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Exploring chromosome evolution in 250 million year old groups of dragonflies and damselflies (Insecta:Odonata)

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Abstract

Using recently published chromosome-length genome assemblies of two damselfly species, *Ischnura elegans* and *Platycnemis pennipes*, and two dragonfly species, *Pantala flavescens* and *Tanypteryx hageni*, we demonstrate that the autosomes of Odonata have undergone few fission, fusion, or inversion events, despite 250 million years of separation. In the four genomes discussed here, our results show that all autosomes have a clear ortholog in the ancestral karyotype. Despite this clear chromosomal orthology, we demonstrate that different factors, including concentration of repeat dynamics, GC content, relative position on the chromosome, and the relative proportion of coding sequence all influence the density of syntenic blocks across chromosomes. However, these factors do not interact to influence synteny the same way in any two pairs of species, nor is any one factor retained in all four species. Furthermore, it was previously unknown whether the micro-chromosomes in Odonata are descended from one ancestral chromosome. Despite structural rearrangements, our evidence suggests that the micro-chromosomes in the sampled Odonata do indeed descend from an ancestral chromosome, and that the micro-chromosome in *P. flavescens* was lost through fusion with autosomes.

KEYWORDS
chromosome evolution, genomics, Odonata, synteny

1 | INTRODUCTION

As more chromosome length genome assemblies are made available, it is increasingly feasible to study the evolution of chromosomes across varying taxonomic levels by aligning genome sequences and analysing synteny (defined in a comparative genomic context as the conserved order of loci on the chromosomes of related species, which originated from a common ancestor (Duran et al., 2009)). It is clear that chromosome evolution patterns vary drastically across the tree of life. In mammals, chromosomes diverge at a rate that is faster than in most other eumetazoan lineages (Bush et al., 1977), with the exception of amphibians (Evans et al., 2012), which have especially fluid sex chromosomes (Dufresnes & Crochet, 2022). Within mammalia, chromosome divergence between lineages is fastest in genera that either form clans or harems, display limited adult vagility, are strongly territorial, or have a patchy geographic distribution (Bush

et al., 1977). The conservation of chromosome structure can also vary dramatically. For example, in primates, the rate of chromosome rearrangements is uneven across chromosomes, with some that are highly conserved within the group while others have undergone numerous inversion events (Stanyon et al., 2008). Chromosome evolution varies dramatically across the ray-finned fishes, class Actinopterygii. Some species have maintained the ancestral karyotype for over 300 million years, while others have undergone such a high number of translocations and other rearrangements that homology with the ancestral karyotype can no longer be identified (Kasahara et al., 2007). Evidence suggests that the karyotype of freshwater fishes evolves at a faster rate than their saltwater counterparts (Nirchio et al., 2014). It has been recently discovered that at least one elasmobranch, the white-spotted bamboo shark, has experienced a number of rearrangements, with 13 of the 51 chromosomes evolving more quickly than the others (Zhang et al., 2020). Among vertebrates, birds are an outlier in that their chromosomes have evolved slowly, with few chromosomal rearrangements since radiating in the late Cretaceous (over 65 mya) (Damas et al., 2018; Ellegren, 2010; Huang et al., 2022; Kretschmer et al., 2018).

In contrast to vertebrates, invertebrate chromosome evolution has been less well studied, including the class Insecta, the most diverse group of animals on the planet. This knowledge gap is problematic considering the rich global biodiversity and almost ~500 my long evolutionary history of insects (Misof et al., 2014). Non-model insects, especially those which inhabit aquatic environments, are dramatically underrepresented in genomics research (Hotaling et al., 2020), with most baseline knowledge about chromosomal evolution coming from the study of karyotype maintenance and rearrangement. Current insect karyotype research suggests that trends in insect chromosome evolution vary greatly across orders. For example, in the superorder Paraneoptera, chromosome evolution is rapid in two of the three orders (Hemiptera and Psocodea) resulting in both the gain and loss of holokinetic chromosomes (chromosomes that lack centromeres), while it is slow in the third order, Thysanoptera (Gavrilov-Zimin et al., 2015). Across the ~28 orders of insecta chromosome number varies greatly, but of the groups studied, the most diverse karyotypes occur in Aphidoidea (aphids), a lineage within the Hemiptera, where chromosome number ranges from $2n=4$ to $2n=192$ (Gavrilov-Zimin et al., 2015). Analysis of the sex chromosomes of Blattodea and Diptera demonstrated high conservation of the sex chromosome between the two orders, and presumably within them (Meisel et al., 2019). Several studies have examined holometabolous (those which undergo complete metamorphosis) insect karyotypes; in the case of scorpionflies (Mecoptera), the family Panorpidae have haploid chromosomes which range from $n=17$ to $n=24$ and diversification within this family is closely related to changes in karyotype (Miao et al., 2019).

Because they are among the first winged lineages (~325 mya), the evolution of chromosomes in Odonata is of particular interest. The ancestor to these insects was likely the first lineage to have developed flight (Misof et al., 2014), an energetically costly key innovation that is considered to be a main driver of evolution in Pterygota. Odonate karyotypes have been studied for over a century (Kuznetsova & Golub, 2020). The chromosome number within Odonata varies from $2n=8$ to $2n=30$ (Blackmon et al., 2017), and the presumed ancestral karyotype, which is found in many lineages of Odonata, is $2n=25$ (Kuznetsova & Golub, 2020). The sex-determination system of Odonates is XO but an XY system has evolved in 20 separate instances, likely from X-autosome fusions (Blackmon et al., 2017). Odonate chromosomes are usually holocentric, but monocentric chromosomes appear to have evolved several times throughout the order (Kuznetsova et al., 2021).

While karyotype number can be used to draw evolutionary conclusions in Odonata, chromosome-level genome assemblies offer additional resolution by illuminating inversion, translocation, and even fission and fusion events often hidden from karyotype analysis (Kuznetsova & Golub, 2020). Genomic data from the damselfly *Ischnura elegans* was used to demonstrate that its sex chromosome is highly conserved when compared to Coleoptera, Lepidoptera and Diptera (Chauhan et al., 2021). However, despite this finding, there remain unresolved questions in Odonate genome evolution that are well-suited to be answered by an analysis of multiple chromosome-length genome assemblies. For example, it is not known how much homology is shared between autosomal chromosomes of Odonata, which have distinct evolutionary histories and pressures as compared to sex chromosomes across the tree of life (Dufresnes & Crochet, 2022; Johnson & Lachance, 2012; Vicoso et al., 2013), or how these chromosomes are shaped by inversions and other rearrangements. It is also not known what specific factors (e.g. repetitive elements, GC content, density of coding sequences) influence the degree of homology across odonate chromosomes or those of other insects.

The pattern of origin and loss of micro-chromosomes (so-called "m" chromosomes), that are notably smaller than autosomes, throughout Odonata is also unclear. Do micro-chromosomes in Odonata descend from an ancestral micro-chromosome? Or have they been independently gained in lineages that contain them (Kuznetsova & Golub, 2020)? In lineages where m chromosomes are not found are they fused to other chromosomes, or lost entirely? Chromosome-level genome assemblies have been used to study chromosome evolution in various insects like Lepidoptera (butterflies and moths). Throughout the 140-million-year history of this order, most species have nearly identical haploid chromosome counts. However, a handful of species vary drastically from the ancestral karyotype due to numerous fusion and fission events, with translocation likely playing a very limited role in the evolution of Lepidopteran chromosomes (Blackmon et al., 2017; Hill et al., 2019; Wright et al., 2023).

The advent of long-read sequencing has resulted in a growing number of publicly available genome assemblies from the order Odonata. As of the time of submission of this manuscript, there were four chromosome length assemblies for Odonata, those of: dragonflies *Tanypteryx hageni* (black petaltail) (Tolman et al., 2023) and *Pantala flavescens* (wandering glider) (Liu et al., 2022), and damselflies *I. elegans* (blue-tailed damselfly) (Price et al., 2022) and *Platycnemis pennipes (white-legged damselfly)* (Price & Allan, 2023). Given the exciting pace of odonate genome research, we anticipate even more assemblies being made available by the time of publication.

Dragonflies and damselflies last shared a common ancestor ~250 mya; *T. hageni* and *P. flavescens* diverged ~170 mya, while

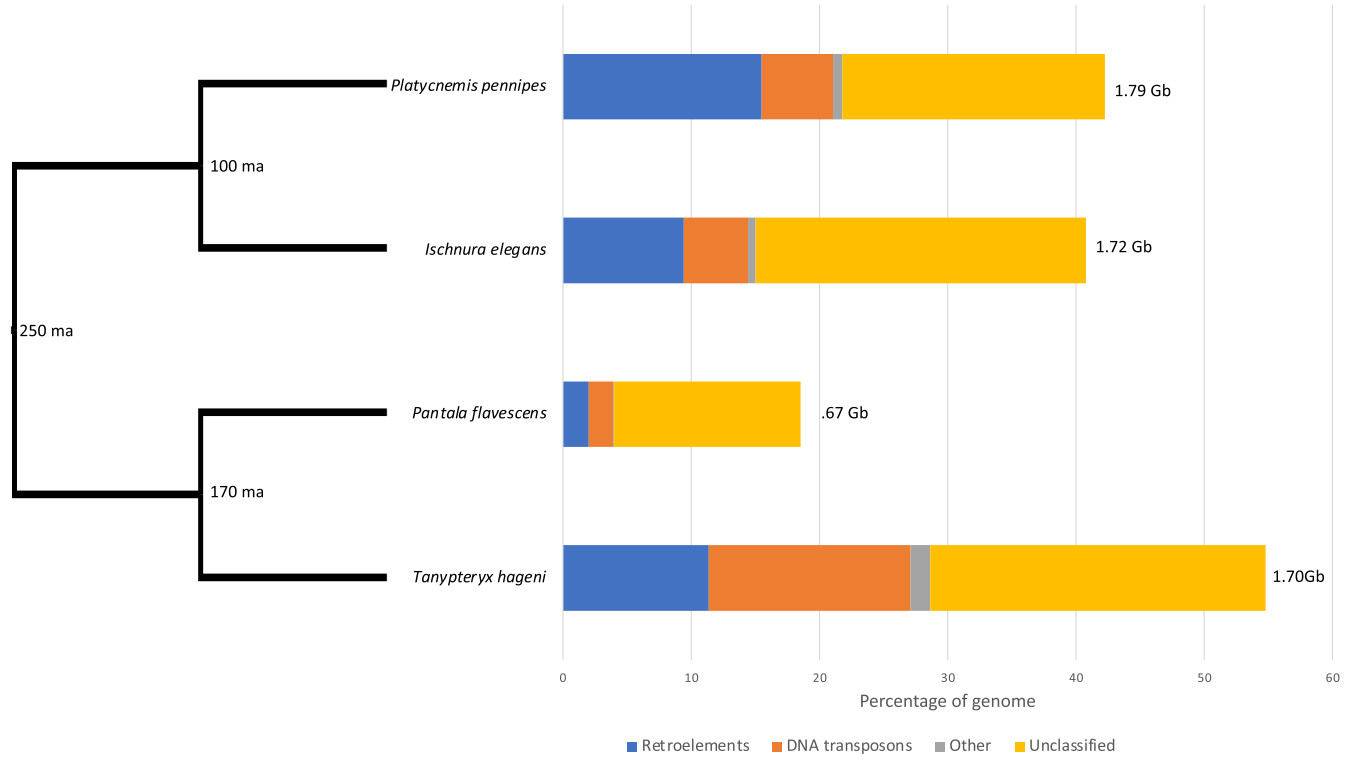

FIGURE 1 Repetitive content of chromosome-level genome assemblies from Odonata.

P. pennipes and *I. elegans* split ~100 mya (Kohli et al., 2021) (Figure 1). *P. pennipes* has retained the putative ancestral karyotype ($2n = 25$) as have all other zygoptera in the family Platycnemididae, making it highly unlikely that the ancestral chromosome number has been retained through a series of fusions, duplications and fissions (Kuznetsova & Golub, 2020). *P. flavescens* ($2n = 23$) and *T. hageni* ($2n = 17$) have a reduced chromosome number, and *I. elegans* ($2n = 27$) has expanded upon the ancestral karyotype (Kuznetsova & Golub, 2020). *P. pennipes*, *T. hageni* and *I. elegans* all have an "m" chromosome, and all four species use an XO sex determination system (Kuznetsova & Golub, 2020). These species are extremely variable in their natural histories. *P. pennipes* and *I. elegans* are highly territorial odonates with relatively limited vagility, while *P. flavescens* is considered the global wanderer, given their globally panmictic population, with individuals potentially crossing oceans (Ware et al., 2022). Further, these 3 species are all relatively young lineages from species rich families (Kohli et al., 2021; Suvorov et al., 2021). Conversely, *T. hageni* is a member of the smallest family within Odonata (Ware et al., 2014) estimated to have split from its sister species (the Japanese Petaltail, *Tanypteryx pryeri*, for which there is currently no genome sequence) some 70 million years ago (Ware et al., 2014). Throughout its history, its morphology has remained largely unchanged, designating it as a "living fossil" (Ware et al., 2014). Other ancient lineages such as the coelocanth (Amemiya et al., 2013), lungfish (Meyer et al., 2021), ginkgo tree (Guan et al., 2016), and mangrove horseshoe crab (Shingate et al., 2020), seem to differ in genomic structure from their more recently derived relatives, through elevated repeat content and a larger genome size, and there is some evidence that *T. hageni* has an elevated repeat content when compared to other Odonata (Tolman et al., 2023). Thus, the prolonged persistence of *T. hageni* is an important factor to consider when viewing its genome in a comparative context. Given the breadth of evolutionary history and life-history traits represented by the four species included in this study, a comparative analysis of their genomes is well-suited for answering outstanding questions regarding the chromosome evolution of Odonata.

Here we identify the synteny among Odonata genomes sampled from four different species and qualitatively describe the genomic architecture influencing synteny. We demonstrate that the autosomes are largely homologous with each other and have undergone few, if any, inversion events. GC content, repetitive elements, and CDS density all influence the degree of homology across chromosomes but interact differently in each species. We also test whether the microchromosomes are descended from a common ancestor, or independently derived. The m chromosomes from each species share many homologous genes but display little synteny. Thus, we infer that, despite rearrangements, they are derived from an ancestral chromosome.

2 | METHODS

2.1 | Dataset

To estimate the amount of conserved synteny across Odonata we used the chromosome level genome assemblies of Zygoptera *P. pennipes* (Price & Louise Allan, 2023) and *I. elegans* (Price et al., 2022)

and Anisoptera *P. flavescens* (Liu et al., 2022) and *T. hageni* (Tolman et al., 2023). As our objective was to look at intra-ordinal patterns, we did not include any other genomes from Insecta. All genomes were assembled using PacBio HiFi sequences and were scaffolded with HiC data. All have been shown to be of relatively high quality (Tolman et al., 2023), and all but *P. pennipes* had been annotated at the time analysis was conducted. We used the remaining Odonata genome assemblies from NCBI (*Rhinocypha anisoptera* (Iridian Genomes, 2020), *Ladona fulva* (Poelchau et al., 2015), *Calopteryx splendens* (Ioannidis et al., 2017), and *Hetaerina americana* (Grether et al., 2023)) to compare repeat content across the order.

2.2 | Genome annotation

To ensure all repetitive elements were assessed using the same software and databases, all four Odonata genome assemblies were modelled using RepeatModeler v2.0.1 (Flynn et al., 2020) and classified with RepeatMasker2 v4.1.2 (Flynn et al., 2020). Following the RepeatModeler pipeline (Flynn et al., 2020), a sequence database was created for each genome using BLAST v2.12.0 (Camacho et al., 2009). RepeatModeler was then run on the BLAST database to identify repetitive sequences. Following this, RepeatMasker was run using the option *--nolow*, which does not mask simple repeats. RepeatMasker then classified the modelled repetitive elements into families and provided a classification summary for each genome.

To annotate the genome of *P. pennipes* we clustered the protein sets of *L. fulva* (Poelchau et al., 2015), *I. elegans* (Price et al., 2022), *P. flavescens* (Liu et al., 2022), and *T. hageni* (Tolman et al., 2023) to 40 percent similarity with CD-HIT v4.8.1 (Fu et al., 2012). To ensure we were using a high-quality reference set, we ran BUSCO (Manni et al., 2021) on the clustered protein set, using the insecta database (odb10) as a reference, and recovered 94.9% of the single and complete BUSCO genes, with an additional 2.7% duplicated and 0.7% fragmented.

We then mapped the clustered reference set onto the masked genome assembly of *P. pennipes* using miniprot v0.4 (Li, 2023). We extracted the protein set from the resulting gff file using gffread (Pertea, 2022), and assessed the annotation completion by running BUSCO on the annotated protein set, with the BUSCO insecta database odb10 as a reference (Manni et al., 2021). The protein sequences are available in Appendix S1.

2.3 | Syntenic analysis

Given the large phylogenetic distance between species, we chose to compare chromosomes based on synteny. To visualize synteny between *T. hageni*, *P. flavescens*, *I. elegans* and *P. pennipes*, we first identified potential homologous genes between species using BLASTp (Camacho et al., 2009) to compare amino acid sequences from each genome annotation to a custom database containing all amino acid sequences from the three other genomes, limiting output to the top five hits between two species per gene, and restricting output to an e-value below 1e−5. All retained BLAST hits were utilized in synteny analysis. We then combined the BLAST output and gff annotation files for each species and used MCScanX, requiring a minimum of five genes to call a syntenic block with no upper limit, and filtering for e-values less than 1e−05 (Wang et al., 2012) to identify areas with conserved synteny.

We visualized conserved syntenic blocks between the chromosomes of each species, highlighting synteny with the ancestral karyotype conserved in *P. pennipes* in a Circos plot (v.0.69-18) (Krzywinski et al., 2009). Syntenic portions of the chromosome are plotted alongside the corresponding proportion of coding sequence (CDS) (min = 0.0, max = 0.13), GC content (min = 0.30, max = 0.43), retroelements (min = 0.0, max = 0.65), transposons (min = 0.0, max = 0.65), unclassified repetitive elements (min = 0.0, max = 0.65), and all other uncategorized repetitive elements (min = 0.0, max = 0.65). All repetitive element proportions were calculated from RepeatMasker2 output (Flynn et al., 2020).

To highlight chromosomal fission and fusion, we generated a second Circos plot (Krzywinski et al., 2009) displaying only the involved chromosomes, as identified by the first plot, and the syntenic blocks between them.

2.4 | Repeat landscape

We generated a repeat landscape for each of the four chromosome-level assemblies by parsing the Repeat Masker output using the script parseRM_GetLandscape.pl from the perl module Parsing-RepeatMasker-Outputs (Hamilton et al., 2016; Kapusta et al., 2017; Kapusta & Suh, 2017; Mitra et al., 2013). We used ggplot2 (Wickham, 2016) in r to visualize the whole repeat landscape for each species. Elements that were classified as PENELOPEs/LINEs by Repeatmasker were classified as LINEs (Long Interspersed Nuclear Elements) in the repeat landscapes.

2.5 | The history of the micro-chromosome

Out of the four species studied here *P. flavescens* is the only one that does not have a micro-chromosome. To further investigate the origins of the micro-chromosomes of *T. hageni*, *I. elegans*, and *P. pennipes* we used BLASTp (Camacho et al., 2009) to identify potentially homologous genes across all the genes located on the m chromosomes, and the entirety of all four genomes. We generated a custom database using the translated amino acid sequences from all four genome annotations and limited the number of BLAST hits per gene to five, and restricted output to an e-value below 1e−5. We visualized the links, and their locations on each chromosome using Circos v.0.69-18 (Krzywinski et al., 2009). We calculated the expected proportion of BLAST hits as a proportion of the size of the micro-chromosomes as compared to all other chromosomes given the size

of the micro-chromosomes, and used a chi-squares test to determine if micro-chromosomes had more BLAST hits with each other than would be expected given their size.

2.6 | Statistical analysis of factors influencing synteny

Synteny may be impacted by numerous factors. To consider this, we tested whether factors including ancestral karyotype, chromosome type (micro, sex, or autosome), chromosome history (whether the partition was on a chromosome that resulted from fusion or fission), CDS content, GC content, and the proportion of unclassified repeats, retroelements, transposons and other repetitive elements influenced proportion of synteny in a given region of a chromosome. We partitioned each chromosome into linear 10 million base pair partitions (dropping the remainder at the end of each chromosome) and calculated the proportion of each partition that was contained within a shared syntenic block with at least one of the three other species. We acknowledge that this method will not capture intergenic divergence, but it does more accurately reflect the length of syntenic regions. As more genomes become available, more research is needed to assess the best statistical methods for comparing anciently diverging lineages.

We first considered if potential relationships between these factors and the proportion of synteny differed among species by constructing models that included main effects of each of these factors (indicating each factor does impact synteny), main effect of these factors and main effects of species (suggesting impacts of factors differed among species but in an additive fashion), and main effect of these factors and main effects of species and interactions among species and factors (suggesting impact of these factors on levels of synteny differed among species). We then generated linear models predicting the proportion of each partition contained in a syntenic block. Since partitions from a given chromosome may be similar to each other in regard to the proportion contained within a syntenic block, we also compared models with and without a random effect of chromosome using AIC values (Burnham et al., 2011). This method allowed comparison of chromosomes of various lengths.

For models with and without random effects, the lowest AIC score was observed when both species and other factors were included but interactions were not (Table S1). Since the lowest overall AIC score was associated with models without random effects and some factors (specifically, state change) were not relevant for all species, we further explored how factors were associated with synteny in each study species using models without random effects. For each species, we used backwards selection procedures based on AIC values to determine final factors to retain in the model (Zuur, 2009). Assumptions regarding multicollinearity, dispersion, and residuals were also checked for all final models, and we also evaluated the final fit using the adjusted R^2 value.

3 | RESULTS

3.1 | Genome annotation

We utilized four long-read chromosome assemblies, representing the breadth of the evolutionary history of Odonata (Tolman et al., 2023) and examined them all in a comparative context here. All four assemblies are highly complete and contiguous (Tolman et al., 2023). All but the genome of *P. pennipes* had been previously annotated, so we annotated this genome using miniprot (Li, 2023) v.0.4, similar to the process used to annotate the genome of *T. hageni* (Tolman et al., 2023). We recovered 22,810 protein coding genes from *P. pennipes*. BUSCO (Manni et al., 2021) analysis showed a high level of completion (C: 91.7% [S: 89.2%, D: 2.5%], F: 2.5%, M: 5.8%, *n*: 1367). This is comparable to other Odonata genome annotations, which have between 15,000 and 27,000 genes, with over 90% of single copy orthologs present (Tolman et al., 2023). Repeat content was widely variable in publicly available chromosome-level Odonata genome assemblies, from approximately 18% of the genome in the dragonfly *P. flavescens*, to over 50% in *T. hageni* (Figure 1). (See Figure S1 for repeat analysis of *all* publicly available Odonata genome assemblies as of May, 2023). The classes of identified repetitive elements are:

1. Unclassified repetitive elements that did not have a match in the RepeatMasker (Flynn et al., 2020) library.
2. Transposable elements that can physically move to other areas of the genome.
3. Retroelements that can "copy and paste" themselves to other areas of the genome through an RNA intermediate.
4. All other repeats making up <5% of Odonata genomes.

The calculated proportions of repetitive elements (including unclassified repetitive elements) in the utilized genomes fell well within the range of what has been found in non-model Insecta (Sproul et al., 2022).

3.2 | Syntenic analysis

To identify homologous regions between the chromosomes of the four species, we compared synteny between the species. Despite being separated by 250 million years of evolution, the chromosomes of Odonata show clear homology (Figure 2). Synteny between chromosomes demonstrates that *I. elegans*, *T. hageni* and *P. flavescens* all have clear orthologs in the ancestral karyotype (Figure 2), despite being separated by ~100 million years of evolution from *I. elegans* and over 250 million years from the two dragonflies (Figure 1) (Kohli et al., 2021). The karyotype of *I. elegans* expanded through one fission event (Figure 2), the chromosome number in *T. hageni* decreased through three fusion events (although these chromosomes still show shared synteny with their original ancestral ortholog) (Figure 2), and the *P. flavescens* chromosome number decreased through the loss of the m Chromosome (Figure 2).

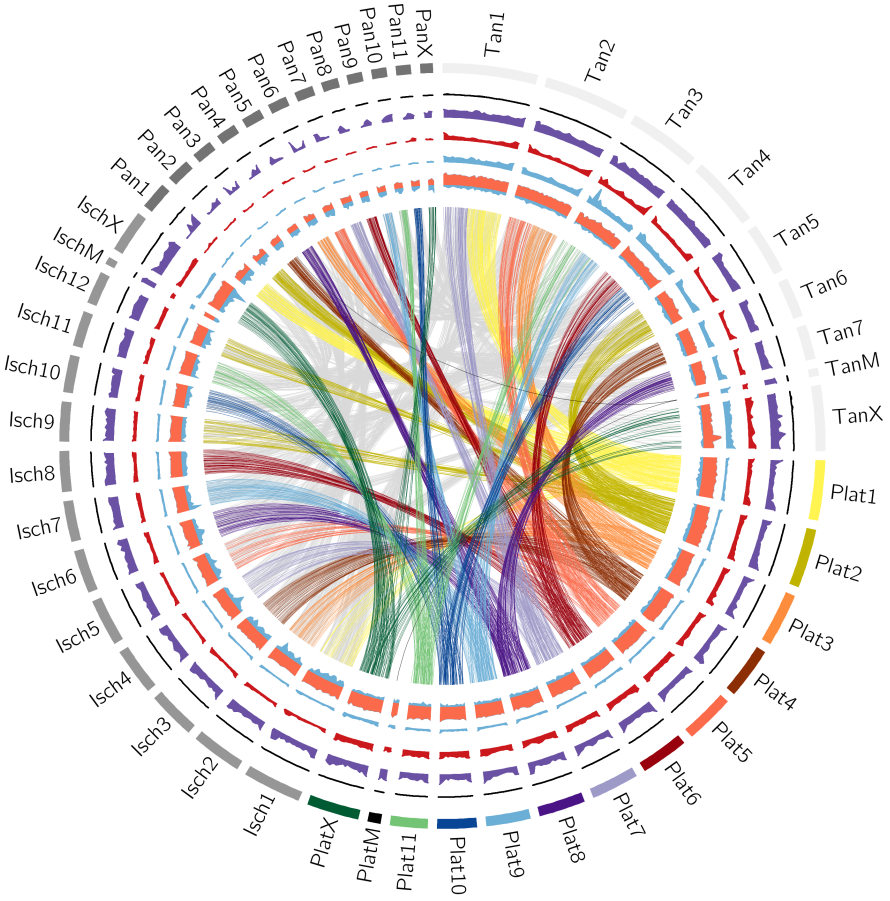

FIGURE 2 Circos (Krzywinski et al., 2009) plot of all four chromosome level Odonata genome assemblies. Lines connect inter-species syntenic regions. Synteny with P. pennipes is color coded according to the color of the P. pennipes chromosome. All other syntenic links are in grey. This is followed by the relative proportion of CDS in blue (min=0, max-.15), and the relative GC content in red (min=.30, max=0.43). The following four tracks display the relative proportion of various repetitive elements in the chromosomes with transposons in blue, retroelements in red, unclassified elements in purple, and all other repeats in black (all elements are displayed on a scale with a minimum proportin of 0, and a maximum proportion of .65)The outer track displays the chromosomes from each genome (Plat=P. pennipes, Tan=T. hageni, Pan=P. Flavescens, and Isch=I. elegans).

There are only three instances of chromosomal regions sharing synteny with multiple chromosomes from the ancestral haplotype. Chromosome three in *T. hageni* (resulting from a fusion of chromosomes 10 and 11 in the ancestral haplotype) shares two syntenic regions with chromosome 11 in the arm of the chromosome that is syntenic with chromosome 10 (Figure 3). Chromosomes 9 and 12 in *I. elegans*, which resulted from the fission of chromosome 2 in the ancestral karyotype, each largely maintain synteny with only one half of chromosome 2 in *P. pennipes* (Figure 3). However, chromosome 12 of *I. elegans* shares one syntenic region with the opposite chromosome arm of *P. pennipes* (Figure 3). There are no other instances of a chromosome, or chromosomal arm in the case of chromosomes resulting from fusion, sharing synteny with more than one chromosome in *P. pennipes*. The overall structure of chromosomes has largely been maintained in these four species. As syntenic analysis is based on cds, inversions or other structural rearrangements that span or fall within a gene region would be detectable; however, intergenic inversions may not be detectable. Given these constraints we find no visible evidence of inversions, translocations, or even exogenous effects (such as transposable elements that can move between different regions of the genome and can reshuffle genes).

3.3 | Repeat landscapes

As repetitive elements play an import role in shaping the homology of the genomes, we further investigated the repeat content by generating repeat landscapes for each species, showing the relative age and abundance of related repetitive elements within the genome. *P. pennipes* (Figure 4a), *I. elegans* (Figure 4b), and *P. flavescens* (Figure 4c) have relatively constant levels of various repetitive elements throughout their repeat landscapes, while the repetitive features in *T. hageni* appear in bursts (Figure 4d). This is also reflected in the genome, where transposons and retroelements especially appear in dense clusters throughout the chromosomes and seem to be associated with a low coding sequence (CDS) content (Figure 2). Repetitive elements have played an especially dramatic role in shaping the X chromosome of *T. hageni*, where unclassified (repetitive elements that did not have a match in the RepeatMasker (Flynn et al., 2020) library), transposable (elements that can physically move to other areas of the genome) and retroelements (elements that can "copy and paste" themselves to other areas of the genome through an RNA intermediate) are densely concentrated across long regions of the chromosome with a low CDS content and little synteny (Figure 2).

3.4 | The history of the micro-chromosome

While the large autosomal chromosomes showed a high level of conservation, the micro-chromosomes (defined here as chromosomes that were much smaller than all other chromosomes in the genome) did not. The m chromosomes of *P. pennipes* and *T. hageni* shared one instance of synteny (Figure 2). When the genes on each m chromosome were BLASTed (Camacho et al., 2009) against all other protein coding

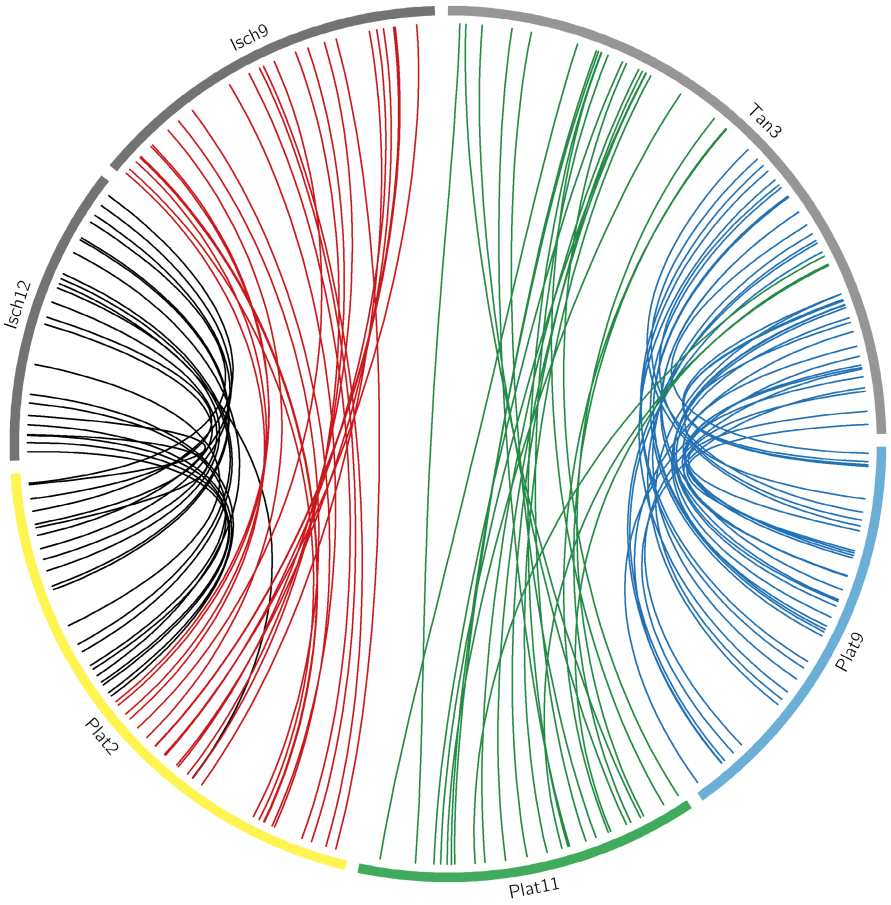

FIGURE 3 Circos (Krzywinski et al., 2009) plot showing chromosomes with mismatched syntenic links. Chromosomes 9 and 12 are displayed from Ischnura elegans (Isch), chromosome 3 is displayed from T. hageni (Tan), and chromosomes 9, 11, and 12 are displayed from P. pennipes (Plat).

sequences in the four genomes we found that they all shared a large number of orthologous genes (Figure 5), and shared a significantly higher number of BLAST hits with each other than would be expected under a normal distribution (*P. pennipes*: chisq = 2026.751, $p < .001$; *I. elegans*: chisq = 1848.900, $p < .001$; *T. hageni*: chisq = 789.311, $p < .001$). All three m chromosomes contain a number of intra-chromosomal homologous genes (Figure 5), suggesting a high amount of duplication of the genes on these chromosomes, which could explain the lack of synteny. While the m chromosome of *P. flavescens* is not present, the telomere of chromosome three of *P. flavescens* does share one syntenic block with the m chromosome of *T. hageni* (Figure 2), and this chromosome contains a number of orthologs to the m chromosomes of *I. elegans*, *P. pennipes*, and *T. hageni* (Figure 5). The three microchromosomes shared a significantly higher number of BLAST hits with the third chromosome than any other chromosome in *P. flavescens* (chisq = 2026.751, $p < .001$). Thus, we infer that, despite rearrangements, they are derived from an ancestral chromosome.

3.5 | Statistical analysis of factors influencing synteny

The models met all necessary assumptions and had no variance inflation factor values greater than 2. No factors were found to impact the proportion of syntenic blocks in all four species, and no two species models retained the same subset of factors (Table 1). However, all four species did have a negative correlation between at least one class of repetitive element and synteny (Table 1).

The corresponding ancestral chromosome was retained in all species but *P. flavescens*. Of the chromosomes retained in *T. hageni*, *I. elegans*, and *P. pennipes*, chromosomes 2,3,4 and 9 all had positive regression coefficients, indicating partitions from these chromosomes contained more synteny on average (Table S2). Chromosomes 6, X and M all had negative regression coefficients (Table S2), meaning partitions from these chromosomes contained less synteny on average in these species. All other chromosomes varied in their directionality and model inclusion (Table S2).

The effect of the different classes of repetitive elements varied across taxa. Higher proportions of retroelements negatively influenced the proportion of synteny in chromosomal partitions in *T. hageni* and *P. pennipes* (Table 1). Transposons had a negative influence upon synteny in *I. elegans* and *P. pennipes* but were positively correlated with synteny in *T. hageni* (Table 1). Unclassified repeats were negatively correlated with synteny in *T. hageni* and *P. flavescens*, and all other repeats were negatively correlated with synteny in *I. elegans* (Table 1).

CDS content was positively correlated with synteny in *I. elegans* and negatively correlated with synteny in *P. flavescens* (Table 1). GC content was positively correlated with synteny in both *P. pennipes* and *P. flavescens* (Table 1). The overall impact of analysed factors on synteny differed among species. Adjusted R^2 values ranged from .37 (*P. flavescens*) to .48 (*T. hageni*) (Table 1).

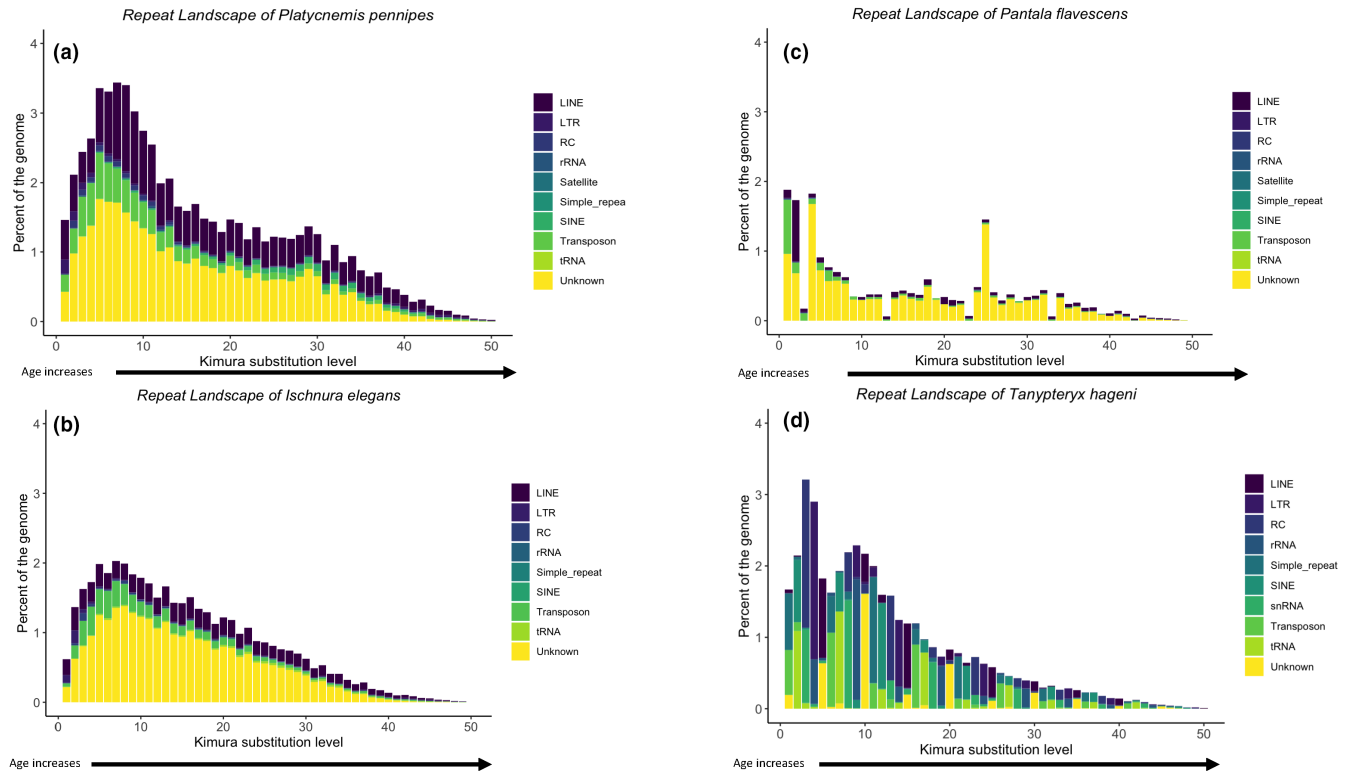

FIGURE 4 Repeat landscapes of A) P. pennipes, B) I. elegans, C) P. flavescens, and D) T. hageni. For each species the Kimura substitution level of the repetitive elements is shown on the x axis as a proxy for age. The proportion of the genome for elements with a given Kimura substitution level is shown on the y axis. Varying repetitive elements are colored according to the legend.

4 | DISCUSSION

4.1 | Chromosome conservation and rearrangement

The level of conserved autosomal synteny shown in these four species of Odonata is comparable to that seen in Aves (Damas et al., 2018; Ellegren, 2010; Huang et al., 2022; Kretschmer et al., 2018) and Lepidoptera (Hill et al., 2019), yet Anisoptera and Zygoptera diverged from each other 185 my and 110 my before the radiation of Aves and Lepidoptera, respectively. In the 250 million years since the split between Anisoptera and Zygoptera, there is scant evidence for any translocation or inversion events, although fusion and fission appear to be somewhat common (Figure 2).

4.2 | Factors influencing chromosomal conservation

It is notable that, in these species of Odonata a higher CDS content is not always retained as a predictor of synteny (Table 1). Conceptually, it would be expected that higher synteny would be associated with elevated CDS content, as observed in *P. pennipes* and *I. elegans*. However, processes such as gene insertion, chromosomal rearrangements and divergence in orthologous coding sequences could break up synteny, even in regions of high CDS content (Liu et al., 2018). Such processes could explain why CDS is not associated with synteny in *P. flavescens* and *T. hageni*. Notably, unclassified repetitive elements are a major negative predictor of synteny in both species. Further research into these repetitive elements will likely be needed to better understand the underlying processes.

Contrary to their static autosomal counterparts, the sex chromosomes of *I. elegans*, *P. pennipes*, and *T. hageni* are fluid. Partitions from the sex chromosomes of these species (Table S2) had a smaller proportion of synteny on average. This is especially intriguing, as previous research has shown that the sex chromosome of *I. elegans* has synteny with several other insect orders (Chauhan et al., 2021). It seems likely that the syntenic blocks are constrained to remain on the sex chromosome, but there is less pressure to maintain elements surrounding them, leading to the observed relative lack of synteny when compared to the autosomes. The turnover of the non-syntenic regions of the sex chromosome could be a possible driver of speciation in Odonata. It is understood that additions and deletions can accumulate more rapidly on sex chromosomes and that sex chromosomes can contain numerous selfish elements that influence genome stability and can quickly lead to reproductive barriers and speciation (O'Neill & O'Neill, 2018).

When compared to the other publicly available genome assemblies from Odonata, *T. hageni* has a high repeat content (Figure 1, Figure S1), which has resulted in a large genome compared to many other Odonata (Tolman et al., 2023). It also has a repeat profile showing punctuated instances of repetitive element activity (Figure 4c), as opposed to the relatively smooth equilibrium seen in the other three species.

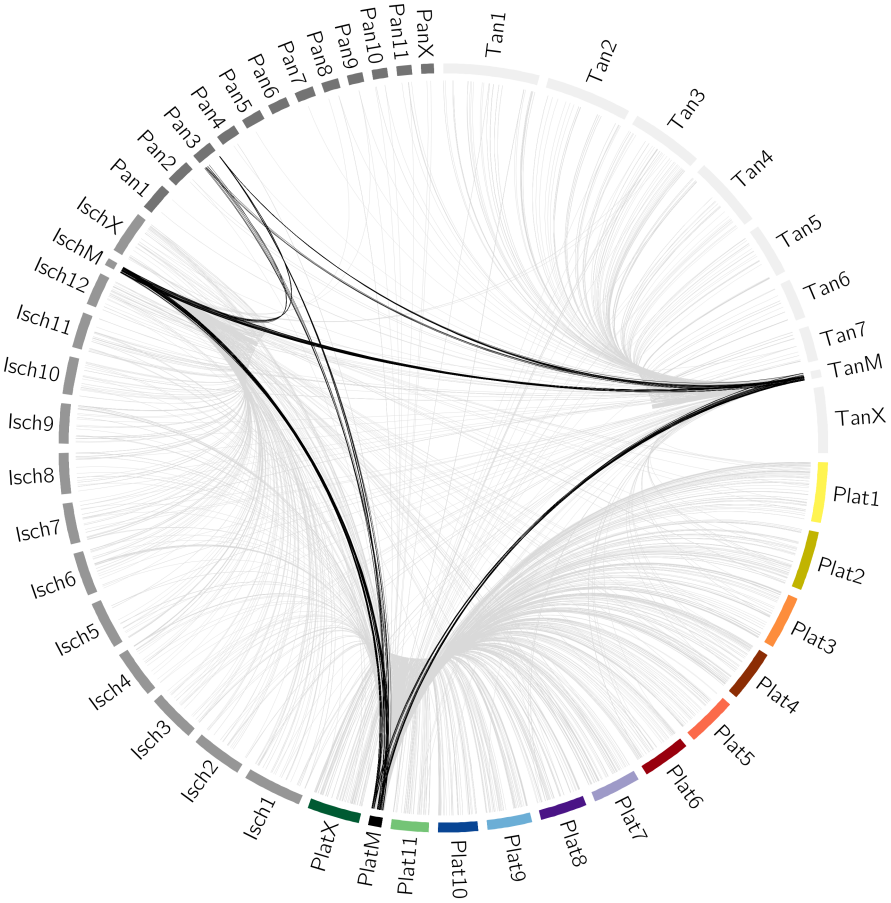

FIGURE 5 Links between genes on the m chromosomes of T. hageni (Tan), I. elegans (Isch), and P. pennipes (Plat) and the third chromosome of P. flavescens (Pan) shown in black. All othersignificant BLAST hits throughout all four genome are shown in grey.

This supports the pattern observed of large areas of repeat insertions on the chromosomes of *T. hageni*. Retroelements are found to disrupt synteny in this species (Table 1) providing evidence that retroelements are disrupting synteny in the genome of *T. hageni*.

Tanypteryx hageni is by far the longest-lived lineage of the four species analysed, so a comparison with other so-called "living fossils" clarifies some of the unique patterns seen in the genome that are not present in the other species. Although it is phylogenetically distant from *T. hageni*, the Giant Australian Lungfish (*Neoceratodus forsteri*) has a similar evolutionary history. Like *T. hageni*, *N. forsteri* has maintained a small population size (Hughes et al., 2015) through much of its history. It has not undergone a whole genome duplication event since the diversification of vertebrates (Meyer et al., 2021), and it diverged from its sister species ~190 mya (Zhao et al., 2021). Both *T. hageni* (Figure S2) and *N. forsteri* (Meyer et al., 2021) have a high proportion of LINEs in their genomes. In *N. forsteri*, LINE activity has caused the size of the genome to consistently grow (Meyer et al., 2021). As *T. hageni* has one of the largest Odonata genome assemblies to date (Tolman et al., 2023), it should be explored whether LINE activity is responsible for the genome size. *N. forsteri* does have a much larger proportion of LINEs in its genome than *T. hageni* (~25% in *N. forsteri* and 12.37% in *T. hageni*). This could be due to phylogenetic differences or the older species age of *N. forsteri*.

It is noteworthy that a recent analysis of chromosome evolution in Lepidoptera found variation in chromosome structure within families (Wright et al., 2023). Previous research has shown that families within Odonata can contain variable karyotypes (Kuznetsova & Golub, 2020), and it is likely that we will observe similar patterns as more genome assemblies from Odonata become available.

4.3 | Investigating the origin of the micro-chromosome

Because the micro-chromosomes of *P. pennipes*, *T. hageni* and *I. elegans* share significantly more orthologous genes with each other than is expected given their size (Figure 5), we infer that they descended from an ancestral micro-chromosome prior to the split of these two suborders. Partitions from the m chromosomes of *T. hageni*, *I. elegans* and *P. pennipes* contained less synteny than autosomes (Table S2) even though they contain a number of orthologous genes. Highly accurate long-read technology, such as the PacBio HiFi reads used for each of these genomes, are able to generate high quality genomes of even highly repetitive Insecta (Hotaling et al., 2021), hence there is no evidence to suggest the lack of synteny is caused by errors in the genome assembly. It is likely that there are fewer selective pressures to maintain the chromosomal structure of the m chromosomes in these Odonata. The three tested micro-chromosomes share significantly more orthologous genes with the third chromosome of *P. flavescens* than

TABLE 1 Factors retained in final model for each species.

		Factors retained							
					Relative proportion of				
Species	Adjusted R^2	Ancestral chromosome	Relative chromosomal position	GC content	CDS content	Unclassified repeats	Retroelements	Transposons	Other repetitive elements
P. pennipes	.47	±			4.88		−.78	−2.19	
I. elegans	.43	±		5.12	2.30			−4.41	−8.91
P. flavescens	.37		0.02	−4.10		−1.12			
T. hageni	.48	±	0.01			−.70	−2.03	.60	

Note: Output from linear models showing regression coefficients of retained factors for each species on the relative proportion of synteny in a chromosome partition. Coefficients for ancestral chromosome varied by chromosome and are shown in Table S2. State change was not retained in any species, and was not an option for *P. pennipes* or *P. flavescens*. Chromosome classification was also not retained for any species. Positive regression coefficients indicate that synteny is more likely to be found on average, while negative coefficients indicate that synteny is less likely to be found on average.

other chromosomes in the *P. flavescens* assembly and chromosome 3 of *P. flavescens* shares one syntenic block with the m chromosome of *T. hageni* (Figure 2), suggesting a fusion event that resulted in the loss of the m chromosome. We acknowledge a larger sample size of Odonata genomes is needed to determine the fate of missing m chromosomes and confirm if all m chromosomes across the order are homologous.

The overall trends in the micro-chromosomes of Odonata are much different than other lineages within the tree of life. In birds, micro-chromosomes contain a large number of genes, are highly conserved, and contain few transposable elements (Waters et al., 2021). Many m chromosomes, however, have been lost in reptiles and mammals (Waters et al., 2021). In the Australian Giant Lungfish the micro-chromosome also contain high CDS content, and a particularly low concentration of LINEs compared to the rest of the genome (Meyer et al., 2021). Further research is needed to determine if the fluid, repeat heavy micro-chromosomes of *P. pennipes*, *T. hageni*, and *I. elegans* are unique to Odonata, or if they can be found more commonly throughout Insecta, or even Arthropoda.

5 | CONCLUSION

To our knowledge, this work represents the first comparison of chromosome level genome assemblies of any non-holometabolous insect, filling in a major taxonomic gap. Our analyses are an important step towards understanding the genome evolution of Odonata, showing high amounts of synteny among species that have been separated for many millions of years. While previous studies have shown that the sex chromosomes of Odonata are conserved (Chauhan et al., 2021), our work demonstrates that this is also true of autosomes, while the micro-chromosomes are much more variable. Evidence suggests that the present micro-chromosomes descend from an ancestral micro-chromosome. It is not known whether this is true of all micro-chromosomes in Odonata, but this finding is a major step towards answering an unknown question in Odonatology (Kuznetsova & Golub, 2020). As more genomes become publicly available, further investigation should be undertaken as to how various genomic elements interact with each other, and perhaps how different classification and annotation methods could impact findings.

While this paper focused on intra-ordinal evolution, further research should explore whether inter-order synteny is maintained in the autosomes of Odonata, as is the case with the sex chromosome (Chauhan et al., 2021). Further work on the genome of *T. hageni* in comparison to other 'living fossil' species may also help elucidate genomic patterns among species with long persistence times.

AUTHOR CONTRIBUTIONS

Ethan R. Tolman: Designed research, performed research, analysed data, wrote paper, edited paper. Christopher D. Beatty: Designed research, wrote paper, edited paper. Jonas Bush: Performed research, analysed data, wrote paper, edited paper. Manpreet Kohli: Designed research, wrote paper, edited paper. Paul B. Frandsen: Designed research, wrote paper, edited paper. J. Stephen Gosnell: Designed research, performed research, analysed data, wrote paper, edited paper. Jessica L. Ware: Designed research, wrote paper, edited paper.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The genome annotation for *Platycnemis pennipes* (including the Repeatmasker output and protein coding sequences), the partitioned chromosome dataset, and the r-markdown and corresponding html file used for statistical analysis have been uploaded to figshare

(https://figshare.com/projects/High_levels_of_chromosomal_orthology_in_250_million_year_old_groups_of_dragonflies_and_damselflies_Insecta_Odonata_/162610) (Tolman, 2023a, 2023b, 2023c, 2023d, 2023e).

BENEFIT-SHARING STATEMENT

Our research benefits the public through the sharing of our data and results on the aforementioned public databases.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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