following joint effort of The International Silkworm Genome Consortium (2008) finally led to the merging of the two datasets and integration of resulting assembly with BAC-based linkage map (Yamamoto *et al.* 2008). Currently, several other lepidopteran genomes are publicly available (Zhan *et al.* 2011, The *Heliconius* Genome Consortium 2012, Jouraku *et al.* 2013, You *et al.* 2013). Of these, only *Heliconius melpomene* scaffolds were successfully assigned to linkage groups by means of restriction-site-associated DNA (RAD) linkage mapping (The *Heliconius* Genome Consortium 2012). Unfortunately, this positional information is still not available (Ensembl Metazoa release 19 - July 2013). Thus in Lepidoptera, *B. mori* genome is still the only resource, which can be used as a reference in comparative genomic studies relying on gene-based linkage (Yasukochi *et al.* 2006, Pringle *et al.* 2007, Beldade *et al.* 2009, Baxter *et al.* 2011, Van't Hof *et al.* 2013) and physical mapping (Yasukochi *et al.* 2009, Sahara *et al.* 2013). These studies revealed highly conserved synteny of genes and supported haploid chromosome number n=31 as the ancestral karyotype in non-tineoid Ditrysia (Baxter *et al.* 2011, Van't Hof *et al.* 2013, Sahara *et al.* 2013). However, though fine-scale comparisons confirmed conserved synteny of genes in Lepidoptera, they suggested remarkably high rates of intrachromosomal rearrangements disrupting gene order and orientation (d'Alençon *et al.* 2010, Conceição *et al.* 2011).

As already mentioned, moths and butterflies, together with their sister order Trichoptera, represent the largest clade with female heterogamety, in which female sex is determined by heterogametic constitution Z0 or WZ. In textbooks, this sex chromosome system is sometimes also called *Abraxas*, as female heterogamety had been inferred from sex-linked inheritance of a geometrid moth, *Abraxas grossulariata* (reviewed by Traut *et al.* 2007). The lepidopteran W chromosome, if present, is usually highly differentiated and can be easily recognized according to its heterochromatin structure (Marec and Traut 1994, Traut and Marec 1997, Fuková *et al.* 2005, Vítková *et al.* 2007, Šíchová *et al.* 2013). The W chromosome carries virtually no genes (Marec *et al.* 2010) and consists mainly of ubiquitous repetitive sequences such as retrotransposons (Sahara *et al.* 2003b, Abe *et al.* 2005, Fuková *et al.* 2007, Vítková *et al.* 2007, Traut *et al.* 2013) and its multiple copies form a sex specific heterochromatin body, the so-called sex chromatin or W chromatin, in interphase nuclei of somatic cells (Traut and Marec 1996). This characteristic can be hence used as an indirect evidence for the presence of a W chromosome. Sex chromatin was detected in 81 percent of

examined moths and butterflies classified in the clade Ditrysia (Traut and Marec 1996), which comprises about 99 percent of all described lepidopteran species (Roe *et al.* 2010). On the contrary, sex chromatin was missing in trichopteran species (Marec and Novák 1998, Lukhtanov 2000) and representatives of basal lepidopteran lineages (Traut and Marec 1996, Traut and Marec 1997, Lukhtanov 2000). Thus it can be inferred that a common ancestor of Lepidoptera and Trichoptera had a sex determination system Z0/ZZ and the W chromosome occurred later in the lepidopteran evolution, most likely in a common ancestor of sister lineages Ditrysia and Tischerioidea (reviewed by Traut *et al.* 2007, Marec *et al.* 2010, Sahara *et al.* 2012). In some species, the W chromosome was secondarily lost or derived variants with multiple W or Z chromosomes occurred (Traut *et al.* 2007, Marec *et al.* 2010, Sahara *et al.* 2012, Yoshido *et al.* 2005b, 2011; Fig. 1). Unfortunately, notable report by Kawazoe (1987) on karyotypes in primitive moth lineages has been overlooked in the debate over the origin of the lepidopteran W chromosome. Kawazoe examined the karyotype of *Endoclyta sinensis*, which belongs to the superfamily Hepialoidea, one of the most basal groups of Lepidoptera (Kristensen *et al.* 2007). Based on absence of sex chromatin, hepialids were believed to lack the W chromosome (Traut and Marec 1996). However, Kawazoe (1987) clearly documented the presence of the W chromosome in the *E. sinensis* mitotic complement of n=32, which suggests that the W chromosome might have arisen repeatedly in different lineages of Lepidoptera.

Two hypotheses were invoked to explain the evolutionary origin of the lepidopteran W chromosome. According to Traut and Marec (1996), the ancestral Z chromosome fused with an autosome, which limited its homologue to females. Due to the female achiasmatic meiosis and thus the absence of meiotic recombination (Suomalainen 1966, Marec and Traut 1993), the sex limited autosome started to degenerate as predicted by classical model of sex chromosome differentiation (see section 1.1 for details). However, chromosome numbers of basal moths correspond to ancestral karyotype n=31 and thus do not support the fusion hypothesis. An alternative scenario suggests that the W chromosome could have arisen from a supernumerary B chromosome which started to pair with the ancestral Z chromosome (Lukhtanov 2000, cf. Nokkala *et al.* 2003).

2. Outline of research

Neither homeology of the lepidopteran W chromosome nor its evolutionary origin, i.e. whether it evolved from autosome or B chromosome, can be studied directly. W-specific probes inspired by comparative chromosome painting used e.g. in birds (Shetty *et al.* 1999, Itoh and Arnold 2005, Nanda *et al.* 2008) were generated from laser-microdissected sex chromatin (Fuková *et al.* 2007, Vítková *et al.* 2007). However, hybridization of such probes to chromosomes of other closely related species showed a rapid loss of homology of W-linked sequences due to the accelerated molecular divergence of the non-recombining W chromosome (Vítková *et al.* 2007, Yoshido *et al.* 2013). The results thus pointed out to a limited use of this approach in comparative studies of lepidopteran sex chromosomes.

A question of the source of the lepidopteran W chromosome can be however answered through the study of its evolutionary companion, the Z chromosome. Since the Z chromosome recombines in males, its molecular composition is presumably more similar to autosomes than to the W chromosomes (Sahara *et al.* 2012, for alternative view see Bellott *et al.* 2010, Gschwend *et al.* 2012) and should preserve a gene content of autosomes from which the sex chromosomes evolved. Comparison of Z-linked genes across Lepidoptera would thus reveal any prospective chromosome fusion and help to reconstruct the evolutionary history of sex chromosomes of moths and butterflies.

Conserved synteny blocks can be identified by means of comparative mapping of orthologous genes. Vertebrate sex chromosomes were physically mapped by FISH with probes derived from cDNA (e.g. Nanda *et al.* 2000, Matsubara *et al.* 2006, Kawai *et al.* 2007). In Lepidoptera, the same technique was used to study neo-sex chromosomes in populations of *Samia cynthia* (Yoshido *et al.* 2011). However, Vicoso *et al.* (2013a) found inconsistencies between results of such mapping and actual genomic data in snakes.

BAC libraries have been considered useful genomic tools, which supposedly sped up and broadened studies of sex chromosomes in vertebrates (Janes *et al.* 2011). BAC clones are widely used for mapping of single-copy genes (e.g. Itoh *et al.* 2006, Veyrunes *et al.* 2008) as they provide reliable signals due to large insert size ranging from 100 to 200 kbp (Yasukochi *et al.* 2009, Janes *et al.* 2011). Recently, FISH with BAC-derived probes (BAC-FISH) has been introduced to lepidopteran cytogenetics as an efficient approach for karyotyping and comparative genomic studies (Yoshido *et al.* 2005a, Yasukochi *et al.* 2009, Sahara *et al.* 2013).

In the following sections, I present results of comparative mapping of sex-linked genes in the peppered moth, *Biston betularia* (section 3.1) and the codling moth, *Cydia pomonella* (section 3.2), representatives of the advanced superfamily Geometroidea and the basal superfamily Tortricoidea, respectively. I took advantage of BAC libraries available in the two species and constructed the

physical maps of their Z chromosomes. The results revealed a synteny block conserved between Tortricoidea and Obtectomera, which most likely corresponds to the ancestral ditrysian Z chromosome. However, the data also suggests unexpected evolutionary dynamics of the lepidopteran Z chromosomes.

3. Original publications

3.1. Chapter one:

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.

Abstract

We have constructed a linkage map for the peppered moth (*Biston betularia*), the classical ecological genetics model of industrial melanism, aimed both at localizing the network of loci controlling melanism and making inferences about chromosome dynamics. The linkage map, which is based primarily on amplified fragment length polymorphisms (AFLPs) and genes, consists of 31 linkage groups (LGs; consistent with the karyotype). Comparison with the evolutionarily distant *Bombyx mori* suggests that the gene content of chromosomes is highly conserved. Gene order is conserved on the autosomes, but noticeably less so on the Z chromosome, as confirmed by physical mapping using bacterial artificial chromosome fluorescence in situ hybridization (BAC-FISH). Synteny mapping identified three pairs of *B. betularia* LGs (11/29, 23/30 and 24/31) as being orthologous to three *B. mori* chromosomes (11, 23 and 24, respectively). A similar finding in an outgroup moth (*Plutella xylostella*) indicates that the *B. mori* karyotype (n=28) is a phylogenetically derived state resulting from three chromosome fusions. As with other Lepidoptera, the *B. betularia* W chromosome consists largely of repetitive sequence, but exceptionally we found a W homolog of a Z-linked gene (*laminin A*), possibly resulting from ectopic recombination between the sex chromosomes. The *B. betularia* linkage map, featuring the network of known melanization genes, serves as a resource for melanism research in Lepidoptera. Moreover, its close resemblance to the ancestral lepidopteran karyotype (n=31) makes it a useful reference point for reconstructing chromosome dynamic events and ancestral genome architectures. Our study highlights the unusual evolutionary stability of lepidopteran autosomes; in contrast, higher rates of intrachromosomal rearrangements support a special role of the Z chromosome in adaptive evolution and speciation.

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ORIGINAL ARTICLE

Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism

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We have constructed a linkage map for the peppered moth (*Biston betularia*), the classical ecological genetics model of industrial melanism, aimed both at localizing the network of loci controlling melanism and making inferences about chromosome dynamics. The linkage map, which is based primarily on amplified fragment length polymorphisms (AFLPs) and genes, consists of 31 linkage groups (LGs; consistent with the karyotype). Comparison with the evolutionarily distant *Bombyx mori* suggests that the gene content of chromosomes is highly conserved. Gene order is conserved on the autosomes, but noticeably less so on the Z chromosome, as confirmed by physical mapping using bacterial artificial chromosome fluorescence *in situ* hybridization (BAC-FISH). Synteny mapping identified three pairs of *B. betularia* LGs (11/29, 23/30 and 24/31) as being orthologous to three *B. mori* chromosomes (11, 23 and 24, respectively). A similar finding in an outgroup moth (*Plutella xylostella*) indicates that the *B. mori* karyotype ($n=28$) is a phylogenetically derived state resulting from three chromosome fusions. As with other Lepidoptera, the *B. betularia* W chromosome consists largely of repetitive sequence, but exceptionally we found a W homolog of a Z-linked gene (*laminin A*), possibly resulting from ectopic recombination between the sex chromosomes. The *B. betularia* linkage map, featuring the network of known melanization genes, serves as a resource for melanism research in Lepidoptera. Moreover, its close resemblance to the ancestral lepidopteran karyotype ($n=31$) makes it a useful reference point for reconstructing chromosome dynamic events and ancestral genome architectures. Our study highlights the unusual evolutionary stability of lepidopteran autosomes; in contrast, higher rates of intrachromosomal rearrangements support a special role of the Z chromosome in adaptive evolution and speciation.
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INTRODUCTION

Linkage maps have been most frequently constructed for the primary purpose of identifying loci controlling specific phenotypic traits (Sturtevant, 1913); however, genome-wide linkage maps are also an efficient starting point for exploring genome architecture and chromosome dynamics (Beldade *et al.*, 2009), complementing the current revolution in next-generation whole-genome sequencing (Wang *et al.*, 2005). Lepidoptera are an interesting group in this respect, characterized by wide variation in chromosome number (Suomalainen and Brown, 1984; Kandul *et al.*, 2007) around a strong, putatively ancestral mode (haploid $n=31$), encountered in approximately one-third of the species (White, 1973). The dynamism implied by high rates of chromosome fission/fusion contrasts with a highly conserved synteny of genes between chromosomes across distantly related taxa (Pringle *et al.*, 2007; Sahara *et al.*, 2007; Beldade *et al.*, 2009; Yasukochi *et al.*, 2009; d'Alençon *et al.*, 2010; Baxter *et al.*, 2011). This latter feature allows unambiguous identification of orthologous chromosomal regions in different species to reconstruct the events that produced the current pattern of structural diversity.

Lepidopteran linkage maps have been published for 8 species of Lepidoptera, within 4 of the 43 (Van Nieukerken *et al.*, 2011) currently recognized superfamilies. This sample is concentrated on species within the Papilionoidea (*Heliconius* spp. (Jiggins *et al.*, 2005; Kapan *et al.*, 2006; The *Heliconius* Genome Consortium, 2012), *Bicyclus anynana* (Van't Hof *et al.*, 2008; Beldade *et al.*, 2009), *Colias* hybrid (Wang and Porter, 2004), *Papilio* hybrid (Winter and Porter, 2010)) and one other macrolepidopteran, *Bombyx mori* (Bombycoidea) (Yasukochi *et al.*, 2006; Yamamoto *et al.*, 2008). Linkage maps are also available for two other distantly related superfamilies, Pyraloidea (*Ostrinia nubilalis*; Dopman *et al.*, 2004) and Yponomeutoidea (*Plutella xylostella* (Heckel *et al.*, 1999; Baxter *et al.*, 2011)). *Biston betularia*, the peppered moth of industrial melanism fame (Majerus, 1998; Cook, 2003), is a significant addition to this list, representing the first linkage map for the Geometroidea, the second largest superfamily of Lepidoptera (after the Noctuoidea), which includes several major crop pests and ecological models.

A comprehensive reconstruction of lepidopteran chromosome evolution is currently lacking as a result of the wide use of anonymous markers, nonoverlapping panels of annotated markers, taxonomic skew and low number of mapped genomes. Linkage map resolution and genome coverage are also important limiting factors in comparative analyses. High marker number allows accurate comparison of chromosome content, whereas high resolution is required for fine-scale reconstruction of intrachromosomal rearrangements. In practice, linkage map construction involves a tradeoff between marker number and resolution, the latter being fundamentally determined by the

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number of recombinant offspring. A further difficulty arises when attempting to reconstruct the sequence of chromosomal rearrangement events on the basis of a minimal sample of independently derived states, such as *B. anynana* ($n=28$) and *Heliconius melpomene* ($n=21$) versus *B. mori* ($n=28$) (Pringle *et al.*, 2007; Beldade *et al.*, 2009). The addition of linkage maps for species with the ancestral $n=31$, of which there is currently only one (Heckel *et al.*, 1999; Baxter *et al.*, 2011), should eliminate much of this uncertainty.

Lepidopteran sex chromosomes and sex-linked genes have historically been investigated more thoroughly than the autosomes, partly because they are easier to identify. Females are the heterogametic sex in Lepidoptera, with most commonly a W/Z female, Z/Z male system (for deviations see Traut *et al.*, 2007). The sex chromosomes stand out in female pachytene spreads by their heterochromatic appearance and produce hemizygous inheritance patterns. In contrast, lepidopteran autosomes are far less distinct because they are cytogenetically uniform as a result of their holokinetic arrangement (that is, lacking a primary constriction, the centromere; Murakami and Imai, 1974; Carpenter *et al.*, 2005), large number, small size and insusceptibility to banding techniques. The gene composition of the Z chromosome is conserved in Lepidoptera, although not as strongly as some of the autosomes (Beldade *et al.*, 2009; Baxter *et al.*, 2011; Kroemer *et al.*, 2011). A number of genes, such as *kettin*, *tpi*, *6-PGD*, *period*, *apterous* and *Ldh*, have been assigned to the Z chromosomes of various Lepidoptera (Johnson and Turner, 1979; Gotter *et al.*, 1999; Suzuki *et al.*, 1999; Dopman *et al.*, 2004; Narita *et al.*, 2006; Pringle *et al.*, 2007; Putnam *et al.*, 2007; Van't Hof *et al.*, 2008; Kroemer *et al.*, 2011; Yoshido *et al.*, 2011) and are now established lepidopteran Z-genes. The silkworm (*Bombyx mori*) whole-genome sequence assembly (The International Silkworm Genome Consortium, 2008) includes a large number of additional Z-linked genes, many of which have since also been found on the Z chromosome in other Lepidoptera (Beldade *et al.*, 2009; Baxter *et al.*, 2011; Kroemer *et al.*, 2011).

There are very few active genes described on lepidopteran W chromosomes (reviewed in Marec *et al.*, 2010), which is consistent with undetectable transcription activity levels of W chromatin in interphase nuclei of somatic cells (Traut and Scholz, 1978). Not a single gene has been found on the W chromosome in *Heliconius* spp., *B. anynana*, or *P. xylostella*, in spite of comprehensive gene-based surveys (Pringle *et al.*, 2007; Beldade *et al.*, 2009; Baxter *et al.*, 2011). An exceptionally small W chromosome may partly explain this paucity of genes in *B. anynana* (Van't Hof *et al.*, 2008), but there is no such association for the *B. mori* W chromosome that is almost entirely composed of repetitive sequences originating from retrotransposons (Abe *et al.*, 2005). Some records of W-linked genes are disputed, such as the dark morph locus in *Papilio glaucus* (Andolfatto *et al.*, 2003) and an egg size determining gene, *Esd*, in the silkworm (Kawamura, 1988; but see Fujii *et al.*, 2010). A genuine example is the sex determining gene *Fem* in the silkworm, mapped to a narrow region of the W chromosome (Abe *et al.*, 2008) that contains two genes encoding a putative zinc-finger protein; however, its role in sex determination remains to be elucidated (Suzuki, 2010).

Industrial melanism in *B. betularia* is perhaps the best-known example of a rapid adaptive response to environmental change (Cook, 2003), although many other moths, especially geometrids, responded similarly (Lees, 1981). The initial motivation for this linkage map was therefore to find the locus determining the difference between the wild-type (typical) and the black (*carbonaria*) forms. The *carbonaria* locus has been localized to a 200-kb region on the ortholog of *B. mori* chromosome 17 but remains to be pinpointed (Van't Hof *et al.*, 2011). The linkage map presented here, for all the chromosomes, featuring the major genes involved in melanin biosynthesis as well as several other important patterning genes, serves as a frame of reference for melanism research in Lepidoptera, providing an essential basis for unraveling the regulatory cascade emanating from the melanism 'switch' locus. The *B. betularia* map also sheds new insight into the likely chromosomal architecture of the ancestral lepidopteran genome, and into interaction and shared gene content of the Z and W chromosomes.

MATERIALS AND METHODS

Linkage mapping

The linkage map is based on family no. 16, a cross between a typical female and a heterozygous *carbonaria* male (the *carbonaria* allele is dominant) and 73 offspring that was used previously to screen candidate genes (Van't Hof and Saccheri, 2010) and to construct the LG17 map that includes the *carbonaria* locus (Van't Hof *et al.*, 2011). The previously published LG17 map was also based on family no. 8, whereas the additional markers presented here are based on family no. 16 alone. Anonymous amplified fragment length polymorphisms (AFLPs) were used to provide a genome-wide recombination-segregation matrix that allowed the positioning of genes, bacterial artificial chromosomes (BACs), microsatellites and the *carbonaria* locus as a morphological marker. A number of genes in the linkage map were specifically included because of their role in insect melanization or pigment pattern formation, and others to provide annotated anchors, improve coverage in the absence of other markers or target regions of special interest (such as the *carbonaria* chromosome, the Z chromosome and putative *B. mori* fusion chromosomes). The linkage map was generated with Joinmap 4 (Kyazma, Wageningen, The Netherlands) using regression mapping with independence LOD (logarithm of the odds) test to group markers and Haldane's mapping algorithm to position markers using Joinmap's default parameters.

The segregation pattern of individual AFLPs determines their utility as mapping markers. Paternally segregating markers (often named male informative (MI) markers), inherited from a heterozygous father and a homozygous mother, have a 1:1 offspring ratio and can be used for positional mapping based on recombination frequencies between markers. Maternally segregating markers (female informative (FI); mother heterozygous, father homozygous) also have a 1:1 ratio and can be assigned to chromosomes, but cannot be positioned because Lepidoptera females do not recombine (see, for example, Traut, 1977; Nokkala, 1987). Interpretation of 3:1 segregating both informative (BI) dominant AFLP markers (mother and father heterozygous) is problematic in Lepidoptera because heterozygotes cannot be distinguished from the dominant homozygotes in the offspring and, moreover, they include the nonrecombinant maternal component. A number of approaches have been used to deal with these dominant BI markers in Lepidoptera (Jiggins *et al.*, 2005; Kapan *et al.*, 2006; Yasukochi *et al.*, 2006; Van't Hof *et al.*, 2008), each predicted to give a reasonable approximation of the actual marker order. We opted not to compromise the reliability of the linkage map and use only markers that are fully informative in all offspring. Dominant BI markers in marker-poor regions were sequenced and used to isolate BAC clones to obtain codominant polymorphisms for these positions (replacing the original dominant BI AFLP). Dominant BI markers that would only increase marker number but not marker coverage were excluded from the final linkage map.

A small proportion of BI AFLPs were codominant owing to an indel within the amplicons and thus segregated with a 1:2:1 ratio (see, for example, Supplementary Figure S1). These markers still contain the nonrecombinant maternal component, which can be easily identified because the maternal alleles are inherited in fixed chromosome-specific patterns named chromosome prints (Yasukochi, 1998). This chromosome print can be used to remove the maternal component from codominant BI markers, leaving only the fully informative paternal segregation pattern. The same BI 1:2:1→MI 1:1 procedure was used to 'clean up' codominant BI genes and BAC segregation patterns (see, for example, following explanations in Jiggins *et al.*, 2005; Kapan *et al.*, 2006).

The Z-chromosome markers were identified from a segregation pattern defined by AFLP peak presence in all sons and half the daughters. The segregation pattern in these daughters was used to identify Z-linked MI

markers that segregate in both sexes. FI markers, which were exclusively present in daughters, are on the W chromosome, and those that were exclusively present in sons were assigned to the Z chromosome.

Marker development

AFLP markers were generated using a protocol modified from Vos *et al.* (1995). Phenol/chloroform extracted genomic DNA from half a thorax was digested with restriction enzyme combinations *Eco*RI/*Mse*I and *Eco*RI/*Hha*I and ligated to standard *Eco*RI and *Mse*I adapters (Vos *et al.*, 1995) and custom-designed *Hha*I adapters (5′-GACGATGAGTCCTGAACG-3′ + 5′-TTCAGG ACTCAT-3′) in a single reaction. The restriction–ligation reactions were performed for 6 h at 37 °C in 40 µl containing 150 ng genomic DNA, 1.5 units of each restriction enzyme, 1 × NEBuffer (NEB2 for *Mse*I/*Eco*RI and NEB4 for *Hha*I/*Eco*RI), 4 µg bovine serum albumin, 64 units T4 ligase, 1 mM ATP, 10 mM dithiothreitol, 68 nM *Eco* adapter and 612 nM *Mse* adapter or *Hha* adapter. Restriction enzymes, ligase, bovine serum albumin, NEBuffers and dithiothreitol were sourced from New England Biolabs (Ipswich, MA, USA). A PCR pre-amplification (preamp) was performed in a total volume of 15 µl containing 2 µl restriction–ligation product, 200 µM of each dNTP, 0.9 units *AmpliTaq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1 × GeneAmp buffer I, 200 nM *Eco* + A primer, 1.5 µM *Mse* + 1 or *Hha* + 1 primer. The primers have a fixed core and an extension that varies in length and nucleotide composition. The core sequences of the *Mse* and *Eco* primers are as in Vos *et al.* (1995), the *Hha* core sequence is 5′-GATGAGTCCTGAACGC-3′ and '+1' represents single-nucleotide extensions, with C and G used for *Hha* primers and all four nucleotides used for *Mse* primers. The subsequent selective PCR amplification (selamp) used the same reaction mix as preamp except that 1.5 µl diluted preamp product replaced restriction–ligation as the template and the primers used were 100 nM fluorescent *Eco* + 3 and 300 nM *Mse* + 2 or *Hha* + 2. Preamp and selamp thermal cycling profiles are described in Van't Hof *et al.* (2008). The codes of the AFLP markers in Figures 1 and 2 refer to the primers and fragment length, with lowercase m, h and e for *Mse*, *Hha* and *Eco* primer cores, respectively, followed by the extensions in uppercase and the fragment size (for example, mAGeACG178). The AFLP genotypes were separated with an ABI3130XL fragment analyzer and scored in Genotyper 3.6 (Applied Biosystems).

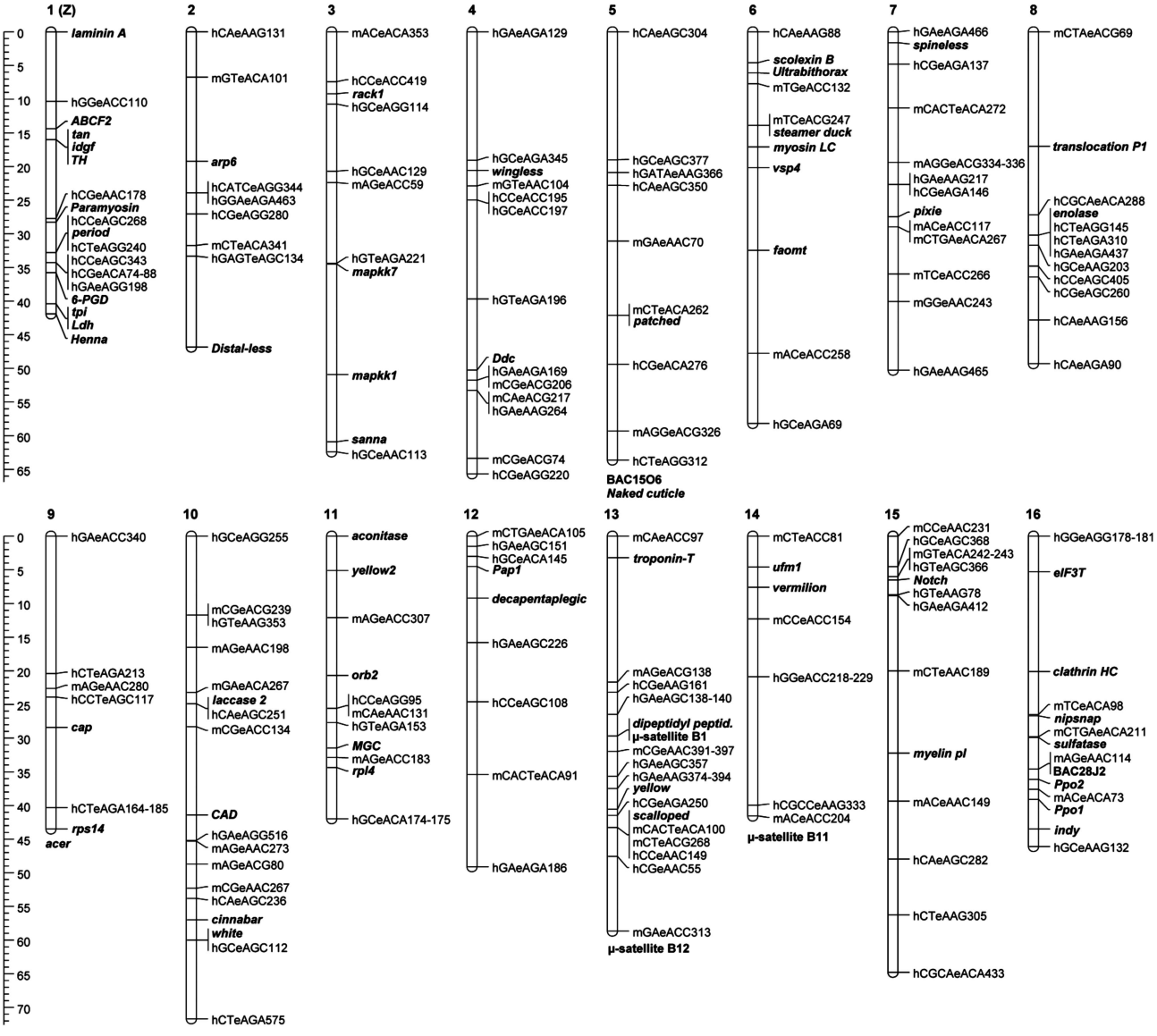

Figure 1 Linkage map of *Biston betularia* chromosomes 1–16. The linkage map is composed of AFLPs, nuclear genes, BACs and microsatellites. AFLP markers are defined by their initial restriction enzymes and selective primer combinations. Nuclear genes are in bold (full gene names are listed in Supplementary Table S1). BACs and microsatellites are also in bold, with BACs named according to library plate position. Assigned but unpositioned (FI) markers are indicated at the base of the LGs.

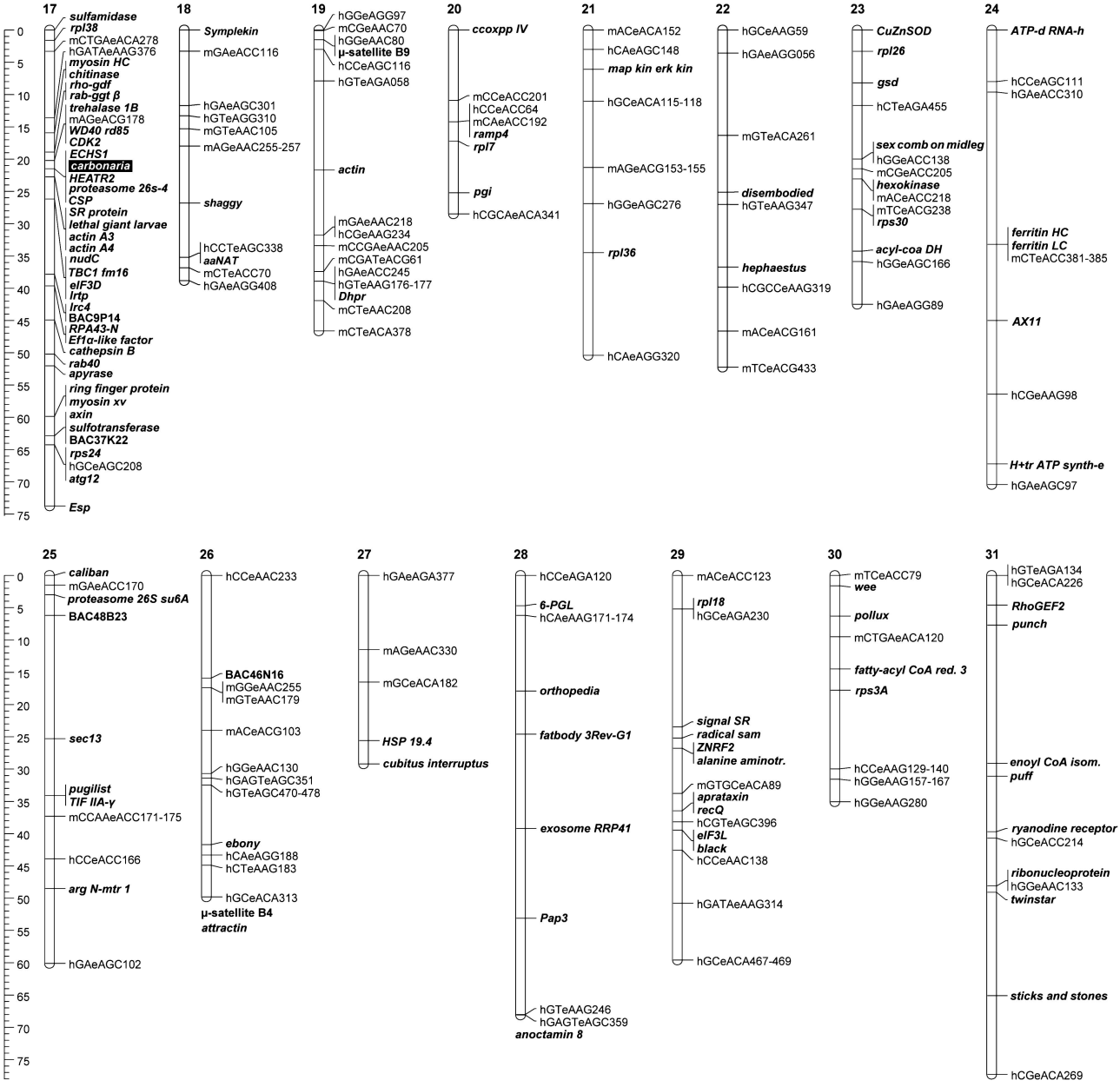

Figure 2 Linkage map of *Biston betularia* chromosomes 17–31. The linkage map has been continued from Figure 1, including the morphological *carbonaria* locus on LG17.

Selective amplification used primers with two selective nucleotides on the '4-cutter' end of the fragment (matching the *Hha* and *Mse* adapters) as the more usual three selective bases generated too low density of fragments in *B. betularia*. A drawback of this strategy was greater variability in marker quality, including weak, inconsistent or overlapping peaks. Such low-quality markers were not included in the final linkage map, but they were screened in a preliminary draft map for their potential to improve linkage map coverage. Low-quality AFLPs that appeared to map to regions with low coverage were made more reliable by extending primers with additional selective bases, which increases peak strength, decreases peak density and differentiates similar sized fragments (Supplementary Figures S1 and S2). Genes were obtained primarily as mRNA-coding sequences either from a transcriptome library (Van't Hof and Saccheri, 2010) or with gene-specific degenerate primers using complementary DNA or genomic DNA as template following the methods described in Van't Hof and Saccheri (2010). All primers were designed specifically for this project, with the exception of *Ddc* and *Pgi* degenerate primers (Regier, 2007) and *Ppo*I_F (Hartzer *et al.*, 2005); the primer sequences are included in Supplementary Table S2. Several gene sequences were characterized from BACs that were isolated with PCR probes derived from mapped and sequenced AFLPs. The segregation patterns of polymorphisms within these BACs were compared against the AFLP segregation to exclude false-positive BACs. Three genes (*enolase*, *CAD* and *period*) were available for *B. betularia* in GenBank (accession numbers in Supplementary Table S2). The polymorphisms used for genotyping genes in the mapping family were usually found within introns, exons or untranslated regions, but occasionally, when such polymorphisms were not available, their segregation patterns were inferred from polymorphisms in end-sequences of BACs that included the gene. The polymorphisms were genotyped either by PCR/restriction fragment length polymorphism (single-nucleotide polymorphisms (SNPs)), separation on agarose gel (large insertion/deletions (indels)) or sequencing (SNPs and small indels).

Rpl36 was also mapped in the butterfly *B. anynana* to investigate the chromosomal origin of this gene. The coding sequence was obtained from expressed sequence tag (EST) sequences deposited in GenBank (Beldade *et al.*, 2006) and an intron (GenBank accession number JF811431) containing a polymorphism was mapped in family 12 of the gene-based linkage map of *B. anynana* (Beldade *et al.*, 2009). The gene was amplified with primer combination: forward 5′-GCGAAAAGGCCACAAAACAACCAAA-3′ and reverse 5′-CCTCGCGGACCAGATCACGGACA-3′, and genotyped by Sanger sequencing.

Gene identity was verified with blastx against GenBank using an e-value threshold of 1e −20, with the higher e-values usually representing relatively short available coding sequence rather than highly diverged orthologous proteins (e-value is not only dependent on similarity, but also on length). To explore shared synteny with *B. mori*, a blastx and tblastx (to cover genes with annotation errors) against SilkDB were used to identify orthologs. Only unambiguous single-copy nuclear genes without known highly or moderately similar paralogs in *B. mori* were used as anchoring genes. Some of the melanization genes are members of gene-families though, and the default best blast hits were tested for being the true ortholog (rather than a paralog) using the stringent criteria described in Van't Hof and Saccheri (2010). The sequences of the genes and BACs are deposited in GenBank with accession numbers listed in Supplementary Table S2. The *B. betularia* microsatellites described in Daly *et al.* (2004) were amplified and sequenced in the parents of the mapping family and screened for polymorphisms. The informative loci were genotyped in the offspring by separation on 2% agarose for μsat-B01 and B09 that have 23-bp and 18-bp indels, respectively, and by Sanger sequencing for the rest.

Cytogenetic procedures

Mitotic and meiotic chromosomes were obtained from testes of mid-late instar male larvae from a cross between a typical Scottish mother and a melanic Austrian father, and from both testes and ovaries of diapausing pupae originating from a cross between a typical female (Scotland) and a typical male (Devon). Spread chromosome preparations were made essentially following the procedure described in Sahara *et al.* (1999). Briefly, gonads were dissected in physiological solution, swollen for 10 min in hypotonic solution (83 mM KCl and 17 mM NaCl), fixed for 15–30 min in Carnoy's fixative (ethanol/chloroform/acetic acid 6:3:1), dissociated in 60% acetic acid and spread on the slide using a heating plate at 45 °C. The preparations were then passed through an ethanol series (70, 80, and 100%; 30 s each) and stored at −80 °C until further use. Shortly before use, selected preparations were removed from the freezer, dehydrated in the graded ethanol series and air-dried. For basic karyotype analysis, the dry preparations were stained with fluorescent dyes, either 0.5 μg ml^{-1} DAPI (4,6-diamino-2-phenylindole; Sigma-Aldrich, St Louis, MO, USA) or 100 nM YOYO-1 (Molecular Probes Inc., Eugene, OR, USA), and mounted in antifade based on DABCO (1,4-diazabicyclo(2.2.2)-octane; Sigma-Aldrich) as described in Van't Hof *et al.* (2008).

BAC probes for fluorescence *in situ* hybridization (FISH) were prepared as follows. BAC-DNA was extracted with the BACMAX DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), amplified using illustra GenomiPhi HY DNA Amplification Kit (GE Healthcare Life Sciences, Milwaukee, WI, USA), and labeled with a fluorochrome, either Amersham Cy3-dUTP (GE Healthcare UK Ltd, Buckinghamshire, UK), Alexa Fluor 488-dUTP (Molecular Probes Inc., Eugene, OR, USA) or Green-dUTP (Abbott Molecular Inc., Des Plaines, IL, USA), using a Nick Translation Mix (Roche Diagnostics GmbH, Mannheim, Germany). Two-color BAC-FISH was carried out following the procedure described in Sahara *et al.* (2003) with some modifications. The probe cocktail for one slide (10 μl; 50% deionised formamide and 10% dextran sulphate in 2 × SSC buffer) contained 160–450 ng of each labeled BAC probe, 3–5 μg of unlabeled sonicated genomic DNA (extracted from *B. betularia* male by standard phenol/chloroform procedure) used as a species-specific competitor and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich). For BAC-FISH mapping of 10 genes on the Z chromosome, we used a protocol for reprobing described in (Shibata *et al.*, 2009). In this case, three different BAC probes were mixed in each cocktail as follows: (1) 160 ng Cy3-labeled BAC, (2) 400 ng Green-labeled BAC and (3) 600 ng two-color labeled BAC (200 ng Cy3 labeled and 400 ng Green labeled). Three BAC probes were hybridized to chromosomes in the first FISH round, and then the preparation was twice reprobed always with three other BAC probes and once reprobed with the last BAC probe. In each BAC-FISH experiment, the probe cocktail was denatured at 95 °C for 5 min. Denaturation of chromosomes was done at 68 °C for 3.5 min in 70% formamide in 2 × SSC. The preparations were hybridized with the probe cocktail for 3 days at 37 °C. The slides were then washed for 5 min in 0.1 × SSC containing 1% Triton X-100 at 62 °C and for 5 min in phosphate-buffered saline containing 1% Triton X-100 at room temperature. The chromosomes were counterstained with 0.5 μg ml^{-1} of DAPI and mounted in antifade as described in Van't Hof *et al.* (2008).

Chromosome preparations were observed in a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) equipped with appropriate fluorescence filter sets. Black-and-white images were recorded with a cooled F-View CCD camera and captured separately for each fluorescent dye with AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany). In BAC-FISH preparations, the images were pseudocolored (light blue for DAPI, green for Alexa Fluor 488-dUTP or Green-dUTP and red for Cy3-dUTP) and superimposed with Adobe Photoshop CS4, version 11.0 (Adobe Systems Inc., San Jose, CA, USA).

To determine the sex chromatin status and thus infer the sex chromosome system in *B. betularia* (see Traut and Marec, 1996), preparations of polyploid nuclei were made from Malpighian tubules of the last instar female and male larvae. The tubules were dissected in physiological solution, briefly fixed in Carnoy's fixative and then stained for 3–5 min with 1.5% lactic acetic orcein. After mounting in the staining solution the preparations were inspected in a light microscope.

RESULTS

Cytogenetic characteristics

The chromosome number was determined by analysis of several tens of mitotic and meiotic chromosome complements on spread preparations from testes. Mitotic spermatogonia showed 62 chromosomes of a holokinetic type (Supplementary Figure S3a). The chromosomes were mostly rod shaped, ∼1–2 μm large, and lacked any morphological signs including primary constrictions (the centromeres) that would allow their identification. In metaphase I spermatocytes, 31 meiotic bivalents were observed (Supplementary Figure S3b). This number was confirmed by counting pachytene bivalents in both sexes (Supplementary Figures S3c and d). Thus, our investigation established that the karyotype of *Biston betularia* consists of $2n = 62$ chromosomes and that the expected number of linkage groups (LGs; that is, the haploid chromosome number) is 31.

Preparations of the Malpighian tubules from larvae of both sexes showed large lobed nuclei. In females, each nucleus displayed a conspicuous spherical heterochromatin body (Supplementary Figure S3e), representing the so-called sex chromatin (W chromatin), which is a characteristic trait in females of the majority of advanced Lepidoptera including Geometridae (Traut and Marec, 1996; Traut *et al.*, 2007). In contrast, somatic polyploid nuclei of males displayed a uniform texture of chromatin grains without any heterochromatin (Supplementary Figure S3f). The presence of sex chromatin in females and its absence in males indicated that *B. betularia* has a WZ/ZZ (female/male) sex chromosome system. In keeping with the sex chromatin status, preparations of pachytene oocytes without a hypotonic treatment showed a WZ bivalent that was discernible from the DAPI-highlighted thread of the W chromosome (Supplementary Figure S3d). However, autosomal bivalents each displayed a regular chromomere pattern that is typical for the synapsis of homologous chromosomes (Traut *et al.*, 2007). In most pachytene oocyte complements seen, the WZ bivalent was one of the longest bivalents. The DAPI-positive staining of the whole W thread indicated that this

chromosome is largely composed of A/T-rich heterochromatin as found in several other lepidopteran species (Fuková *et al.*, 2005; Marec *et al.*, 2010). It should be noted that *B. betularia* is the first geometrid species in which the sex chromosomes have been identified (cf. Table 1 in Traut *et al.*, 2007).

The linkage map

The map (Figures 1 and 2) is defined by 227 AFLPs (17 of which are 1:2:1 segregating codominant markers, the remainder being paternally 1:1 segregating backcross markers), 156 genes, 6 BACs, 5 microsatellites and the *carbonaria* phenotype, included as a morphological backcross marker. The ortholog of *B. mori kettin* was positioned on the Z chromosome by means of BAC-FISH only, as no polymorphisms were available in the mapping family (making a total of 157 genes).

The *carbonaria* morph maps to chromosome 17 (Van't Hof *et al.*, 2011). Interestingly, none of the major genes known to be involved in insect melanization (*aaNat*, *black*, *Ddc*, *Dhpr*, *ebony*, *ferritinLC*, *ferritinHC*, *henna*, *laccase2*, *punch*, *Pap1*, *Pap3*, *Ppo1*, *Ppo2*, *tan*, *TH*, *yellow*, *yellow2*; see Supplementary Table S1 for full names) map to this chromosome. This confirms earlier findings, based on cosegregation, of a lack of association between genetic variation in these genes and the *carbonaria* morph (Van't Hof and Saccheri, 2010). In addition to the melanization candidates, *vermilion, cinnabar*, *white*, *cubitus interruptus*, *decapentaplegic*, *patched*, *Ultrabithorax*, *wingless*, *Distal-less*, *naked cuticle*, *Notch* and *scalloped* were specifically mapped because of their role in lepidopteran pigment patterning (Carroll *et al.*, 1994; Keys *et al.*, 1999; Weatherbee *et al.*, 1999; Beldade *et al.*, 2002; McMillan *et al.*, 2002; Reed and Serfas, 2004; Reed and Nagy, 2005), but none of these candidates maps to chromosome 17 either.

Consistent with the karyotype, the maternal backcross markers define 31 LGs with the reliability of marker assignment supported by the maximum possible independence test LOD of 10, and (independently of FI markers) paternally segregating markers also separate into 31 LGs with LOD $\geqslant 7$ (average LOD = 9.35). These LOD values are high because (1) low-density regions were specifically targeted with additional genes predicted to fill gaps based on shared synteny with *B. mori*; (2) 3:1 dominant markers that can diminish the reliability of marker order were excluded; and (3) for AFLP, which typically generate markers with a wide quality range, only those that produced unambiguous genotypes were included. Manual inspection of the recombinants per chromosome ('graphical genotypes' in JoinMap 4, Supplementary Table S2) show marker orders that are consistent with the predictions of the mapping algorithm, most positions being defined by single recombinations per chromosome and a small fraction of double recombinations. The effect of missing values on the linkage map is negligible because only 221 genotypes out of $\sim$28 000 ($<$1%) remained unscored. A few genotypes suggesting short-range double recombinations (12 out of $\sim$28 000) were presumed to be genotyping errors because nearby crossovers are inconsistent with rules of interference (Muller, 1916). The low number of inconsistencies reflects the stringent marker quality threshold that was used.

The total map length is 1638 cM, with LG size ranging from 28.5 cM (LG20) to 77.2 cM (LG31) and averaging 52.9 cM. Variation in recombinational length estimates among LGs reflects differences in the physical size of the chromosomes, but is also influenced by chromosome-specific recombination frequencies, and by the degree of coverage. The pachytene spreads (Supplementary Figures S3c and d) suggest that there is less variation in chromosome size than implied by LGs, reflecting incomplete coverage for several chromosomes (that is, some chromosomes may not have markers near one or both telomeric regions). The total recombinational length estimate should therefore be taken as an underestimate, but implies that, on average, a cM is equivalent to $\sim$250–300 kb, assuming a genome size of 500 Mb, the average for geometrid moths (Gregory and Hebert, 2003).

The previously published map of LG17, which includes the *carbonaria* locus (Van't Hof *et al.*, 2011), has been supplemented with three additional genes, including *epidermal stripes and patches* (*Esp*), which extends the LG at the 'lower' end. This is the gene closest to the 'bottom' end of *B. mori* chromosome 17 and, based on shared synteny, *Esp* is assumed to also mark the lower end in *B. betularia.* With *sulfamidase* close to the other end in *B. betularia* (demonstrated with BAC-FISH in Van't Hof *et al.*, 2011), LG17 is expected to be fully covered.

Conserved synteny (autosomes)

Every chromosome in the linkage map is anchored with two or more single-copy nuclear genes, allowing presumptive orthologous links to be made to each of the 28 chromosomes in *Bombyx mori*, the least taxonomically distant reference genome currently available (Xia *et al.*, 2004) (Supplementary Table S1). In all, 25 *B. betularia* LGs were numbered according to *B. mori* single-chromosome orthologs. The six *B. betularia* LGs with orthologous links to the three remaining *B. mori* chromosomes follow the numbering scheme used for *P. xylostella* (Baxter *et al.*, 2011), which also possesses 31 chromosomes. Comparison of annotated anchoring markers with the positions of their orthologs in *B. mori* demonstrates that genes are occasionally rearranged within a chromosome, but rarely translocated to other chromosomes. Although the density of genes across the linkage map is in general too sparse to allow extensive analysis of synteny, chromosomes with two mapped genes can be used to verify co-occurrence and those with three or more can reveal gene order.

Genes that are on the same LG in *B. betularia* are always grouped together on the same chromosome in *B. mori* except for *rpl36* (*B. betularia* LG21) and *recQ* (*B. betularia* LG29) that are located on *B. mori* chromosomes 1 (Z) and 17, respectively. *Rpl36* also mapped to the LG21 ortholog of *B. anynana* (Bany LG21), which implies that this gene translocated to the Z chromosome in *B. mori*, rather than away from it in both *B. anynana* and *B. betularia*. Conversely, the location of *recQ* is similar in *B. mori* chromosome 17 and its *H. melpomene* ortholog (Ferguson *et al.*, 2010), suggesting that *recQ* is relocated in *B. betularia* relative to its ancestral position. The position of the melanization gene *punch* is ambiguous in the silkworm genome assembly with the 5′-untranslated region and part of the coding sequence of the GenBank deposited sequence on chromosome 24 and the remainder on chromosome 13. An attempt to map *punch* in *B. mori* was unsuccessful (Futahashi *et al.*, 2008), but we nevertheless assume the sequence on chromosome 24 to be in the correct position, implying that *B. mori* and *B. betularia* share synteny for this gene. In total, only 2 out of 157 mapped genes reside on different chromosome orthologs between these two species (Supplementary Table S1).

Of the 17 LGs with three or more genes mapped, 12 have gene orders that are identical to *B. mori*. This includes the more densely mapped '*carbonaria* chromosome', LG17, as described in Van't Hof *et al.* (2011). The exceptions are LG1 (Z), LG11, LG16, LG28 and LG29, in which several genes are rearranged by means of inversions such as (*PpoI*, *PpoII*, *indy*) and (*eIF3T*, *nipsnap*, *sulfatase*) on LG16 (Supplementary Figure S4) and the upper half of LG29 (Figure 3). Other intrachromosomal rearrangements are caused by transpositions, as demonstrated by *clathrin HC* on LG16 (Supplementary Figure S4) and *orb2* on LG11 (Figure 3).

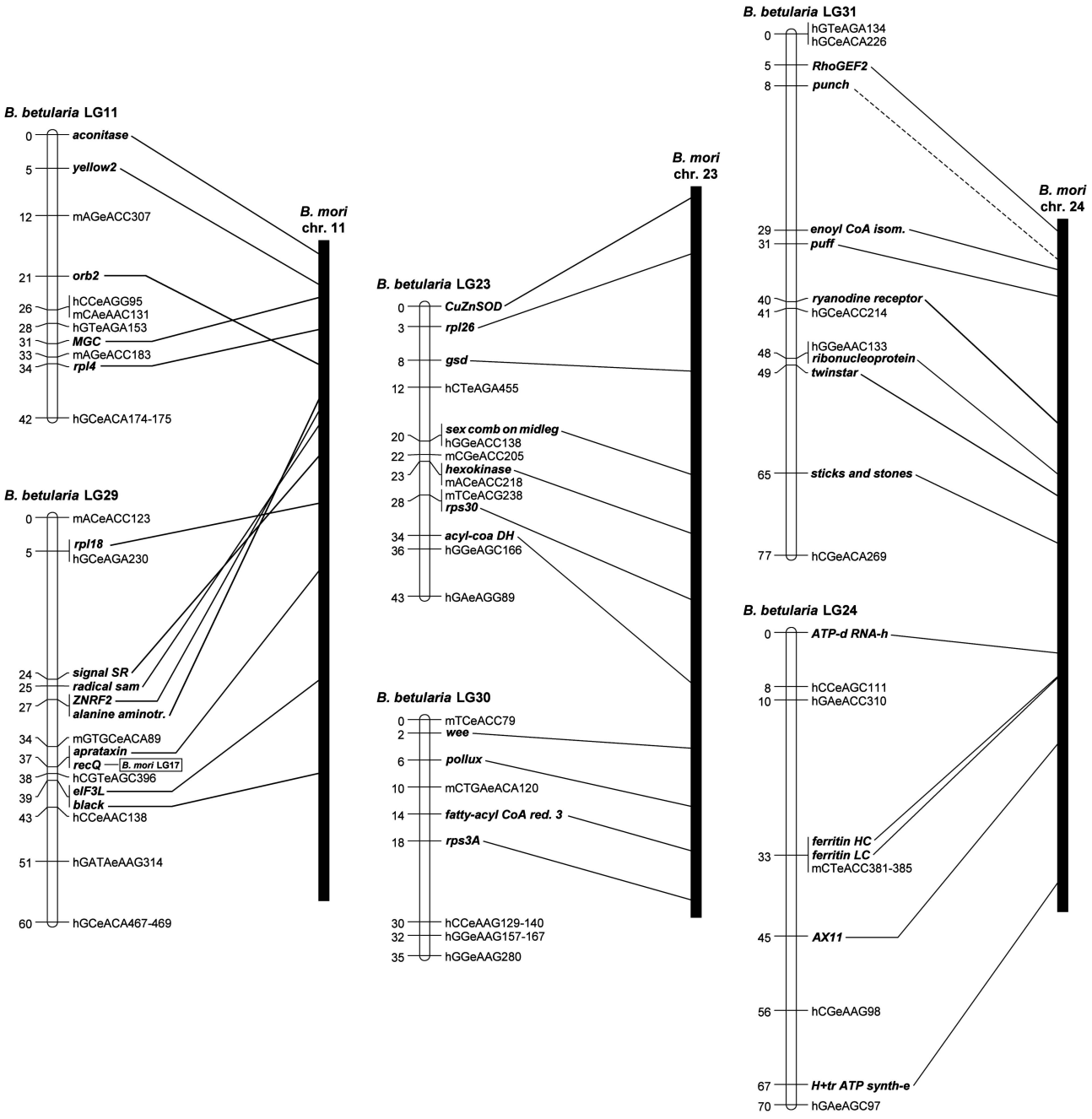

Figure 3 Putatively fused chromosomes in *B. mori*. Six *B. betularia* LGs share gene content with distinct sections on three *B. mori* chromosomes. This suggests three fusion events in *B. mori* that would account for the haploid chromosome number reduction from the presumed ancestral $n=31$ to $n=28$. The gene order between *B. betularia* LG11 and its orthologous region in *B. mori* is rearranged such that the upper half of *B. betularia* LG29 is inversed relative to *B. mori*. The remaining LGs (23, 24, 30 and 31) have gene orders identical to *B. mori*. *Punch* is linked with an interrupted line because part of the gene is assigned to a different chromosome in the *B. mori* genome sequence, presumably because of a sequence assembly error.

B. mori chromosome fusions

As *B. mori* has three fewer autosomes than *B. betularia* (28 vs 31) we expected to find genes that are linked in *B. mori* to occur on different LGs in *B. betularia*. Moreover, as 31 is considered to be the ancestral chromosome number for Lepidoptera (Suomalainen, 1969; Lukhtanov, 2000), these patterns suggest fusions between specific ancestral chromosomes. *B. betularia* LGs 11/29, 23/30 and 24/31 had links to *B. mori* chromosomes 11, 23 and 24, respectively. These six LGs were targeted with additional genes to test the initial evidence for three chromosome fusions in the ancestors of *B. mori*, and to determine the orientation and approximate position of the fusion points (Figure 3). The links between orthologous genes clearly demonstrate how orthologous regions of the two species match, although *B. betularia* LG11 and LG29 experienced a transposition and an inversion, respectively.

Sex chromosomes

The Z-chromosome map (LG1) is defined by 11 anchoring orthologs of *B. mori* Z-linked genes, which include 3 melanization genes

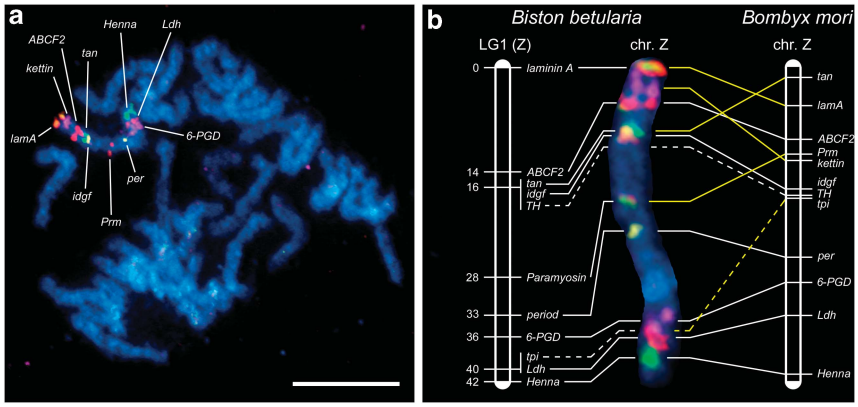

Figure 4 BAC-FISH and linkage mapping of the Z chromosome in *B. betularia*. Meiotic chromosomes from males were counterstained with DAPI (light blue). The BAC probes were labeled with Cy3-dUTP, Green-dUTP and with a combination of both Cy3-dUTP and Green-dUTP, respectively. Hybridization signals indicate the physical positions of the 10 loci: *laminin A* (orange), *kettin* (violet), *ABCF2* (red), *tan* (green), *idgf* (yellowish to orange), *Paramyosin* (red and green), *period* (yellow), *6-PGD* (violet), *Ldh* (red) and *Henna* (green). (**a**) Pachytene spermatocyte complement with the ZZ bivalent identified by hybridization signals of 10 Z-linked gene-containing BAC probes. Scale bar = 10 μm. (**b**) Chromosomal localization and synteny of 10 Z-linked loci between *B. betularia* and *B. mori* Z chromosomes. The recombinational positions of genes mapped to *B. betularia* linkage group 1 (LG1; left-hand vertical bar) are related to their physical positions in the BAC-FISH-painted Z chromosome of *B. betularia* (middle image) and in a scheme of the *B. mori* Z chromosome (right-hand vertical bar) by interconnecting lines; white lines indicate preserved gene order and yellow lines rearranged gene order between the two species.

(*Henna*, *tan* and *TH*), 3 other enzyme-coding genes (*6-PGD*, *Ldh* and *tpi*) and 5 protein-coding genes without enzymatic function (*ABCF2*, *idgf*, *laminin A*, *Paramyosin* and *period*). The gene *kettin*, encoding insect muscle protein, was screened for nearly 10 kb of genomic sequence, but did not contain polymorphisms in the mapping family and was positioned by means of BAC-FISH instead (Figure 4).

Out of 13 Z-linked *B. mori* gene orthologs, 12 mapped to the Z chromosome of *B. betularia* (Figures 4a and b), with *rpl36* being autosomal in *B. betularia*. However, although Z linkage of most genes is conserved between these species, gene order within the Z chromosome differs in some respect. Comparison of the Z-chromosome map of *B. betularia*, constructed by linkage and BAC-FISH mapping, with the physical map of *B. mori* Z chromosome (Figure 4b) revealed conserved gene order in a Z-chromosome segment between the *period* and *Henna* loci (that is, *period*, *6-PGD*, *Ldh* and *Henna*), except the *tpi* locus that is positioned near *Ldh* in a subterminal region of the *B. betularia* Z, but located in the middle part of the *B. mori* Z. In contrast, the upper half of the Z chromosome showed altered order of four genes, namely *laminin A*, *kettin*, *tan* and *Paramyosin*. The observed pattern of divergence can be hypothetically accounted for by two inversions in the upper part of the chromosome and two transpositions (Supplementary Figure S5).

Distances between some genes differ considerably in the linkage and physical (BAC-FISH) maps of the *B. betularia* Z chromosome (Figure 4b). In particular, the *ABCF2* locus is separated from the terminal *laminin A* locus by a large gap covering one-third of the LG, whereas BAC-FISH positions these loci relatively close to each other. In contrast, the physical distance between the *period* and *6-PGD* loci is much larger than the cM distance. The discrepancies between physical and cM distances suggest a recombination hot spot near the upper end of the Z chromosome and a reduced recombination region at the lower end.

Exceptionally for a Z-linked gene, *laminin A* also occurs on the W chromosome, as revealed by the observation that an exonic SNP within the gene was heterozygous A/C (not hemizygous) in both the mother and father. The same heterozygous genotype was shared by approximately half of the sons, the other half being homozygous for their mother's Z allele, whereas (WZ) daughters were either heterozygous or homozygous for the alternate allele (Table 1). Additionally, the *laminin A* alleles cosegregated with the Z-linked markers at the 'upper' end of the chromosome. This pattern of inheritance is only possible for a Z-linked/W-linked locus. Full-length gene identity and functionality of the W representative remain to be confirmed but the available sequence lacks characteristics of a pseudogene; in particular, the W and Z copies are identical over the full length of a 566-bp PCR fragment apart from the synonymous SNP (that is, identical amino-acid sequence) and there are no frameshifts or premature stop codons relative to the *B. mori* sequence. Moreover, the observation that the W-linked SNP allele also occurs on the Z chromosome highlights that there is no unique feature in the W sequence.

Table 1 Genotype frequencies of the *laminin A* WZ single-nucleotide polymorphism (SNP) in sons and daughters inherited from mother A_ZC_W × father A_ZC_Z

Genotype	*Sons (ZZ)*	*Daughters (WZ)*
A/A	17 (A_ZA_Z)	0
A/C	17 (A_ZC_Z)	17 (A_ZC_W)
C/C	0	22 (C_ZC_W)

Four BAC clones containing *laminin A* sequence were identified and used as probes for BAC-FISH to verify the presence of the gene on the W chromosome. Each BAC clone provided a different hybridization pattern on spread chromosomes of pachytene oocytes (Figures 5a–f). The clone 2I7 hybridized to the ends of both the W and Z chromosomes (Figures 5a and b), indicating remnants of sequence homology at one end of both sex chromosomes. Two clones provided a discrete hybridization signal at the end of one sex chromosome only, either Z (clone 1A21) or W (clone 8M12) (Figures 5e and f). This suggests that clone 1A21 is derived from the Z chromosome, whereas clone 8M12 originates from the W chromosome. It should be noted that in the two BAC-FISH experiments, pairing orientation of the sex chromosomes in the

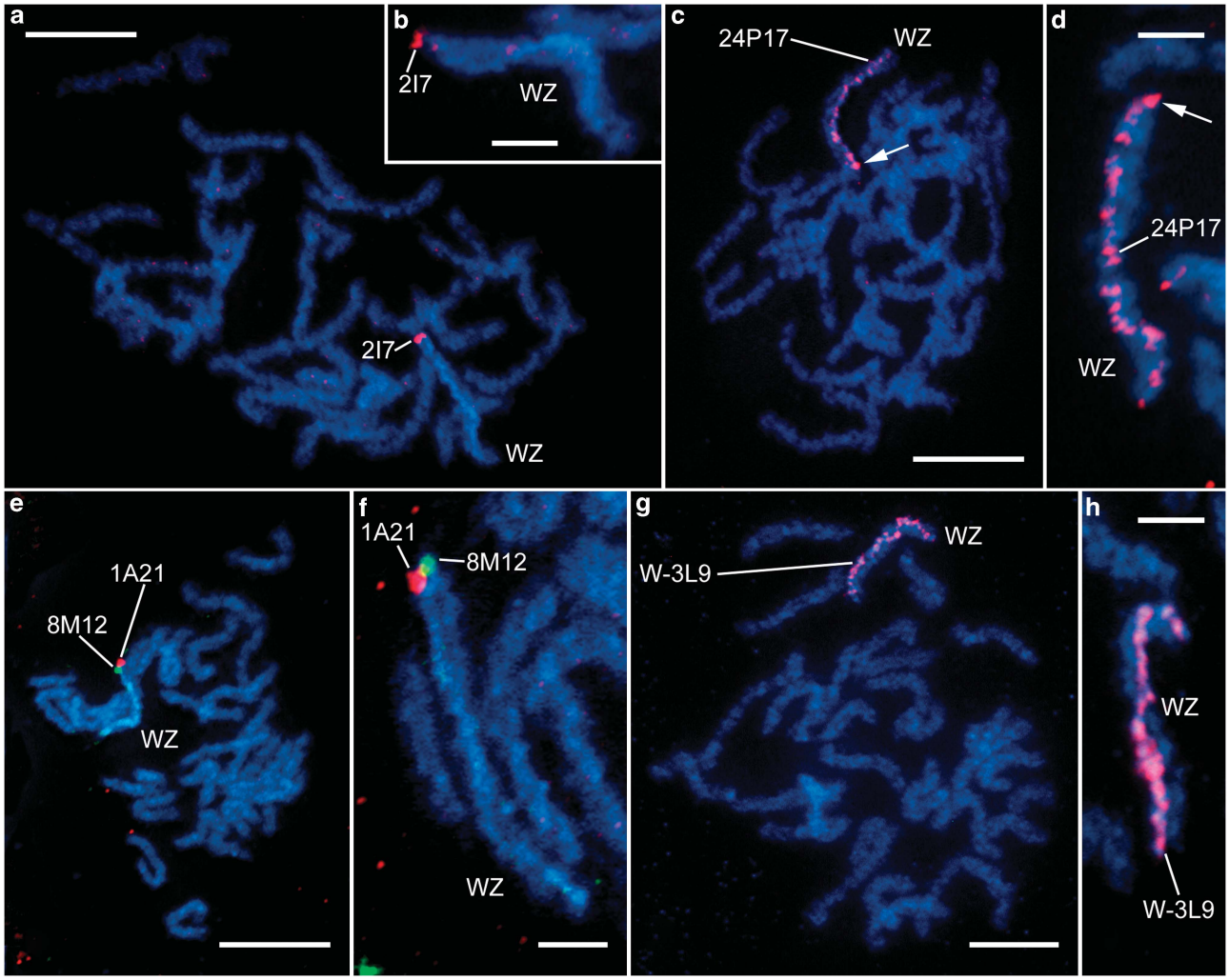

Figure 5 BAC-FISH mapping on both sex chromosomes (W and Z) in female *B. betularia*. Hybridization patterns of four laminin *A*-containing clones and one W-linked AFLP marker-derived clone on W and Z chromosomes. (**a**, **c**, **e**, **g**) Pachytene oocyte complements. (**b**, **d**, **f**, **h**) Details of WZ bivalents. Chromosomes were counterstained with DAPI (light blue); BAC probes were labeled with Cy3-dUTP (red) or Alexa Fluor 488-dUTP (green). The sex chromosome bivalent (WZ) is discernible from a DAPI-positive heterochromatic thread of the W chromosome and also by hybridization signals of the BAC probes. (**a**, **b**) *laminin A* BAC probe 2I7 (red) shows hybridization signals at the end of both the W and Z chromosomes. (**c**, **d**) *laminin A* BAC probe 24P17 (red) shows scattered hybridization signals along the whole W-chromosome thread; a stronger signal at one end of the W chromosome (arrow) possibly indicates a physical position of the BAC clone. (**e**, **f**) *laminin A* BAC probes 1A21 (red) and 8M12 (green) indicate physical positions of *laminin A* at the ends of the Z and W chromosomes, respectively. (**g**, **h**) AFLP-derived BAC probe W-39L (red) paints the entire W-chromosome thread. Scale bars = 10 μm (**a**, **c**, **e**, **g**) or 3 μm (**b**, **d**, **f**, **h**).

WZ bivalent was identical in all pachytene complements examined, with either two 2I7 signals or 1A21 plus 8M12 signals always at one end of the bivalent, which suggests specific pairing. Unlike the other *laminin A*-containing clones, which showed discrete signals, the clone 24P17 painted the whole W chromosome in a scattered manner (Figures 5c and d). This suggests that this particular clone is rich in repetitive sequences spread throughout the W chromosome. For a comparison, we also mapped two W-derived BAC clones, W-3L9 and 38L11, identified with W-specific AFLP markers. The clone W-3L9 showed uniform, almost continuous painting of the whole W chromosome (Figures 5g and h), whereas clone 38L11 showed scattered signals on the whole W chromosome (not shown), similar to the *laminin A*-containing clone 24P17.

One end sequence of BAC 8M12 contains two additional *laminin A* exons (and four introns) upstream of the exon used to map family no. 16. The mother of family no. 67, whose daughters were used for BAC construction, has a FI SNP in one of these introns. One of the alleles is inherited by family no. 67 daughters exclusively (11 daughters, 16 sons), allowing unambiguous assignment of BACs 8M12 and 24P17 as W derived and 1A21 and 2I7 as Z derived, respectively, confirming the BAC-FISH results. The presence of additional *laminin A* exons on a W-BAC (8M12) suggests that the W chromosome contains a substantial section of the *laminin A* gene. Obtaining full sequence from W-BACs would reveal whether the W representative of *laminin A* is complete and intact, but is not yet available owing to the large genomic size of the gene (66 and 21 kb in *B. mori* and *Danaus plexippus*, respectively).

DISCUSSION

In the age of whole-genome sequence assembly, linkage mapping remains an important tool for analyzing the genesis of genome architecture. The *Biston betularia* linkage map, the first for a

geometrid moth, illustrates the value of broadening the taxonomic coverage to reconstructing chromosome dynamics (inversion, transposition, fission and fusion). Such a phylogenetic perspective is necessary to distinguish ancestral from derived states, but has until recently been limited by the availability of a single lepidopteran (*B. mori*) whole-genome reference sequence or comprehensive linkage map against which to make comparisons. (Two additional lepidopteran whole genomes are now available for *H. melpomene* (The *Heliconius* Genome Consortium, 2012) and *D. plexippus* (Zhan *et al.*, 2011), although the latter's scaffolds are not assigned to chromosomes). A case in point is the reconstruction of the direction of a translocation event that led to *rpl36* being Z linked in *B. mori* but autosomal in *Ostrinia nubilalis*. On the basis of pairwise comparison, Kroemer *et al.* (2011) suggested that Z linkage is the ancestral state. However, a three-way comparison of the position of *rpl36* between *B. mori* (Z), *B. betularia* (LG21) and *B. anynana* (mapped for this study to the ortholog of *B. mori* chromosome 21) provides stronger support for the alternative hypothesis. In contrast, the same approach, using *B. mori*, *B. betularia* and *Heliconius melpomene* (Ferguson *et al.*, 2010), applied to *recQ*, the only other gene (out of 157 mapped genes) whose LG differed between *B. betularia* and *B. mori*, implies that the position in *B. mori* is the ancestral state.

Phylogenetically informed reconstruction is also essential for properly accounting for differences in chromosome number among species by means of fissions or fusions. In this respect, *B. mori* is not an ideal reference for Lepidoptera, having three fewer chromosomes than the accepted ancestral 31 (Suomalainen, 1969; Lukhtanov, 2000). The problem of reconstructing the sequence of fission/fusion events is compounded in comparisons between independently derived chromosome complements, such as between *B. mori* and *B. anynana* (Beldade *et al.*, 2009) or *B. mori* and *H. melpomene* (Pringle *et al.*, 2007). We have established that *B. betularia* has 31 chromosomes, and that three pairs of these chromosomes are orthologous to three *B. mori* chromosomes (*Bb*LG11/29-*Bm*11; *Bb*LG23/30-*Bm*23; and *Bb*LG24/31-*Bm*24). This pattern is entirely consistent with the RAD (restriction site associated DNA)-based, $n = 31$, linkage map for *P. xylostella* (Baxter *et al.*, 2011), but is additionally based on gene order and not just content. Moreover, the large phylogenetic distance between *B. betularia* and *P. xylostella* (*P. xylostella* is a distant outgroup to *B. betularia* and *B. mori*) effectively establishes that the difference in chromosome number is the result of three fusions in the *B. mori* lineage, rather than the same three chromosome fissions, independently, in the *B. betularia* and *P. xylostella* lineages. A recent comparison between *H. melpomene* and *B. mori* genomes arrived at the same conclusion (The *Heliconius* Genome Consortium, 2012).

The *B. betularia* linkage map provides further evidence for deep conservation of synteny and gene order within the Lepidoptera. The degree of shared synteny among representatives of the Lepidoptera, currently all within the infraorder Heteroneura (diverged ~160 Myr ago; Labandeira *et al.*, 1994), is comparable to that found within the single, 65-Myr-old genus *Drosophila* (Singh *et al.*, 2009). By comparison, the genomes of two distantly related dipterans, *Anopheles gambiae* and *Drosophila melanogaster*, have been extensively reshuffled during 250 Myr of divergence (Bolshakov *et al.*, 2002). The chromosome scale patterns in Lepidoptera may mask higher rates of gene order rearrangement at the finest scale (that is, neighboring genes), as revealed by interspecific comparisons of contiguous sequence (d'Alençon *et al.*, 2010; Conceição *et al.*, 2011), possibly associated with holocentrism and transposable elements.

According to the minimum-interaction hypothesis, karyotypes consisting of high number of small elements, as is typical for Lepidoptera, suffer fewer deleterious rearrangements such as translocations because the likelihood of interaction among chromosomes during meiosis is reduced (Imai *et al.*, 1986; Hirai *et al.*, 1994). The centromere-drive hypothesis (nonrandom segregation based on centromere activity differences in asymmetric female meiosis) was formulated to explain karyotype evolution in mammals (Pardo-Manuel de Villena and Sapienza, 2001). It has been speculated that holokinetic chromosomes evolved as suppressors of meiotic drive (Talbert *et al.*, 2009; Zedek and Bureš, 2012). Thus, the holokinetic structure of lepidopteran chromosomes might contribute to their evolutionary stability. This is a paradox because holokinetic chromosomes are expected to facilitate karyotype evolution via fusion and fission by reducing the risk of the formation of dicentric and/or acentric chromosomes (Wrensch *et al.*, 1994; Carpenter *et al.*, 2005). These hypotheses suggest that small and numerous holokinetic chromosomes predispose lepidopteran genomes to structural stasis.

The *B. betularia* Z map extends previous efforts of mapping genes on non-silkworm lepidopteran Z chromosomes (Beldade *et al.*, 2009; Kroemer *et al.*, 2011) and suggests that the synteny conserving mechanism is weaker for the Z chromosome than the autosomes, with five gene order differences and one gene content difference (*rpl36*) compared with *B. mori*. Taking into account the available data on Z-linked genes in other Lepidoptera (Supplementary Table S3), the emerging picture is that although its gene content is highly conserved, the Z may be characterized by higher rates of intrachromosomal rearrangement. This may reflect its disproportionate role in adaptive evolution and speciation, the so-called Large-Z effect (Prowell, 1998; Naisbit *et al.*, 2002; Presgraves, 2002). The rearranged order of Z-linked genes between *B. mori* and *B. betularia* suggests two inversions and two transpositions during divergence of their Z chromosomes from a common ancestor (Supplementary Figure S5). Inversions in particular can play an important role in speciation by restricting meiotic recombination in the whole inverted region, protecting favorable haplotypes from being broken down, and allowing the accumulation of genetic incompatibilities in the loci involved (Faria and Navarro, 2010). The loss of Z-chromosome colinearity in comparison with the autosomes, recently found between scaffolds of *B. mori* and *D. plexippus*, supports this hypothesis (Zhan *et al.*, 2011).

A special feature of lepidopteran genomes is the W chromosome. Being inherited via females, the complete absence of recombination has resulted in its independent evolution accompanied with extensive genetic erosion and accumulation of repetitive DNA sequences (Marec *et al.*, 2010). So far, the repetitive nature of the W chromosome, together with a lack of functional genes, have obstructed detailed structural analysis, even in *B. mori* (Abe *et al.*, 2010). The relatively high number of W-linked AFLPs (18) and one gene (*laminin A*) in *B. betularia* allowed the identification of W-linked BACs as a tool to examine the characteristics of a lepidopteran W chromosome further. Three W-linked BAC clones (two AFLP derived and one *laminin A* derived) did not localize discrete sites with BAC-FISH but painted the whole W chromosome (Figures 5c, d, g and h), similar to W-derived BAC clones in *B. mori* (Sahara *et al.*, 2003). Although unsuitable for positional mapping, these hybridization patterns do add further evidence of the repetitive nature and uniformity of the lepidopteran W chromosome (Marec *et al.*, 2010). The chromosome-wide hybridization signals, especially of 3L9, suggest the presence of repetitive elements that exist (almost) exclusively on the W chromosome.

The identification of a W homolog of the *laminin A* gene, revealed by nonhemizygous segregation (Table 1), and its localization to the

end of the *B. betularia* W chromosome (Figures 5a, b, e and f) is a significant addition to the very small number of confirmed protein-coding sequences on lepidopteran W chromosomes (although transcription of the W representative remains to be confirmed). As the Z-linked *laminin A* is also located at the end of the Z chromosome, the W-linked copy of this gene could have arisen and be maintained by ectopic recombination between subtelomeric (terminal) regions resulting in gene conversion (Schlecht *et al.*, 2004; Linardopoulou *et al.*, 2005). Besides the putative zinc-finger protein (*Fem*) in the silkworm (Suzuki, 2010), the only W-linked genes with known sequence are in *Antheraea pernyi*, which has two variants of the *period* gene on the W chromosome, one producing a truncated protein and the other antisense RNA transcript (Gotter *et al.*, 1999). These W-linked *period* variants might originate from the functional *period* gene, which is also Z linked in *A. pernyi*, as in *B. mori* and *B. betularia* (Figure 4b).

Valid synteny comparisons are dependent on linkage map quality, which in turn depends on the choices made for experimental design and analysis. The linkage mapping strategies for *P. xylostella* (Baxter *et al.*, 2011) and *B. anynana* (Beldade *et al.*, 2009), based on RAD and EST polymorphisms, respectively, used single or multiple small families (20 and 22 offspring), thus prioritizing the number of markers over resolution. In the *P. xylostella* map, marker order is reliable but markers are clustered in $\sim$5 cM intervals. In the *B. anynana* map, the reliability of gene order may be partially compromised in LGs lacking polymorphic anchoring markers to join the maps from each of the 12 mapping families. RAD mapping (using a single family of 43 offspring) and *de novo* genome assembly were powerfully combined to order sequence scaffolds onto chromosomes in *H. melpomene* (The *Heliconius* Genome Consortium, 2012). For the *B. betularia* map, we opted for a single relatively large family (73 offspring) at modest marker density (a second family was additionally used for LG17). Marker number was supplemented to fill specific gaps and for LGs of particular interest (Z, *carbonaria* locus, *B. mori* fusions) using genes from orthologous *B. mori* chromosomes. The resulting chromosome maps are highly reliable in terms of marker order and, conditional on variable marker density, are relatively well resolved. Parallel patterns of orthology for *B. betularia* and *P. xylostella* against *B. mori*, across all of the 31 LGs, strongly suggest that the genome structure of these two species closely resembles the ancestral chromosome print for the Lepidoptera (direct comparison between *B. betularia* and *P. xylostella* cannot be made because only uniref90 blastx positives are available for *P. xylostella*). Furthermore, this implies that the strong mode of $n = 31$ chromosomes in Lepidoptera is maintained through stasis rather than a dynamic fusion/fission equilibrium.

As the number of lepidopteran linkage maps and mapped genomes expands, it will be possible to reconstruct the architecture of ancestral lepidopteran genomes at increasingly fine taxonomic and genetic scales. The significance of this exercise lies not only in a better understanding of chromosome dynamics *per se*, but is highly relevant to analyzing the role of genetic rearrangements and LG conservation in phenotypic evolution and reproductive isolation. An elegant example of this is the inversion polymorphism defining the wing-pattern supergene (*P*) in *Heliconius numata*, which acts to suppress low-fitness (nonmimic) recombinant phenotypes (Joron *et al.*, 2011). Through an unlikely coincidence, the *carbonaria* locus controlling melanism in *B. betularia* is orthologous to *P*, suggesting the existence of a deeply conserved switch of pattern regulation in this region. The positional information provided by the *B. betularia* linkage map for the majority of genes known to be directly involved in melanin biosynthesis (and some additional patterning genes) sets the genomic context for unraveling the cascade of interactions underlying this widespread polymorphism.

DATA ARCHIVING

Sequence data have been submitted to GenBank (accession numbers are listed in Supplementary Table S1). Genotypes of the *Biston betularia* mapping family have been deposited at Dryad, doi:10.5061/dryad.6r826.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)

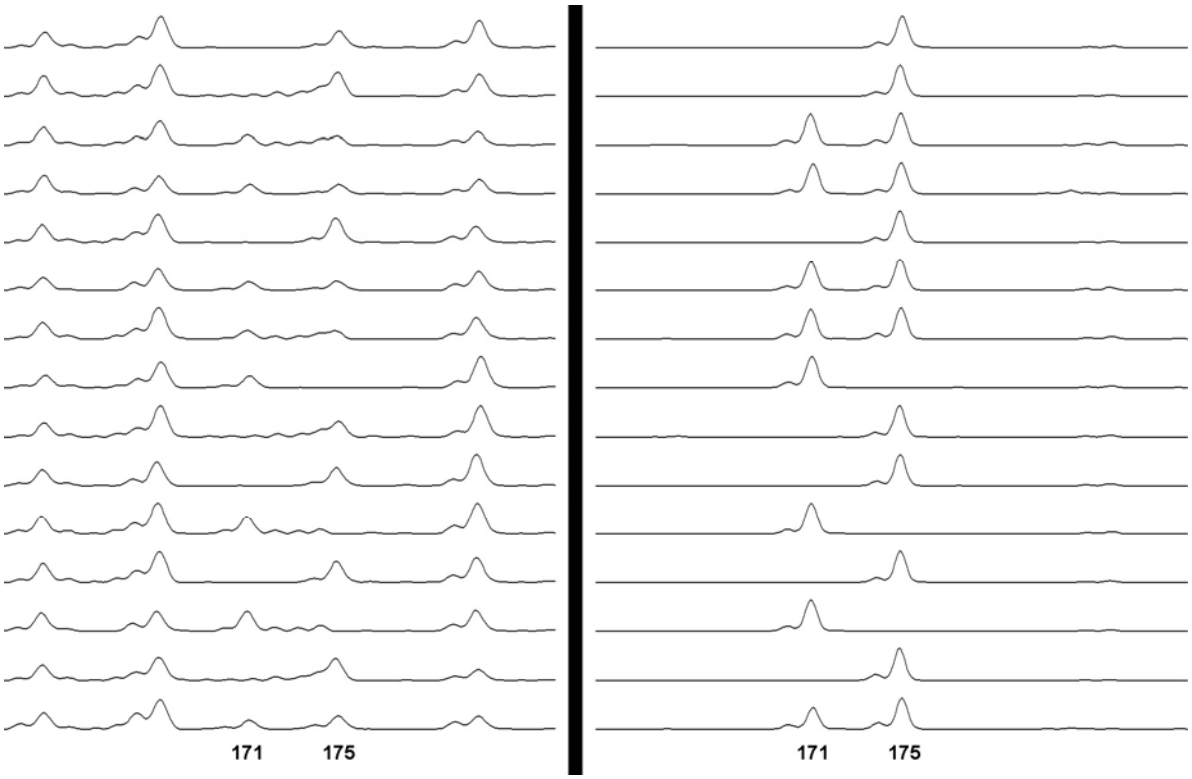

Figure S1 Quality improvement of a co-dominant AFLP. mCCeACC171-175 was a co-dominant AFLP marker with two distinct alleles of 171 and 175 bp, but with a pattern that is somewhat ambiguous. The original AFLP traces to the left of the vertical black bar were generated with the Mse+CC primer, whereas the traces to the right of the bar are for the same individuals amplified with Mse+CCAA, which was used to generate a reliable genotype (mCCAAeACC171-175 marker on LG25).

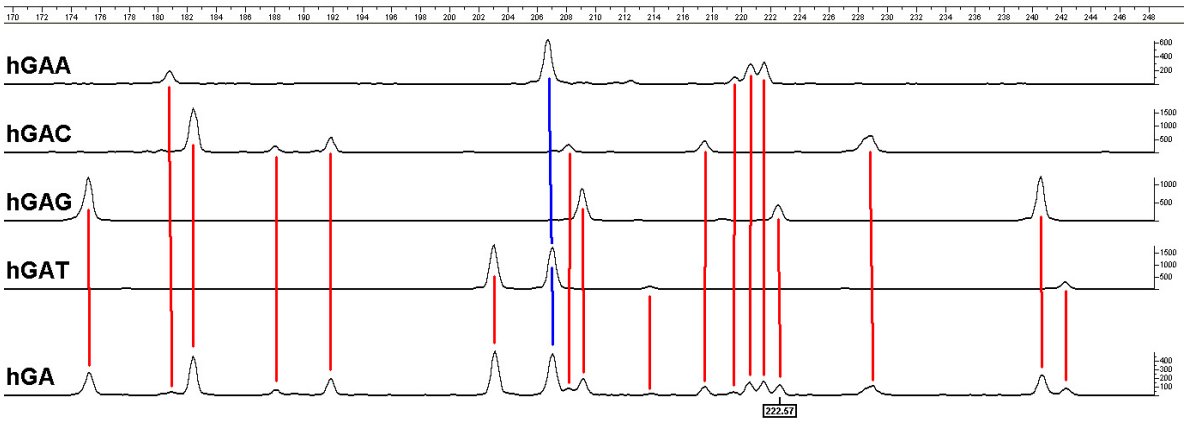

Figure S2 Separation of a complex AFLP trace by selective primer extension. The bottom trace shows a region of the original Hha+GA trace with 18 peaks, including a 222 bp peak that was selected for sequencing. The four traces above have alternative 3-base (GAN) extensions. The original peaks separate into the four groups, except for the peak indicated with the blue line, which is an overlapping peak in the +2 trace (size homoplasy), and splits into two peaks in the +3 profiles. The +3 peaks are higher (vertical scales are not identical) and more isolated.

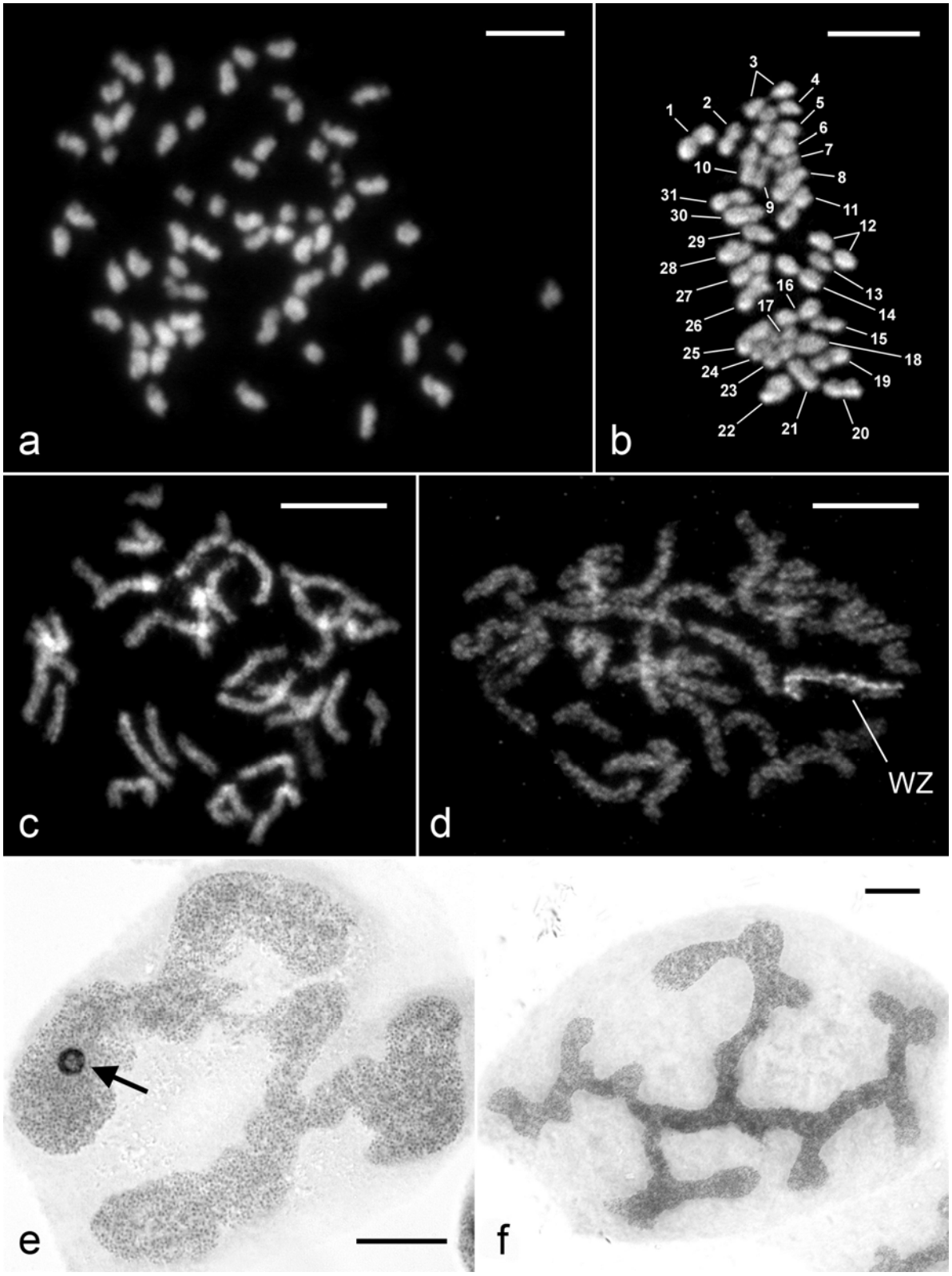
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Figure S3 Basic cytogenetic characteristics of *Biston betularia*. (a) DAPI-stained mitotic spermatogonial metaphase showing 2n = 62 chromosomes; (b) YOYO-1 stained spermatocyte complement showing n = 31 meiotic metaphase I bivalents (individual bivalents are arbitrarily numbered to facilitate their distinction); (c) DAPI-stained male pachytene complement showing n = 31 meiotic bivalents (chromomere pattern is not seen due to the pretreatment in hypotonic solution); (d) DAPI-stained female pachytene complement (n = 31) showing chromomere patterns; note the WZ bivalent is easily identified from the DAPI-highlighted heterochromatic thread of the W chromosome; (e) Orcein-stained polyploid nucleus of the female Malpighian tubule cell showing a large spherical sex-chromatin body (arrow), representing multiple copies of the heterochromatic W chromosome; (f) orcein-stained polyploid nucleus of the male Malpighian tubule cell without sex chromatin. Scale bars indicate 5 μm in (a, b), 10 μm in (c, d), and 20 μm in (e, f).

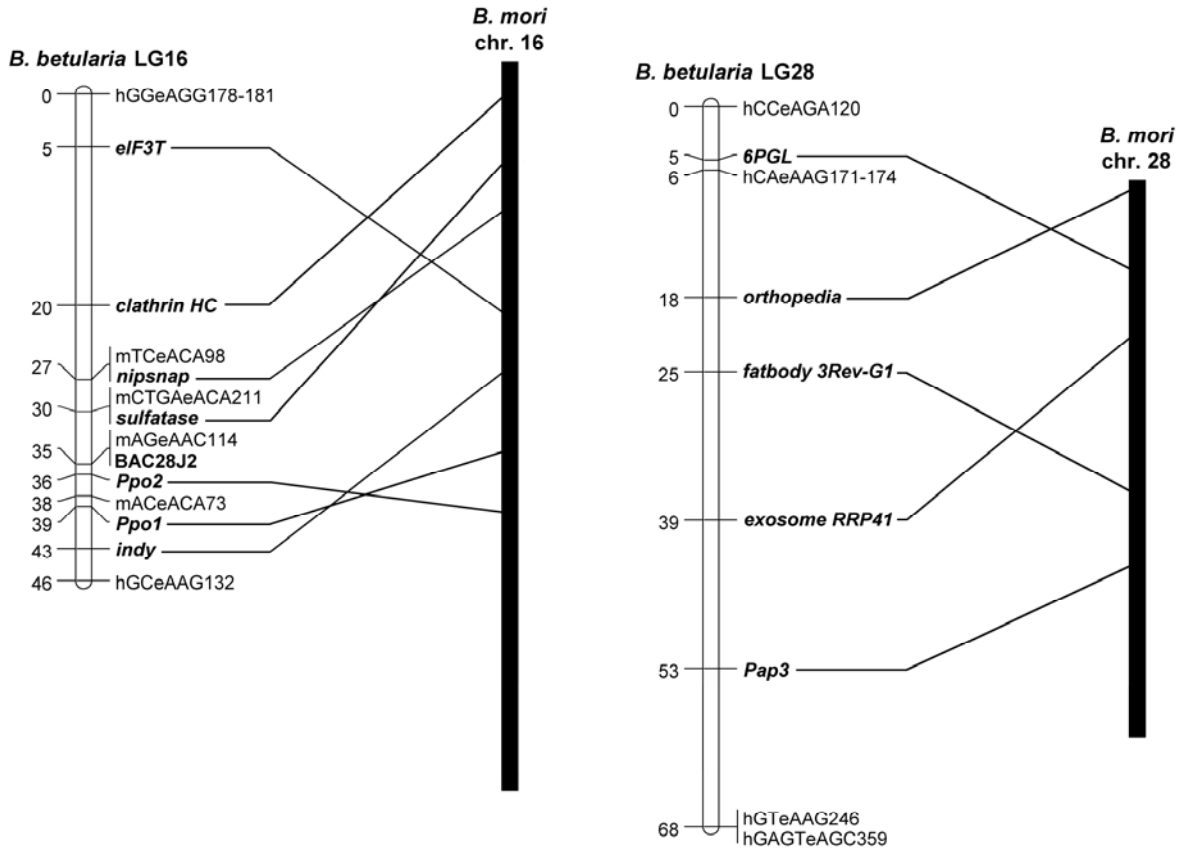

Figure S4 Gene order rearrangements between *B. betularia* and *B. mori*. Gene order rearrangements were detected between *B. betularia* and *B. mori* in five linkage groups. Two of these are shown in this figure, the others are included in Figure 3 (LG11 and LG29) and in Figure 4 (Z). The *B. mori* chromosomes are scaled according to scaffold sizes in SilkDB using estimated gap sizes, which differ slightly from the fixed gap sizes in Kaikobase.

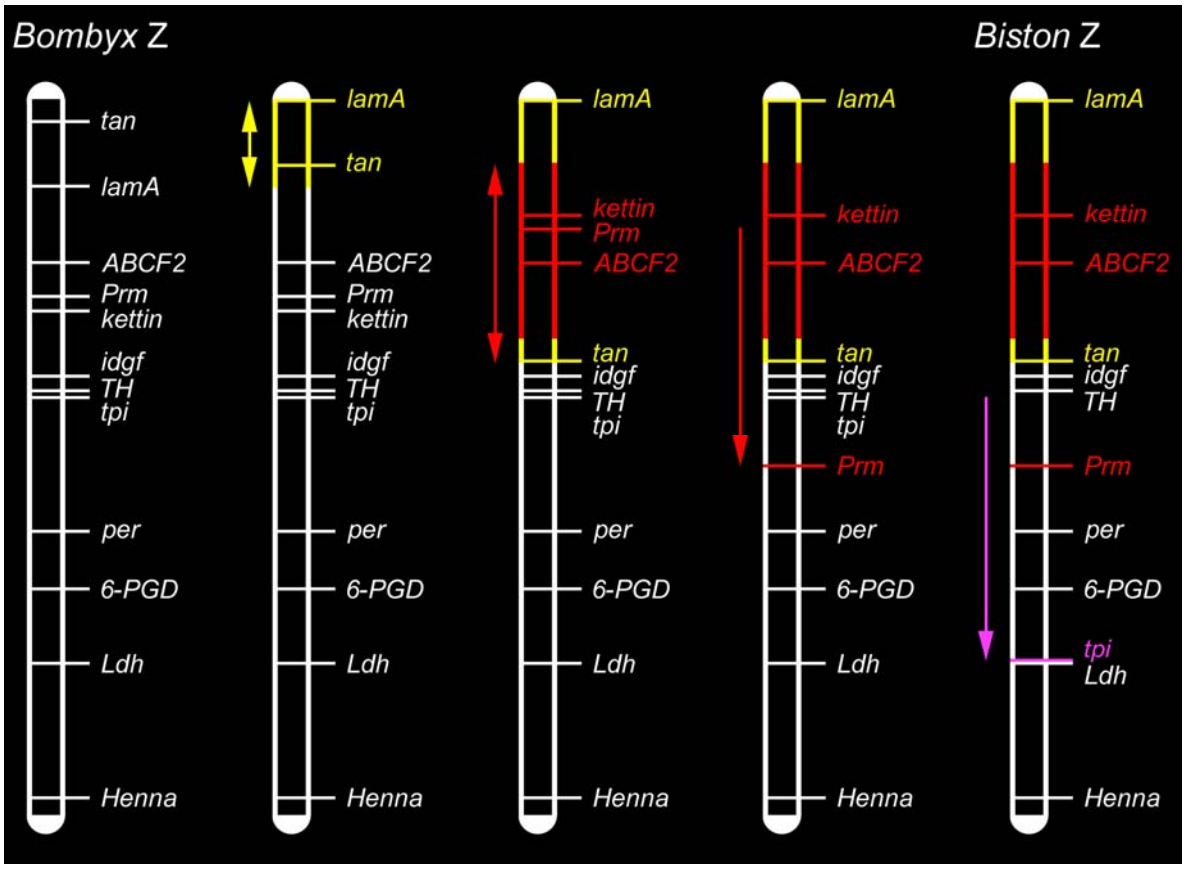

Figure S5 Reconstruction of Z chromosome rearrangements between *Biston betularia* and *Bombyx mori*. Hypothetical scheme of rearrangements, which could explain the different gene order that differentiated the Z chromosomes of *Bombyx mori* (left) and *Biston betularia* (right) from a common ancestor. Double-headed arrows indicate inversions, single-headed arrows transpositions; colour of arrows corresponds to colour of repositioned genes. For simplicity, unidirectional alterations from *B. mori* to *B. betularia* (from left to right) are illustrated; however, as the ancestral structure of the Z chromosome is unknown, reverse unidirectional alterations (from right to left) or bidirectional alterations from any intermediate structure are equally possible.

Table S1 Blast results and primers for the mapped genes. Details of the genes included in the linkage map: gene identity, GenBank accession number, NCBI and SilkDB blast results and primers used to isolate and genotype the genes. The SilkDB blast results present the best blastx hit, unless tblastx produced a better hit that was not annotated. The tblastx hits have no *Bombyx* gene identifier. **[VantHof-et-al_2012_SupplementaryTable1.xls]**

Table S2 Paternal segregation pattern of mapping family ('16') offspring. The alleles have been translated from SNP nucleotides, indel large/small, or AFLP present/absent into a binary 1 or 0 code representing the chromosomal origin of the allele. "md" stands for missing data. Conditional formatting is used to demonstrate the recombination breakpoints per individual (vertical orientation) by means of a green/white change. For example, individual 1 does not have any recombination over the full length of LG1, while individual 2 recombined between *6-PGD* and *tpi*. **[VantHof-et-al_2012_SupplementaryTable2.xls]**

Table S3 List of published lepidopteran Z-linked genes (excluding *Bombyx mori*). Gene names are listed in the first column with alternative identifiers or frequently used abbreviations in brackets. Methods used to confirm Z-linkage are abbreviated as: FISH (fluorescence *in situ* hybridization); South (Southern hybridization); LM (linkage mapping); Inh (hemizygous inheritance). References are numbered in square brackets and listed in full below the table; all *B. betularia* data originate from this study. Only confirmed Z-linked genes are given in *O. nubilalis* and only the two genes specifically mentioned in the text are included for *P. xylostella* since only uniref90 best hits are available for this species. All except two genes (31/33) are also Z-linked in *B. mori*. *MYST histone acetyltransferase* (BGIBMGA006995) is located on chromosome 17 in *B. mori* and the identity of *Papilio spp. acid phosphatase* is unclear; however no *acid phosphatase* homologues occur on the Z-chromosome scaffolds of *B. mori*.

Table S3 List of published lepidopteran Z-linked genes (excluding *B. mori*).

Species → / Gene ↓	*Samia cynthia ricini*	*Antheraea pernyi*	*Manduca sexta*	*Agrius convolvuli*	*Biston betularia*	*Ostrinia nubilalis*	*Heliconius melpomene*	*Bicyclus anynana*	*Papilio glaucus + canadensis*	*Cydia pomonella*	*Plutella xylostella*
laminin A					LM, FISH						
kettin	FISH [1]				FISH	LM [4,5]			Inh [10]		
titin			FISH [3]	FISH [3]					Inh [10]		
ATP-binding cassette sub-family F member 2 (ABCF2)					LM, FISH						
tan					LM, FISH						
imaginal disc growth factor (idgf) (BaC2115)					LM, FISH			LM [9]			
tyrosine hydroxylase (TH)					LM						
Paramyosin					LM, FISH	LM [4]					
period		South [2]			LM, FISH				Inh [10]	South [12]	
6-phosphogluconate dehydrogenase (6-PGD)					LM, FISH				Inh [11]		
triosephosphate isomerase (tpi)					LM	LM [5,6]	LM [7]	LM [8]	Inh [10]		
lactate dehydrogenase (Ldh)					LM, FISH	LM [4,5]			Inh [10,11]		
Henna (PAH)					LM, FISH			LM [9]			
Y-box protein (BYB)	FISH [1]										
catalase								LM [9]			LM [13]
ephrin receptor			FISH [3]	FISH [3]							
epidermal growth factor receptor (EGF rec)			FISH [3]								
NADH dehydrogenase (ubiquinone)			FISH [3]	FISH [3]							
P-glycoprotein			FISH [3]	FISH [3]							
apterous							LM [7]				
acid phosphatase (Acp)									Inh [11]		
shaker						LM [4]					
MiniParamyosin						LM [4]					
FK506-binding protein						LM [4]					
Cuticular protein						LM [4]					
Coatomer protein subunit						LM [4]					
Stretchin											LM [13]
MYST histone acetyltransferase (MOF) (BaC1211)								LM [9]			
BaC2745								LM [9]			
BaC2817								LM [9]			
BaC4049								LM [9]			
BaC5197								LM [9]			
BaC5904								LM [9]			

Gene names are listed in the first column with alternative identifiers or frequently used abbreviations in brackets. Methods used to confirm Z-linkage are abbreviated as: FISH (fluorescence in situ hybridization); South (Southern hybridization); LM (linkage mapping); Inh (hemizygous inheritance). References are numbered in square brackets and listed in full below the table; all *B. betularia* data originate from this study. Only confirmed Z-linked genes are given in *O. nubilalis* and only the two genes specifically mentioned in the text are included for *P. xylostella* since only uniref90 best hits are available for this species. All except two genes (31/33) are also Z-linked in *B. mori*. *MYST histone acetyltransferase* (BGIBMGA006995) is located on chromosome 17 in *B. mori* and the identity of *Papilio spp. acid phosphatase* is unclear; however no *acid phosphatase* homologs occur on the Z-chromosome scaffolds of *B. mori*.

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3.2. Chapter two:

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

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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*,

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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).

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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

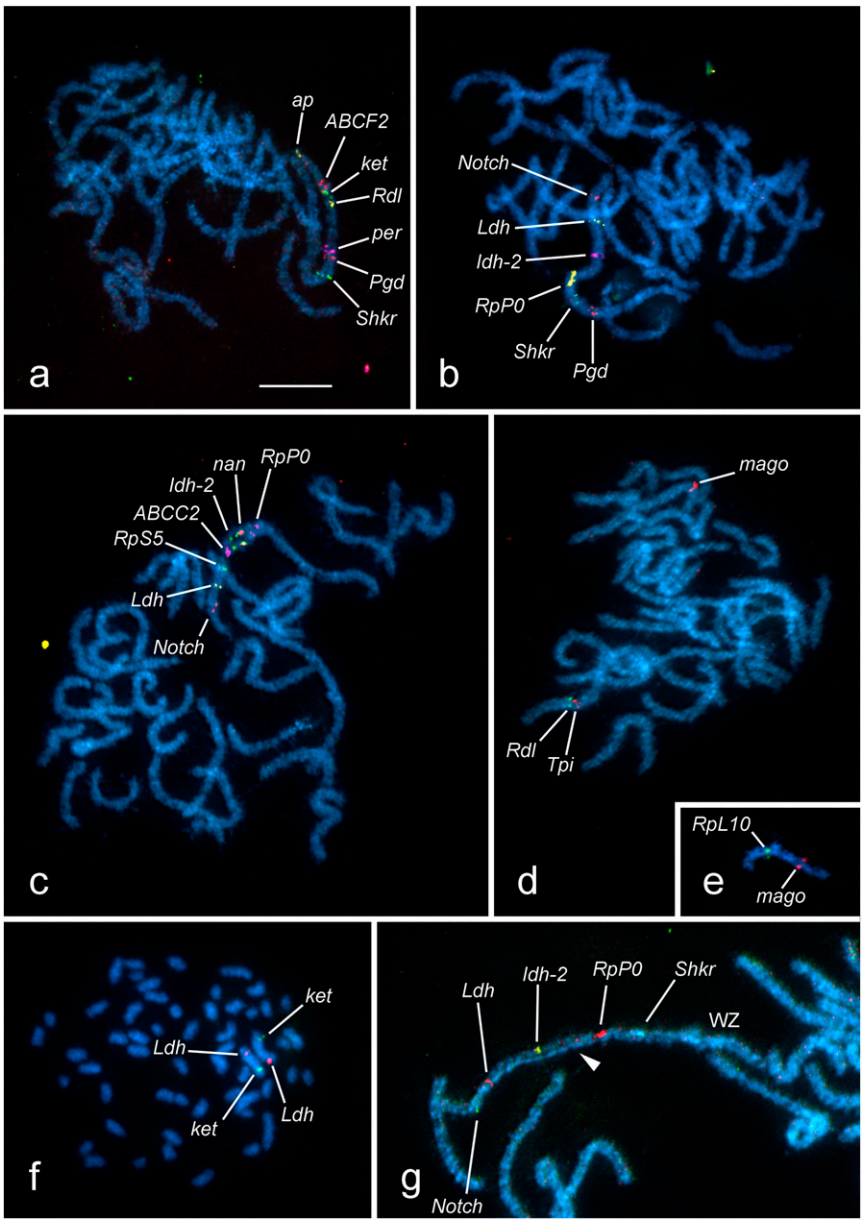

Fig. 1. BAC-FISH mapping of genes on chromosome preparations of the codling moth, *C. pomonella*. Chromosomes were counterstained with DAPI (light blue). Hybridization signals of BAC probes (yellow, green, red, and violet) indicate the physical positions of loci marked by abbreviated names. (*A–D*) Pachytene 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

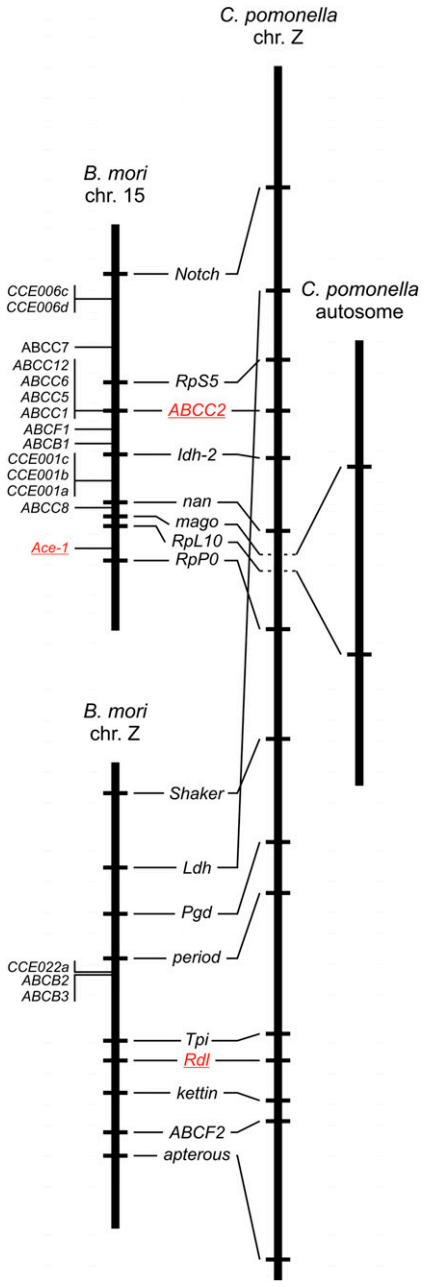

Fig. 2. A gene-based scheme of the Z chromosome of the codling moth, *C. pomonella*, integrating all BAC-FISH mapping data (Fig. 1) and its comparison with the *B. mori* chromosomes Z and 15. Locations of *B. mori* genes were retrieved from KAIKObase (Table S1). The mean relative positions of loci in 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

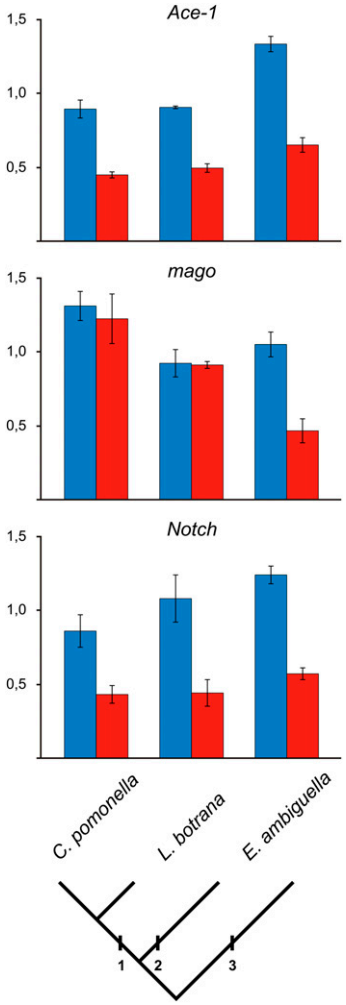

Fig. 3. Quantitative PCR comparison of male (blue columns) and female (red columns) doses of *Ace-1*, *mago*, and *Notch* genes normalized to the autosomal reference gene *EF-1α* in *C. pomonella* (Olethreutinae, Grapholitini), *L. botrana* (Olethreutinae, Olethreutini), and *E. ambiguella* (Tortricinae, 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.

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Supporting Information

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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).

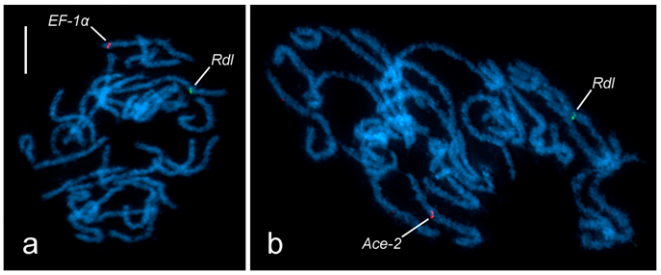

Fig. S1. BAC-FISH localization of two unmapped genes in comparison with a Z-linked gene on chromosome preparations of the codling moth, *Cydia pomonella*. Chromosomes were counterstained with DAPI (light blue). Hybridization signals of BAC probes (green and red) indicate the physical positions of loci 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.)

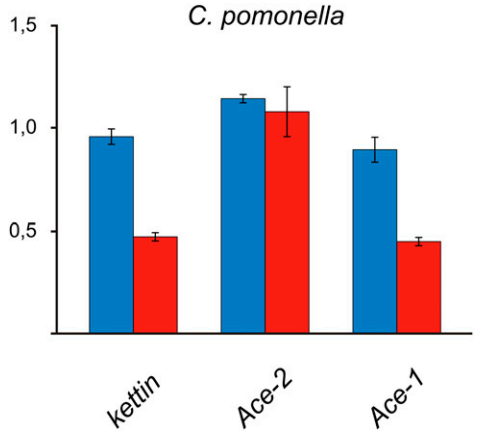

Fig. S2. qPCR determination of sex-linkage of the *Ace-1* gene in the codling moth, *Cydia pomonella*. Male (blue columns) and female (red columns) doses of *kettin, Ace-2*, and *Ace-1* genes normalized to the autosomal reference gene *EF-1α* are compared. A vertical bar at each column indicates the 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

4. Synthesis and perspectives

4.1. Conserved synteny of genes between lepidopteran Z chromosomes

In this thesis, the physical Z chromosome maps were developed in two species of the superfamilies Geometroidea and Tortricoidea, *Biston betularia* and *Cydia pomonella*, respectively. In *B. betularia*, BAC-FISH just added to the immense linkage mapping effort. Yet it proved itself a useful tool as it confirmed a gene order of the *B. betularia* Z-linked markers and provided their physical distances. Physical mapping also clearly demonstrated its advantage over genetic linkage analysis by anchoring the conserved *kettin* gene (cf. Yasukochi *et al.* 2009). However, it is necessary to keep in mind limitations of this approach. It is indeed a powerful tool for testing synteny of genes, i.e. co-localization of a set of markers, but it cannot *per se* identify any addition to a synteny block (cf. Sahara *et al.* 2007 and Yasukochi *et al.* 2009). At least not without time-consuming and laborious approach requiring large number of clones (Janes *et al.* 2011). BAC-FISH would most likely fail to uncover the neo-sex chromosomes in *C. pomonella* unless we had a prior hint that sex chromosomes-autosome fusion occurred in tortricids (cf. Nguyen 2009).

The resulting Z chromosome map of *B. betularia* altogether consists of 12 genes distributed along the whole chromosome. Its comparison with the genes known to be Z linked in other Lepidoptera (Sahara *et al.* 2012, 2013, Van't Hof *et al.* 2013; Fig. 1) suggests that the Z chromosome gene content is conserved between *B. betularia* (Geometroidea) and the representatives of four other advanced superfamilies, namely Pyraloidea, Papilionoidea, Noctuoidea, and Bombycoidea which are all comprised in the lepidopteran clade Obtectomera *sensu* van Nieukerken *et al.* (2011) (Fig. 1). A comparison with the Z chromosome map of *C. pomonella*, representative of the basal ditrysian superfamily Tortricoidea, further suggests that the synteny block corresponding to the obtectomeran Z chromosome is conserved also between the lineages Obtectomera and Tortricoidea (Fig. 1), which diverged more than 150 Mya (Wheat and Wahlberg 2013). However, in the vast majority of tortricoids, this putative ancestral Z chromosome fused with the autosome homeologous to *B. mori* chromosome 15.

What does it tell us about the origin of the lepidopteran W chromosome? It is not possible to draw any conclusion from available data. As mentioned in section 1.2, we cannot rule out a possibility that W chromosome arose independently in different lepidopteran lineages. For example, the W chromosome could have evolved from female-limited homolog of the chromosome 15, i.e. autosome, in tortricoids and from supernumerary B chromosome in obtectomeran moths and butterflies. Thus, at first a hypothesis on the common origin of the W chromosome needs to be tested. In order to do that, more data from basal ditrysian lineages is necessary. Sahara *et al.* (2012) provide also a list of genes, which are supposed to be Z-linked in one such suitable outgroup, *Plutella*

xylostella (Yponomeutoidea). The data comes from the RAD linkage map constructed by Baxter *et al.* (2011). The authors reported 15 Z-linked protein-coding sequences. However, only 8 of these sequences had a match on the *B. mori* sex-linked scaffolds. Furthermore, it is very difficult to check this data, because only about 46 bp long RAD alleles are available in supplementary information, though the original Blast search was performed using much longer RAD contigs. The very same RADseq data failed to assign about two thirds of *P. xylostella* genomic sequences to chromosomes (You *et al.* 2013). Thus caution is in place and synteny of sex-linked genes should be carefully re-examined in *P. xylostella*.

4.2. Intrinsic dynamism of the lepidopteran Z chromosomes

Sahara *et al.* (2012) came to a conclusion that the lepidopteran Z chromosome is evolutionary conserved as the X and Z chromosomes of placental mammals and birds, respectively (Murphy *et al.* 1999, Raudsepp *et al.* 2004, Nanda *et al.* 2008, Ellegren 2010). However, the lack of colinearity observed in some representatives of the clade Obtectomera as well as the sex chromosome-autosome fusion common to the majority of the superfamily Tortricoidea do not support this view.

The presented comparative analysis of gene order suggests that, compared to autosomes, the Z chromosome of *B. betularia* experienced a higher rate of intrachromosomal rearrangements (Van't Hof *et al.* 2013). Hypothetical reconstruction of the events, which broke down colinearity between Z chromosomes of *B. betularia* and *B. mori*, involved two inversions and two translocations. Inversions are often associated with sex chromosomes as they are supposedly responsible for the suppression of recombination and its spread during the initial phase of sex chromosome differentiation (see section 1.1). Yet inversions have no say in this process in Lepidoptera due to the female achiasmatic meiosis. However, they can still effectively reduce male recombination in inverted regions and thus play an important role in limiting gene flow and facilitating speciation (Faria and Navarro 2010).

Several other mechanisms can help to explain the pattern observed in *B. betularia*. Chromosomal rearrangements are mediated by ectopic recombination between repetitive motives, and thus the amount and distribution of repetitive sequences can affect rearrangement rates (Montgomery *et al.* 1991, Bergero *et al.* 2008, d'Alençon *et al.* 2010). It was shown that the accumulation of repetitive sequences is not confined to sex-limited chromosome (Y or W) but occurs also in the X or Z chromosomes (Bellott *et al.* 2010, Gschwend *et al.* 2012), most likely because of reduced recombination (Gschwend *et al.* 2012). Furthermore, providing an equal number of breeding males and females, the effective population size of the Z chromosome is reduced to three quarters compared to an autosome and further drops due to the mating system, i.e. due to decreased number of males involved in reproduction (Mank *et al.* 2010b). The reduced effective population size enhances the effect of genetic drift which seems to be responsible also for the so-

called Faster-Z effect, i.e. a higher rate of nonsynonymous substitutions in Z-linked loci in birds and snakes (Mank *et al.* 2010a, Vicoso *et al.* 2013a). A chromosomal rearrangement can be indeed fixed in a population by genetic drift, though it is less clear how it could spread throughout a species range (Veltsos *et al.* 2008, Ironside 2010). However, it should be noted that unlike in birds and snakes, accelerated rates of functional change were suggested to be driven by positive selection, not genetic drift, in Lepidoptera (Sackton *et al.* 2013).

Finally, Diaz-Castillo (2013) hypothesized that the stability of the gene order is affected by differences in nuclear architecture between male and female germ lines. Since the Z chromosome supposedly carries the largest number of testis-specific genes, it is assumed to be located in the peripheral and internal nuclear domains in female and male germ lines, respectively. These two domains differ in their accessibility to cellular machinery and more importantly in the mechanism of DNA break repair. The interplay of different repair of DNA breaks and non-random nuclear architecture is supposed to influence the overall stability of the gene order. In Lepidoptera, non-random chromosome arrangement was studied only in blue butterflies of the genus *Agrodietus*. Lukhtanov and Dantchenko (2002) noticed that central position in the metaphase plate is always occupied by the largest bivalent. Many of the examined species had derived karyotype formed by repeated chromosome fissions. In such karyotype, the largest chromosome pair allegedly represents sex chromosomes (Ennis 1976). However, no data on the sex chromosome territories in male and female interphase nuclei are available in Lepidoptera.

4.3. Neo-sex chromosomes in lower Ditrysia

Taking the advantage of available BAC library, orthologs of *B. mori* genes linked to the chromosomes Z and 15 were mapped in the codling moth, *Cydia pomonella* (Tortricoidea). The results presented in section 3.2 revealed that the Z chromosome of this species arose by a fusion between an ancestral Z chromosome and an autosome corresponding to *B. mori* chromosome 15, henceforth referred to as F(Z;15). Furthermore, this rearrangement (i.e. neo-Z chromosome) was confirmed in two representatives of distant tortricid lineages, the European grapevine moth (*Lobesia botrana*) and the vine moth (*Eupoecilia ambiguella*), which strongly suggests that the fusion occurred in a common ancestor of the main subfamilies of Tortricidae, Olethreutinae and Tortricinae, comprising 97% of extant species of this diverse family (Gilligan *et al.* 2012).

Detailed cytogenetic analyses of several tortricids, including all species examined in section 3.2, were carried out and chromosome data available in the family Tortricidae was comprehensively reviewed (Fuková *et al.* 2005, Šíchová *et al.* 2013). In the subfamily Tortricinae, the vast majority of karyotypes consists of n=30 elements, whereas reduced chromosome numbers, predominantly n=28, were observed in the subfamily Olethreutinae. Providing that the chromosome print of n=31

represents an ancestral karyotype of non-tineoid Ditrysia (see section 3.1), F(Z;15) seems to be the very first chromosomal rearrangement differentiating the common ancestor of Olethreutinae and Tortricinae. This poses an intriguing question about its role in divergence and radiation of tortricid moths, since it was shown that changes in genome architecture can significantly affect ecological specialization and speciation, even more so if sex chromosomes are involved (Faria and Navarro 2010, Loxdale 2010, but see section 4.2 for the role of genetic drift in fixation of chromosomal rearrangements).

Now it is clear that the neo-sex chromosomes manifested as a conspicuously large chromosome pair in published tortricid karyotypes (e.g. Saitoh 1966, Ennis 1976, Kawazoe 1987, Fuková *et al.* 2005, Šíchová *et al.* 2013). Ennis (1976) noted that a larger chromosome pair is characteristic not only for the family Tortricidae but also for the families Pyralidae, Oecophoridae, and Gelechiidae, the latter two belonging to the largest microlepidopteran superfamily Gelechioidea. The presence of the large chromosome pair has been repeatedly reported in several gelechioid families (as distinguished by Brown *et al.* 2012), namely Xyloryctidae (Kawazoe 1987), Elachistidae (Ennis 1976), Coleophoridae (Lukhtanov and Puplesiene 1999), and Gelechiidae (Ennis 1976, Bedo 1984, Bartlett and Del Fosse 1991). Due to the presence of the large chromosome pair in species with reduced chromosome numbers, Ennis (1976) concluded that it may "merely represent the autosomal fusion product". Heteromorphic sex chromosomes have not been observed in Gelechioidea (Bedo 1984, Kawazoe 1987a, Bartlett and Del Fosse 1991). However, genomic *in situ* hybridization (GISH) clearly identified sex chromosomes as the largest pair in a female mitotic complement (2n=58) of the tomato leaf miner, *Tuta absoluta* (Gelechiidae) (Carabajal Paladino *et al.* 2013).

According to recent phylogenetic hypotheses on higher-level relationships in Lepidoptera (Kristensen *et al.* 2007, Mutanen *et al.* 2010, Regier *et al.* 2013), Tortricoidea and Gelechioidea are not closely related. Thus, providing homeology of the largest chromosome pair discussed above, ancestral gelechioid karyotype n=30 (Lukhtanov and Puplesiene 1999), and phylogenetic relationships and species numbers within the superfamily (Kaila *et al.* 2011) we can hypothesize that the sex chromosome-autosome fusion occurred independently in a common ancestor of 90% described species of the superfamily Gelechioidea. In such case, it would be again the first rearrangement, which differentiated gelechioid genome from an ancestral karyotype of non-tineoid Ditrysia. Is it just coincidence or do the neo-sex chromosome indeed play a role in radiation of lower Ditrysia?

Both the chromosomes Z and 15 were shown to bear a remarkable set of genes. The Z chromosome of moths and butterflies is known to be involved in postzygotic reproductive isolation (Presgraves 2002) and was reported to harbor female host preference (Thompson 1988, Nygren *et al.* 2006), components of the chemosensory system (Olsson *et al.* 2010, Lassance *et al.* 2011, Yasukochi

et al. 2011), and a disproportionally high number of genes underlying differences between closely related species (Sperling 1994, Prowell 1998).

The chromosome 15 of the silkworm, *B. mori*, contains genes presumably involved in detoxification of plant secondary metabolites, namely a major cluster of ABC transporters and two clusters of Lepidoptera-specific carboxylesterases (Yu *et al.* 2009, Xie *et al.* 2012). Carboxylesterases were shown to be responsible for metabolism of phenolic glycosides (Lindroth 1989) and ubiquitous aliphatic esters (Zangerl *et al.* 2012). Dietary nicotine was shown to be actively excreted by ABC transporter mechanism in the Malpighian tubules of the tobacco hornworm, *Manduca sexta* (Gaertner *et al.* 1998). Expansion of ABC transporters observed in the genome of the diamondback moth, *Plutella xylostella* (Yponomeutoidea), was highlighted as a basis for evolutionary success of this herbivore (You *et al.* 2013). Furthermore, genome-wide analysis of ABC transporters in polyphagous spider mite *Tetranychus urticae* linked its broad plant host range with evolution of this gene family (Dermauw *et al.* 2013). Thus it is reasonable to assume that the F(Z;15) fusion physically linked the whole battery of the so-called performance genes, which affect growth and survival of larvae on their host plants, to the tortricid Z chromosome.

Physical linkage between performance and either preference or host-independent isolation genes mentioned above is expected to generate genetic covariance between traits and facilitate thus ecological specialization and speciation under divergent selection (Hawthorne and Via 2001, Matsubayashi *et al.* 2010). Furthermore, as recombination ceases due to the achiasmatic meiosis in lepidopteran females, maternally transmitted (neo-W-linked) alleles of performance genes slowly deteriorate. Selection for dosage compensation, caused by increasing environmental stress, is then expected (see section 1.1). Providing that global dosage compensation is absent in Lepidoptera (Harrison *et al.* 2012), beneficial duplications of performance genes are supposed to be fixed by positive selection (Kondrashov *et al.* 2002, Innan and Kondrashov 2010). This scenario is well supported by the role of gene amplification in metabolic resistance to insecticides (Li *et al.* 2007, Bass and Field 2011) and contrasts with presumed expansion of gene families in a response to reduced population size (Feyereisen 2011). Amplification of detoxification genes and their functional diversification would increase a detoxification capacity of lepidopteran larvae and thus facilitate their shift to novel host plants. Therefore, the F(Z;15) fusion could be taken as a key innovation conferring physiological advantage in plant-herbivores interactions and resulting in adaptive radiation of the species-rich tortricid subfamilies Tortricinae and Olethreutinae. Since early angiosperms probably used more primitive and less toxic chemicals (Cornell and Hawkins 2003), the proposed mechanism had indeed potential to overcome defenses of contemporary plants and lead to radiation of tortricid lineage.

The interactions of moths and butterflies with their host plants were suggested as a driving force generating the present lepidopteran diversity. Ehrlich and Raven (1964) hypothesized that secondary plant metabolites play a central role in co-evolutionary arm races of herbivorous insects and plants. In their concept, novelty in biochemical defense allows plants entry into a new adaptive zone and selects for a physiological innovations in processing the plant allelochemicals, which, in turn, leads to adaptive radiation of herbivore groups (Ehrlich and Raven 1964, Futuyma and Agrawal 2009, Janz 2011). Several such key innovations have been identified in Lepidoptera. The *Papilio* swallowtails adapted to their host plants containing toxic furanocoumarins by diversification of cytochrome P450 monooxygenases (Berenbaum *et al.* 1996, Li *et al.* 2003), whereas Pierinae white butterflies radiated due to the novel nitrile-specifier protein disarming the defensive glucosinolate-myrosinase system of Brassicales (Wheat *et al.* 2007). Yet another example is pyrrolizidine alkaloid N-oxygenases crucial for alkaloid sequestration in arctiid moths (Sehlmeyer *et al.* 2010).

An alternative though not mutually exclusive premise, so-called oscillation hypothesis, was proposed in order to explain the complex pattern of host-plant use observed in nymphaline butterflies, where diversity of host use positively correlates with species richness. According to this hypothesis, diversification is fuelled by repeated expansion of host range, which facilitates an expansion in geographical distribution, followed by fragmentation of population and secondary ecological specialization on different hosts (Janz *et al.* 2006, Nylin and Wahlberg 2008).

Recently, great attention has been paid to the role of neo-sex chromosomes in speciation. Based on the remarkable diversity of sex chromosomes in stickleback fishes, it was suggested that differences in sex chromosome systems caused by sex chromosome-autosome fusions promote speciation (reviewed by Kitano and Peichel 2012). Furthermore, Pala *et al.* (2012) reported a sex chromosome-autosome fusion in Sylvioidea passerine birds and hypothesized that the autosomal gene content may be relevant in maintenance of the neo-sex chromosomes. Neo-sex chromosomes observed in the superfamily Tortricoidea support this hypothesis. Assumed independent genesis of neo-sex chromosomes in the superfamilies Tortricoidea and Gelechioidea indicates possible parallel evolution, which presents unique opportunity to dissect forces responsible for formation and evolutionary success of neo-sex chromosomes in these two speciose lineages. It is noteworthy that all above mentioned hypotheses explaining staggering diversity of Lepidoptera were proposed and tested in advanced macrolepidopteran taxa. Although it is clear that the rapid radiation of Lepidoptera was associated with their transition to herbivory and the great diversification of angiosperm plants (Grimaldi and Engel 2005, Whitfield and Kjer 2008, Menken *et al.* 2010), nothing has been known about mechanistic basis of substantial radiations in lower Ditrysia. Nguyen *et al*. (2013) thus provide an entirely new framework for future evolutionary studies on microlepidopteran diversity, which is built solely upon the extraordinary features of sex chromosomes.

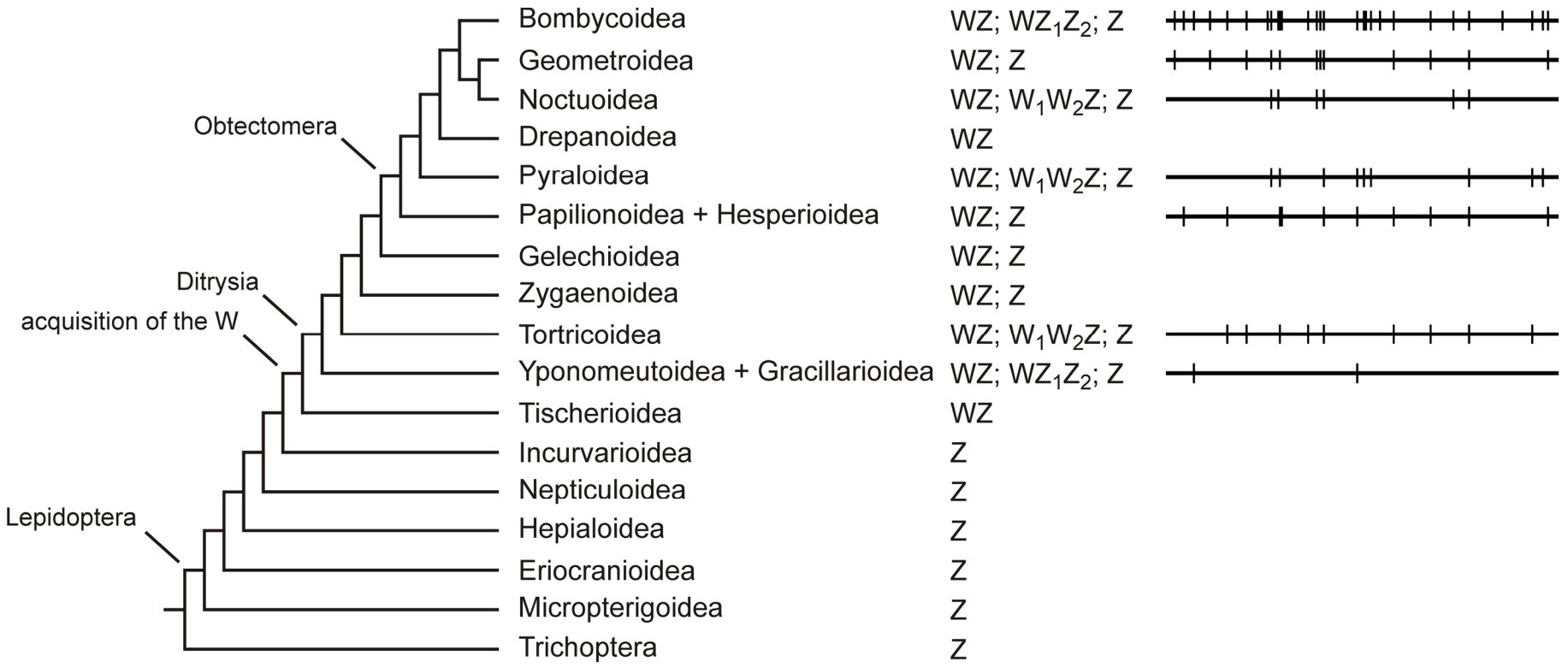

Figure 1 Phylogeny of the sex chromosome system and conserved synteny of the Z-linked genes in Lepidoptera. The cladogram depicts superfamilies in which either sex chromosomes or W chromatin were examined along with a list of inferred female sex chromosome constitutions (based on Sahara *et al*. 2012). Diagrams on the right represent gene content of the *B. mori* Z chromosome (Bombycoidea) conserved, i.e. sex-linked, in other superfamilies. Physical positions of Z-linked loci in the *B. mori* genome were acquired from KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase). The list of published lepidopteran Z-linked genes was obtained from Van't Hof *et al*. (2013) and completed by data from Nguyen *et al*. (2013) and Sahara *et al*. (2013). Phylogenetic relationships are based on Mutanen *et al*. (2010).

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6. *Curriculum vitae*

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Research Interests:

Karyotype evolution, evolution of sex chromosomes, comparative genomics of Lepidoptera

University Education:

Since 2009	Ph.D. studies in Molecular and Cell Biology and Genetics at the Faculty of Science, University of South Bohemia in České Budějovice, and the Institute of Entomology, Biology Centre of the Academy of Sciences of the Czech Republic. Thesis: Comparative mapping of sex-linked genes in Lepidoptera.
2009	Master's degree in Genetics and Gene Engineering at the Faculty of Biological Sciences, University of South Bohemia in České Budějovice. Thesis: Z chromosome synteny in codling moth, *Cydia pomonella* (L.), as revealed by BAC-FISH.
2007	Bachelor's degree in Biology at the Faculty of Biological Sciences, University of South Bohemia in České Budějovice. Thesis: Chromosomal distribution of rDNA in Lepidoptera.

Professional and Research Experience:

2013	BAC-FISH mapping of the Z chromosome of the diamondback moth (*Plutella xylostella*); Iwate University, Faculty of Agriculture, Morioka, Japan. (c/o Ken Sahara, 3 weeks)
2012	Molecular analysis of sex chromosomes of the diamondback moth (*Plutella xylostella*); University of Liverpool, Institute of Integrative Biology, Liverpool, UK (c/o Ilik Saccheri and Alistair C. Darby, 3 months).
2012	Comparative analysis of molecular differentiation of sex chromosomes in Heteroptera and Lepidoptera, the insects with holokinetic chromosomes; University of Buenos Aires, Faculty of Exact and Natural Sciences, Buenos Aires, Argentina (c/o María José Bressa, 1 month).
2010	Evolutionary repositioning of nucleolar organizer region (NOR) in Lepidoptera; Hokkaido University, Graduate School of Agriculture, Sapporo, Japan. (c/o Ken Sahara, 1 month).
2008	Synteny mapping of Z chromosomes in Lepidoptera using BAC-FISH; Hokkaido University, Graduate School of Agriculture, Sapporo, Japan (c/o Ken Sahara, 3 months).

Publications:

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Supervised or Consulted Theses:

Hladová I (since 2013) Genetic mapping of CpGV resistance in codling moth, *Cydia pomonella*. Mgr. Thesis in progress.

Voleníková A (since 2013) Synteny of sex-linked genes in the ghost moth, *Hepialus humuli* (Hepialidae). Mgr. Thesis in progress.

Blažková B (2012) Characterization of molecular composition of the codling moth (*Cydia pomonella*) genome using reassociation kinetics. Mgr. Thesis, in Czech, 34 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

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Sýkorová M (2011) Isolation and chromosomal localization of acetylcholinesterase genes in the codling moth, *Cydia pomonella*. Mgr. Thesis, in Czech, 34 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Grants:

Grant 059/2010/P "Mechanism of rDNA mobility in lepidopteran genome" of the Grant Agency of the University of South Bohemia in České Budějovice.

Grant SGA2008/001 "Z chromosome evolution in Lepidoptera" of the Student Grant Agency of the Faculty of Science, University of South Bohemia in České Budějovice.

Grant SGA2006/01 "Mechanism of karyotype evolution in *Polyommatus* blue butterflies studied by mapping of rRNA genes clusters" of the Student Grant Agency of the Faculty of Biological Sciences, University of South Bohemia in České Budějovice.

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