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Original Article

Genome assembly, sex-biased gene expression and dosage compensation in the damselfly *Ischnura elegans*

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ABSTRACT

The evolution of sex chromosomes, and patterns of sex-biased gene expression and dosage compensation, are poorly known among early winged insects such as odonates. We assembled and annotated the genome of *Ischnura elegans* (blue-tailed damselfly), which, like other odonates, has a male-hemigametic sex-determining system (X0 males, XX females). By identifying X-linked genes in *I. elegans* and their orthologs in other insect genomes, we found homologies between the X chromosome in odonates and chromosomes of other orders, including the X chromosome in Coleoptera. Next, we showed balanced expression of X-linked genes between sexes in adult *I. elegans*, i.e. evidence of dosage compensation. Finally, among the genes in the sex-determining pathway only *fruitless* was found to be X-linked, while only *doublesex* showed sex-biased expression. This study reveals partly conserved sex chromosome synteny and independent evolution of dosage compensation among insect orders separated by several hundred million years of evolutionary history.

1. Introduction

Sex chromosomes are derived from ordinary autosomes, harbour genes that determine sex (e.g. *SRY* in mammals) and segregate differently in males and females [1–3]. They play important roles in central evolutionary processes, including sexual conflict and speciation [4–6], and contain genes with sex-specific and sexually antagonistic functions [7–9]. Accordingly, sex chromosomes have a long research tradition and are well-characterised molecularly, functionally and evolutionary in several lineages, including in mammals and insects [1–3].

Sex chromosomes frequently evolve recombination suppression with subsequent decay and massive gene loss of the sex-limited sex chromosome (Y or W in respectively XY and ZW heterogametic sex-determining systems), which sometimes may be completely lost (X0 and Z0 hemigametic systems; [10–14]). This degenerative process leads to differences in the number of gene copies between the sexes, with the hetero−/hemigametic sex (XY/X0 males and ZW/Z0 females) having only a single copy of sex-linked genes. This drop in gene copy number (gene dose) in the hetero−/hemigametic sex was thought to be detrimental because it could lead to imbalanced expression between sex-linked and autosomal genes [3,13,15,16]. However, a process known as dosage compensation may evolve to balance the expression of sex-linked genes between the sexes as well as between sex chromosomes and autosomes [13,17,18]. Dosage compensation is taxonomically widespread and occurs e.g. in both therian mammals and true flies (Diptera), and was thought to be a necessary regulatory process in species with genetic sex determination until disproved by data from e.g. birds [13].

It is becoming increasingly clear that the mechanisms of dosage compensation, and even dosage compensation per se, are far from universal and more flexible than than previously assumed [13,19–21]. In Diptera, dosage compensation is achieved through sex-specific epigenetic finetuning resulting in hyper-expression of the single X chromosome in heterogametic males and counteracting downregulation of the two X-linked gene copies in the homogametic females [22–24], and in therian mammals epigenetic silencing of one entire X chromosome in females plays an important role for equalizing the expression between the sexes [25–27]. In contrast, all birds, and some fish and amphibians, lack similar chromosome-wide dosage compensation mechanisms and

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consequently show pronounced sex-bias in a majority of sex-linked genes [13,18,28]. In addition to such taxonomical contrasts, the level of dosage compensation may vary across gene classes, between tissues and through development [13,29,30]. Single genes sometime escape chromosome-wide dosage compensation and evolve sex-specific expression [29,31]; potentially to resolve intralocus sexual conflicts driven by sexually antagonistic selection [32]. Many questions regarding dosage compensation still remain, e.g., it is little understood what is causing the wide variation in sex chromosome gene regulation within and between taxa [13,16,21]. Moreover, the underlying genetic basis of sexual conflict is still largely unknown, and how sexual conflict is intertwined with sex determination and dosage compensation is not fully understood [6,16,32,33].

In insects, dosage compensation and sex-biased gene expression are well characterised in species representative of the orders Hemiptera, Strepsiptera, Coleoptera, Lepidoptera and Diptera [21,23,34–39], all within infraclass Neoptera, but remain poorly known in other lineages (Fig. 1; [21]). To expand our knowledge on the evolution of sex chromosomes and their gene regulation in insects, a more comprehensive view of the existing mechanisms in a wider range of taxa is needed. The order Odonata (dragonflies and damselflies), infraclass Palaeoptera, is of particular interest in this respect due to its early-branching phylogenetic position within winged insects (subclass Pterygota [40]). Characterization of the X chromosome in representatives of the first winged insects would allow an unprecedented exploration of orthologous relationships between the sex chromosomes of other insects with available genomes [41]. Ecological and evolutionary research in odonates has a long history [42,43], and such research include the consequences of selection and sexual conflict in natural field populations and the maintenance of body colour polymorphism that is present in some families [44–48]. More recently, there has also been an increasing number of phylogenetic comparative studies on odonates investigating questions about the

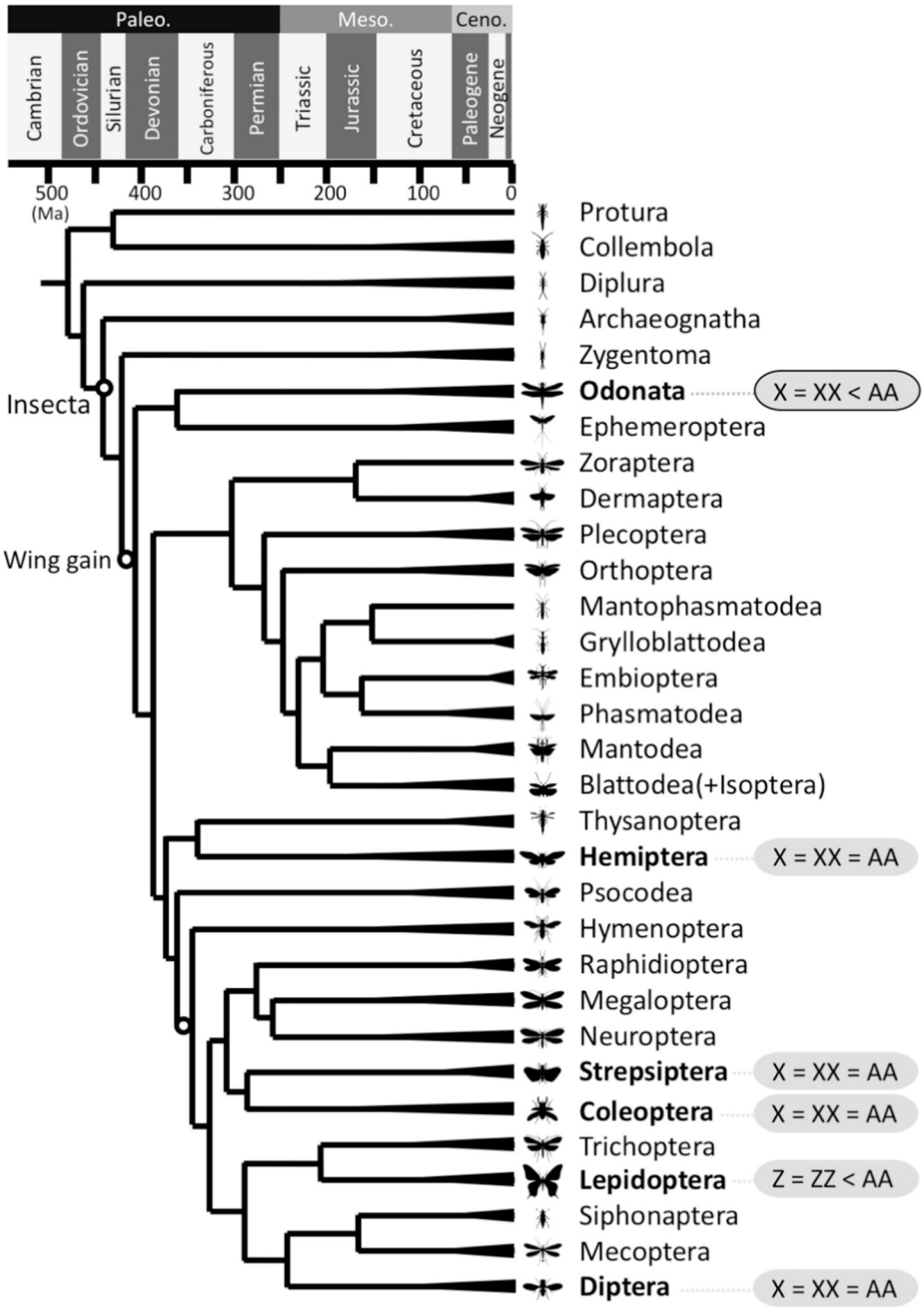

Fig. 1. Phylogeny of Insecta with dosage compensation type indicated for available lineages. Dosage compensation types (sensu Gu and Walters [21]) and sex chromosome systems are denoted based on the type generally encountered in respective insect order: X = XX = AA: balanced and complete; X = XX < AA (or Z = ZZ < AA): balanced but incomplete. The result of the current study (*Ischnura elegans*; Odonata) is denoted with a circled diagram. The phylogeny is based on previous publications [43,122], and references for dosage compensation type in each order are given in the main text.

macroevolutionary dynamics of several life-history traits [49,50], as well as phylogeographical and landscape genomic studies addressing the adaptive consequences of recent range expansions [51,52].

Two odonate species have had their genomes assembled (*Libellula fulva*, BioProject PRJNA194433; *Calopteryx splendens*, [53]), but neither of them included information of the sex chromosome. Thus, limited genomic resources for odonates have so far impeded the progress towards understanding their pattern of dosage compensation, sex-biased gene expression and sex determination system [43]. For this reason, we assembled and annotated a draft genome of the blue-tailed damselfly *Ischnura elegans* (Odonata: Coenagrionidae). *Ischnura elegans* has a male hemigametic sex chromosome system (XX females and X0 males; [54]), a feature shared with many other odonate species [55]. Moreover, *I. elegans* exhibits several features of evolutionary and ecological interest including a female-specific, genetically determined colour polymorphism (with three distinct morphs [47,56]) and a well-documented range expansion in northern Europe in response to recent (anthropogenic) climate change [52,57]. With the resulting genome assembly and associated characterization of the X chromosome in *I. elegans*, and by analysing transcriptome-wide gene expression in adult males and females, we address three key questions. First, does the X chromosome of this old insect order show homologies to more recently evolved insect orders? Second, does dosage compensation occur, and if so by which mechanism [21]? Third, are genes with sex-biased expression enriched on the X chromosome in this lineage? We also investigated whether known sex-determining genes are expressed in adult *I. elegans* and whether these are sex-biased and/or X-linked.

2. Results

2.1. Genome assembly and annotation

Our initial *I. elegans* genome assembly consisted of 45,562 scaffolds with a total length of 1.8 Gbp and a N50 of 613 kbp (Supplementary material 1, sheet 1). Gap-filling filled 20% of the gaps and merged, together with transcriptome-assisted scaffolding, 800 scaffolds. After removing short scaffolds (< 10 kbp; possibly containing repeats and mainly fragmented genes), the final genome assembly contained 8936 scaffolds with a total size of 1.67 Gbp, a N50 of 744 kbp, and a maximum scaffold length of 5.4 Mbp (Supplementary material 1, sheet 1). This genome assembly included 98.2% of the BUSCO genes (complete: 96.3% [single: 95.7%, duplicate: 0.6%], fragment: 1.9%, missing: 1.8%; n = 1658 genes). Our assembled genome size (1.67 Gbp) is similar to the size of 1.7 Gbp that we estimated in Allpaths-LG [58] and similar to the 1.63 Gbp assembled genome of *Calopteryx splendens* (an odonate species in family Calopterygidae; [53]). The 1000 longest scaffolds constituted 64% of the genome (Fig. 2A). De novo repeats identified constituted 24% of the genome, with the most common repeats being LINEs: RTE/Bov-B (2.4%) and L2/CR1/Rex (1.9%) (Supplementary material 1, sheet 2).

Our annotation predicted 25,841 protein-coding isoforms consisting of 24,777 protein-coding genes. Among the 25,841 isoforms, 19,506 (75.5%) were annotated by BLASTp, 20,105 (77.8%) contained an InterProScan protein domain and 10,207 (39.5%) had gene ontology (GO) annotations. The BLASTp top-hits (Supplementary material 2, Fig. S1) included hits to *Zootermopsis nevadensis* (Isoptera: Termopsidae) and *Nilaparvata lugens* (Hemiptera: Delphacidae). The most widely distributed InterProScan families and domains were P-loop-containing-nucleotide-triphosphate-hydrolase (IPR027417) and Zinc-finger-C2H2-

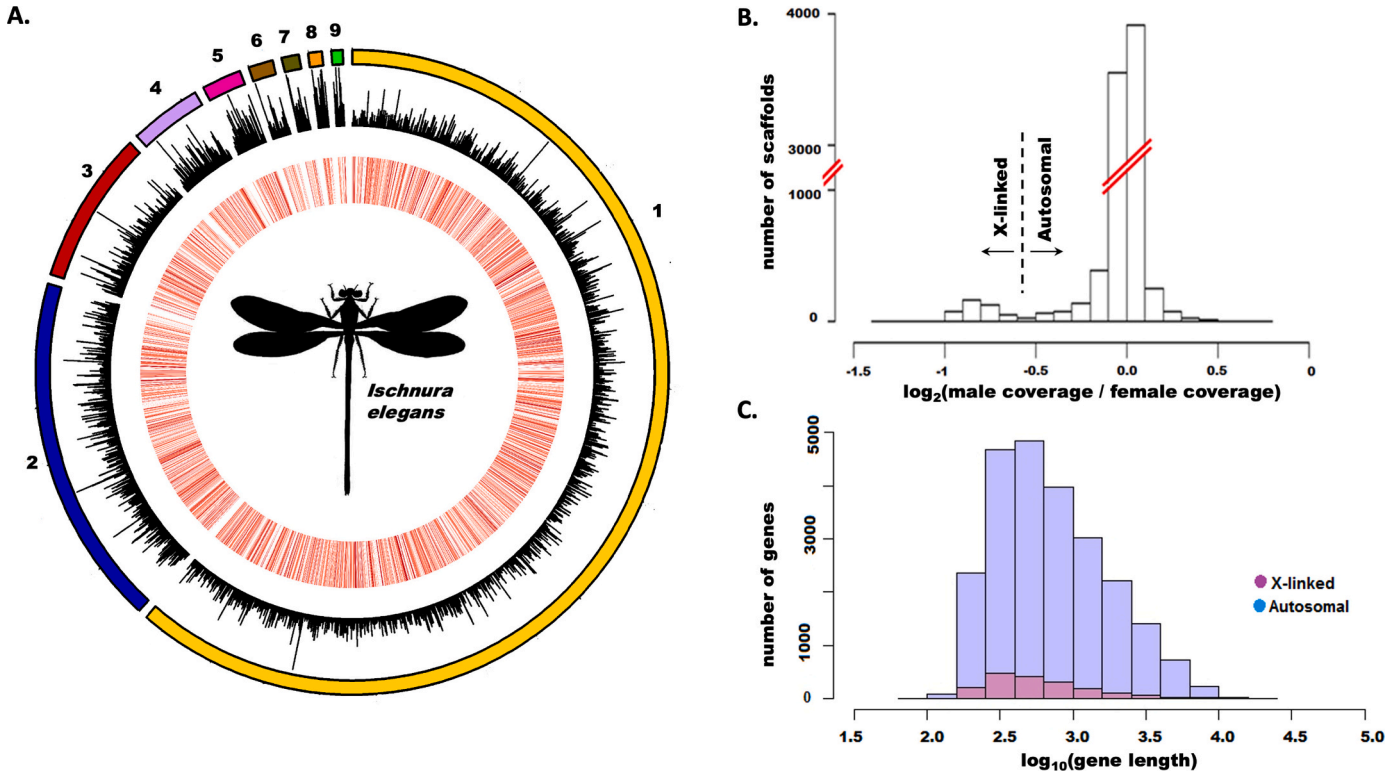

Fig. 2. The draft genome assembly of *Ischnura elegans*. **A.** Circular plot illustrating the draft genome of *I. elegans* consisting of 8936 scaffolds. Scaffolds are arranged in descending size order (5.4 Mbp to 10 kpb), and the outer circle shows bins of 1000 scaffolds (except for bin 9 that contains the 936 shortest scaffolds). The middle circle indicates the position of genes and their length (black bars), and the inner circle is a heat-map demonstrating the gradient of gene annotation (based on BLASTx and InterProScan result). **B.** Histogram showing the frequency of scaffolds in bins of $\log_2$(male coverage / female coverage). Scaffolds with $\log_2$(male coverage / female coverage) < −0.62 were classified as X-linked. **C.** Histogram showing the frequency of $\log_{10}$(gene length) for autosomal and X-linked genes.

type (IPR013087) (a complete list of InterProScan domains and families is given in Supplementary material 2, Fig. S2-S3). GO annotation classification into three major GO categories generated 5750 (56.3%) genes belonging to biological process, 3225 (31.6%) to cell component and 8895 (87.1%) to molecular function categories (functional descriptions of GO terms of each category are given in Supplementary material 2, Fig. S4–S6). In total, 1973 protein-coding genes were annotated as enzymes belonging to six major classes of oxidoreductase (188 genes), transferase (407), hydrolases (1217), lyases (55), isomerase (52) and ligases (96). The gene length of the 25,841 annotated isoforms ranged between 93 and 20,365 bp and long genes were found on both long and short scaffolds (Fig. 2A).

To identify possible contaminations in the genome assembly, we performed a species scan of the predicted genes. We detected 393 genes with BLASTp hits to bacteria (Supplementary material 1, sheet 3). Among them, 341 (87%) showed high similarity with *Wolbachia*, while the other 52 (13%) were from *Candidatus Nephrothrix* sp. *EaCA, E.coli,* etc. The genes that showed similarity to *Wolbachia* were annotated as *Wolbachia*-endosymbionts of *Drosophila simulans*, *Nasonia vitripennis, Laodelphax striatella, Dactylopius coccus, Diaphorina citri, and Cimex lectularius* (Supplementary material 1, sheet 3). These might reflect ongoing parasitic infection and/or bacterial sequences integrated in the genome due to historical horizontal gene transfer events [53,59–63]. All these 393 genes were excluded from further downstream analyses.

2.2. X chromosome scaffolds and X-linked genes

We utilised the difference in X chromosome copy number between males (X0) and females (XX) to search for X-linked scaffolds. A sequencing read coverage analysis based on resequencing data of four males and four females mapped to the 8936 scaffolds identified a bimodal distribution of $\log_2$(male coverage / female coverage)-values, with a valley at around −0.62 (corresponding to a male/female coverage-ratio of approximately 0.65). Accordingly, we categorised scaffolds with $\log_2$(male coverage / female coverage) < −0.62 as X-chromosomal and with $\log_2$(male coverage / female coverage) > −0.62 as autosomal. An inspection of the coverage values along all scaffolds identified 50 mis-assembled scaffolds that consisted of fused parts of X and autosomal scaffolds. The mis-assembled scaffolds were visualized in Aliview [64] and clipped manually to separate the X and autosomal parts (details are given in Supplementary material 1, sheet 4). After clipping the mis-assembled scaffolds, the number of scaffolds increased from 8936 to 8988. The coverage analysis of all 8988 scaffolds again showed a bimodal distribution of $\log_2$(male coverage / female coverage)-values with a valley at around −0.62 corresponding to separation of X-linked and autosomal scaffolds (Fig. 2B). In total, 423 X chromosome (4.7%) and 8565 autosomal scaffolds (95.3%) were identified.

The annotation of these scaffolds showed that there were 1835 (7.2%) X-linked and 23,613 (92.8%) autosomal genes (including isoforms), and the lengths of the X-linked and autosomal genes were similar (Fig. 2C). Among the 1835 X-linked genes, 1390 were annotated by BLASTx and 675 had GO annotations. Gene set enrichment analysis of the X-linked genes showed that the top three gene functions were cellular-protein-modification function, calcium-ion transport and oxidoreductase activity (all enriched X-linked gene functions are given in Supplementary material 2, Fig. S7).

2.3. Comparative analysis of orthologs and chromosome homology

We identified 159, 322 and 381 single-copy genes that were orthologous to the X-linked *I. elegans* genes in *Drosophila melanogaster*, *Tribolium castaneum* and *Bombyx mori*, respectively (Fig. 3). In all three species, the distribution of orthologs over the chromosomes differed significantly from what was randomly expected by the size of the chromosomes. In *D. melanogaster*, there were more orthologs than

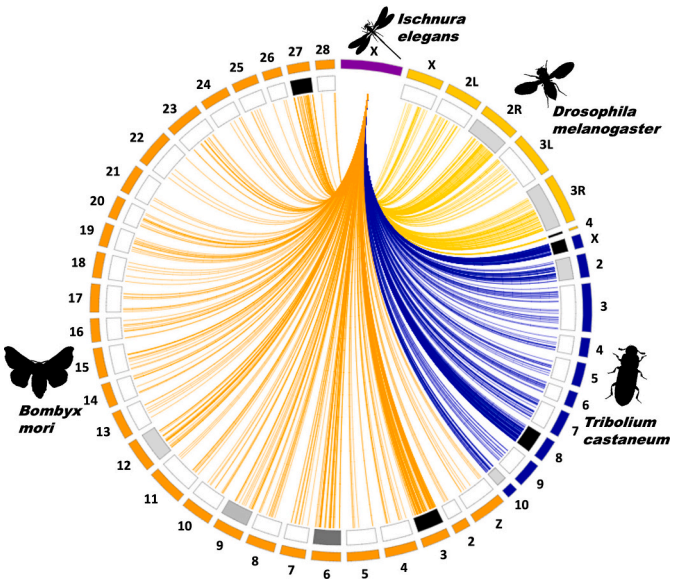

Fig. 3. Synteny of X-linked genes. Chromosomal position of genes in *Drosophila melanogaster*, *Tribolium castaneum* and *Bombyx mori* that are orthologous to the X-linked *I. elegans* genes. The outer circle shows chromosome number for each species, and the inner circle indicates the proportion of orthologous genes per chromosome or chromosome arm (ranging from low (white) to high (black) proportion of orthologs; for actual values per species and chromosome, see Supplementary material 1, sheet 5).

expected by chance on chromosome arm 2R (observed:expected, 46:30.0; binomial test: $p = 0.0014$, $p_{adj} < 0.01$) and chromosome 4 (7:1.6; $p = 0.0012$, $p_{adj} < 0.01$). In *T. castaneum*, high and significant ortholog numbers were seen for chromosome X (70:18.9; $p = 1.19e^{-21}$, $p_{adj} < 0.001$) and chromosome 8 (106:31.8; $p = 1.15e^{-29}$, $p_{adj} < 0.001$). Finally, in *B. mori*, there were more orthologs than expected by chance on chromosome 3 (96:13.8; $p = 1.62e^{-51}$, $p_{adj} < 0.001$), chromosome 6 (35:14.4; $p = 1.62e^{-6}$, $p_{adj} < 0.001$), and chromosome 27 (33:11.0; $p = 3.15e^{-8}$, $p_{adj} < 0.001$) (Supplementary material 1, sheet 5). Interestingly, *vitellogenin*, a X-linked gene in *I. elegans*, had an ortholog on the X chromosome in *T. castaneum* and on chromosome 27 in *B. mori*, and the *I. elegans* X-linked gene *fruitless* was shared by *B. mori* at chromosome 6.

2.4. Sex-biased gene expression and dosage compensation

After removing 6951 genes with low expression support (sum of normalized read count of all samples <1), gene expression was evaluated between males and females for 1222 X-linked and 17,275 autosomal genes (the average expression for males and females per gene, given as mean($\log_2$(normalized read count +1)), is shown in Fig. 4A; a gene density plot for X-linked and autosomal genes is given as Supplementary material 2, Fig. S8). The overall expression pattern between the X chromosome and the autosomes was similar for males and females with (i) a X-to-autosome median expression ratio of 0.93 for males ($p = 0.017$) and 0.92 for females ($p = 0.007$) (Fig. 4B,C; p-values from Wilcoxon rank sum tests with continuity correction), and (ii) a male-to-female–X-to-autosome mean expression ratio ($M{:}F_{X\text{-linked}}/M{:}F_{autosomal}$ ratio) of 1.00.

Differential gene expression analysis in DEseq2 showed that 32 of the 1222 X-linked genes (2.6%), and 510 of the 17,275 autosomal genes (3.0%), were significantly differentially expressed between males and females (Supplementary material 1, sheet 6). The majority of these significantly sex-biased genes were female-biased: 68.8% (22 of 32) of the X-linked genes (Fig. 4D), and 83.1% (424 of 510) of the autosomal genes (Fig. 4E).

Gene enrichment analysis of the female-biased X-linked genes

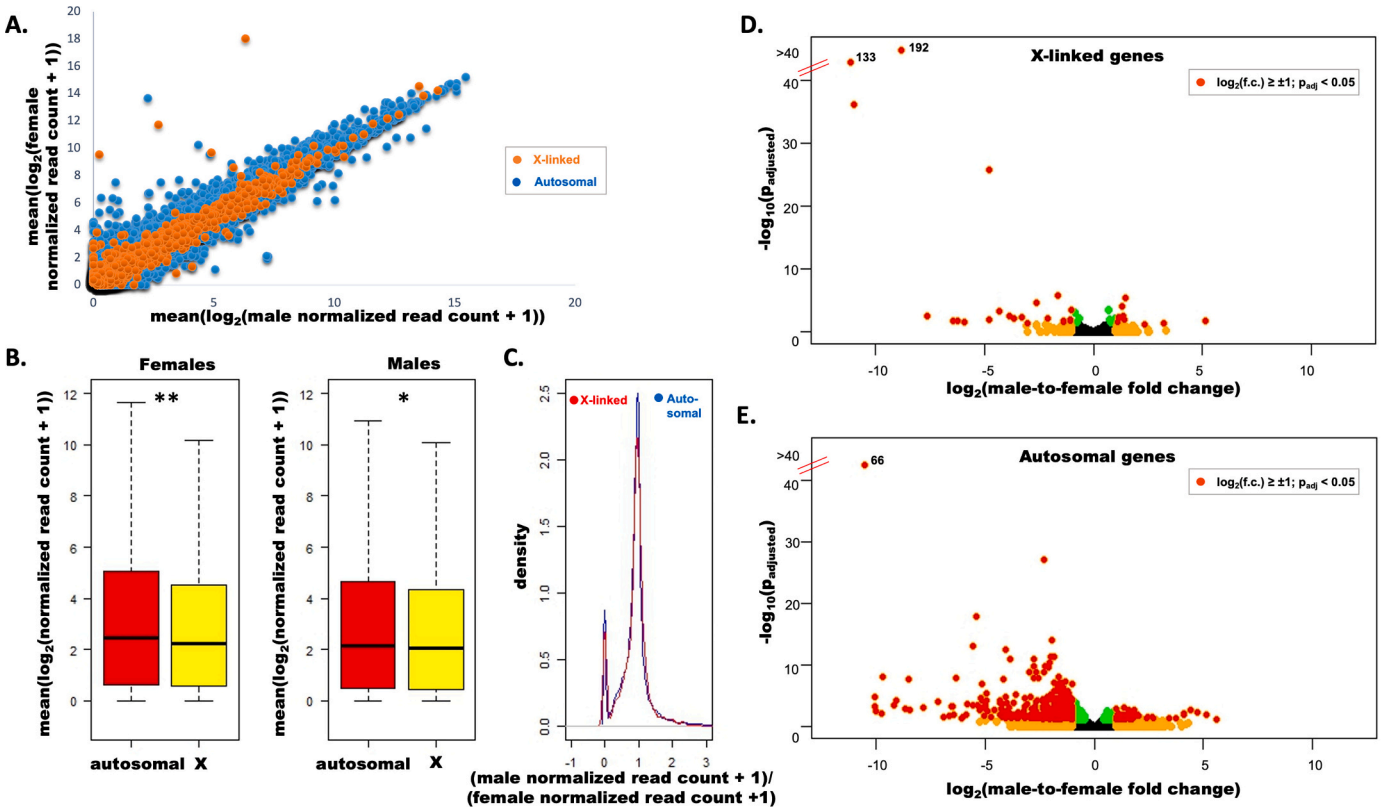

Fig. 4. Sex-biased gene expression. **A.** Female vs. male gene expression (mean($\log_2$(normalized read count +1))) of autosomal (blue; n = 17,275) and X-linked (red-brown; n = 1222 genes). **B.** Box-plots showing the distribution of gene expression (mean($\log_2$(normalized read count +1))) of autosomal and X-linked genes in females and males (median, 25th percentile (Q1), 75th percentile (Q3), Q1–1.5IQR and Q3 + 1.5IQR (where IQR is the interquartile range) are given). Asterisks denote significant difference between X:A in females (**: p = 0.007) and males (*: p = 0.017). **C.** Also shown is the male to female expression ratio (i.e. (male normalized read count +1)/(female normalized read count +1)) of autosomal and X-linked genes. **D,E.** Volcano plots showing male to female expression ratio ($\log_2$(male-to-female fold change)) and significance values based on DEseq2-analysis ($-\log_{10}(p_{adjusted})$) for X-linked (D) and autosomal (E) genes (red dots indicate genes with $\log_2$(male-to-female fold change) $\geq \pm 1$ and $p_{adj} < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed two functions (lipid transport and lipid transported activity), while the male-biased X-linked genes had three functions (integral component of membrane, neurotransmitter transport and neurotransmitter sodium symporter activity; Supplementary material 2, Fig. S9). For the enriched autosomal genes there were 26 different functions for female-biased genes and six functions for male-biased genes (Supplementary material 2, Fig. S9). Eleven out of the 26 functions for the female-biased genes were involved in regulation of mitotic cell-cycle (mitotic cell-cycle process, regulation of mitotic cell-cycle, and spindle organization) and protein binding and post-translational modifications (protein binding and regulation of protein modification process), which suggests involvement of female-biased autosomal genes in cell-cycle regulation and protein modification processes. Most of these female upregulated autosomal genes are associated with condensin- and histone-mediated chromosome inactivation (Supplementary material 1, sheet 6), which is relevant for the possible mechanism of dosage compensation in *I. elegans*.

2.5. Genes in the sex determination pathway and their splice variants

In the assembled genome, we identified most genes known to be present in the sex-determining pathway of insects (Supplementary material 1, sheet 7), including *sex-lethal* (*sxl*), *male-specific lethal 3* (*msl3*), *transformer 2* (*tra2*), *fem, fruitless* (*fru*) and *doublesex* (*dsx*). All these genes were located on autosomal scaffolds, except *fru*, which was X-linked. Only *dsx* was significantly differentially expressed between the sexes, with higher expression in males than in females ($\log_2$(male-to-female fold change) = 1.7, p_{adj} = 0.05; Supplementary material 2, Fig. S10).

Both *tra2* and *msl3* existed in different splice variants. *Tra2* has 9 exons, and all these 9 exonic regions were present in *tra2* isoform-1, whereas *tra2* isoform-2 contained only 8 exons (Supplementary material 2, Fig. S11). *Msl3* has 5 exons, and *msl3* isoform-1 was composed of 4 of these exons and *msl3* isoform-2 of 3 exons (Supplementary material 2, Fig. S12). Several studies on sex determination have shown an important role for sex-specific splice variants in the development of sex-specific phenotypes. However, we found no evidence for differential exon-usage of the sex-determining genes with multiple splice variants between the sexes in *I. elegans* ($p_{adj} > 0.1$; Supplementary material 2, Fig. S13–S14).

3. Discussion

3.1. Genome assembly and X chromosome homology

So far only two odonate species have had their genomes assembled: *Libellula fulva* (BioProject PRJNA194433) and *Calopteryx splendens* [53]. The present genome assembly of *I. elegans* captures a larger or similar genome size (1.67 Gbp) compared to these two assemblies (1.16 and 1.63 Gbp, respectively; Supplementary material 1, sheet 8). Our annotation shows high completeness of core insect BUSCO genes, with annotated genes being equally distributed over scaffolds of different lengths. Moreover, in addition to assembling and in detail annotating the genome, we also identified X-linked genes and evaluated sex-biased

expression of such genes for the first time in odonates. The X chromosome scaffolds accounted for 6.1% of the genome, which corresponds well to karyotype estimates of chromosome sizes: *I. elegans* has 14 chromosomes of which X is one of the smallest [54].

Our comparative genomic analysis showed that orthologs of the X-linked *I. elegans* genes were unevenly distributed over the chromosomes of Diptera, Coleoptera and Lepidoptera, with enrichment of orthologs on chromosome arm 2R and chromosome 4 in *D. melanogaster*, chromosome X and 8 in *T. castaneum*, and chromosome 3, 6 and 27 in *B. mori*. As Odonata is an extant representative of an early-branching clade of winged insects (Fig. 1; [40]) our results suggest some degree of deep phylogenetic conservation of genome structure in general, and of sex chromosome synteny in particular, among insects. The shared synteny of the X chromosomes of *I. elegans* and *T. castaneum* is particularly striking, since although deep chromosome homology has been found previously in insects [41], sex chromosome synteny between different insect orders has to our knowledge not been detected before. Perhaps equally curious is the link between the X chromosome in *I. elegans* and chromosome 4 in *Drosophila*, since this chromosome acts as the sex chromosome in more basal Diptera [65]. Thus, our present study and previous studies indicate highly dynamic insect genome evolution with deep conservation on the one hand, and frequent chromosome rearrangements, translocations and sex chromosome turnovers on the other (cf., [41,65]).

3.2. Sex-biased gene expression and dosage compensation

Despite the fact that male (X0) and female (XX) *I. elegans* have different X chromosome copy numbers, they showed equal expression levels at both X-linked and autosomal genes ($M{:}F_{X\text{-linked}}/M{:}F_{autosomal}$ ratio = 1.00). Also, the proportion of significantly sex-biased genes was similar between the X chromosome (2.6%) and the autosomes (3.0%). These results strongly suggest that the expression of X-linked genes is overall balanced between the sexes and that dosage compensation occurs in adult *I. elegans*. However, for both sexes the expression level of X-linked genes was significantly lower (92–93%) than for autosomal genes, which suggests that dosage compensation is balanced but somewhat incomplete (i.e., a "type II" sex chromosome dosage compensation pattern according to Gu and Walters' classification [21]). This is similar to the situation in Lepidoptera, a clade with female heterogametic sex determination system (ZZ/ZW) (e.g. [66]), whereas several insect orders with male heterogamy (Hemiptera, Coleoptera and Diptera) have balanced and complete dosage compensation ("type I"; [21]). Both these types of dosage compensation patterns are, however, consistent with dosage compensation through different degrees of upregulation of genes on the single X chromosome in the hetero−/hemigametic sex in combination with counteracting downregulation of the two X-linked gene copies in the homogametic sex. Results from *Drosophila* and the hemipteran *Acyrthosiphon pisum* suggest that hyper expression in the heterogametic males occurs through increased global chromatin accessibility of the single X chromosome [24,67], and in *Drosophila* and the nematode *Caenorhabditis elegans* maintained low expression levels in the homogametic females have been associated with condensin- and histone-mediated X chromosome inactivation. In line with the latter suggestion, we observed significant female-biased gene expression of several autosomal genes that have been associated with condensin- and histone-mediated inactivation in *Drosophila* and *C. elegans* (Supplementary material 2, Fig. S15; cf., [68–76]).

Sex chromosomes have been suggested to be a hotspot for the location of sexually antagonistic genes and might also show high degree of sex-biased gene expression as an evolutionary response to resolve sexual antagonisms [6–9,19] (but see [77]). Accordingly, in most insect species, including several species with chromosome-wide mechanisms of dosage compensation, sex-biased gene expression is often more frequent on the sex chromosomes [35,36,66,67,78,79]. In contrast, in *I. elegans* this enrichment was not detected as genes with significant sex-biased expression (of which the majority showed female-biased expression) were found in similar proportions on the X chromosome and the autosomes.

3.3. Genomic location and expression of sex-determining genes

Research conducted in *Drosophila* suggests that insect sex determination is regulated by a cascade induced by a dose-dependent expression signal of X and autosomal genes (X:A ratio) affecting the master-regulator *sex-lethal* and subsequent regulators *transformer*, *fruitless* and *doublesex* [80]. Key in this sex-determining cascade is alternative splicing: by including or excluding specific exons in the mRNA of the latter effectors, sex differentiation is established [81]. For example, the presence of two copies of the X chromosome induces female-specific splicing of *sex-lethal* precursor messenger RNA. However, in several other insect systems with heteromorphic sex chromosomes, including other Diptera species (such as *Anopheles* and *Aedes* mosquitos; [82,83]), the sex determination cascade deviates from this pathway [81]. Nevertheless, the mechanism of alternative splicing induced by X-to-autosomal expression ratio, and also controlling sex-specific expression, seems to be a common factor for insect sex determination in all studies so far [81].

In the assembled *I. elegans* genome, we identified most genes that are known to be involved in insect sex determination [81,84], including *sex-lethal* (*sxl*), *male-specific lethal 3* (*msl3*; a downstream gene associated with dosage compensation in *Drosophila*; [24]), *transformer 2* (*tra2*), *fem* (a *tra* homologue; [85]), *fruitless* (*fru*) and *doublesex* (*dsx*) (Supplementary material 1, sheet 7). Of these genes only *fru* was located on the X chromosome in *I. elegans*, and none of them, except *dsx*, were significantly differentially regulated between the sexes nor exhibited differences in exon usage (i.e., splicing). This was, however, not surprising since we have analysed adult *I. elegans*, whereas sex-biased expression and sex-specific usage of splice-site variants of sex-determining genes in insects are most prominent at earlier developmental stages [81,84,86,87]. In the future it would be interesting to evaluate how the expression of these sex-determining genes (particularly the X-linked *fru*) or their splice-site variants (the *msl3* and *tra2* isoforms) are sexually differentially expressed during the early developmental stages of sex determination in odonates.

It is interesting that *dsx* was significantly differentially upregulated in males compared to females in *I. elegans*. This gene is the last member in the sex-determining pathway [81,88] and has been suggested to play a key role in the development and evolution of sexual dimorphism [89–91]. For example in *Drosophila* there are several regulatory pathways that are directly or indirectly influenced by *dsx*, including regulation of *vitellogenin* that in turn controls yolk protein synthesis [92,93] and *fru* that in turn controls sex-specific neural circuits and male courtship behaviours [94,95].

4. Conclusion

In conclusion, in this study we have presented a well-annotated draft genome assembly and identified sex-linked genes in *I. elegans*. This enabled us to demonstrate some degree of deep phylogenetic conservation of the synteny of genomes and sex chromosomes of insects, and for the first time show evidence of dosage compensation in odonates. The availability of the draft genome and our findings presented in this paper about sex chromosome homology and dosage compensation in *I. elegans* have significant value for insect comparative genomics.

5. Materials and methods

5.1. Genome sequencing

DNA extracted from a male *Ischnura elegans* sampled in Scania (Sweden) was used to build three short-insert paired-end libraries (two 180 and one 600 bp insert size) and five long-insert mate-pair libraries

(two 2–3 kbp and one each of 5, 8 and 20 kbp). DNA extraction, library preparation and 100 bp (short-insert) or 50 bp (long-insert) paired-end Illumina sequencing was performed by BGI (Hongkong). Sequencing details, and usage of these libraries for the genome assembly, are found in Supplementary material 1, sheet 9. Raw reads obtained from these libraries are deposited at NCBI short read archive (SRA) under Bioproject PRJNA575663 and Biosample SAMN12906381.

In addition, four male and four female *I. elegans* sampled in Spain were whole-genome resequenced. DNA extraction, short-insert library preparation and 150 bp paired-end Illumina sequencing were performed by the SNP&SEQ Technology Platform in Uppsala (which is part of the National Genomics Infrastructure and Science for Life Laboratory, Sweden). Raw reads for these eight individuals are deposited at NCBI SRA under Bioproject PRJNA575663 and Biosample SAMN12920919–20.

5.2. De novo genome assembly and annotation

The *I. elegans* de novo genome assembly was generated in three steps. First, all short- and long-insert libraries were assembled into a first draft genome using Allpath-LG version Allpathslg/52485 with parameter settings: maxpar = 2, min_contig = 300 and haploidity = true [58]. Second, gaps were filled in the first assembly using Sealer version Abyss/1.9.0 at default parameters [96]. Lastly, Rascaf [97] was used to further scaffold the assembled genome using transcripts obtained from RNA-seq data generated from our previous projects (NCBI PRJNA245854, SRR1265958; PRJNA305793, SRR2990848-SRR2990851; [98,99]). De novo repeats were identified, annotated and masked in the *I. elegans* genome using RepeatModeler version 1.0.11 and RepeatMasker version 4.1.0 [100,101].

The assembly was annotated with Maker2 version 2.31.9 [102] in two iterations (https://github.com/sujaikumar/assemblage/blob/master/README-annotation.md). As extrinsic evidence sets, we used our previous de novo-assembled transcripts (ESTs) of adult *I. elegans* [98,99], and arthropod proteomes obtained from OthoDB v8 [103]. For the first iteration, Maker2 was provided with these extrinsic evidence sets and CEGMA-SNAP, Genemark HMM-files [104–106]. For the second iteration, the result from the first iteration was converted to Maker-SNAP and Augustus HMM-files, and Maker2 was run with our extrinsic evidence sets (i.e. ESTs and proteins) and these HMM-files from MakerSNAP, Genemark and Augustus [107]. Only genes having an annotated edit distance (AED) < 0.5 were kept for further analysis. Functional annotation of the genes was conducted with BLASTX against NCBI's non-redundant database (accessed November 2017) at an E-value cutoff of 1E-5. Interproscan [108] was further used to identify the conserved protein domains. The BLASTX and Interproscan results were imported in BLAST2GO [109] for gene ontology (GO) annotations and KEGG-pathway analysis. GO annotations were refined into biological processes, cellular components and molecular functional annotations. Further, functional annotation and GO classification of the genes was also performed in PANNZER [110]. The quality assessment of the assembled and annotated genome was performed with BUSCO version 2.0.1 for class Insecta using orthologous database odb9 [103,111].

5.3. Identification of X chromosome scaffolds

Adapters, low-quality bases and sequence reads shorter than 20 bp were removed from each of the eight resequenced individuals using Nesoni clip (http://thegenomefactory.blogspot.com/2012/11/cleaning-illumina-fastq-reads-with.html). Next, the reads of each individual were mapped to the draft genome assembly with BWA mem version 0.7.8 using default parameters [112]. Duplicates and mismatches were removed using Bamutils in NGSUtils version 0.5.9 [113] and the read-coverage depth (i.e. number of mapped reads) was calculated for each individual by Multicov in Bedtools version 2.26.0 [114] at a window size of 3 kbp. The X0 sex determination system of *I. elegans*, with males having one and females two X chromosomes, makes it possible to use male/female read-coverage ratio to distinguish between X-linked and autosomal scaffolds (X-linked scaffolds: $male_{coverage}/female_{coverage} \approx ½$; autosomal scaffolds: $male_{coverage}/female_{coverage} \approx 1$). After normalising for variation in read numbers between individuals, we calculated the average coverage of the four males and the four females, respectively, over each 3 kbp window, which was then used for calculating male/female coverage ratio and categorising scaffolds as either X-linked or autosomal (scaffolds with an average $\log_2(male_{coverage}/female_{coverage}) < -0.62$ were categorised as X linked; see Results). Gene set enrichment analysis of the X-linked genes was performed with BLAST2GO at default parameters (Fishers exact test, false discovery rate FDR < 0.05), the list of enriched functioned were further reduced to specific functions at FDR < 0.05 [109].

5.4. Comparative analysis of orthologous genes and chromosome homology

The complete sets of proteins of insect species with well-annotated, chromosome-level genomes (i.e. *Drosophila melanogaster*, *Tribolium castaneum* and *Bombyx mori*) were downloaded from Ensembl metazoa database [115]. In these genomes, orthologs to the X-linked *I. elegans* genes were identified using Orthofinder version 2.2.7 at default parameters [116]. For each species, two-tailed binomial tests were used to test for unequal distribution of orthologs among chromosomes after accounting for the variation in chromosome length. In *Drosophila*, which has fewer chromosomes ($n = 4$) than the other two species (*Tribolium*, $n = 10$; *Bombyx*, $n = 28$), we separated the arms of chromosome 2 and 3 in this analysis. Circos version 0.69–6 was used to make circular plot [117].

5.5. Dosage compensation and differential gene expression analysis

RNA-seq reads from whole-body extractions of seven females and seven males (collected in Sweden; see [99] for sample information) were trimmed for adapter and low-quality bases (Q < 20) using Nesoni clip version 0.109 (www.thegenomefactory.blogspot.com/2012/11/cleaning-illumina-fastq-reads-with.html). Trimmed reads <25 bp were discarded from the dataset. Next, the RNA-seq reads were mapped to the X-linked or autosomal scaffolds using Bowtie 2 version 2.3.3.1 [118]. Abundance estimation was performed with RSEM version 1.3.1 [119]. The raw counts were normalized with DESeq2 (median of ratios method), a bioconductor package in R [120], and genes with low expression support (sum of normalized read count of all samples <1) were dropped from downstream analysis.

Dosage compensation was studied using different estimates. First, we calculated, for each sex separately, the X:A expression ratio, i.e. mean $\log_2$(X-linked gene expression)/mean $\log_2$(autosomal gene expression). The difference in expression between X-linked and autosomal genes was tested by Wilcoxon rank sum test with continuity correction for each sex separately. Second, we calculated, for X-linked and autosomal genes separately, the M:F expression ratio, i.e. mean $\log_2$(male gene expression)/mean $\log_2$(female gene expression). Third, we calculated the ratio between the M:F ratio of X-linked and autosomal genes (i.e., $M{:}F_{X\text{-linked}}/M{:}F_{autosomal}$).

Differential expression analysis between females and males at all genes was performed with DESeq2. Genes with $\log_2$(male/female fold change) > ±1 and $p_{adj} < 0.05$ were considered to be significantly differentially expressed between sexes. We identified several genes known to be present in the sex-determining pathway in insects, and for some of them we detected different isoforms (splice variants). For these genes we analysed differential exon usage using RNA-exon counts in DEXSeq version 1.24.4 at default parameters [121].

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

Illumina raw reads are deposited at NCBI short read archive (SRA) under accession numbers Bioproject PRJNA575663 and Biosample SAMN12906381 and SAMN12920919–20. The annotated genome assembly is available at Zenodo (DOI: 10.5281/zenodo.4679993).

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Authors' contributions

PC and BH performed analyses. PC, JS and BH wrote the paper with input from all coauthors.

Declaration of Competing Interest

No competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.04.003.

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