A Chromosome-length Assembly of the Black Petaltail (*Tanypteryx hageni*) Dragonfly

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Abstract

We present a chromosome-length genome assembly and annotation of the Black Petaltail dragonfly (*Tanypteryx hageni*). This habitat specialist diverged from its sister species over 70 million years ago, and separated from the most closely related Odonata with a reference genome 150 million years ago. Using PacBio HiFi reads and Hi-C data for scaffolding we produce one of the most high-quality Odonata genomes to date. A scaffold N50 of 206.6 Mb and a single copy BUSCO score of 96.2% indicate high contiguity and completeness.

Significance

We provide a chromosome-length assembly of the Black Petaltail dragonfly (*Tanypteryx hageni*), the first genome assembly for any non-libelluloid dragonfly. The Black Petaltail diverged from its sister species over 70 million years ago. *T. hageni*, like its confamilials, occupies fen habitats in its nymphal stage, a life history uncommon in the vast majority of dragonflies. We hope that the availability of this assembly will facilitate research on *T. hageni* and other petaltail species, to better understand their ecology and support conservation efforts.

Introduction

The Black Petaltail dragonfly (*Tanypteryx hageni*) (fig. 1*A*), found in montane habitats from California to British Columbia, is something of an evolutionary enigma. It is a member of the odonate family Petaluridae (known as "petaltails' due to the broad, petal-like claspers at the end of the male abdomen), which is estimated to have originated approximately 150 million years ago (Ware et al. 2014).

While the family originated long before most recognized insect families, the geologic ages of its member species are even more extreme in relation with other animal species, leading members of Petaluridae to be considered as "living fossils." Most extant petaltails are estimated to have appeared in the mid- to late-Cretaceous, with speciation being driven by major events like continental drift; *T. hageni* is estimated to have diverged from the sister species

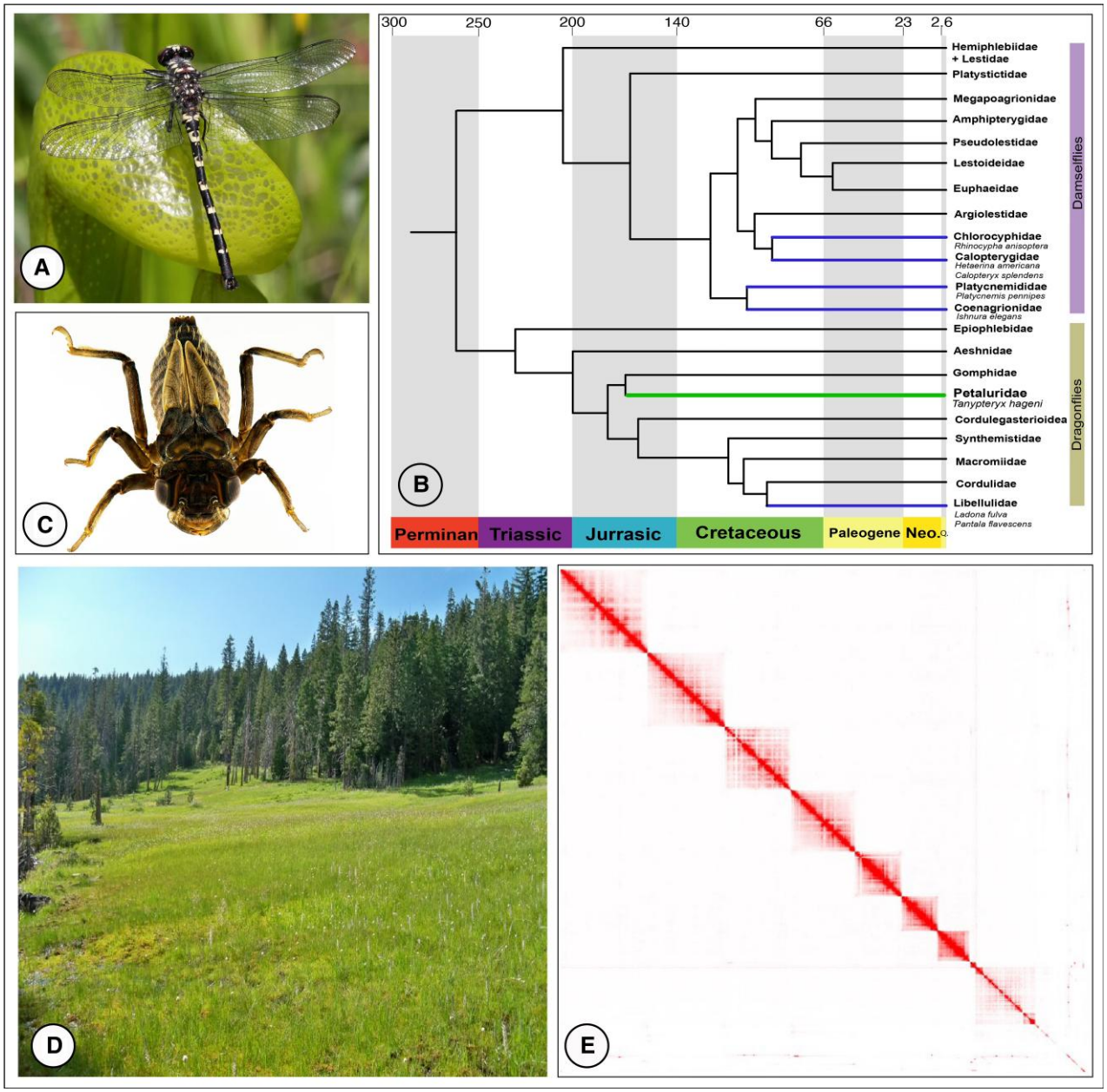

Fig. 1.—(*A*) *T. hageni*, adult. E. HiC contact map with the chromosome level assembly. (*B*) Phylogeny of Odonata (modified from Kohli et al. 2021). Families with a reference genome (The zygopterya Cholorcyphidae, Calopterygidae, Platycnemididae and Anisoptera Libellulidae) highlighted in purple, Petaluridae highlighted in green. (*C*) *T. hageni*, larvae, credit Marla Garrison. (*D*) A fen in Lassen National Forest (California, USA) where *T. hageni* was collected. (Upper right) E. Hi-C contact map visualizing the alignment to the reference genome assembly.

Tanypteryx pryeri (found in Japan) ~73 million years ago, potentially diverging when the Beringian land bridge disappeared in the late Cretaceous (fig. 1*B*) (Fiorillo 2008; Ware et al. 2014). Often, species with long geological persistence have wide geographic ranges (Powell 2007; Hopkins 2011), and tend to be habitat generalists, or give rise to habitat generalists (Colles et al. 2009), but for *T. hageni* this is not the case—the persistence of this species (as well as other petaltails) is puzzling as the nymphs of *T. hageni* exclusively inhabit fens (fig. 1*C*) (Baird 2012), groundwater-driven habitats which host a number of specialist animals and plants. These habitats are characterized by soils saturated by groundwater, commonly found around springs and in riparian areas of headwater streams. Nymphs (fig. 1*D*) dig and maintain a burrow (a behavior displayed by a number of other petaltail species) that fills with water.

The research on fens in North America is sparse, but it is known that while fens make up a tiny fraction of the North American landscape, they contain a surprising proportion of the continent's biodiversity. The US Department of Agriculture observes that between 15% and 20% of the rare and uncommon plant species found in Deschutes National Forest (Oregon, USA) are found in fen ecosystems (US Department of Agriculture). It is estimated that fens are the most floristically diverse wetlands in the United States, and contain a high number of rare and endangered species (Bedford and Godwin 2003). Research on fens across the range of *T. hageni* is minimal but it is known that fens are degrading across the continental United States (Bedford and Godwin 2003). Thus, we have concerns not only for the survival of the Black Petaltail, but for the specialized habitats in which they live. There is little research regarding genomic adaptations to life in fens, so it is paramount to establish a baseline of understanding for this declining habitat.

Here, we present a chromosome-length assembly of the Black Petaltail. This genome will be a valuable tool for studying an organism that may be especially hard hit by climate change and habitat destruction. Additionally, this genome will shed light on an evolutionary enigma: the petaltail dragonflies have persisted for tens of millions of years, despite exclusively occupying fragile fen habitat as nymphs (Ware et al. 2014). Lastly, this genome will be an important resource in resolving the phylogeny of early divergences within Odonata, as no genome of any basal anisopteran is currently available.

Results and Discussion

Sequencing and Genome Size Estimation

We recovered >44.6 Gbp of sequence contained in HiFi reads, generated from 730 Gbp of raw sequence in subreads from two PacBio SMRT cells with a read N50 of 14.2 kbp. Smudgeplot showed strong evidence of a diploid genome (supplementary fig. S1, Supplementary Material online) and reported 1n = 14 monoploid coverage. The estimated genome size using kmers from HiFi reads with GenomeScope 2.0 (Ranallo-Benavidez et al. 2020) ranged from 1.47Gbp to 1.63 Gbp depending on the upper limit used, with an estimated 60.0–53.9% of unique sequence dependent upon the upper limit, (Ranallo-Benavidez et al. 2020), resulting in approximately 25× coverage (supplementary fig. S2, Supplementary Material online).

In addition to the sequencing-based method, the *T. hageni* genome size was estimated by flow cytometry analysis using *Drosophila melanogaster* as the standard (supplementary fig. S3*A*, Supplementary Material online). The genome size of *D. melanogaster* has been estimated using next-gen sequencing methods and flow cytometry. According to the NCBI genome database, the median size of the *D. melanogaster* genome is 138.9 Mbp, whereas the lowest flow cytometry estimate is 156 Mbp (Nardon et al. 2005; Boulesteix et al. 2006). The *T. hageni* genome size estimates were calculated as the ratio of red mean fluorescence intensity (MFI) of the G1 and G2 peaks of the *T. hageni* samples (supplementary fig. S3*B*, Supplementary Material online) to the G1 and G2 peaks of the *D. melanogaster* standards (supplementary fig. S3C, Supplementary Material online) multiplied by the genome size estimates of *D. melanogaster*. Thus, the Seq control group estimates the *T. hageni* genome size using the *D. melanogaster* sequencing estimate, and the flow control group estimates the *T. hageni* genome size using the *D. melanogaster* flow cytometry genome size estimate. The first *T. hageni* genome size (2.26 Gbp) was calculated using *Drosophila* genome size 138.9 Mbp and the second *T. hageni* genome size (2.54 Gbp) was calculated using *Drosophila* genome size 156 Mbp.

While these estimates were quite a bit larger than our sequencing-based estimation, there is precedent for genome size estimates from flow cell cytometry being more than twice the size of sequencing based methods in insecta (Pflug et al. 2020). The largest genome size estimate for an Odonata is 2.36 pg (~2.3 Gbp), with a mean size of 1.01pg (~.99 Gbp) in Anisoptera (Ardila-Garcia and Gregory 2009), so it is likely that the flow cytometry estimates are overestimating the genome size of *T. hageni*.

Genome Assembly and QC

Our contig assembly was generated with hifiasm v.0.16.1 (Cheng et al. 2021) and submitted to NCBI to identify possible contaminants. Following the removal of two possible contaminants (identified as Illumina adapters by NCBI), the assembly was 1.69 Gbp in length, contained 2,133 contigs, and had a contig N50 > 4 Mbp (supplementary Table S1, Supplementary Material online). After scaffolding

with Hi-C data, we generated a highly contiguous assembly that was 1.70 Gbp in length with a scaffold N50 > 206.6 Mbp, with 90.5% of base pairs assigned to nine chromosome, matching previous estimates of the haplotype of *T. hageni* (Kuznetsova and Golub 2020) (supplementary Table S1 and fig. S7, Supplementary Material online, fig. 1*E* for contact maps). After filtering out contigs that were assigned to proteobacteria, mollusca, cnidaria, and bacteroidetes by megablastv.2.9.0 (Camacho et al. 2009) and had a GC content >42% or <34%, and replaced mitochondrial contigs with the mitochondrial genome assembly, resulting in a final assembly length of 1.68 Gbp with a scaffold N50 > 206.6 Mbp (supplementary Table S1, Supplementary Material online) and an overall GC content of 37.98% (supplementary Table S2, Supplementary Material online). We note that this final assembly size is slightly larger than the sequencing based estimates (supplementary fig. S2, Supplementary Material online), and smaller than the flow cytometry estimates (supplementary fig. S3, Supplementary Material online). As far as we are aware, *Calopteryx splendens* and *Pantala flavescens*, are the only other Odonata genome assembly for which there are genome size estimates. In the case of C. splendens, the authors estimated the genome assembly size to be 1.7 Gbp using flow cytometry, while the (illumina based) assembly length was 1.6 Gbp (Ioannidis et al. 2017). The genome assembly size of *Pantala flavescens* was slightly larger than a k-mer-based genome size estimation, generated using Feulgen image analysis.

We recovered 96.8% of universal single-copy orthologs (including 96.2% single and complete, 0.6% duplicated, and an additional 1.06% fragmented) from the BUSCO (Manni et al. 2021) Insecta database indicating a high level of completeness, especially when compared with most publicly available Odonata genomes (Table 1). The low level of duplicated single copy orthologs also indicates that the built-in haplotype purging step in hifiasm successfully removed duplicate contigs.

After blobtools analysis, 19.22% of our genome was assigned to chordata. (supplementary fig. S4, Supplementary Material online). This included two of the chromosome-length scaffolds, which had nearly identical GC proportions and coverage to the other chromosome-length scaffolds (supplementary fig. S4, Supplementary Material online). It is unlikely that this was due to contamination, as our Hi-C experiment assigned these to chromosomes. We hypothesized that this could be due to a lack of coverage of the lineage Petaluridae in the BLAST database. To test this, we characterized the top BLAST hits of publicly available Petaluridae transcriptomes. In the transcriptomes of *Phenes raptor* (Suvorov et al. 2021), *T. pryeri* (Suvorov et al. 2021), and *T. hageni* (Misof et al. 2014) 23%, 10%, and 10% of contigs had a top blast hit of chordata (supplementary fig. S5, Supplementary Material online). This suggests that a lack of database coverage for *Petaluridae* could be a likely explanation for this phenomenon. It appears that the genomes of *Petaluridae* have greatly diverged from what is covered in databases, leading to erroneous BLAST characterizations. We also observed this phenomenon when blasting the genome of the Atlantic Horseshoe Crab (*Limulus polyphemus*), where 9.7% of BLAST hits mapped to Chordata (supplementary fig. S6, Supplementary Material online). The Atlantic Horseshoe Crab also has a highly repetitive genome, and lack of database coverage may be resulting in BLAST results outside of the phylum.

Annotation

Here 55.12% of the genome was classified as repetitive using RepeatModeler2v2.0.1 (Flynn et al. 2020) and RepeatMasker v4.1.2 (Smit et al. 2013). 26.14% of the genome was classified as "unclassified repetitive elements'', 15.73% as DNA transposons, 11.38% as retroelements, and 1.53% as rolling circles. We identified 22,261 protein coding genes. The annotated protein set contained 89.4% of BUSCO insecta genes, with 6.7% of the BUSCO genes duplicated, and another 4.7% fragmented.

Mitochondrial Genome

Conclusion

Here we present one of the most complete Odonata genomes to date. As the first non-libelluloid Anisopteran genome, and the first genome of an odonate habitat specialist, this assembly will be a valuable tool for understanding the biology of the Black Petaltail, resolving the phylogeny of Anisoptera, and will provide general insights into long-persisting species.

Materials and Methods

Specimen Collection, DNA Extraction, and Sequencing

We collected an immature nymph live from a burrow near Cherry Hill Campground (Lassen National Forest, California, USA) in fall of 2020. The specimen was flash frozen in liquid nitrogen and stored in a −80 °C freezer prior to extraction. High molecular weight DNA was extracted from a single individual using the Qiagen Genomic-tip kit.

Another specimen was collected from the same location in spring 2022 for Hi-C library generation. It was also flash frozen in liquid nitrogen, and sent for Hi-C analysis by DNA Zoo, who used the hemolymph for Hi-C library preparation.

High molecular weight DNA was sheared to 18 kbp using a Diagenode Megaruptor and prepared into a sequencing library using the PacBio HiFi SMRTbell Express Template Kit 2.0. The library was sequenced on two

Table 1.
Comparison of Publicly Available Odonata Genomes

	Family	Assembly level	Assembly size (Mb)	N50 (Mb)	Scaffolds	Number of Annotated Genes	Single copy complete BUSCO Score of Assembly	Source
Tanypteryx hageni	Petaluridae	Chromosome	1.68	206.6	1,033	22,261	C:96.8%[S:96.2%, D:0.6%],F:1.0%, M:2.2%,n:1367	Authors
Ladona fulva	Libellulidae	Contig	1.16	0.06	9,411	18,817	C:95.5%[S:93.6%, D:1.9%],F:2.1%, M:2.4%,n:1367**	NCBI
Pantala flavescens	Libellulidae	Chromosome	0.66	553	43	15,354	C:96.9%[S:93.6%, D:3.3%],n:1367	(Liu et al. 2022)
Ischnura elegans	Coenagrionidae	Chromosome	1.66	123.6	110	27,076	C:97.2%[S:96.4%, D:0.8%], F:1.3%, M:1.5%,n:1367	(Price et al. 2022)
Hetaerina americana	Calopterygidae	Scaffold	1.63	86.1*	1583*	N/A	C:97.8%[S:96.3%, D:1.5%],F:0.5%, M:1.7%,n:1367	NCBI
Platycnemis pennipes	Platycnemididae	Chromosome	1.65	144.8	88	N/A	C:96.9%[S:96.3%, D:0.6%],F:1.4%, M:1.7%,n:1367**	NCBI
Rhinocypha anisoptera	Chlorocyphidae	Contig	1.55	0.41*	754,445*	N/A	C:87.7%[S:71.0%, D:16.7%],F:4.2%, M:8.1%,n:1367**	NCBI
Calopteryx splendens	Calopterygidae.	Contig	1.63	0.42*	8,896*	22,523	C:95.0%[S:94.6%, D:0.4%],F:3.0%, M:2.0%,n:1367**	(Ioannidis et al. 2017)

PacBio Sequel II 30 h SMRT cells in CCS mode at the BYU sequencing center.

D. melanogaster heads and *T. hageni* head and thorax tissue were placed into ice cold Galbraith's buffer containing RNase A and crushed using a Dounce homogenizer. Lysate was then transferred to FACS tubes, through a filter. Samples were stained with propidium iodide (PI) diluted in Galbraith's buffer (Galbraith et al. 2001) (1 mg/mL) for 3 h. Two experiments were performed, one on the BD Accuri C6 cytometer and another on the Beckman Cytoflex cytometer, with two data points being retrieved from the Accuri experiment and one from the Cytoflex experiment. During the Accuri experiment, two data points were obtained from the ratios of red mean fluorescence intensities of the G1 and G2 peaks of one *Drosophila* sample and one *T. hageni* sample. During the cytoflex experiment, only the G1 peak of the *Drosophila* and *T. hageni* samples were compared. Thus, two biological replicates are presented with an additional technical replicate. The total quantity of DNA content within the *T. hageni* samples was calculated as the ratio of red MFI of the G1 and G2 peaks of the *T. hageni* samples to the G1 and G2 peaks of the *D. melanogaster* standards multiplied by the genome size estimates of *D. melanogaster*. As demonstrated by other insect genome estimates obtained by next-generation sequencing technology and flow cytometry, including *D. melanogaster*, flow cytometry analysis may result in larger genome size estimates compared to sequencing methods (Pflug et al. 2020).

Sequencing QC and Genome Assembly

We generated HiFi reads from raw subreads with PacBio SMRTlink. We then used the HiFi reads to estimate genome size with Genomescope 2.0 (Ranallo-Benavidez et al. 2020). We generated read histograms with KMC3 (Kokot et al. 2017), and tested three different higher upper bounds of 10,000, 100,000, and 1,000,00 with a k of 21. We also assessed the reads with smudgeplot (Ranallo-Benavidez et al. 2020) by using the script "smudgeplot.py" to estimate the upper and lower bounds of the plot, which returned estimates of 970 and 10 respectively, and used these estimates, and a k of 21, when generating the final smudgeplot. We generated an initial contig assembly with hifiasm v.0.16.0 (Cheng et al. 2021), and submitted the assembly to NCBI, through the genome submission tool, to check for contamination.

To generate chromosome length scaffolds, we used High-throughput chromosome conformation capture (Hi-C). The DNA Zoo consortium (dnazoo.org) generated an in situ Hi-C library using the protocol described in Rao et al. (2014). Hi-C data were then aligned to the draft

assembly using Juicer (Durand et al. 2016), and the candidate chromosome length genome assembly was built using 3D-DNA (Dudchenko et al. 2017). The resulting contact maps (fig. 1*E*, supplementary fig. S7, Supplementary Material online) were manually reviewed using Juicebox Assembly Tools (Durand et al. 2016; Dudchenko et al. 2018). Manual curation involves identify visually anomalous patterns in the heatmap (see e.g., Rao et al. 2014; Durand et al. 2016; Dudchenko et al. 2017, 2018; Harewood et al. 2017; Lapp et al. 2018)). The full process has been described in detail (Dudchenko et al. 2018). Interactive contact maps were generated using juicebox.js (Robinson et al. 2018), for both the draft and reference assembly and are publicly available at www.dnazoo.org/assemblies/Tanypteryx_hageni.

We screened for contamination with taxon-annotated GC-coverage plots using BlobTools v1.1.1 (Laetsch and Blaxter 2017). We mapped all Hi-Fi reads against the final assembly using minimap2 v2.1 (Li 2018), sorted the bam file with samtools v1.13 (Danecek et al. 2021) using the command *samtools sort*, and assigned taxonomy with megablast (Shiryev et al. 2007) using the parameters: *task megablast and -e-value 1e-25*. We calculated coverage using the blobtools function *map2cov*, created the blobtools database using the command *blobdb*, and generated the blobplot with the command *blobtools plot*. After examining the blobplot we removed contigs blasting to proteobacteria, bacteroides, cnidaria and mollusca, and had a GC content >42% or < 34%.

To investigate whether excessive megablast assignments to chordata were due to a lack of database coverage for *Petaluridae*, we used BLAST to classify the transcriptomes of *T. hageni* (Misof et al. 2014), and other members of Petaluridae, *T. pryeri* and *P. raptor* (Suvorov et al. 2021), against the Genbank nucleotide database, using the same parameters as above: *task megablast* and *-e-value 1e-25*.

Quality Control

We generated all contiguity stats with assembly-stats (Trizna 2020). We also ran BUSCO (Manni et al. 2021) on the other publicly available Odonata genomes for comparison (Table 1) using the *Insecta ODB10* database, in genome mode with the flag *–long* to retrain BUSCO for more accurate identification of genes.

Annotation

We first modeled and masked the repetitive elements of the scaffold and chromosome-level assemblies using RepeatModeler2 (Flynn et al. 2020). We then annotated the masked, scaffold-level assembly using MAKER v3.01.03 (Campbell et al. 2014). We ran a homology-only MAKER run using the 1kite *T. hageni* transcriptome (Misof et al. 2014), the transcriptomes of the Petaluridae *T. pryeryi* and *P. raptor* (Suvorov et al. 2021), and the complete annotated protein sets of *Ladona fulva*, *Pantala flavescens*, and *Ischnura elegans*. We trained Augustus (Stanke et al. 2008, 2006) on the identified protein sets using BUSCO and the insecta dataset (Manni et al. 2021), and ran MAKER a second time to generate *ab-initio* gene predictions. We then mapped these proteins to the chromosome level assembly using miniprot v0.4 (Li 2022) and re-trained Augustus (Stanke et al. 2008, 2006) using the scaffold-level coding sequences, with 1000 base pairs surrounding each sequence as the training set. As this annotation resulted in a high number of genes, and a less-than ideal BUSCO score we also mapped the protein set of *Pantala flavescens* (Liu et al. 2022) to the masked chromosome level assembly using miniprot v0.4(Li 2022), and extracted the mapped *Pantala* proteins, the protein set from the augustus annotation, and the protein set of the mapped proteins from their respective gff files with *gffread* (Pertea 2022). We combined all three protein sets and clustered the proteins at 80* similarity with CD-HIT v4.8.1 (Fu et al. 2012). We then mapped this clustered protein set back to the chromosome-level assembly with miniprot (Li 2022), and used BLAST (Shiryev et al. 2007) to align the candidate proteins to all Arthropoda proteins available on NCBI using the parameters *-outfmt "6 sseqid pident evalue qseqid -max_target_seqs 1 -max_hsps 1 -num_threads 16 -evalue 1e-15"*. We only retained proteins with a significant BLAST hit for our final annotation, and used BUSCO, using the *Insecta* database, to assess annotation completeness.

Mitochondrial Genome Assembly

We assembled and annotated the mitochondrial genome using Mitohifi (Allio et al. 2020; Uliano-Silva et al. 2021) on the scaffolded assembly, using the default parameters and the mitochondrial genome of *Anax parthenope* (Ma et al.) as a reference. We aligned the mitochondrial genome with the mitochondrial genomes of the Corduliidae *Epothalmia elegans* (Wang et al. 2019), the Macromiidae *Macromia daimoji* (Kim et al.), the Libellulidae *Pantala flavescens* (David et al.), the Gomphidae *Gomphus vulgatissimus*, the Corduligastridae *Cordulegaster boltonii* (Hovmöller 2006), the Aeshnidae *Anax parthenope* (Ma et al.), and the zygopteran *Pseudolestes mirabilis* (Yu et al. 2021), representing each of the families of Anisoptera for which there is a mitochondrial genome assembly, in AliView (Larsson 2014) using MUSCLE v3.8.31(Edgar 2004).

Supplementary material

Supplementary data are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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Data Availability

The draft and unfiltered reference assembly are publicly available on DNA Zoo's website (www.dnazoo.org/assemblies/Tanypteryx_hageni). The final assembly file, annotation files, and the mitochondrial genome alignment are available on figshare (project link: https://figshare.com/projects/A_Chromosome-length_Assembly_of_the_Black_Petaltail_Tanypteryx_hageni_Dragonfly/151584. DOI: genome assembly 10.6084/m9.figshare.21390975, genome annotation file 10.6084/m9.figshare.21390966, annotated proteins 10.6084/m9.figshare.21390972, repeatmasker output 10.6084/m9.figshare.21391029, and mitochodnrial genome alignment 10.6084/m9.figshare.21824424). In addition to the figshare repository, the final assembly file is also being processed by NCBI and will be available under the accession number JAJPWW000000000. Illumina reads used for HiC scaffolding are available at https://www.ncbi.nlm.nih.gov/sra/SRX18440492%5Baccn%5D. The Pacbio Hifi reads are available under the NCBI BioProject accession PRJNA743558.

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