Deep ancestral introgression shapes evolutionary history of dragonflies and damselflies

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Abstract

Introggression is an important biological process affecting at least 10% of the extant species in the animal kingdom. Introggression significantly impacts inference of phylogenetic species relationships where a strictly binary tree model cannot adequately explain reticulate net-like species relationships. Here we use phylogenomic approaches to understand patterns of introggression along the evolutionary history of a unique, non-model insect system: dragonflies and damselflies (Odonata). We demonstrate that introgression is a pervasive evolutionary force across various taxonomic levels within Odonata. In particular, we show that the morphologically “intermediate” species of Anisozygoptera (one of the three primary suborders within Odonata besides Zygoptera and Anisoptera), which retain phenotypic characteristics of the other two suborders, experienced high levels of introgression likely coming from zygopteran genomes. Additionally, we find evidence for multiple cases of deep inter-superfamilial ancestral introgression.

Key words: reticulate evolution, gene flow, phylogenomics, Odonata

Running title: Reticulate evolution of Odonata

Introduction

In recent years numerous studies have showed that multiple parts of the Tree of Life did not evolve according to a strictly bifurcating phylogeny (Hallstrom and Janke 2010, Mallet, et al. 2016). Instead, many organisms experience reticulate network-like evolution that is caused by an exchange of inter-specific genetic information via various biological processes. In particular, lateral gene transfer, incomplete lineage sorting (ILS) and introgression can result in gene trees that are discordant with the species tree (Degnan and Rosenberg 2009, Maddison 1997, Posada and Crandall 2001). Lateral transfer and introgression both involve gene flow following speciation, thereby producing “reticulate” phylogenies. Incomplete lineage sorting (ILS), on the other hand, occurs when lineages fail to coalesce within their ancestral population. Since this process does not involve any post-speciation gene flow, it does not contribute to reticulate evolution, even though it often results in discordant gene trees. Phylogenetic species-gene tree incongruence observed in empirical data can provide insight into underlying biological factors that shape the evolutionary trajectories of a set of taxa. The major source of reticulate evolution for eukaryotes is introgression where it affects approximately 25% of flowering plant and 10% of...
animal species (Mallet 2005, Mallet, et al. 2016). Introgressed alleles can be fitness-neutral, deleterious (Petr, et al. 2019) or adaptive (Norris, et al. 2015, Oziolor, et al. 2019). For example, adaptive introgression has been shown to provide an evolutionary rescue from polluted habitats in gulf killifish (*Fundulus grandis*) (Oziolor, et al. 2019), yielded mimicry adaptations among *Heliconius* butterflies (Heliconius Genome 2012) and archaic introgression has facilitated adaptive evolution of altitude tolerance (Huerta-Sanchez, et al. 2014), immunity and metabolism in modern humans (Gouy and Excoffier 2020). Additionally, hybridization and introgression are important and often overlooked mechanisms of invasive species establishment and spread (Perry, et al. 2002).

Odonata, the insect order that contains dragonflies and damselflies, lacks a strongly supported backbone tree to clearly resolve higher-level phylogenetic relationships (Carle, et al. 2015, Dijkstra, et al. 2014a). Current evidence places odonates together with Ephemeroptera (mayflies) as the living representatives of the most ancient insect lineages to have evolved wings and active flight (Thomas, et al. 2013). Odonates possess unique anatomical and morphological features such as a specialized body form, specialized wing venation, a distinctive form of muscle attachment to the wing base (Busse, et al. 2013) allowing for direct flight and accessory (secondary) male genitalia that support certain unique behaviors (e.g., sperm competition). They are among the most adept flyers of all animals and are exclusively carnivorous insects relying primarily on vision to capture prey (Chauhan, et al. 2014, Suvorov, et al. 2017). During their immature stage they are fully aquatic and spend much of their adult life in flight. Biogeographically, odonates exhibit species ranges varying from worldwide dispersal (Troast, et al. 2016) to island-endemic. Odonates also play crucial ecological roles in local freshwater communities, being a top invertebrate predator as both adults and immatures (Dijkstra, et al. 2014b). Due to this combination of characteristics, odonates are quickly becoming model organisms to study specific questions in ecology, physiology and evolution (Bybee, et al. 2016, Cordoba-Aguilar 2008). However, the extent of introgression at the genomic scale within Odonata remains largely unknown.

In various biological systems, the empirical evidence shows that hybridization can potentially lead to intermediate phenotypes (Runemark, et al. 2019) observed at molecular level (e.g. semidominant expression in inter-specific hybrids (Landry, et al. 2005)) as well as organismal morphology (e.g. (Kaldy, et al. 2020, Lemmon and Lemmon 2010, Rothfels, et al. 2019)).
The Anisozygoptera suborder, which contains only three extant species, retains traits shared with both dragonflies and damselflies (hence its taxonomic name), ranging from morphology and anatomical structures (Busse, et al. 2015) to behavior and flight biomechanics (Ruppell and Hilfert 1993). These characteristics could suggest either a hybrid origin of this suborder or substantial introgression at loci governing key morphological and behavioral traits shortly after the suborder’s formation. The potential introgression scenario for Anisozygoptera is yet to be formally tested using genome-wide data. Two early attempts to tackle introgression/hybridization patterns within Odonata were undertaken in (Monetti, et al. 2002, Sánchez-Guillén, et al. 2005). The studies showed that two closely related species of damselflies, *Ischnura graellsii* and *I. elegans*, can hybridize under laboratory conditions and that genital morphology of male hybrids shares features with putative hybrids from *I. graellsii–I. elegans* natural allopatric populations (Monetti, et al. 2002). The existence of abundant hybridization and introgression in natural populations of *I. graellsii* and *I. elegans* has received further support from an analysis of microsatellite data (Sanchez-Guillen, et al. 2011). Putative hybridization events have also been identified in a pair of calopterygoid damselfly species, *Mnais costalis* and *M. pruinosa* based on the analyses of two molecular loci (mtDNA and nucDNA) (Hayashi, et al. 2005), and between *Calopteryx virgo* and *C. splendens* using 16S ribosomal DNA and 40 random amplified polymorphic DNA (RAPD) markers (Tynkkynen, et al. 2008). A more recent study identified an inter-specific hybridization between two cordulegasterid dragonfly species, *Cordulegaster boltonii* and *C. trinacriae* using two molecular markers (mtDNA and nucDNA) and geometric morphometrics (Solano, et al. 2018).

Here we present a comprehensive analysis of transcriptomic data from 83 odonate species. First, we reconstruct a robust phylogenetic backbone using up to 4341 genetic loci for the order and discuss its evolutionary history spanning from the Carboniferous period (~360 Ma) to present day. Furthermore, in light of the “intermediate” phenotypic nature of Anisozygoptera, we investigate phylogenetic signatures of introgression within Odonata. Most notably, we identify a strong signal of deep introgression in the Anisozygoptera suborder, species of which possess traits of both main suborders, Anisoptera and Zygoptera. Although the strongest signatures of introgression are found in Anisozygoptera, we find evidence that introgression was pervasive in Odonata throughout its entire evolutionary history.
Materials and Methods

Taxon Sampling and RNA-seq
In this study, we used 85 distinct species (83 ingroup and 2 outgroup taxa). 35 RNA-seq libraries were obtained from NCBI (Supplementary Table S1 available on Dryad). The remaining 58 libraries were sequenced in the Bybee Lab (some species have several RNA-seq libraries; Supplementary Table S1 available on Dryad). Total RNA was extracted for each taxon from eye tissue using NucleoSpin columns (Clontech) and reverse-transcribed into cDNA libraries using the Illumina TruSeq RNA v2 sample preparation kit that both generates and amplifies full-length cDNAs. Prepped mRNA libraries with insert size of ~200bp were multiplexed and sequenced on an Illumina HiSeq 2000 producing paired-end reads with average length of 275-bp by the Microarray and Genomic Analysis Core Facility at the Huntsman Cancer Institute at the University of Utah, Salt Lake City, UT, USA. Quality scores, tissue type and other information about RNA-seq libraries are summarized in Supplementary Table S1 available on Dryad and NCBI BioProject PRJNA641626.

Transcriptome Assembly and CDS Prediction
RNA-seq libraries were trimmed and de novo assembled using Trinity (Grabherr, et al. 2011, Haas, et al. 2013) with default parameters. Then only the longest isoform was selected from each gene for downstream analyses using the Trinity utility script. In order to identify potentially coding regions within the transcriptomes, we used TransDecoder with default parameters specifying to predict only the single best ORF. Each predicted proteome was screened for contamination using DIMOND BLASTP (Buchfink, et al. 2015) with an E-value cutoff of $10^{-10}$ against custom protein database. Non-arthropod hits were discarded from proteomes (amino acid, AA sequences) and corresponding CDSs. To mitigate redundancy in proteomes and CDSs, we used CD-HIT (Fu, et al. 2012) with the identity threshold of 0.99. Such a conservative threshold was used to prevent exclusion of true paralogous sequences; thus, reducing possible false positive detection of 1:1 orthologs during homology searches.

Homology Assessment
In the present study three types of homologous loci (gene clusters), namely conserved single-copy orthologs (CO), all single-copy orthologs (AO) and paralogy-parsed orthologs (PO) identified by BUSCO v1.22 (Simao, et al. 2015), OrthoMCL (Li, et al. 2003), and Yang and Smith’s (2014) pipelines, respectively, were used in phylogenetic inference.

BUSCO arthropod Hidden Markov Model Profiles of 2675 single-copy orthologs were used to find significant COs matches within CDS datasets by HMMER’s `hmmersearch v3` (Eddy 2011) with group-specific expected bit-score cutoffs. BUSCO classifies loci into complete [duplicated] and fragmented. Thus, only complete single-copy loci were extracted from CDS datasets and corresponding AA sequences for further phylogenetic analyses. Since loci were identified as true orthologs if they score above expected bit-score, and complete if their lengths lie within ~95% of BUSCO group mean length, many partial erroneously assembled sequences were filtered out.

OrthoMCL v2.0.9 (Li, et al. 2003) was used to compute AOs in all species using predicted AA sequences by TransDecoder. AA sequences were used in an all-vs-all BLASTP with an E-value cutoff of $10^{-10}$ to find putative orthologs and paralogs. The Markov Cluster algorithm (MCL) inflation point parameter was set to 2. Only 1:1 orthologs were used in further analyses. In order to exclude false-positive homology clusters identified by OrthoMCL, we applied machine learning filtering procedure (Fujimoto, et al. 2016a) implemented in OGCleaner software v1.0 (Fujimoto, et al. 2016b) using a metaclassifier with logistic regression.

Finally, to identify additional clusters, we used Yang’s tree-based orthology inference pipeline (Yang and Smith 2014) that was specifically designed for non-model organisms using transcriptomic data. Yang’s algorithm is capable of parsing paralogous gene families into “orthology” clusters that can be used in phylogenetic analyses. It has been shown that paralogous sequences encompass useful phylogenetic information (Hellmuth, et al. 2015). First, the Transdecoder-predicted AA sequences were trimmed using CD-HIT with the identity threshold of 0.995. Then, all-vs-all BLASTP with an E-value cutoff of $10^{-5}$ search was implemented. The raw BLASTP output was filtered by a hit fraction of 0.4. Then, MCL clustering was performed with an inflation point parameter of 2. Each cluster was aligned using iterative algorithm of PASTA (Mirarab, et al. 2015) and then was used to infer a maximum-likelihood (ML) gene tree using IQ-TREE v1.5.2 (Nguyen, et al. 2015) with an automatic model selection. Tree tips that were longer than relative and absolute cutoffs of 0.4 and 1 respectively were removed. Mono-
and paraphyletic tips that belonged to the same species were masked as well. To increase quality of homology clusters realignment, tree inference and tip masking steps were iterated with more stringent relative and absolute masking cutoffs of 0.2 and 0.5 respectively. Finally, POs (AA sequences and corresponding CDSs) were extracted by rooted ingroups (RI) procedure using Ephemera danica as an outgroup (for details see (Yang and Smith 2014)).

Cluster Alignment, Trimming and Supermatrix assembly
For most of the analyses only clusters with \( \geq 42 \) (~50%) species present were retained. In total, we obtained five cluster types, namely DNA (CDS) and AA COs, AA AOs and DNA and AA POs. Each cluster was aligned using PASTA (Mirarab, et al. 2015) for the DNA and AA alignments and PRANK v150803 (Loytynoja 2014, Loytynoja and Goldman 2008) for the codon alignments and alignments where either 1\(^{st}\) and 2\(^{nd}\) or 3\(^{rd}\) codon positions were removed. In order to reduce the amount of randomly aligned regions, we implemented ALISCORE v2.0 (Misof and Misof 2009) trimming procedure (for PASTA alignments) followed by masking any site with \( \geq 42 \) gap characters (for both PASTA and PRANK alignments). Also, since fragmentary data may have a negative effect on accuracy of gene and hence species tree inference (Wickett, et al. 2014), sequence fragments with \( >50\% \) gap characters were removed from clusters that were used to estimate trees with ASTRAL v4.10.12 (Mirarab, et al. 2014) estimation. For each of the cluster type, we assembled supermatrices from trimmed gene alignments. Additionally, completely untrimmed supermatrices were generated from DNA and AA COs with \( \geq 5 \) species present.

Phylogenetic Tree Reconstruction
Four different in spirit tree building methods (ML:IQTREE, Bayesian:ExaBayes, Supertree:ASTRAL, Alignment-Free (AF): Co-phylog) were used to infer odonate phylogenetic relationships using different input data types (untrimmed and trimmed supermatrices, codon supermatrices, codon supermatrices with 1\(^{st}\) and 2\(^{nd}\) or 3\(^{rd}\) positions removed, gene trees and assembled transcriptomes). In total we performed 48 phylogenetic analyses and compared topologies to identify stable and conflicting relationships (Supplementary Table S2 available on Dryad).
We inferred phylogenetic ML trees from each supermatrix using IQTREE implementing two partitioning schemes: single partition and those identified by PartitionFinder v2.0 (three GTR models for DNA and a large array of protein models for AA) (Lanfear, et al. 2016a) with relaxed hierarchical clustering option (Lanfear, et al. 2014). In the first case, IQTREE was run allowing model selection and assessing nodal support with 1000 ultrafast bootstrap (UFBoot) (Minh, et al. 2013) replicates. In the second case, IQTREE was run with a given PartitionFinder partition model applying gene and site resampling to minimize false-positives (Gadagkar, et al. 2005) for 1000 UFBoot replicates.

For Bayesian analyses implemented in ExaBayes (Aberer, et al. 2014), we used highly trimmed (retaining sites only with occupancy of ≤ 5 gap characters) and original DNA and AA CO supermatrices assuming a single partition. We initiated 4 independent runs with 4 Markov Chain Monte Carlo (MCMC) coupled chains sampling every 500\textsuperscript{th} iteration. Due to high computational demands of the procedure, only the GTR and JTT substitution model priors were applied to DNA and AA CO supermatrices respectively with the default topology, rate heterogeneity and branch lengths priors. However, all supported protein substitution models as a prior were specified for the trimmed AA CO supermatrix. For convergence criteria, an average standard deviation of split frequencies (ASDSF) (Lakner, et al. 2008), a potential scale reduction factor (PSRF) (Brooks and Gelman 1998) and an effective sample size (ESS) (Lanfear, et al. 2016b) were utilized. Values of 0% < ASDSF < 1% and 1% < ASDSF < 5% indicate excellent and acceptable convergence respectively; ESS > 100 and PSRF ~ 1 represent good convergence (see ExaBayes manual, (Aberer, et al. 2014)).

ASTRAL analyses were conducted using two input types: (i) gene trees obtained by IQTREE allowing model selection for fully trimmed DNA and AA clusters and (ii) gene trees obtained from the alignment-tree coestimation process in PASTA. Nodal support was assessed by local posterior probabilities (Sayyari and Mirarab 2016). In addition to standard phylogenetic inferential approaches, we applied an alignment-free (AF) species tree estimation algorithm using Co-phylog (Yi and Jin 2013). Raw Transdecoder CDS outputs were used in this analysis using k-mer size of 9 as the half context length required for Co-phylog. Bootstrap replicate trees were obtained by running Co-Phylog with the same parameter settings on each subsampled with replacement CDS Transdecoder libraries and were used to assess nodal support.
Assessment of Phylogenetic Support via Quartet Sampling

As an additional phylogenetic support, we implemented quartet sampling (QS) approach (Pease, et al. 2018). Briefly, this method provides three scores for internal nodes: (i) the quartet concordance (QC) score gives an estimate of how sampled quartet topologies agree with the putative species tree; (ii) quartet differential (QD) estimates frequency skewness of the discordant quartet topologies, which can be indicative of introgression if a skewed frequency is observed and (iii) quartet informativeness (QI) quantifies how informative sampled quartets are by comparing likelihood scores of alternative quartet topologies. Finally, QS provides a quartet fidelity (QF) score for terminal nodes that measures a taxon “rogueness”. We performed QS analysis with all 48 putative species phylogenies using the SuperMatrix_50BUSCO_dna_pasta_ali_trim supermatrix, specifying the IQTREE engine for quartet likelihood calculations with 100 replicates (i.e. number of quartet draws per focal branch).

Fossil Dating

A Bayesian algorithm of MCMCTree v4.9h (Yang 2007) with approximate likelihood computation was implemented to estimate divergence times within Odonata using 20 crown node fossil constraints with corresponding prior distributions (Supplementary Table S3 available on Dryad). First, we estimated branch lengths by ML and then the gradient and Hessian matrix around these ML estimates in MCMCTree using SuperMatrix_50BUSCO_dna_pasta_alitrim supermatrix. Second, we used these gradient and Hessian matrix to construct an approximate likelihood function by Taylor expansion (Dos Reis and Yang 2011) and perform fossil calibration in MCMC framework under the uncorrelated clock model. For this step we specified GTR+Γ substitution model with 4 gamma categories, along with birth, death and sampling parameters of 1, 0.5 and 0.01, respectively. To ensure convergence, the analysis was run independently five times for $6 \times 10^7$ generations, logging every 1000th generation and then removing 50% as a burn-in. Convergence (ESS > 200) of the MCMC chains was verified using Tracer v1.7.1 (Rambaut, et al. 2018). Visualization of the calibrated tree was performed in R using the MCMCtreeR package (Puttick 2019).

Analyses of Introgression
In order to address the scope of possible reticulate evolution across odonate phylogeny, we used various methods of introgression detection such as HyDe/D (Blischak, et al. 2018), D_{FOIL} (Pease and Hahn 2015), $\chi^2$ goodness-of-fit test, branch length test (BLT), QuIBL (Edelman, et al. 2019) and PhyloNet (Than, et al. 2008, Wen, et al. 2018). Furthermore, we used the methodological consensus of HyDe/D, D_{FOIL}, $\chi^2$ goodness-of-fit test and BLT approaches to provide more conservative inferences of introgression across the order (see Results). Specifically, we compared sets of unique introgressing species pairs that were identified by each of the aforementioned methods. The significance of overlap among the signals from these different methods was then assessed using an exact test of multi-set interactions (Wang, et al. 2015).

The HyDe framework allows detection of hybridization events which relies on quantification of phylogenetic site patterns (invariants). HyDe estimates whether a putative hybrid population (or taxon) H is sister to either population P1 with probability $\gamma$ or to P2 with probability $1-\gamma$ in a 4-taxon (quartet) tree (((P1,H,P2),O), where O denotes an outgroup. Then, it conducts a formal statistical test of $H_0: \gamma = 0$ vs. $H_1: \gamma > 0$ using Z-test, where $\gamma = 0$ (=1) is indicative of non-significant introgression. We applied HyDe to the concatenated supermatrix SuperMatrix_50BUSCO_dna_pasta_ali_trim of 1603 BUSCO genes under default parameters specifying *Ephemera danica* as an outgroup. Under this setup HyDe evaluates all possible taxa quartets. Since HyDe only allows indication of a single outgroup taxon (i.e. *Ephemera danica*), we excluded all quartets that contained the *Isonychia kiangsinensis* outgroup from the HyDe output. Additionally, we calculated Patterson’s $D$ statistic (Patterson, et al. 2012) for every quartet from the frequency ($p$) of ABBA-BABA site patterns estimated by HyDe as $D = \frac{p_{ABBA} - p_{BABA}}{p_{ABBA} + p_{BABA}}$. To test significance of $D$ statistics we used a $\chi^2$ test to assess whether the proportions $p_{ABBA}$ and $p_{BABA}$ were significantly different. To minimize effect of false positive cases (type I error) in the output, we first applied a Bonferroni correction to the $P$ values derived from Z- and $\chi^2$ tests and then filtered the results based on a significance level of 0.05 and $10^{-6}$ for $D$ and $\gamma$, respectively. Additionally, we excluded all quartets that did not match the species topology. Further, we ran HyDe on SuperMatrix_50BUSCO_dna_prank_trim excluding 3rd codon position to investigate a potential impact of the saturation effect on introgression inference. 

$D_{FOIL}$ is an alternative site pattern-based approach that detects introgression using symmetric 5-taxon (quintet) trees, i.e. (((P1,P2),(P3,P4),O). $D_{FOIL}$ represents a collection of
statistics for quintet trees that are similar in spirit to the Patterson’s $D$ statistic; if considered simultaneously, these statistics provide a powerful approach to identify introgression including ancestral as well as donor and recipient taxa (i.e. introgression directionality). Moreover, $D_{\text{FOIL}}$ exhibits exceptionally low false positive rates (Pease and Hahn 2015). Since the number of possible quintet topologies for a phylogeny of 85 taxa is $> 32 \times 10^6$, for analysis we extracted them only for every odonate suborder individually using custom R scripts. Note, that for Anisozygoptera we only considered quintets that can be formed between Anisozygoptera, Anisoptera, and Zygoptera taxonomic groups. As the number of Anisozygoptera quintets is highly disproportional (34619 out of all 72971 tested Odonata quintets), for downstream analyses we randomly selected 4000 Anisozygoptera quintets which approximately matches the number of quintets for an individual species. Analogously to HyDe, we applied $D_{\text{FOIL}}$ to the concatenated supermatrix SuperMatrix_50BUSCO\_dna\_pasta\_ali\_trim of 1603 BUSCO genes under default parameters specifying *Ephemera danica* as an outgroup. Also, since $D_{\text{FOIL}}$ requires that every quintet has a symmetric topology we considered only those quintets within our phylogeny that met this criterion (Fig. 1). Additionally, $D_{\text{FOIL}}$ requires that the divergence time of P3 and P4 precedes divergence of P1 and P2, i.e. $T_2 > T_1$, thus we filtered out quintets that violated this assumption using divergence times from our fossil calibrated phylogeny. In order to correct the $P$ values resulted from $D_{\text{FOIL}}$ analysis for multiple testing, we applied the Benjamini-Hochberg procedure at a false discovery rate cutoff (FDR) of 0.05.

As an alternative test for introgression, we performed a simple yet conservative $\chi^2$ goodness-of-fit test on the gene count values for each triplet. Specifically, we asked whether one of the two possible discordant gene tree topologies was supported by a greater number of genes than the other discordant topology (i.e. a significant difference between the number of discordant gene trees showing ((P1,P3),P2) versus ((P2,P3),P1), where the ((P1,P2),P3) topology corresponds to the species tree). Under ILS alone, the fractions of genes supporting each discordant topology are expected to be the same, while in the presence of introgression they may differ. We therefore used a $\chi^2$ test to determine if these fractions differed significantly, and we considered triplets where the null hypothesis was rejected to be suggestive of introgression. Because we tested many triplets for introgression, we corrected the $P$ values resulted from these $\chi^2$ tests using the Benjamini-Hochberg procedure and applied a false discovery rate cutoff (FDR) of 0.05. Second, we used a branch length test (BLT) to identify cases of introgression (Suvorov,
This test examines branch lengths to estimate the age of the most recent coalescence event (measured in substitutions per site). Introgression should result in more recent coalescences than expected under the concordant topology with complete lineage sorting, while ILS yields older coalescence events. Importantly, ILS alone is not expected to result in different coalescence times between the two discordant topologies, and this forms the null hypothesis for the BLT. For a given triplet, for each gene tree we calculated the distance $d$ (a proxy for the divergence time between sister taxa) by averaging the external branch lengths leading to the two sister taxa under that gene tree topology. We calculated $d$ for each gene tree and denote values of $d$ from the first discordant topology $d_{T_1}$ and those from the second discordant topology $d_{T_2}$. We then compared the distributions of $d_{T_1}$ and $d_{T_2}$ using a Wilcoxon Rank Sum Test. Under ILS alone the expectation is that $d_{T_1} = d_{T_2}$, while in the presence of introgression $d_{T_1} < d_{T_2}$ (suggesting introgression consistent with discordant topology $T_1$) or $d_{T_1} > d_{T_2}$ (suggesting introgression with consistent with discordant topology $T_2$). The BLT is conceptually similar to the D3 test (Hahn and Hibbins 2019), which transforms the values of $d_{T_1}$ and $d_{T_2}$ in a manner similar to the $D$ statistic for detecting introgression. As with the $\chi^2$ test, we performed the BLT on all triplets within a clade and used a Benjamini-Hochberg correction with a false discovery rate cutoff (FDR) of 0.05. We note that both the $\chi^2$ test and BLT may be conservative in cases where there is introgression between both tested species pairs (i.e. introgression between P1-P3 and P2-P3 for a given species topology $((P1,P2),P3)$) depending on the fraction of affected loci (affects the $\chi^2$ test) and timing of introgression between each species pair (affects the BLT).

QuIBL is based on the analysis of branch length distributions across gene trees to infer putative introgression patterns. Briefly, under coalescent theory internal branches of rooted gene trees for a set of 3 taxa (triplet) can be viewed as a mixture of two distributions with the underlying parameters. Each mixture component generates branch lengths corresponding to either ILS or introgression/speciation. Thus, estimated mixing proportions ($\pi_1$ for ILS and $\pi_2$ for introgression/speciation; $\pi_1 + \pi_2 = 1$) of those distribution components show what fraction of the gene trees were generated through ILS or non-ILS processes. For a given triplet, QuIBL computes frequency of gene trees that support three alternative topologies. Then for every alternative topology QuIBL estimates mixing proportions along with other relevant parameters via Expectation-Maximization and computes Bayesian Information Criterion (BIC) scores for ILS-only and introgression models. For concordant topologies elevated values of $\pi_2$ are expected.
whereas for discordant ones $\pi_2$ can vary depending on the severity of ILS/intensity of introgression. In extreme cases when the gene trees were generated exclusively under ILS, $\pi_2$ will approach zero and the expected gene tree frequency for each alternative topology of a triplet will be approximately 1/3. To identify significant cases of introgression here we used a stringent cutoff of $\Delta BIC < -30$ (Edelman, et al. 2019). We ran QuIBL on every triplet individually under default parameters with number of steps (numsteps parameter) is equal to 50 and specifying one of the Ephemeroptera species ($Isonychia kiangsinensis$ and $Ephemera danica$) for triplet rooting. For computational efficiency we extracted triplets only from the odonate superfamilies in a similar manner as we did for $D_{FOIL}$ (see above). For this analysis we used 1603 ML gene trees estimated from CO orthology clusters. We note that most of the phylogenomic-based introgression detection methods, including approaches used here (namely HyDe, $D_{FOIL}$, $\chi^2$ test, BLT and QuIBL) are not able to infer gene flow between sister lineages (Hibbins and Hahn 2021) as they rely on topological discordance at either the gene or site level, which can only be examined for topologies with more than two taxa.

To identify patterns of reticulate evolution for Anisozygoptera we estimated phylogenetic networks from the 1603 ML gene trees estimated from CO orthology clusters using pseudolikelihood (InferNetwork_MPL, (Yu and Nakhleh 2015)) and likelihood (CalGTProb) approaches implemented in PhyloNet (Than, et al. 2008, Wen, et al. 2018). For scalability purposes we subsampled our taxon set to eight Zygoptera species, nine Anisoptera species and $Epiophlebia superstes$. For all network searches we explicitly indicated $Epiophlebia superstes$ as a putative hybrid ($-h$ option). For both pseudolikelihood and likelihood analyses we only selected gene trees that had at least one of the outgroup species ($Isonychia kiangsinensis$ and $Ephemera danica$) and at least three ingroup taxa. For pseudolikelihood analysis, we ran PhyloNet allowing a single reticulation event, with the starting tree that corresponds to the species phylogeny ($-s$ option), 100 iterations ($-x$ option), 0.9 bootstrap threshold for gene trees ($-b$ option) and optimization of branch lengths and inheritance probabilities on the inferred networks ($-po$ option). To ensure convergence, the network searches were repeated 3 times. For the full likelihood estimation, we fixed the topology (equivalent to the species tree topology) and calculated likelihood scores for possible networks with a single reticulation (generated with a custom script) using CalGTProb. Additionally, to assess significance of networks, we used
difference of BIC scores ($\Delta$BIC) derived from network without reticulation (i.e. tree) and a network with a reticulation (Supplementary Table S4 available on Dryad).

Dimensionality reduction and visualization

To uncover and visualize complex relationships between site pattern frequencies and Patterson’s D statistic and HyDe $\gamma$ parameter we implemented a dimensionality reduction technique t-distributed stochastic neighbor embedding (tSNE) (Van Der Maaten and Hinton 2008) under default parameters in R. Specifically we estimated tSNE maps from counts of 15 quartet site patterns calculated by HyDe (“AAAA”, “AAAB”, “AABA”, “AABB”, “AABC”, “ABAA”, “ABAB”, “ABAC”, “ABBA”, “BAAA”, “ABBC”, “CABC”, “BACA”, “BCAA”, “ABCD”).

Results

Phylogenetic Inference

We compiled transcriptomic data for 83 odonate species including 49 new transcriptomes sequenced for this study (Supplementary Table S1 available on Dryad). To assess effects of various steps of our phylogenetic pipeline on species tree inference, we examined different methods of sequence homology detection, multiple sequence alignment strategies, postprocessing filtering procedures and tree estimation methods. Specifically, three types of homologous loci (gene clusters) were used to develop our supermatrices, namely 1603 conserved single-copy orthologs (CO), 1643 all single-copy orthologs (AO) and 4341 paralogy-parsed orthologs (PO) with $\geq$42 (~50%) species present (for more details, see Material and Methods).

To date, our data represents the most comprehensive resource available for Odonata in terms of gene sampling. Each gene cluster was aligned, trimmed and concatenated resulting in five main supermatrices, CO (DNA/AA), AO (AA) and PO (DNA/AA), which included 2,167,861 DNA (682,327 amino acid (AA) sites), 882,417 AA sites, 6,202,646 DNA (1,605,370 AA) sites, respectively. Thus, the largest alignment that we used to infer the odonate phylogeny consists of 4341 loci concatenated into a supermatrix with >6 million nucleotide sites. All supermatrices are summarized in Supplementary Table S2 available on Dryad; the inferred odonate relationships are shown in Supplementary Fig. S1a available on Dryad whereas topologies of all inferred
phylogenies are plotted in Supplementary Fig. S1b available on Dryad and topologies of 1603 CO gene trees are shown in Supplementary Fig. S1c available on Dryad. Additionally, we performed nodal dating of the inferred phylogeny using 20 fossil calibration points (Supplementary Table S3 available on Dryad).

The inferred ML phylogenetic tree of Odonata using DNA supermatrix of 1603 BUSCO loci (Fig. 1) was used as a primary phylogenetic hypothesis throughout this study as it agrees with the majority of relationships inferred by other methods (Supplementary Figs. S1a-b available on Dryad). Divergence of Zygoptera and Epiprocta (Anisozygoptera+Anisoptera) from the Most Recent Common Ancestor (MRCA) was estimated to have occurred in the Middle Triassic ~226 Ma (95% Credible Interval [CI]: 221.8 – 231.1 Ma, Fig. 1), which is in line with recent estimates (Misof, et al. 2014, Thomas, et al. 2013). Comprehensive phylogenetic coestimation of subordinal relationships within Odonata showed that the suborders were well supported (Supplementary Fig. S1a available on Dryad), as they were consistently recovered as monophyletic clades in all analyses. In several previous studies, paraphyletic relationships of Zygoptera had been proposed based on wing vein characters derived from fossil odonatoids and extant Odonata (Trueman 1996), analysis of 12S (Saux, et al. 2003), analysis of 18S, 28S, Histone 3 (H3) and morphological data (Ogden and Whiting 2003) and analysis of 16S and 28S data (Hasegawa and Kasuya 2006). In most of these studies, Lestidae was inferred to be sister to Anisoptera. Functional morphology comparisons of flight systems, secondary male genitalia and ovipositors also supported a non-monophyletic Zygoptera with uncertain phylogenetic placement of multiple groups (Pfau 1991). Nevertheless, the relationships inferred from these previous datasets seem to be highly unlikely due to apparent morphological differentiation (e.g., eye spacing, body robustness, wing shape) between the suborders and support for monophyletic Anisoptera and Zygoptera from more recent morphological (Busse, et al. 2015), molecular (Carle, et al. 2008, Kim, et al. 2014, Suvorov, et al. 2017, Thomas, et al. 2013) and combined studies using both data types (Bybee, et al. 2008). Our analyses recover Zygoptera as monophyletic consistently (Supplementary Fig. S1a available on Dryad).

Divergence time estimates suggest a TMRCA of Anisoptera and Anisozygoptera (we occasionally refer these two suborders as “Epiprocta”) in the Late Triassic (~204 Ma; 95% CI 201.7 – 207.8 Ma; Fig. 1). Epiprocta as well as Anisoptera were consistent with more recent studies and recovered as monophyletic with very high support. We also note here that our
divergence time estimates of Anisoptera tend to be younger than those found by Letsch, et al. (2016). The fossil-calibration approach based on penalized likelihood that was used by Letsch, et al. (2016) has been shown to overestimate true nodal age (Britton, et al. 2007) preventing direct comparison between our dates derived from the Bayesian framework MCMCTree and those estimated by Letsch, et al. (2016). Additionally, our divergence time estimate for Epiprocta is older than inferred by Misof, et al. (2014), which can be explained by the differences in calibration schemes. Specifically, for the Epiprocta crown node we specified *Liassophlebia sp.* fossil using an informative skewed normal distribution prior (see Supplementary Table S3 available on Dryad).

The phylogenetic position of Gomphidae and Petaluridae, both with respect to each other and the remaining anisopteran families, has long been difficult to resolve. Several phylogenetic hypothesis have been proposed in the literature based on molecular and morphological data regarding the placement of Gomphidae as sister to the remaining Anisoptera (Blanke, et al. 2013b) or to Libelluloidea (Misof, et al. 2001). Petaluridae has exhibited stochastic relationships with different members of Anisoptera, including sister to Gomphidae (Misof, et al. 2001), sister to Libelluloidea (Carle, et al. 2008), sister to Chlorogomphidae+Cordulegasteridae (Bybee, et al. 2008) and sister to all other Anisoptera (Rehn 2003, Trueman 1996). The most recent analyses of the major anisopteran lineages using several molecular markers (Carle, et al. 2015) suggest Gomphidae and Petaluridae as a monophyletic group, but without strong support. Here the majority of our supermatrix analyses (Supplementary Fig. S1a available on Dryad) strongly support a sister relationship between the two families, and in our phylogeny (Fig. 1) they split from the MRCA ~161 Ma (95% CI: 156.6 – 165.5 Ma) in the Middle Jurassic (Fig. 1). We further investigated the species tree topologies that were estimated by the coalescent-based tree summary method, ASTRAL. We found that almost all these species trees reject such a relationship with high confidence (Supplementary Fig. S1a available on Dryad). In the presence of incomplete lineage sorting, concatenation methods can be statistically inconsistent (Roch and Steel 2014) leading to an erroneous species tree topology with unreasonably high support (Kubatko and Degnan 2007). This inconsistency in the recovery of a sister group relationship between Gomphidae and Petaluridae can be explained by elevated levels of incomplete lineage sorting between the families and/or possible introgression events (Maddison 1997).
New zygopteran lineages originated in the Early Jurassic ~189 Ma (95% CI: 182.5 – 197.7 Ma) with the early split of Lestoidea and the remaining Zygoptera (Fig. 1). A subsequent occurrence of two large zygopteran groups, Calopterygoidea and Coenagrionoidea, was estimated within the Cretaceous (~67 Ma; 95% CI: 61.5 – 71.6 Ma and 115.8 Ma; 95% CI: 112.7 – 121.2 Ma for Calopterygoidea and Coenagrionoidea, respectively) and culminated with the rapid radiation of the majority of extant lineages in the Paleogene in the interval between ~23 Ma and ~66 Ma. Our calibrated divergences generally agree with estimates in Thomas, et al. (2013). However, any further comparisons are precluded by the lack of comprehensive divergence time estimation for Odonata in the literature. The backbone of the crown group Calopterygoidea that branched off from Coenagrionoidea ~129 Ma (95% CI: 121.9 – 134.8 Ma) in the Early Cretaceous was well supported as monophyletic in most of our inferred phylogenies (Fig. 1 and Supplementary Fig. S1a available on Dryad). Previous analyses struggled to provide convincing support for the monophyly of the superfamily (Bybee, et al. 2008, Carle, et al. 2008, Dijkstra, et al. 2014a), whereas only 11 out of 48 phylogenetic reconstructions rejected Calopterygoidea (Supplementary Fig. S1a available on Dryad).

We used quartet sampling (Pease, et al. 2018) to provide additional information about nodal support and investigate biological explanations for alternative evolutionary histories that received some support. We found that for most odonate key radiations, the majority of quartets (i.e. Frequency > 0.5) support the proposed phylogenetic hypothesis with Quartet Concordance (QC) scores > 0 (Supplementary Fig. S2 available on Dryad) across all estimated putative species trees (Supplementary Fig. S1b available on Dryad). The few exceptions consist of the Gomphidae+Petaluridae split and the A+B split, where we have Frequency < 0.5 and QC < 0, which suggests that alternative relationships are possible. Quartet Differential (QD), inspired by \( f^2 \) and \( D \) statistics for introgression, provides an indicator of how much the proportions of discordant quartets are skewed (i.e. whether one of the two discordant relationships is more common than the other), suggestive of introgression and/or substitution rate heterogeneity (Pease, et al. 2018). Interestingly, we identified skewness (i.e. QD < 0.5) for almost every major radiation (Supplementary Fig. S2 available on Dryad), which suggests that alternative relationships can be a result of additional underlying processes (e.g. introgression) rather than ILS alone (Pease, et al. 2018); however, this score may not be highly informative if the majority of quartets agree with the focal topology (i.e Frequency > 0.5 and QC > 0). For both the
Gomphidae+Petaluridae and the A+B splits, we have Frequency > 0.5, QC < 0 and QD < 0.5, implying that alternative phylogenetic relationships are plausibly not only due to ILS but also possible ancestral introgression.

**Major Trends in Evolutionary History of Odonata**

Investigation of diversification rates in Odonata highlighted two major trends correlated with two mass extinction events in the Permian-Triassic (P-Tr) ~251 Ma and Cretaceous-Paleogene (K-Pg) ~66 Ma. First, it appears that P-Tr mass extinction event might have reduced the extent of biodiversity that had been present in Odonata as reflected in the fossil record (Rohde and Muller 2005) for that period (see the temporal distribution of fossil samples in Fig. 1) as was also the case for multiple insect lineages (Labandeira and Sepkoski 1993). According to the fossil record at least two major odonatoid lineages went extinct (Protodonata and Protanisoptera (Grimaldi and Engel 2005)) and likely many genera from other lineages as well (e.g., Kargalotypidae from Triadophlebimorpha (Nel, et al. 2001)). The establishment of major odonate lineages was observed during the Cretaceous starting ~135 Ma (Fig. 1, red line). This coincided with the radiation of angiosperm plants that, in turn, triggered the formation of herbivorous insect lineages (Misof, et al. 2014). Odonates are exclusively carnivorous insects, and their diversification was likely driven by the aforementioned sequence of events. Interestingly, molecular adaptations in the odonate visual systems are coupled with their diversification during the Cretaceous as well (Suvorov, et al. 2017).

**Overview of Introgression Hypotheses Tested**

The scope of introgression within Odonata remains largely unknown, where previous studies looked for its patterns only within certain species relying on inference from a limited number of genetic loci. Thus, we searched for signatures of introgression using genome-scale datasets between lineages at several different taxonomic levels: between different suborders, between superfamilies and within superfamilies. We used six different methods to test for introgression...
within Odonata, as exemplified for the Anisozygoptera suborder in Fig. 2. Specifically, we searched for signatures of ancestral inter-superfamilial introgression within the Zygoptera and Anisoptera suborders. Also, we tested the hypothesis of inter-subordinal gene flow between Anisozygoptera and Zygoptera. Finally, we tested introgression within superfamilies of Zygoptera and Anisoptera that included several species (Lestoidea, Calopterygoidea, Coenagrionioidea, Aeshnoidea (Aeshnidae), Aeshnoidea (Gomphidae+Petaluridae), Cordulegaastroidea and Libelluloidea; Fig. 1). The introgression results for the entire Odonata order either comprise of all tests performed within the entire phylogeny (HyDe/D) or of a union of tests performed within Anisoptera, Zygoptera and between Anisozygoptera-Zygoptera (D_{FOIL}, QuiBL and $\chi^2$ count test/Branch Length Test (BLT)).

**Site pattern-based Methods Strongly Suggest Multiple Instances of Introgression within Odonata**

Initially, we tested the above hypotheses of introgression in quartet topologies (Supplementary Fig. S3 available on Dryad) within Odonata using two site pattern-based methods: the ABBA-BABA test (Patterson, et al. 2012) and HyDe (Meng and Kubatko 2009). The ABBA-BABA test and HyDe rely on computation of $D$ and $\gamma$ statistics, respectively, where their significant deviation from 0 may indicate the presence of introgression between the tested pair of taxa. Additionally, estimated $\gamma$ and $(1-\gamma)$ of HyDe’s hybrid speciation model (Meng and Kubatko 2009) corresponds to the parental fractions in a putatively hybrid genome. Note that HyDe’s hybrid speciation model is appropriate for detecting introgression with sufficient statistical power and can produce reasonable estimates of $\gamma$ (Blischak, et al. 2018, Kong and Kubatko 2020). The analysis of the ABBA-BABA test results revealed possible gene flow events throughout the entire evolutionary history of Odonata (Supplementary Table S5, Fig. S4a and Results available on Dryad). We also highlight the positive relationship between the values of $D$ and $\gamma$ statistics (Spearman’s rank correlation test, $\rho = 0.308$, $P = 0$), demonstrating their broad concordance in identifying signatures of introgression (Supplementary Fig. S4b available on Dryad).

There are a variety of other test statistics that have been developed to detect introgression (e.g., (Durand, et al. 2011, Green, et al. 2010, Kubatko and Chifman 2019, Martin, et al. 2015)).
Because, like $D$ and $\gamma$, many of these statistics are computed from different invariants, we attempted to visualize the relationships between all 15 site patterns computed by HyDe using $t$-distributed stochastic neighbor embedding (tSNE; (Van Der Maaten and Hinton 2008)) for dimensionality reduction, along with the corresponding values of $D$ and $\gamma$ (Supplementary Figs. S4c and S4d available on Dryad, respectively). We found that the clustering of quartets with significant introgression according to a two-dimensional representation of their site patterns may suggest the presence of additional site pattern signatures of introgression (Supplementary Figs. S4c-d available on Dryad). These results suggest that powerful dimensionality reduction techniques could serve as a useful tool for the exploration and visualization of complex signatures of introgression simultaneously estimated from a large set of site patterns.

In order to assess the extent of preservation of ancestrally introgressed genetic material within contemporary taxa, we compared inferred average values of significant $\gamma$ statistic from Odonata with the averages derived from different intra- and inter-superfamilial taxonomic levels (Fig. 3a). We found significantly higher values of $\gamma$ for several intra- and inter-superfamilial comparisons including those that involve Anisozygoptera (Wilcoxon rank-sum test [WRST], all $P < 0.05$, Fig. 3a, Supplementary Table S5 and Results available on Dryad). Additionally, Anisozygoptera exhibits the largest average $\gamma$ (0.27) across all the inter-superfamilial comparisons (Fig. 3a, Supplementary Table S5 available on Dryad). We also found an excess (Fisher exact test [FET], all $P < 0.05$, Supplementary Table S5 available on Dryad) of significant triplets that support introgression (Fig. 3b) based on both the ABBA-BABA and HyDe hybrid speciation model (Supplementary Fig. S3 available on Dryad) tests for Anisozygoptera, Aeshnoidea (Aeshnidae), Lestoidea and Calopterygoidea (between and within). We note that the accuracy of introgression detection for site pattern-based methods may be impaired by saturation, which will be exacerbated on larger timescales. Thus, we additionally performed HyDe analysis using only the 1$^{st}$ and 2$^{nd}$ codon positions and obtained highly similar results for $D$ and $\gamma$ (Supplementary Fig. S5 available on Dryad) suggesting that the potential saturation effect in 3$^{rd}$ codon position did not severely impact our inferences. Despite these results, the $D$ statistic distributions, if considered individually, should be interpreted with caution: the ABBA-BABA test can produce false positives within genomic regions of reduced inter-specific divergence, and can also be significantly affected by demographic parameters, genetic drift and variation in recombination rates (Martin, et al. 2015).
Further, we tested introgression within Odonata using an alternative site pattern-based method, $D_{\text{FOIL}}$ (Supplementary Fig. S6a and Results available on Dryad), which is capable of detecting ancestral as well as inter-group introgression and inferring its polarization (see Materials and Methods). Specifically, we observed a highly skewed distribution of $D_{\text{FOIL}}$ statistics for Anisozygoptera (Supplementary Fig. S6b and Results available on Dryad) that may suggest specific directionality of introgression: all 61 (1157 out of 1158 without FDR correction) quintets with positive evidence for ancestral introgression suggest that Anisozygoptera is the recipient lineage whereas Zygoptera is the donor (Fig. 2, red and blue arrows). Additionally, 1030 out of 1091 (4473 out of 4738 without FDR correction) evaluated quintets with significant inter-group introgression are indicative of one-way introgression from Zygoptera lineages to Anisozygoptera as well, whereas the directionality of remaining 59 (254 without FDR correction) quintets support Anisozygoptera as a donor and Zygoptera as a recipient and only two (11 without FDR correction) show introgression between Zygoptera and Anisoptera.

Signatures of Introgression Revealed by Phylogenetic Gene Tree-based Discordance Methods

Besides specific site patterns, introgression will also generate certain patterns of gene tree-species tree discordance and reduce the genetic divergence between introgressing taxa, which is reflected in gene tree branch lengths. Thus, the footprints of introgression can be detected using phylogenetic discordance methods. First, for a triplet of species, under ILS alone one would expect equal proportions of gene tree topologies supporting the two topologies disagreeing with the species tree, and any imbalance may suggest introgression (Supplementary Fig. S7 available on Dryad). Thus, deviation from equal frequencies of gene tree counts among discordant gene trees can be assessed using a $\chi^2$ test, similar to previously proposed methods leveraging discordant gene tree counts (Degnan and Rosenberg 2009, Huson, et al. 2005). One would expect the average distance between putatively introgressing taxa in discordant trees to be significantly smaller than the distances derived from the concordant as well as alternative discordant triplets (see Materials and Methods and Supplementary Fig. S7 available on Dryad). We used both the $\chi^2$ test of discordant gene tree counts and a test based on the distribution of branch lengths for concordant and discordant gene tree triplets (BLT) to identify introgression within Odonata.
testing different scenarios (Fig. 4a). With this combination of methods, we identified a significant fraction of triplets that support ancestral introgression for scenario 1 that involves inter-subordinal gene flow between Anisozygoptera and Zygoptera as well as scenarios 2 through 4, which correspond to inter-superfamilial instances of introgression within Epiprocta (Fig. 4b). Within superfamilies we found signatures of introgression for Calopterygoidea and Libelluloidea (Fig. 4b). For scenario 1 (introgression between Zygoptera and Anisozygoptera), examination of the genetic divergence distributions (Fig. 4c) for concordant and discordant triplets showed that discordant triplets that may have resulted from gene flow between Anisozygoptera and Zygoptera (the topology labeled “discord2”), have markedly smaller average divergence between these two taxa, as expected in the presence of introgression. Similarly, based on the distribution of mean divergence between putatively introgressing taxa (Supplementary Fig. S8 available on Dryad) as well as fraction of significant triplets (Fig. 4b) gene flow is supported for scenarios 2, 3 and 4.

Additionally, we used a gene tree branch length-based approach, QuIBL (Edelman, et al. 2019), which also detected multiple instances of introgression within the entire order (Supplementary Fig. S9 available on Dryad). We note that particularly for introgression involving Anisozygoptera we observed a larger fraction of triplets suggestive of introgression than for any other scenario tested.

**Phylogenetic Network Analyses Support Anisozygoptera-Zygoptera Introgression**

As an alternative approach to identify the lineage experiencing gene flow with Anisozygoptera, we performed phylogenetic network inference in PhyloNet (Than, et al. 2008, Wen, et al. 2018). For this analysis we specified that Anisozygoptera was involved in a reticulation event—the only such event occurring on the tree—and inferred for the other two nodes that were most likely to be involved in the reticulation as well as the value of $\gamma$, the fraction of Anisozygoptera’s genetic material derived from this reticulation. The full maximum likelihood (Fig. 4d) and maximum pseudolikelihood (Supplementary Fig. S10 available on Dryad) approaches recovered topologically similar networks with comparable values of $\gamma$. Pseudolikelihood analysis suggests a reticulation event between Aeshnidae (a family within Anisoptera) and Zygoptera with 38% of...
genetic material coming from Zygoptera (Supplementary Fig. S10 available on Dryad), whereas
full likelihood infers reticulation between Anisoptera and Zygoptera suborders 33% (Fig. 4d) of
genetic material from Zygoptera. Overall, we note that the full likelihood approach returned
networks with higher log-likelihood scores. This observation is most likely due to the fact that
we performed full likelihood analysis in an exhaustive manner (Supplementary Table S4
available on Dryad) testing every possible reticulation event within a focal clade topology
congruent with the species tree (Fig. 1), whereas pseudolikelihood analysis used the hill-
climbing algorithm to search the full network space but is not guaranteed to retrieve the most
optimal solution. Moreover, differences in objective function within likelihood and
pseudolikelihood frameworks could also lead to distinct network topologies and $\gamma$ estimates.

### Discussion

Using a comprehensive multi-locus transcriptomic dataset, we reconstructed a fossil-calibrated
deep evolutionary history of dragonflies and damselflies (Odonata) (Fig. 1). Although our
phylogenetic analyses resolve many major radiations within the order with high confidence, we
note that a strictly bifurcating phylogeny could be positively misleading (i.e. asserting erroneous
relationships with high support) or provide a poor fit for genomic data undergoing biological
processes such as ILS and introgression, respectively. Thus, the phylogenies presented in this
paper should be interpreted with a degree of caution. Untangling patterns of phylogenetic
discordance within our dataset revealed multiple gene flow events that have been impacted the
course of Odonata evolution for the past 200 myr. We examined the agreement across site- and
gene tree-based methods for detecting introgression (i.e. HyDe/D, and $D_{\text{FOIL}}$ and the $\chi^2$ test/BLT;
Fig. 5 and Supplementary Fig. S11 available on Dryad). Overall, all methods individually were
able to identify abundant introgression within the entire Odonata order with strong agreement.
We found considerable overlap across methods in their support of introgression within Epiprocta
(scenarios 2 and 3), gene flow involving Anisozygoptera (scenario 1), and intra-superfamilial
introgression within Libelluloidea. Within Zygoptera our methods showed strong overlap in
identifying signatures of introgression for Calopterygoidea only. Notably, our $\chi^2$ test /BLT
produced very few predictions that were not in agreement with HyDe, $D$ and/or $D_{\text{FOIL}}$ across
different comparisons. Our deep-time introgression analyses suggest that ancestral introgression
events may leave genetic footprints that are preserved in the genomes and observed in the phenotypes of contemporary species. Below, we discuss the implications of our phylogenetic results in the context of previous studies in Odonata, before turning to the importance and challenges associated with the detection and interpretation of signatures of introgression within phylogenomic studies in light of our findings.

**Major radiations on Odonata phylogeny**

Several competing hypotheses of evolutionary relationships within Odonata have been proposed by multiple authors regarding various taxonomic levels (Supplementary Fig. S12a available on Dryad) (Blanke, et al. 2013b, Bybee, et al. 2008, Carle, et al. 2008, Carle, et al. 2015, Dijkstra, et al. 2014a, Dumont, et al. 2010, Hasegawa and Kasuya 2006, Misof, et al. 2001, Saux, et al. 2003, Suvorov, et al. 2017, Trueman 1996, Ware, et al. 2007). Our phylogenetic analyses recovered Epiprocta (Anisoptera+Anisozygoptera) and Zygoptera as monophyletic with high support (Supplementary Fig. S1a available on Dryad) agreeing with other recent studies (Bybee, et al. 2008, Carle, et al. 2008, Dumont, et al. 2010, Suvorov, et al. 2017). Our estimated superfamilial relationships within Zygoptera support hypothesis of Dijkstra, et al. (2014a) recovering monophyly of Lestoidea, Platystictoidea, Coenagrionoidea and Calopterygoidea (Supplementary Fig. S12b available on Dryad) with high support (Supplementary Fig. S1a available on Dryad). Inferred higher-level phylogenetic classification of anisopteran families was highly congruent with Carle, et al. (2015) and well-supported (Supplementary Fig. S1a available on Dryad) with the exception of Gomphidae and Petaluridae radiations. The phylogenetic position of Gomphidae and Petaluridae, both with respect to each other and the remaining anisopteran families, has long been difficult to resolve (Supplementary Fig. S12c available on Dryad). The most recent analyses of major anisopteran lineages, using several molecular markers (Carle, et al. 2015), suggest Gomphidae and Petaluridae as a monophyletic group, but without strong branch support. The multi-species coalescent model (MSC) provides a probabilistic framework for estimation of species trees that accounts for genealogical discord occurring as a result of ILS. Close scrutinization of Gomphidae and Petaluridae relationships inferred under concatenation and and ASTRAL (a supertree method that is statistically consistent under MSC) showed that the majority of the supermatrix analyses strongly support a sister relationship between the two
families (Supplementary Fig. S1a available on Dryad); however, almost all the ASTRAL species
tree analyses reject such a relationship with high confidence (Supplementary Fig. S1a available
on Dryad). In the presence of incomplete lineage sorting concatenation methods can be
statistically inconsistent (Roch and Steel 2014) leading to an erroneous species tree topology
with unreasonably high support (Kubatko and Degnan 2007). Thus, inconsistency in the recovery
of a sister group relationship between Gomphidae and Petaluridae between concatenation and
ASTRAL could be a result of elevated levels of incomplete lineage sorting between the families.
Furthermore, gene tree-species tree conflict can occur in the presence of non-sister species gene
flow (Leaché, et al. 2014), which violates assumptions of the MSC by skewing frequency
distributions of incongruent gene tree topologies thereby making the MSC framework
inconsistent for species tree estimation (Eckert and Carstens 2008, Solís-Lemus, et al. 2016),
although we note that studies of this phenomenon have examined gene flow at shallower
phylogenetic timescales than those investigated here. We found that the Gomphidae and
Petaluridae lineages exhibited inter-superfamilial introgression events (scenario 3, Fig. 5)
involving the Cordulegastroidea + Libelluloidea clade. Additionally, the familial relationships
within Calopterygoidea significantly varied between estimated phylogenies using either
ASTRAL or concatenation methods (Supplementary Fig. S1a available on Dryad). For example,
within clade C, monophyly of the Calopterygidae and Chlorocyphidae families was suggested
by ASTRAL trees but rejected by the majority of ML phylogenies estimated from the
supermatrices (Fig. 1 and Supplementary Fig. S1a available on Dryad). Interestingly, for this
superfamily further analyses similarly yielded abundant instances of inter-familial introgression
(Fig. 5). Together, these results may support the proposition that purely bifurcating species
topologies inferred under concatenation/MSC methods in the presence of post-speciation
introgression on recent as well deep temporal scales may inadequately model taxonomic
relationships (Fontaine, et al. 2015, Mcvay, et al. 2017, Zhang, et al. 2021). To alleviate this
problem, a unifying multi species coalescent network model, implemented in the program
PhyloNet (Yu, et al. 2011), has been developed to describe evolutionary history via species
networks where reticulation nodes correspond to instances of introgression. Unfortunately, we
were not able to infer a full species network topology using PhyloNet because the taxon and gene
sampling presented in our study would render this analysis unfeasible even when using the more
tractable pseudolikelihood implementation (Yu and Nakhleh 2015). However, we were able to use PhyloNet to test patterns of reticulate evolution involving Anisozygoptera (see below).

We also note that discordance caused by ILS, if not accounted for, may lead to inaccurate inference of substitution rates (Mendes and Hahn 2016) and thus overestimation of divergence times using relaxed clock models (Ogilvie, et al. 2016). Moreover, a failure to incorporate introgression effects into a probabilistic framework of a time calibration method will result in underestimated divergence times (Leaché, et al. 2014). Several recent fully Bayesian approaches have been proposed to perform divergence time estimation using the MSC model alone (Heled and Drummond 2010, Ogilvie, et al. 2017) or together with introgression (Jones 2019), however they would not be scalable to our dataset.

**Widespread introgression within Odonata**

Introgression among Odonata has been previously thought to be uncommon (Lowe, et al. 2008, Tennessen 1982) as a result of probable reproductive isolation mechanisms such as ethological barriers, phenotypic divergence (e.g., variable morphology of genitalia (Barnard, et al. 2017, Hosken and Stockley 2004)) and habitat and temporal isolation. Most of the introgression and hybridization research in Odonata has been done within the zygopteran Coenagrionidae family and especially between populations of genus *Ishnura* focusing on mechanisms of reproductive isolation (e.g. (Sanchez-Guillen, et al. 2014, Sanchez-Guillen, et al. 2011)). These studies established “hybridization thresholds” for these closely related species and showed positive correlation between isolation and genetic divergence. Furthermore, rapid karyotype evolution can also contribute to post-mating isolation (Lai, et al. 2005); however, recent cytogenetic studies across Odonata phylogeny indicate very stable chromosome number with most prevalent karyotype of \(2n = 25\) (Kuznetsova and Golub 2020). Additionally, in recent years there has been a growing body of evidence for introgression throughout evolutionary histories of various groups. This includes more recent hybridization events observed in *Heliconius* butterflies (Edelman, et al. 2019), cats (Li, et al. 2016), cichlid fishes (Svardal, et al. 2020) as well as deeper ancestral introgression in primates (Vanderpool, et al. 2020), *Drosophila* (Suvorov, et al. 2021) and vascular plants (Pease, et al. 2018).
The extent of gene flow and maintenance of introgressed variation together determine the fraction of introgressed material, or $\gamma$, in a focal genome (Martin and Jiggins 2017). However, with increased divergence time the fixation or loss of the ancestrally introduced genetic material will primarily depend on the strength and direction of selection (Norris, et al. 2015, Oziolor, et al. 2019, Petr, et al. 2019). Here, our taxon sampling allowed to test ultra-deep ancestral introgression scenarios where the MRCA of tested taxa can be traced as far back as to the Triassic period (~251 Ma to ~201 Ma). We argue that for the cases where we infer that a large amount of introgressed genetic material was preserved (e.g. >25% in Anisozygoptera) the ancestral effective migration rates (Martin and Jiggins 2017) were high (similar conclusions can be made for Aeshnoidea (Aeshnidae), Calopterygoidea and within Libelluloidea and Lestoidea; Fig. 3a); whereas for the remaining taxa with lower or non-significant deviations of $\gamma$ (Fig. 3a) the rates of introgression may have been substantially lower or its signatures were purged from the genome by selection.

We found compelling evidence for patterns of ancestral introgression (Fig. 5) among distantly related odonate taxa. Our conservative analysis of agreement between different introgression tests shows the presence of gene flow within the entire order (Fig. 5 and Supplementary Fig. S11 available on Dryad). This observation may be explained by reduced sexual selection pressures in the early stages of odonate evolution that may have inhibited rapid genital divergence (Eberhard 2004), which is probably a primary source of reproductive isolation in Odonata (Cordero Rivera, et al. 2004).

**Phenotypic consequences of deep time introgression**

Perhaps the most compelling evidence that we uncovered in this study was for deep ancestral introgression between the Zygoptera and Anisozygoptera suborders, which based on the fossil record most likely became genetically isolated after the Lower Jurassic (Carle 2012). Species of Anisozygoptera exhibit anatomical characteristics of both Anisoptera and Zygoptera suborders. Some general features of Anisozygoptera that relate them to Zygoptera include dorsal folding of wings during perching in adults, characteristic anatomy of proventriculus (a part of digestive system that is responsible for grinding of food particles) and absence of specific muscle groups in the larval rectal gills; whereas abdominal tergite shape, rear wing geometry and larval
structures are similar to Anisoptera (Asahina, et al. 1954). More recent studies also revealed that Anisozygoptera ovipositor morphology shares similarity with Zygoptera (Matushkina 2008); muscle composition of the head resemble characteristics of both Anisoptera and Zygoptera (Blanke, et al. 2013a); thoracic musculature of Anisozygoptera larva exhibit similarity between Anisoptera and Zygoptera (Busse, et al. 2015). Thus, Anisozygoptera represent a morphological and behavioral “intermediate” (Liu, et al. 2011), which is supported by our findings where we recovered strong evidence of introgression between Zygoptera and Anisozygoptera (Figs. 2-4). Moreover, according to the $D_{FOIL}$ method the Anisozygoptera was inferred to be a recipient taxon from a zygopteran donor, though we cannot rule out a lesser degree of gene flow in the opposite direction as well. In fact, such a phenotypic “intermediate” can occur as a result of homoploid hybrid speciation (Elgvin, et al. 2017), which generates recombinant (mosaic) genotypes from two parental genomes while preserving their ploidy. For example, empirical studies have suggested that sex chromosome mosaicism in hybrid tiger swallowtail butterflies was linked to phenotypic mosaicism (e.g. female dimorphism and duration of a life cycle) (Kunte, et al. 2011), and that hybrid speciation is the cause of an intermediate plumage phenotype in sparrows (Hermansen, et al. 2011). A significant fraction of genetic material from both parental genomes is one of the main conditions required to establish hybrid speciation (Schumer, et al. 2014). Strikingly, we found that the average HyDe probability parameter $\gamma \sim 0.27$ (Fig. 3a) inferred from multiple quartets is very similar to PhyloNet’s inheritance probability $\gamma \sim 0.33$. This may suggest that a sizeable fraction of the Anisozygoptera genome descends from zygopteran lineages. However, we note that genetic mosaicism and intermediate traits can be also acquired via post-speciation gene flow (Bonfante, et al. 2021, Vonholdt, et al. 2011). Thus, to fully test the Anisozygoptera hybrid speciation hypothesis the other two conditions need to be examined: (i) the presence of reproductive barriers between hybrid and parental lineages and (ii) the establishment of reproductive isolation was caused by hybridization events (Schumer, et al. 2014). It is impossible to empirically test these conditions for gene flow events as ancient as the one we identified for Anisozygoptera.

Taken together, our observations strongly suggest a xenoplasious origin (Wang, et al. 2020) of Anisozygopteran traits (i.e. traits introduced to a recipient taxon via introgression) that are shared with Zygoptera. However, we do not reject the possibility that some trait hemiplasy may have resulted from ILS (Guerrero and Hahn 2018). Based on the gathered evidence for
introgression, we suggest that ancestral lineages that gave rise to modern day Anisozygoptera and Zygoptera experienced introgression in their past evolutionary history.

According to the hybrid swarm hypothesis (Seehausen 2004), introgression can trigger a rapid cladogenesis resulting in the establishment of ecologically different species with niche-specific adaptive phenotypes. Some of the notable examples can include adaptive radiations caused by ancestral introgression in cichlid fishes (Meier, et al. 2017), and intricate inter-specific introgression patterns in big cats that could be linked to rapid diversification of modern-day species in that lineage (Figueiro, et al. 2017). Interestingly, the large fraction of introgressed material estimated within the Anisozygopteran lineage does not appear to have facilitated any adaptive radiation. This proposition is supported by an extremely low species diversification within the suborder (just three extant species) and also by the lack of fossil record for Anisozygoptera.

Conclusions

A rapidly growing body of empirical evidence strongly indicates that introgression is a widespread phenomenon observed not only in plants but also in the animal kingdom. Over the past decade this biological process has received tremendous attention from the phylogenetics community, as it fundamentally changes how we view and reconstruct evolutionary histories of different organisms and may even redefine our understanding of species concepts (Wang, et al. 2020). Introgression is an important source of novel variation that can lead to speciation, trigger rapid species diversifications, facilitate adaptation to novel environments (Nolte and Tautz 2010) as well as affect the course of species boundary establishment (Harrison and Larson 2014). From a practical perspective, introgression is one of the key factors that needs to be considered during the development of biodiversity protection and conservation programs (Quilodrán, et al. 2020). Furthermore, a failure to accurately detect introgression scenarios may also lead to ineffective domestication and breeding programs (Dempewolf, et al. 2017, Glémin, et al. 2019, Janzen, et al. 2019).

Here, we investigated a unique and ancient insect lineage, Odonata, and provided the first insight into global patterns of introgression that were pervasive throughout their evolutionary history. Moreover, we note that signatures of introgression were most likely underestimated as
majority of phylogenomic methods (including those used in the current study) are unable to
detect gene flow between sister lineages (Hibbins and Hahn 2021). Our findings further
exemplify the evidence that post-speciation gene flow can be a fairly common process occurring
in various taxa, including the most diverse group of animals, i.e. insects. The abundance of this
biological phenomenon across the Tree of Life creates patterns of reticulate evolution which
make the tree model itself ill-suited for explaining historic relationships between species
(Doolittle and Bapteste 2007). It is possible that ancestral sequence reconstruction methods may
also be affected by introgression as they typically assume character evolution along a single
species tree (Mendes and Hahn 2016). Additionally, introgression poses new challenges for
phylogenetic time calibration by biasing age estimates (Leaché, et al. 2014). The fraction of the
introgressed variation from a donor to a recipient taxon can vary significantly (Runemark, et al.
2019), including cases where the most of the ancestry originates from donor genomes (Fontaine,
et al. 2015). In our case we show that in Odonata introgressed genetic material can account for a
large fraction of the genome (around ~ 30% in Anisozygoptera) and persist for millions of years
following the gene flow event(s). This pattern is indicative of a high rate of gene flow and
potentially of positive selection in favor of some hybrid genotypes.

We provide a roadmap of analyses to help identify introgression at both shallow and deep
phylogenetic scales that is practical, timely and much needed. Our research highlights the
importance of development of tractable models and methods that scale to modern phylogenomic
datasets derived from high throughput molecular data. Moreover, we argue there is an urgent
need to develop a theoretical framework that unifies various sources of phylogenetic discordance
for more accurate and comprehensive inferences in phylogenetics (Charles-Elie, et al. 2020).

Data Availability

All raw RNA-seq read libraries generated for this study have been uploaded to the Short
Read Archive (SRA) (SRR12086708-SRR12086765). All assembled transcriptomes, alignments,
inferrered gene and species trees, fossil calibrated phylogenies (including paleontological record
assessed from PaleoDB used to plot the fossil distribution in Fig. 1), PhyloNet results, QS results and introgression results are available on figshare (https://doi.org/10.6084/m9.figshare.12518660). Custom scripts that were used to create various input files as well as the analysis pipeline are available on GitHub https://github.com/antonysuv/odo_introgression.

Supplementary Material
Data available from the Dryad Digital Repository: XXX

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Author Contributions
AS, CS, DRS and SMB conceived the research. AS, CS, MSF and PB analyzed the data. MC, KAC, MFW and DRS supervised the project. AS, CS, DRS and SMB wrote the manuscript.

Declaration of Interests
The authors declare no competing interests.
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Figure captions

Figure 1. Evolutionary History of Odonata
Fossil calibrated ML phylogenetic tree of Odonata using a DNA supermatrix consisting of 1603 BUSCO genes with a total of 2,167,861 aligned sites. The blue densities at each node represent posterior distributions of ages estimated in MCMCTree using 20 fossil calibration points. The red dashed vertical line indicates the beginning of establishment for major odonate lineages originating in and spanning the Cretaceous. The histogram (blue bars) represents temporal distribution of fossil Odonatoptera samples. The black dashed vertical lines mark major extinction events, namely Permian-Triassic (P-Tr, ~251 Ma), Triassic-Jurassic (Tr-J, ~201.3 Ma) and Cretaceous-Paleogene (K-Pg, ~66 Ma).

Figure 2. Detection of Introgression/Hybridization Trajectories by Different Methods in Anisozygoptera.
Three site pattern-based (D statistic, HyDe and D_{FOIL}), gene tree count/branch length-based (χ² test/BLT and QuIBL) and network ML inference (PhyloNet) methods were used to test for introgression/hybridization. Arrows denote introgression. The figure panel represents larval and adult stages for three Odonata suborders. Species from top to bottom: Lestes australis, Epiophlebia superstes and Anax junius. Image credit: Epiophlebia superstes adult by Christian Dutto Engelmann; Lestes australis and Anax junius adults by John Abbott.

Figure 3. Distributions of HyDe γ and Quartets Fractions Supporting Introgression Across Odonate Taxonomic Levels.
(a) Distribution of significant (Bonferroni corrected P < 10⁻⁸) γ values for each quartet estimated by HyDe. In general, γ values that are not significantly different from 0 denote no relation of a putative hybrid species to either of the parental species P₁ (1-γ) or P₂ (γ) in a quartet. Asterisks indicate significantly greater (red) or lower (blue) γ averages of various tested cases compared to γ average of the entire order.
(b) Proportions of quartets that support introgression based on simultaneous significance of D statistics and γ. Asterisks indicate significantly greater (red) and smaller (blue) fraction of quartets that support introgression compared to the entire order.

Figure 4. Results of the χ² Count-Branch Length Test (BLT) for Odonate Taxonomic Levels and PhyloNet result for Anisozygoptera.
a) Scenarios of deep (numbered red arrows) and intra-superfamilial (white triangles) introgression in Odonata tested using χ² test and BLT. Red arrows mark the location of ancestral introgression events that were tested between lineages (e.g. for scenario 8, we tested whether contemporary species of Platystictoidea shared introgressed genetic material with either Coenagrionoidea or Calopterygoidea).
b) Classification of triplets based on the χ² test and BLT results. Introgression+ILS cases are those significant according to both the χ² test and BLT (FDR corrected P < 0.05); all the remaining cases were those where any discordance was inferred to be due to ILS alone.
c) Normalized genetic divergence between sister taxa (shown in triplets above the panel) averaged across all BUSCO gene trees supporting each topology involving three lineages representing Anisoptera, Anisozygoptera, and Zygoptera.
d) Phylogenetic network estimated from a set of ML gene trees using maximum likelihood. Epiophlebia superstes, the sole representative of Anisozygoptera in our study, was specified to be involved in a reticulation with two other (unspecified) lineages. Blue lines indicate the reticulation event and are labeled with PhyloNet’s estimate of γ. The number above the network indicates the log-likelihood score.

Figure 5. Overlap between Putatively Introgressed Species Pairs Inferred by HyDe/D, D_{FOIL} and BLT/χ² test.
The tree shows tested scenarios of deep (numbered red arrows) introgression for Anisoptera, Anisozygoptera and Zygoptera. The numbers within sets represent the number of unique introgressing species pairs identified by a corresponding method. Significance of an overlap between all methods (intersection of all sets) for each scenario was determined by the exact multi-set interactions test. Significant P values are indicated in red. Note that due to the limitations of D_{FOIL}, introgression could not be tested for scenarios 7 and 8 using this method.
