Insights into the Structure of the Spruce Budworm (Choristoneura fumiferana) Genome, as Revealed by Molecular Cytogenetic Analyses and a High-Density Linkage Map

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ABSTRACT Genome structure characterization can contribute to a better understanding of processes such as adaptation, speciation, and karyotype evolution, and can provide useful information for refining genome assemblies. We studied the genome of an important North American boreal forest pest, the spruce budworm, Choristoneura fumiferana, through a combination of molecular cytogenetic analyses and construction of a high-density linkage map based on single nucleotide polymorphism (SNP) markers obtained through a genotyping-by-sequencing (GBS) approach. Cytogenetic analyses using fluorescence in situ hybridization methods confirmed the haploid chromosome number of n = 30 in both sexes of C. fumiferana and showed, for the first time, that this species has a WZ/ZZ sex chromosome system. Synteny analysis based on a comparison of the Bombyx mori genome and the C. fumiferana linkage map revealed the presence of a neo-Z chromosome in the latter species, as previously reported for other tortricid moths. In this neo-Z chromosome, we detected an ABC transporter C2 (ABCC2) gene that has been associated with insecticide resistance. Sex-linkage of the ABCC2 gene provides a genomic context favorable to selection and rapid spread of resistance against Bacillus thuringiensis serotype kurstaki (Btk), the main insecticide used in Canada to control spruce budworm populations. Ultimately, the linkage map we developed, which comprises 3586 SNP markers distributed over 30 linkage groups for a total length of 1720.41 cM, will be a valuable tool for refining our draft assembly of the spruce budworm genome.

KEYWORDS Choristoneura fumiferana linkage map genotyping-by-sequencing karyotype neo-Z chromosome

The order Lepidoptera (moths and butterflies) is one of the most diverse taxa of insects, with at least 157,000 species described to date (Kristensen et al. 2007; van Nieukerken et al. 2011). Among these species, many have been extensively studied due to their pest status, whereas others have been used as experimental models in fields as diverse as physiology, genetics, evolution and ecology (Roe et al. 2010). For example, the diamondback moth, Plutella xylostella, has been used in many studies examining the development of insecticide resistance (You et al. 2013), while the squinting bush brown, Bicyclus anynana, whose wing color patterns vary depending on the season, has proven a valuable system to study the evolution of phenotypic plasticity (Brakefield et al. 2007).

Genomic characteristics unique to Lepidoptera have also drawn much attention. Moths and butterflies have holokinetic chromosomes (i.e., chromosome lacking a primary constriction, the centromere; Marec et al. 2010; Sahara et al. 2012) and meiotic recombination is limited to the male sex (Suomalainen et al. 1973; Turner and Sheppard 1975; Traut 1977; Traut 1977; Nokkala 1987; Traut et al. 2007). Moreover, lepidopteran chromosome numbers show a high degree of variability, ranging from n = 5 to n = 224-226 in the species examined (Marec et al. 2010; Lukhtanov 2015; Blackmon et al. 2017), with an average around the inferred ancestral number of n = 31 chromosomes (Suomalainen 1969; Lukhtanov 2000; Ahola et al. 2014; Yasukochi et al. 2016), found in ca.
one-third of species (Suomalainen 1969; Robinson 1971; De Prins and Saitoh 2003; Blackmon et al. 2017). Interestingly, a high degree of gene synteny has been observed among homologous chromosomes across distantly related taxa (Pringle et al. 2007; Yasukochi et al. 2009; Baxter et al. 2011; Yoshido et al. 2011; Van’t Hof et al. 2013; Ahola et al. 2014), and the patterns of synteny described so far indicate that the numerically altered karyotypes evolved via fusion and/or fission events (Marcé et al. 2010; Ahola et al. 2014; Sichová et al. 2015). Finally, the order Lepidoptera, along with its sister group Trichoptera (caddisflies) and some tephritid fruit flies, is part of a restricted assemblage of insects in which the female is the heterogametic sex (ZW or ZW) (Bush 1966; Traut et al. 2007; Marcé et al. 2010; Sahara et al. 2012; Blackmon et al. 2017).

The spruce budworm, Choristoneura fumiferana, is another lepidopteran species that has attracted much research attention. Belonging to the family Tortricidae, it is native to North America, and its larvae mainly feed on the foliage of spruces and true firs. One of the hallmarks of spruce budworm population dynamics is the development of severe outbreaks lasting ca. 10 years and recurring every 30 to 40 years (Jardon et al. 2003; Boulanger et al. 2012). During these outbreaks, intense defoliation typically results in major growth reductions, and several years of severe infestations can cause widespread tree mortality (MacLean 1985). While considered a very serious pest of North American spruce and fir stands, the spruce budworm is also seen as playing a positive, essential role in forest renewal (MacLean 2016). Since 2006, a new outbreak has gradually developed and spread over several regions of the province of Quebec, affecting 7.1 MHa as of 2017 (Ministère des forêts, de la faune et des parcs 2017). Due to its significant economic impact on the pulp and lumber industry across the Canadian boreal forest, the spruce budworm has been the subject of numerous studies and has imposed itself as a model for the study of insect population dynamics in North America (Pureswaran et al. 2016).

The spruce budworm has also been at the center of many studies examining various aspects of its biology, including its cytogenetic characteristics. It was reported that its karyotype consists of 30 pairs of chromosomes, including a pair of large chromosomes, presumably sex chromosomes (Ennis 1976; Harvey 1997). According to recent data, large sex chromosomes are a typical feature of most tortricids, with Z chromosomes having arisen from the fusion between an ancestral Z chromosome and an autosome corresponding to chromosome 15 of Bombyx mori (Nguyen et al. 2013; Síchová et al. 2013). However, the occurrence of a similar fusion in C. fumiferana has yet to be confirmed. In addition, the structure and composition of the W chromosome varies greatly among lepidopteran species, and characterization of these features can provide novel insights into the formation and evolution of sex chromosomes in the Lepidoptera (Traut and Marcé 1997; Yoshido et al. 2005; Vítková et al. 2007; Síchová et al. 2013; Dalíková et al. 2017; Mongue et al. 2017). In C. fumiferana, composition of the W chromosome has not yet been examined.

In the present work, we developed a SNP-based high-density linkage map of C. fumiferana and used cytogenetic techniques to study the composition and structure of its genome. More specifically, (i) we confirmed the presence of 30 pairs of chromosomes in both sexes of this species, including a large pair of sex chromosomes, (ii) we used synteny analysis to confirm the existence of a neo-Z chromosome and (iii) we conducted cytogenetic characterization of the structure of the W chromosome. The present work was initiated as part of a C. fumiferana genome project, whose main goal is to generate a high-quality reference genome for this species. The high-density linkage map developed here will help to position scaffolds on chromosomes.

MATERIALS AND METHODS

Karyotype analysis

Chromosome number for Choristoneura fumiferana was determined from mitotic metaphase cells stained by fluorescence in situ hybridization (FISH) using (TTAGG)n telomeric probe (tel-FISH), which enables identification of chromosome ends. Mitotic chromosomes were obtained from wing imaginal discs of 5th instar male and female larvae using the spreading technique described in Mediouni et al. (2004). The telomeric probe was generated by non-template PCR according to the protocol of Sahara et al. (1999) and labeled with Cy3-dUTP (Jena Bioscience, Jena, Germany) using a Nick Translation Kit (Abbott Molecular Inc., Des Plaines, USA) with 75 min incubation at 15°C. FISH with the (TTAGG)n telomeric probe was performed following the protocol of Yoshido et al. (2005). The hybridization mixture contained 100 ng of telomeric probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, USA) in 10 μL of 50% formamide and 10% dextran sulfate in 2 x SSC.

To identify the sex chromosomes of C. fumiferana, we used genomic in situ hybridization (GISH) combined with tel-FISH as described in Yoshido et al. (2005) and Síchová et al. (2015). GISH is based on hybridization of a fluorescently labeled female genomic DNA (gDNA) in the presence of an excess of unlabeled competitor DNA (i.e., male gDNA). With this approach, the W chromosome is identified by strong binding of female labeled DNA due to its repetitive character. This approach has successfully identified the W chromosome in several lepidopteran species (Mediouni et al. 2004; Fuková et al. 2005; Yoshido et al. 2006).

Spread chromosome preparations were obtained from testes and ovaries of 5th instar larvae as described in Mediouni et al. (2004). For GISH, total gDNA from males and females was isolated using the CTAB protocol adapted from Winnepenninckx et al. (1993). Female gDNA was labeled with fluorescein-12-dUTP (Jena Bioscience) using the Nick Translation Kit (Abbott Molecular Inc.), with 4 h of incubation at 15°C, and male gDNA was sonicated using a Sonopuls HD 2070 (Bandelin Electric, Berlin, Germany) and used as competitor DNA. For GISH combined with tel-FISH, the probe mixture contained fluorescein-labeled female gDNA (300 ng), Cy3-labeled telomeric probe (100 ng), sonicated male gDNA (3 μg) and sonicated salmon sperm DNA (25 μg).

Preparations for both FISH experiments were counterstained with 0.5 mg/mL DAPI, mounted in antifade based on DABCO (Sigma-Aldrich), and observed under a Zeiss Axiosplan 2 microscope (Carl Zeiss Jena, Germany). Black-and-white images were recorded with a cooled monochrome CCD camera XM10 using cellSens Standard software, version 1.9 (Olympus Europa Holding, Hamburg, Germany). All images were captured separately for each fluorescent dye, pseudocolored (light blue for DAPI, green for fluorescein and red for Cy3) and superimposed with Adobe Photoshop, version 7.0.
Insect material and crossing strategy for the linkage map

Parents were derived from *C. fumiferana* 4th-6th instar larvae collected in 2013 from two Canadian populations in Alberta (AB; north of Conklin, 55.6985 N, -111.0841 W) and Quebec (QC; Gaspésie region, 48.4675 N, -68.1946 W). These two populations were selected on the basis of the large distance separating them (~3000 km), each being near the longitudinal extremes of this species’ range in North America (Figure 1). We reasoned that genetic differentiation would likely be maximal between these two populations, thereby maximizing the number of informative loci between parents. Larvae were shipped to the Laurentian Forestry Centre (Quebec City, Canada) and reared to the adult stage. To obtain F1 families, we made 67 crosses according to the following combinations: QC male x QC female (n = 20), QC male x AB female (n = 27), AB male x QC female (n = 12) and AB male x AB female (n = 8). Of these crosses, 39 were successful in producing progeny. This F1 generation was reared for up to two years to determine the numbers of descendants tend to maximize the number of detectable recombination events used to build an informative linkage map, we selected the four largest unrelated F2 families available, representing thousands of markers from several outbred families and takes into account the absence of recombination in lepidopteran females (Robinson 1971; Suomalainen et al. 1973; Turner and Sheppard 1975). First, we employed the module EstimateLOD Limit to choose the logarithm-of-odds (LOD) score threshold, which is used to determine whether or not markers belong to the same linkage group (LG). Based on 100 datasets randomly generated from the input data, an empirical distribution of maximum LOD scores is calculated from which the LOD value can be chosen with a desired significance level. Estimation of the LOD limit was made considering only maternal information (maleprior = -1). Then, assignment to LGs was carried out using the SeparateChromosomes module, on the basis of an LOD score limit of 6.8 (p value = 0.01) and considering only maternal information (maleprior = -1). At this step, only LGs with five or more markers were kept (sizeLimit = 5). Then, singular markers were added to the defined LGs, using the JoinSingles module with all informative markers and an
LOD score limit of 8.4 (obtained by EstimateLODDLimit with 7% significance and all informative markers). After the JoinSingles step, markers with more than 15 missing genotypes (13%) were removed from the LGs. Finally, ordering and positioning of markers was computed using the OrderMarkers module of Lep-MAP, with constant rates for genotype errors and recombination. For each LG, ten independent runs were carried out, at the end of which the marker ordering with the best likelihood was kept. LG-assigned markers with a genotype error rate estimate \( \geq 0.1 \) were then removed (genotype error rate estimation is given for each marker in the output of the OrderMarkers module). In addition, markers near the ends of LGs were also removed if they contributed to \( >1\% \) of map length.

**Synteny with the Bombyx mori genome**

Reads containing SNPs used in generating the linkage map were mapped onto the genome of the silkworm, *Bombyx mori* (KAIKObase v. 3.2.2, http://sgp.dna.affrc.go.jp/KAIKO), using tblastx analysis (e-score cut-off value of 1.0e-03). KAIKObase has the advantage of providing chromosome assignments for the scaffolds forming the genome assembly of *B. mori*. As a complement to the above analysis, we also conducted a tblastx analysis against the genome of the Glanville fritillary, *Melitaea cinxia* (Lepbase version, http://lepbase.org), which has \( n = 31 \) chromosomes; this number corresponds to what is considered to be the ancestral number of chromosomes for the Lepidoptera (Suomalainen 1969; Lukhtanov 2000; Ahola et al. 2014; Yasukochi et al. 2016), thus making *M. cinxia* a valuable subject for syntenic analysis. Scaffold chromosome assignment for *M. cinxia* was obtained from the website of the Glanville fritillary genome project (Metapopulation research center, 2017, https://www.helsinki.fi/en/beta/metapopulation-research-centre/downloads; last consulted July 3, 2018). A graphical representation of the results of our *C. fumiferana–B. mori* synteny analysis was generated using the R package circlize (Gu et al. 2014).

**Data availability**

File S1 details the experimental design used to generate spruce budworm families for the linkage map. File S2 summarizes linkage map information i.e., number of markers and size in cM, for each linkage group. File S2 also provides details on position, errors and sequence for each marker included in the linkage map, and the significant hits in tblastx.
searches against the *Bombyx mori* and the *Melitaea cinxia* genomes. File S3 details assignment of the *Choristoneura fumiferana* linkage groups to *B. mori* and the *M. cinxia* chromosomes. Supplemental material and SNP data are available at Figshare: https://doi.org/10.25387/g3.6686543.

**RESULTS**

**Karyotype analysis**

The molecular cytogenetic analyses of mitotic chromosomes of *Choristoneura fumiferana* confirmed the presence of 2n = 60 chromosomes in both males and females (Figure 2), i.e., the haploid chromosome number is n = 30. In both sexes, two chromosomes stood out by their larger size (Figure 2); in female preparations, one of these large chromosomes was more intensely stained with DAPI (blue) (Figure 2a; Figure 3e) and homogeneously highlighted with the green-labeled female gDNA probe (Figure 3d, f). These two large chromosomes represent the ZZ pair of sex chromosomes in males, and the WZ pair in females, where the intensely stained chromosome is the W chromosome composed of heterochromatin. Interestingly, in preparations of pachytenic oocyte chromosomes stained by GISH and tel-FISH, the Z chromosome was clearly observed to wrap around the W chromosome to adjust its length for correct pairing; as a consequence, it appeared larger than its W counterpart (Figure 3d, e – see schematic representation). With GISH, strong binding of the female-derived genomic probe over the entire length of the W chromosome showed a high degree of molecular differentiation between the W and Z sex chromosomes (Figure 3d, f). The GISH analysis also highlighted a heterochromatic double-dot in one autosome bivalent in both sexes (Figure 3a-c; not shown for females) and an interstitial heterochromatic region of another autosome bivalent associated with the nucleolus (Figure 3d-f). This region obviously represents the nucleolus organizer region (NOR).

**SNP identification and filtering**

The *de novo* UNEAK pipeline identified 208,098 polymorphic SNPs (Table 1), 1958 of which remained after eliminating those that did not genotype in at least 80% of individuals or did not present a minor allele frequency > 0.01. We then discarded an additional 679 SNPs that showed significant deviation from Mendelian or sex chromosome (Z-linked) inheritance patterns. In comparison, the reference-genome-based Fast-GBS pipeline generated 30,854 SNPs (SNPs having passed quality criteria in Platytpus v0.8.1). Among these, we discarded markers that were not genotyped in at least 80% of individuals, presented a minor allele frequency < 0.01 or were triallelic. Of the remaining 22,094 SNPs, we discarded 9473 that showed significant deviation from Mendelian or sex chromosome (Z-linked) inheritance patterns. One descendant of family 24 (Figure 1) revealed Z-linked SNP segregation incompatible with the sex determined at the pupal stage; for the further analyses this individual was discarded. At the end of this filtering process, we were left with 13,900 SNPs, 12,621 and 1279 of which were identified using the Fast-GBS and UNEAK pipelines, respectively (Table1).

**Construction of a Choristoneura fumiferana linkage map**

We ran Lep-MAP on 13,900 SNPs, 7610 and 8012 of which were maternally and paternally informative, respectively (i.e., heterozygous in the mother and the father of one of the crosses). The SeparateChromosomes module initially allocated 3180 SNPs to 30 LGs, while the JoinSingles module added 1853 SNPs to these 30 LGs, for a total of 5192 SNPs. After these two steps, 99 markers were removed from the LGs because more than 15 individuals presented missing genotypes (13%) for these markers. Then, the OrderMarkers module successfully ordered 3741 SNPs across 30 LGs. Among these 3741 SNPs, we discarded 50 markers presenting a genotype error estimate > 0.1 and 14 markers located near LG tips and contributing over 20 cM (>1.2%) to map length. In addition, we found 78 duplicated SNPs on the linkage map, a situation arising from our dual SNP identification approach (i.e., UNEAK + Fast-GBS pipelines). In these cases, we retained the SNP copy presenting the least amount of missing data, typically the one identified using the Fast-GBS pipeline. Finally, close examination of the SNPs identified using the UNEAK pipeline indicated that 13 of them were in fact duplicates that escaped detection due to the presence of an indel within a mono-nucleotide repetitive region of both alleles of a locus (for details on this property of UNEAK, see Picq et al. 2018). Once again, the SNP copy presenting the least amount of missing data were retained.

In the end, the GBS/SNP-based linkage map we developed for C. *fumiferana* is made up of 3586 markers forming 30 linkage groups (LGs), for a total length of 1720.41 cM (Figure 4, File S2). The number of LGs found is in agreement with the number of chromosomes identified by our cytogenetic analyses. The genetic length of each LG ranges from 24.72 to 110.22 cM, with an average inter-locus distance varying between 0.31 and 1.23 cM among LGs, and a number of SNPs ranging between 22 to 352 per LG. The longest LG, with 352 SNPs along 110.22 cM, revealed a SNP segregation pattern characteristic of the Z chromosome (female offspring appear homozygous for one of the father’s alleles, indicating hemizygosity). LG numbering is that provided by the Lep-Map software. Among the 3586 markers used to generate the linkage map, 3394 (94.65%) were identified using the reference-genome-dependent Fast-GBS pipeline, while the 192 remaining SNPs (5.35%) were found using *de novo* UNEAK pipeline; 35 of these SNPs were in regions absent from our reference genome, confirming an earlier assessment of the bw6 assembly’s completeness. Thus, combining the two SNP identification approaches used here proved to be both appropriate and efficient, as judged by the quality and density of markers found, making it possible to obtain the expected number of linkage groups.

It must be pointed out that the SeparateChromosomes module, parameterized with an LOD score limit of 6.8, identified a 31st LG featuring 39 SNP markers. However, the ordermarker module could order only 9 of these markers. After removing three markers that...
displayed a genotype error estimate > 0.1, we ran again the ordermarker module on the remaining markers of the 31st LG; this time, the ordermarker module was unable to order markers (blank output). To further explore this phenomenon, we ran the SeparateChromosomes module again, with a lower LOD score limit of 6.2. This time, the 39 SNP markers were added to the end of the 27th LG. After a first run of the ordermarker module followed by the deletion of markers displaying a genotype error estimate > 0.1, the ordermarker module was run a second time on this elongated LG. In the end, only 6 markers out of 39 could be successfully ordered, but they were 40 cM away from the nearest marker on LG 27 and spanned 91.41 cM. With these 6 additional markers, the LG 27 doubles in size. Eventually, the sequences associated with these 6 ordered markers did not generate significant hits against the B. mori genome or against the NCBI nr database (TBLASTX analyses, expected value cut-off: 1.0e-03). In light of these results, we discarded the 31st LG obtained with the SeparateChromosomes module, parameterized with an LOD score limit of 6.8.

Chromosomal homology through synteny analysis

Among the sequences associated with the 3586 markers making up the C. fumiferana linkage map, 285 (7.9%) generated a significant hit in TBLASTX searches against the B. mori genome (expected value cut-off: 1.0e-03; Figure 5, File S2 and File S3). We inferred chromosomal homology between C. fumiferana and B. mori on the basis of 2 to 20 significant hits per chromosome. This analysis revealed one interesting feature: the Z chromosome of C. fumiferana corresponds to the fusion of chromosomes Z and 15 in the B. mori genome (Figure 5).

A similar synteny analysis targeting the M. cinxia genome yielded 176 significant TBLASTX hits and, overall, confirmed the above inferences about chromosome homology based on the B. mori genome (File S2 and File S3). However, LG 30 of C. fumiferana generated no significant hits in TBLASTX searches against M. cinxia chromosomes; in this case, chromosome homology was first inferred, and then confirmed with an earlier M. cinxia-B. mori synteny analysis (Aholá et al. 2014; see File S3 for details).

Given that the fusion of the Z chromosome with an autosome can significantly increase the adaptive potential of a species (Orr 2010), we conducted TBLASTX analyses against the NCBI nr database using, as queries, C. fumiferana sequences associated with markers located in the Z chromosome region corresponding to the B. mori chromosome 15 (Table 2). One Z-linked marker sequence revealed a significant match (expect value 5.10^-6) with an ABC transporter C2 (ABCC2) protein of B. mori, which confers resistance to Bacillus thuringiensis toxin Cry1Ab (Atsumi et al. 2012).

**DISCUSSION**

We have combined cytogenetic approaches and a high-density SNP-based linkage map to explore the organization of the spruce budworm
genome. Our karyotype analyses confirmed the presence of 30 pairs of chromosomes in Choristoneura fumiferana, a number commonly found in Tortricidae, one of three subfamilies recognized in the Tortricidae (Robinson 1971; Ennis 1976; Harvey 1997; Šírová et al. 2013). Our molecular cytogenetic analyses also enabled identification of a noticeably larger pair of chromosomes in both sexes. In females, one of these two large chromosomes was strongly stained by both DAPI and fluorescently-labeled female gDNA (GISH analysis). In Lepidoptera, this staining pattern is typical of the W chromosome (Traut and Marec 1987; Robinson 1971; Ennis 1976; Blackmon et al. 2004; Fuková et al. 2005; Yoshido et al. 2006). As such, our findings provide evidence for the existence of a WZ/ZZ (female/male) sex chromosome system in the spruce budworm; C. fumiferana can now be added to the modest list of ~90 lepidopteran species for which the sex chromosome system has been identified [F. Marec, unpublished data; Traut et al. (2007) and Marec et al. (2010) reported ~40 species]. Interestingly, the number of lepidopteran species for which the sex chromosome system has been described is much smaller than the number for which chromosomes have been quantified (~1,000; Robinson 1971; Ennis 1976; Blackmon et al. 2017). This difference may be attributed to the fact that many earlier studies used male germ cells, which are appropriate for chromosome counts, but not for identifying the sex chromosomes (Traut et al. 2007; Sahara et al. 2012). In recent years, the use of female meiotic pachytenic chromosomes combined with techniques of molecular cytogenetics (FISH, CGH, GISH, etc.), has facilitated the characterization of sex chromosomes. Thus, in coming years, we can anticipate an increase in the number of species for which the sex chromosome system has been characterized (Sahara et al. 2012).

The ancestral chromosome number in Lepidoptera is inferred to be n = 31 (Suomalainen 1969; Lukhtanov 2000; Ahrola et al. 2014; Yasukochi et al. 2016). From this ancestral state, the number of chromosomes has evolved through chromosome fission/fusion events (De Prins and Saitoh 2003; Brown et al. 2004; Marec et al. 2010). In tortricids, the reduced number of chromosome pairs relative to the ancestral state (i.e., n = 28 in Olethreutinae and n = 30 in Tortricinae, instead of 31) and the presence of large sex chromosomes suggest a sex chromosome-autosome fusion event during the evolution of their karyotypes (Fuková et al. 2005; Šírová et al. 2013). Indeed, a recent cytogenetic study confirmed that the Z chromosome in three different tortricid species (Cydia pomonella, Lobesia botrana, and Eupoecilia ambiguella) arose from the fusion of an ancestral Z chromosome and an autosome (Nguyen et al. 2013). In our study, synteny analysis comparing the 30 linkage groups of C. fumiferana with the 28 chromosomes comprising the B. mori genome provided strong evidence for the presence of the same neo-Z chromosome in the spruce budworm genome. A Z chromosome-autosome fusion can have a significant impact on the adaptive capacity of a species. Indeed, the hemizygous state of the Z-linked genes in the female Lepidoptera increases the efficiency of natural selection acting on recessive mutations (faster X/Z evolution; Charlesworth et al. 1987; Bachtrog et al. 2009). In tortricids, the Z chromosome-autosome fusion is considered to have contributed to the adaptive success and the important radiation of this taxon (Nguyen et al. 2013). Indeed, the part of the neo-Z chromosome derived from an autosome corresponds to chromosome 15 in B. mori, which is known to bear clusters of genes involved in detoxification of plant metabolites and genes conferring insecticide resistance (Nguyen et al. 2013), such as the ABC transporter.
Table 2 SNP markers located in the Z chromosome region corresponding to B. mori chromosome 15 and identified in sequences displaying significant TBLASTX hits for known proteins

| Gene product | Function | Position on chromosome 15 (cM) | E-value | Protein sequence access. No. (organism) | No. of hits |
|--------------|----------|---------------------------------|---------|----------------------------------------|-------------|
| Cap-specif... | Molecular scaffold protein; ... | 23188   | 10^-10 <1 | XM_012692141.1 (Bombyx mon) | 10          |
| Neoplastic... | Membrane lipid homostasis | 45.7    | 10^-10 <1 | XM_012570849.1 (Papilio xuthus) | 4           |
| AP-3 complex... | Facilitate the budding of vesicle | 4.5    | 10^-10 <1 | XM_012592435.1 (Bombyx mon) | 4           |
| DNA binding... | Regulation of MHCII molecule | 55.6    | 10^-10 <1 | XM_012570849.1 (Papilio xuthus) | 19          |
| C2 (ABCC2)... | Transmembrane transport/... | 62.3    | 10^-10 <1 | XM_012697398 (Bombyx mon) | 203         |

C2 (ABCC2) gene associated with recessive resistance to the Bacillus thuringiensis (Bt) toxin (Atsumi et al. 2012; Park et al. 2014; Xiao et al. 2014). In our study, the sequence of a marker located on the Z linkage group showed significant similarity (expect value: 5 *10^-6) to the ABCC2 protein located on chromosome 15 in B. mori. Thus, Z-linkage of the ABCC2 gene in C. fumiferana provides a genomic context favorable to the selection and rapid spread of Bt resistance in spruce budworm populations. Since there is significant variation in Bt tolerance among spruce budworm populations (van Frankenhuyzen et al. 1995), our results stress the importance of making rational use of Bt, as it is the main insecticide used to suppress spruce budworm populations in Canadian provinces (van Frankenhuyzen 2013).

The W chromosome of C. fumiferana displayed distinctive features relative to other chromosomes, including the Z chromosome. Indeed, the staining patterns obtained with our FISH assays revealed a W chromosome that is thoroughly and homogeneously heterochromatinized, i.e., constituted of condensed chromatin. Studies examining the composition of the heterochromatinized W chromosome in other lepidopteran species have pointed to a high content of repetitive sequences, transposable elements, degenerated protein-coding genes, and sequences of mitochondrial origins (Abe et al. 2005; Fuková et al. 2007; Vítková et al. 2007; Traut et al. 2013; Lammermann et al. 2016). Such an unusual composition raises questions about the involvement of this chromosome in sex determination in Lepidoptera. For instance, in Samia cynthia, the W chromosome does not seem to be involved in sex determination (Yoshido et al. 2016). However, in B. mori, a dominant female-determining factor (Fem) located on the W chromosome has been shown to promote female-bias (Fuji and Shimada 2007). Recently, this feminizing factor was determined to be a small RNA (Fem piRNA, Kiuchi et al. 2014). Considering the above mentioned examples, the sex determination mechanism in the spruce budworm has a high chance of being specific to this species. In C. fumiferana and other pest species, identification of the sex determination mechanism has relevance beyond questions of basic evolutionary biology, as this knowledge could enable development of alternative pest management approaches based on the sterile insect technique (SIT) (Whyard et al. 2015; Darrington et al. 2017). The SIT method is based on rearing and releasing a large number of sterile males into wild populations with the aim to decrease reproductive success (Marcé et al. 2005). RNA interference (RNAi) targeting genes involved in female sex determination has been used successfully to eliminate females during the rearing process, while RNAi could also be used to silence genes involved in spermatogenesis and induce male sterility (Whyard et al. 2015). SIT could thus offer an interesting alternative to the insecticide Btk (see above) to manage spruce budworm populations.

Concerning the heterochromatinization patterns of the W chromosome, those observed here in the spruce budworm are similar to patterns reported for the tortricid Cydia pomonella (Fuková et al. 2005; Síchová et al. 2013). However, heterochromatinization of the W chromosome is partial in two other tortricid species, Lobesia botrana and Eupoecilia ambigua (Síchová et al. 2013). According to Síchová et al. (2013), this pattern suggests that the tortricid W chromosome originated from the fusion of an ancestral W chromosome with an autosome, most likely the homolog of chromosome 15 in B. mori, as shown for the Z chromosome (see above). Thus, the spruce budworm appears to have a neo-W chromosome whose complete heterochromatization may have resulted from more rapid molecular degeneration of its neo-part than that affecting the W chromosomes of L. botrana and E. ambigua.

To our knowledge, linkage maps have been published for 10 different lepidopteran species, half of which belong to the Papilionoidea superfamily (Tobler et al. 2004; Wang and Porter 2004, Jiggins et al. 2005;
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LITERATURE CITED

Abe, H., K. Mita, Y. Yasukochi, T. Oshiki, and T. Shimada, 2005 Retrotransposable elements on the W chromosome of the silkworm, Bombyx mori. Cytogenet. Genome Res. 110: 144–151. https://doi.org/10.1159/000084946

Ahola, V., R. Lehtonen, P. Somervuo, L. Salmela, P. Koskinen et al., 2014 The Glanville fritillary genome retains an ancient karyotype and reveals selective chromosomal fusions in Lepidoptera. Nat. Commun. 5: 4737. https://doi.org/10.1038/ncomms5737

Andrews, S., 2016 FastQC A quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Atsumi, S. K., Miyamoto, K. Yamamoto, J. Narukawa, S. Kawai et al., 2012 Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, Bombyx mori. Proc. Natl. Acad. Sci. USA 109: E1591–E1598. https://doi.org/10.1073/pnas.1126090109

Bachtrog, D., J. D. Jensen, and Z. Zhang, 2009 Accelerated adaptive evolution on a newly formed X chromosome. PLoS Biol. 7: e1000082. https://doi.org/10.1371/journal.pbio.1000082

Baxter, J., W. L. Baxter, J. W. Davey, J. S. Johnston, A. M. Shetlon, D. G. Heckel et al., 2011 Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. PLoS One 6: e19315. https://doi.org/10.1371/journal.pone.0019315

Beldade, P., S. V. Saenko, N. Pul, and A. D. Long, 2009 A gene-based linkage map for Bicyclus anynana butterflies allows for a comprehensive analysis of synten with the lepidopteran reference genome. PLoS Genet. 5: e1000366. https://doi.org/10.1371/journal.pgen.1000366

Blackmon, H., L. Ross, and D. Bachtrog, 2017 Sex determination, sex chromosomes, and karyotype evolution in insects. J. Hered. 108: 78–93. https://doi.org/10.1093/jhered/esw047

Boudanger, Y., D. Arsenault, H. Morin, T. Jardou, P. Bertrand et al., 2012 Dendrochronological reconstruction of spruce budworm (Choristoneura fumiferana) outbreaks in southern Quebec for the last 400 years. Can. J. For. Res. 42: 1264–1276. https://doi.org/10.1139/s2012-069

Brakefield, P. M., J. Pijpe, and B. J. Zwaan, 2007 Developmental plasticity and acclimation both contribute to adaptive responses to alternating seasons of plenty and of stress in Bicyclus butterflies. J. Biosci. 32: 465–475. https://doi.org/10.1007/s12038-007-0046-8

Brown, K. S., Jr, B. Von Scholtz, and E. Suomalainen, 2004 Chromosome evolution in Neotropical Danaine and Ithomiinae (Lepidoptera). Hereditas 141: 216–236. https://doi.org/10.1111/j.1601-5223.2004.01868.x

Brunet, B. T. M., 2014 Genomic analysis of hybridization between the spruce budworm species Choristoneura fumiferana, C. occidentalis, and C. biennis (Lepidoptera: Tortricidae). PhD thesis, University of Alberta, Edmonton, AB, Canada.

Brunet, B. T. M., G. S. Blackburn, K. Muirhead, L. M. Lumley, B. Boyle et al., 2017 Two’s company, three’s a crowd: new insights on spruce budworm species boundaries using genotyping-by-sequencing in an integrative species assessment (Lepidoptera: Tortricidae). Syst. Entomol. 42: 317–328. https://doi.org/10.1111/syen.12111

Bush, G. L., 1966 Female heterogamy in the family Tethephridae (Acalyptratae, Diptera). Am. Nat. 100: 119–126. https://doi.org/10.1086/282405

Cartwright, D. A., M. Troggio, R. Velasco, and A. Guti, 2007 Genetic mapping in the presence of genotyping errors. Genetics 176: 2521–2527. https://doi.org/10.1534/genetics.106.063982

Charlesworth, B., J. A. Coyne, and N. H. Barton, 1987 The relative rates of evolution of sex chromosomes and autosomes. Am. Nat. 130: 113–146. https://doi.org/10.1086/284701

Chen, N., C. V. Van Hout, S. Gottipati, and A. G. Clark, 2014 Using Mendelian inheritance to improve high-throughput SNP discovery. Genetics 198: 847–857. https://doi.org/10.1534/genetics.114.169052

Cheng, T., J. Wu, Y. Wu, R. V. Chilukuri, L. Huang et al., 2017 Genomic adaptation to polyphagy and insecticides in a major East Asian noctuid pest. Nat. Ecol. Ecol. 1: 1747–1756. https://doi.org/10.1038/s41559-017-0314-4

Dalkóvíc, M., M. Zravá, I. Hladová, P. Nguyen, I. Sonský et al., 2017 New insights into the evolution of the W chromosome in Lepidoptera. J. Hered. 108: 709–719. https://doi.org/10.1093/jhered/ess063

Danecek, P., A. Auton, G. Abecasis, C. A. Albers, E. Banks et al., 2011 The variant call format and VCFtools. Bioinformatics 27: 2156–2158. https://doi.org/10.1093/bioinformatics/btr330

Darrington, M., T. Dalmay, N. I. Morrison, and T. Chapman, 2017 Implementing the sterile insect technique with RNA interference – a review. Entomol. Exp. Appl. 164: 155–175. https://doi.org/10.1111/eea.12575

Davey, J. W., M. Chouteau, S. L. Barker, L. Maroja, S. W. Baxter et al., 2016 Major improvements to the Heliconius melpomene genome assembly used to confirm 10 chromosome fusion events in 6 million years of butterfly evolution. G3 (Bethesda): 695–708. https://doi.org/10.1534/g3.16.1.2655

Dupuis, J. R., B. M. T. Brunet, H. M. Bird, L. M. Lumley, G. Fagua et al., 2017 Genome-wide SNPs resolve phylogenetic relationships in the North American spruce budworm (Choristoneura fumiferana) species complex. Mol. Phylogenet. Evol. 111: 158–168. https://doi.org/10.1016/j.ympev.2017.04.001

Dopman, E. B., S. M. Bogdanowicz, and R. G. Harrison, 2004 Genetic mapping of sexual isolation between E and Z pheromone strains of the European corn borer (Ostrinia nubilalis). Genetics 167: 301–309. https://doi.org/10.1534/genetics.167.1.301

Ennis, T. J., 1976 Sex chromatin and chromosome numbers in Lepidoptera. Can. J. Genet. Cytol. 18: 119–130. https://doi.org/10.1139/g-76.017

van Frankenhuyzen, K., 2013 Cross-order and cross-phylum activity of Bacillus thuringiensis pesticidal protein. J. Invertebr. Pathol. 114: 76–85. https://doi.org/10.1016/j.jip.2013.05.010

van Frankenhuyzen, K., C. W. Nystrom, and B. E. Tabashnik, 1995 Variation in tolerance to Bacillus thuringiensis among and within populations of the spruce budworm (Lepidoptera: Tortricidae) in Ontario. J. Econ. Entomol. 88: 97–105. https://doi.org/10.1093/jee/88.1.97

Fuji, T., and T. Shimada, 2007 Sex determination in the silkworm, Bombyx mori: A female determinant on the W chromosome and the sex-determining gene cascade. Semin. Cell Dev. Biol. 18: 379–388. https://doi.org/10.1016/j.semcdb.2007.02.008
Van’t Hof, A. E., P. Nguyen, M. Daliková, N. Edmonds, F. Marec et al., 2013 Linkage map of the peppered moth, Biston betularia (Lepidoptera, Geometridae): a model of industrial melanism. Heredity 110: 283–295. https://doi.org/10.1038/hdy.2012.84

Vítková, M., I. Fuková, S. Kubíčková, and F. Marec, 2007 Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera). Chromosome Res. 15: 917–930. https://doi.org/10.1007/s10577-007-1173-7

Wang, B., and A. H. Porter, 2004 An AFLP-based interspecific linkage map of sympatric, hybridizing Colias butterflies. Genetica 168: 215–225. https://doi.org/10.1023/A:1009297729547

Wühr, S., C. N. Erdelyan, A. L. Partridge, A. D. Singh, N. W. Beebe et al., 2015 Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. Parasit. Vectors 8: 96. https://doi.org/10.1186/s13071-015-0716-6

Winnenpeinickx, B., T. Backeljau, and R. De Wachter, 1993 Extraction of high molecular weight DNA from molluscs. Trends Genet. 9: 407. https://doi.org/10.1016/0168-9525(93)90102-N

Winter, C. B., and A. H. Porter, 2010 AFLP linkage map of hybridizing swallowtail butterflies, Papilio glaucus and Papilio canadensis. J. Hered. 101: 83–90. https://doi.org/10.1093/hered/esp067

Xiao, Y., T. Zhang, C. Liu, D. G. Heckel, X. Li et al., 2014 Mis-splicing of the ABC2 gene linked with Bt toxin resistance in Helicoverpa armigera. Sci. Rep. 4: 6184. https://doi.org/10.1038/srep06184

Yamamoto, K., J. Nohata, K. Kadono-Okuda, J. Narukawa, M. Sasanna et al., 2008 A BAC-based integrated linkage map of the silkworm Bombyx mori. Genome Biol. 9: R21. https://doi.org/10.1186/gb-2008-9-1-r21

Yasukochi, Y., L. A. Ashakumary, K. Baba, A. Yoshido, and K. Sahara, 2006 A second-generation integrated map of the silkworm reveals synteny and conserved gene order between lepidopteran insects. Genetics 173: 1319–1328. https://doi.org/10.1534/genetics.106.055541

Yasukochi, Y., M. Ohno, F. Shibata, A. Jouraku, R. Nakano et al., 2016 A FISH-based chromosome map for the European corn borer yields insights into ancient chromosomal fusions in the silkworm. Heredity 116: 75–83. https://doi.org/10.1038/hdy.2015.72

Yasukochi, Y., M. Tanaka-Okuyama, F. Shibata, A. Yoshido, F. Marec et al., 2009 Extensive conserved synteny of genes between the karyotypes of Manduca sexta and Bombyx mori revealed by BAC-FISH mapping. PLoS One 4: e7465. https://doi.org/10.1371/journal.pone.0007465

Yoshido, A., F. Marec, and K. Sahara, 2005 Resolution of sex chromosome constitution by genomic in situ hybridization and fluorescence in situ hybridization with (TTAGG)ₙ telomeric probe in some species of Lepidoptera. Chromosome Res. 11: 193–202. https://doi.org/10.1007/s10577-005-0013-9

Yoshido, A., F. Marec, and K. Sahara, 2016 The fate of W chromosomes in hybrids between wild silkmots, Samia cynthia spp.: no role in sex determination and reproduction. Heredity 116: 424–433. https://doi.org/10.1038/hdy.2015.110

Yoshido, A., Y. Yamada, and K. Sahara, 2006 The W chromosome detection in several lepidopteran species by genomic in situ hybridization (GISH). J. Insect Biotechnol. Sericology 75: 147–151.

Yoshido, A., Y. Yasukochi, and K. Sahara, 2011 Samia cynthia vs. Bombyx mori: Comparative gene mapping between a species with a low-number karyotype and the model species of Lepidoptera. Insect Biochem. Mol. Biol. 41: 370–377. https://doi.org/10.1016/j.ibmb.2011.02.005

You, M., Z. Yue, W. He, X. Yang, G. Yang et al., 2013 A heterozygous moth genome provides insights into herbivory and detoxification. Nat. Genet. 45: 220–225. https://doi.org/10.1038/ng.2524

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