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A molecular phylogeny of the Petaluridae (Odonata: Anisoptera): A 160-Million-Year-Old story of drift and extinction

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ABSTRACT

Petaluridae (Odonata: Anisoptera) is a relict dragonfly family, having diverged from its sister family in the Jurassic, of eleven species that are notable among odonates (dragonflies and damselflies) for their exclusive use of fen and bog habitats, their burrowing behavior as nymphs, large body size as adults, and extended lifespans. To date, several nodes within this family remain unresolved, limiting the study of the evolution of this peculiar family. Using an anchored hybrid enrichment dataset of over 900 loci we reconstructed the species tree of Petaluridae. To estimate the temporal origin of the genera within this family, we used a set of well-vetted fossils and a relaxed molecular clock model in a divergence time estimation analysis. We estimate that Petaluridae originated in the early Cretaceous and confirm the existence of monophyletic Gondwanan and Laurasian clades within the family. Our relaxed molecular clock analysis estimated that these clades diverged from their MRCA approximately 160 mya. Extant lineages within this family were identified to have persisted from 6 (*Uropetala*) to 120 million years (*Phenes*). Our biogeographical analyses focusing on a set of key regions suggest that divergence within Petaluridae is largely correlated with continental drift, the exposure of land bridges, and the development of mountain ranges. Our results support the hypothesis that species within Petaluridae have persisted for tens of millions of years, with little fossil evidence to suggest widespread extinction in the family, despite optimal conditions for the fossilization of nymphs. Petaluridae appear to be a rare example of habitat specialists that have persisted for tens of millions of years.

1. Introduction

Extinction is a part of the evolutionary process (Jablonski, 2008) and tends to occur at a higher rate in habitat specialists that are susceptible to isolation and local extirpation (Tilman et al., 1994). The dragonfly family Petaluridae (Odonata, Anisoptera), is a notable exception to this trend (Ware et al., 2014a). Members of this family are habitat specialists that exclusively inhabit fen and bog habitats in North America, Japan, New Zealand, South America and Australia (Baird, 2012; Turner, 1970). Fens are defined as areas where the groundwater is discharged into the biologically active soil zone at a sufficient rate for continual peat accretion to occur (Aldous et al., 2015). Despite this habitat dependence, a previous phylogenetic hypothesis based on a small number of genetic loci suggested the persistence of some Petaluridae lineages for tens of

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millions of years (Ware et al., 2014a). Divergence times were putatively in line with continental drift patterns and inland sea development, making Petaluridae a compelling study system in biogeography (Ware et al., 2014a). In addition to the wide geographic distribution, evolution, and ecology of Petaluridae, they have long intrigued humans due to their size as the largest dragonflies and some of the largest insects, and for their tendency to land on humans (Fig. 1A). Petaltails also hold significance in some cultures. For example, in Māori tradition kapokapowai (*U. carovei*) are said to have aided the chief Rakeiao by flying into the faces, noses and ears of the chief's enemies in battle (Rongoma, n.d.).

Petaluridae are among the earliest diverging families of Anisoptera (dragonflies), and are sister to family Gomphidae (Bybee et al., 2021; Kohli et al., 2021; Suvorov et al., 2021). Petaluridae is one of the most species poor families within dragonflies, comprising only five genera. *Petalura* (five species in Australia), *Uropetala* (two species in New Zealand) and *Phenes* (one species in Chile and Argentina) form a Gondwanan clade. *Tanypteryx* (one species each from Western North America and Japan) and *Tachopteryx* (one species found in Eastern North America) (Baird, 2012; Turner, 1970) together form a Laurasian clade. The two clades within this family are estimated to have split in the late Jurassic or early Cretaceous (Kohli et al. 2021; Suvorov et al., 2021; Ware et al., 2014a). Although Petaluridae have a widespread pan-Pacific distribution, no two genera overlap in their distributions (Fig. 1B). Additionally, the abundance of suitable habitat for Petaluridae is often extremely small within their respective species ranges (Baird, 2012, 2019, 2013). As a result, population densities and absolute population sizes of Petaluridae are low compared to other insects (Tolman et al., 2023b).

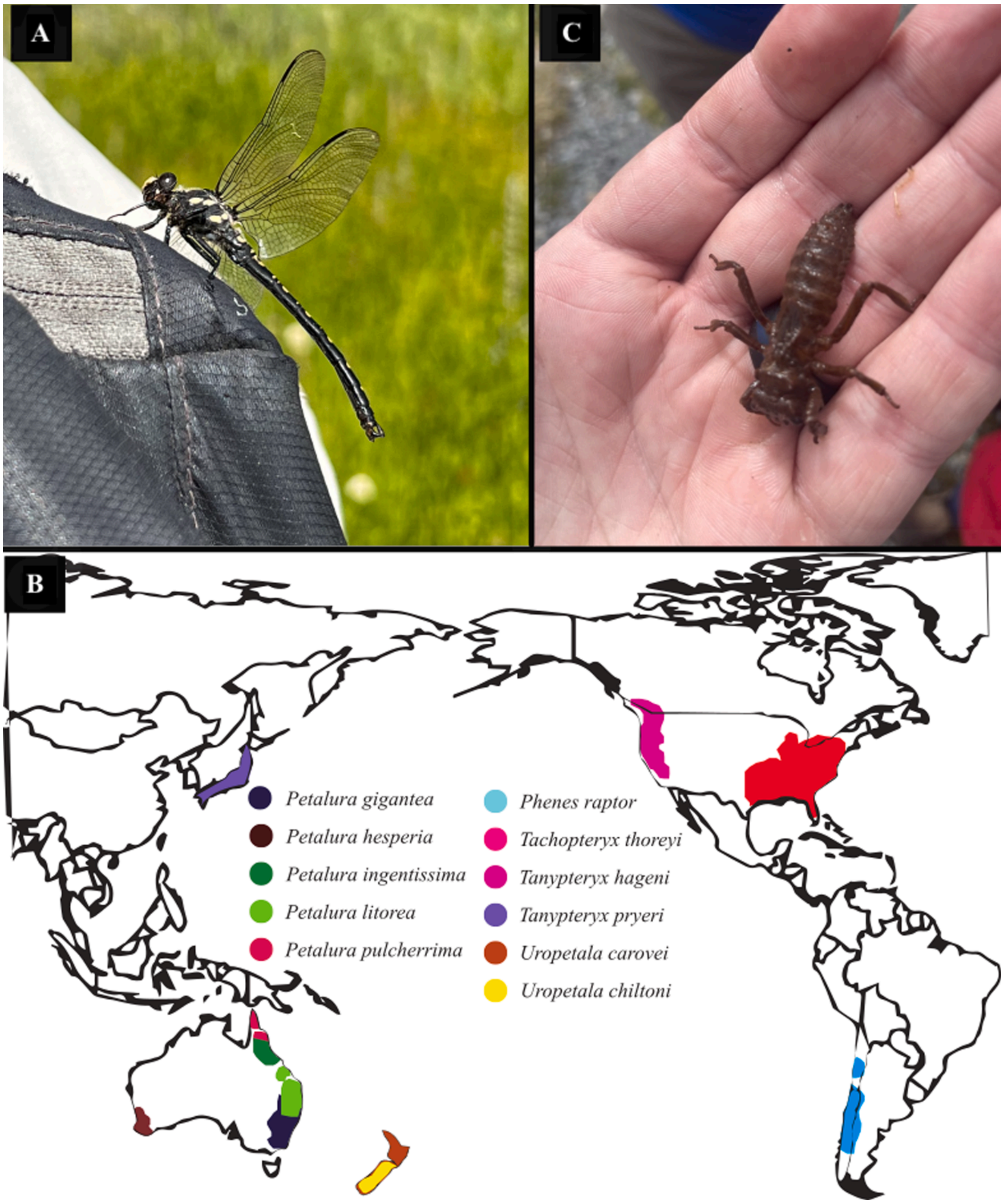

Fig. 1. Distribution of Petaluridae. A). Adult *Tanypteryx hageni* perched on the backpack of a hiker. B). Maps GBIF ("GBIF Occurence Download," 2023) occurrences of the extant Petaluridae. *Tachopteryx thoreyi* is shown in Eastern North America, *Tanypterx hageni* in Western North America, *Tanypteryx pryeri* in Japan, *Phenes raptor* in Patagonia, *Uropetala carovei* on both islands of New Zealand, *U. chiltoni* on the South island of New Zealand, *Petalura hesperia* in Southwest Australia, *P. gigantea* in Southeastern Australia, and then *P. litorea*, *P. ingentissima*, and *P. pulcherrima* moving northward across the eastern coast of Australia. C). Nymph of *U. chiltoni*.

As nymphs, nine of the eleven species burrow in highly saturated soil (Fig. 1C), while the remaining two can be found in the moist substrate around fens and bogs (Baird, 2012, 2019, 2013). Nymphs of several of the species are known to leave their burrows, and are semi-terrestrial (Baird, 2012). Petaluridae are estimated to spend between five and 20 years as nymphs, a remarkably long lifespan for an insect (Baird, 2012). The sequenced genomes of Petaluridae are reflective of their unique life history, as expanded gene families related to vision and taste are plausibly indicative of their semi-terrestrial lifestyle. Further, a recent study of two Petaluridae genomes identified gene family expansions related to DNA damage response as likely related to the longevity of Petaluridae (Tolman et al., 2023b).

Estimating divergence dates for Petaluridae has proven challenging because there is considerable debate about the crown and stem group status of clade Petalurida fossils (defined by Nel et al. (1998) to include stem and crown Petaluridae) (Kohli et al., 2021), making it difficult to generate accurate fossil calibrations. Petaluridae are the only remaining extant family of the previously diverse super-family Petaluroidea based on fossil evidence (Coram and Nel, 2009; Nel and Bechly, 2009). While some extinct Petalurida have been found within or near the distributions of extant Petaluridae, three species have been discovered in Asia, and another seven in Europe, far beyond the geographic distribution of extant Petaluridae (Fig. 1). The definition of and relationships within Petalurida are contentious, and it is debated whether some extinct Petalurida should even be considered within crown Anisoptera (Martill et al., 2007; Nel et al., 1998). Only one fossil Petaluarida, *Argentinopetala archangelski* (Petrulevicius, 2003), is widely considered to belong to the crown Petaluridae. Despite these discrepancies, fossil Petalurida have not been analyzed in a systematic context, and hence much uncertainty surrounding the timing and biogeography of this family remains.

We used anchored-hybrid enrichment (AHE) data for ten of the eleven extant species, alongside gene tree likelihoods, likelihood mapping, quartet concordance, and multinomial logistic regressions to better understand the processes driving discordant topologies with in Petaluridae. We also consider the relationships of extinct Petalurida through the systematic analysis of wing characters, enabling calibration of relaxed molecular clock models to estimate divergence times. Together, our analyses establish Petaluridae as a model system for studying deep divergence and biogeography, as a family with deep speciation events, and little subsequent branching that is highly influenced by drift and changes in geography.

2. Materials and Methods

2.1. DNA extraction and sequencing

A total of 19 specimens, representing 10 Petaluridae and one Gomphidae, were used for extraction (supplementary table 1). A single leg from each specimen was removed with sterilized forceps for DNA extraction using the Zymo DNA Micro-prep kit (Hilden, Germany). The extraction procedure was modified from the manufacturer's protocol in the following way: we included a weeklong incubation with extra proteinase K added. RAPID Genomics (Gainesville, Florida) performed library preparation and sequencing using a previously described 1300 loci Anchor Hybrid Enrichment probe set (Bybee et al., 2021; Goodman et al., 2023) for 19 specimens, and a 90 locus subset (Newton et al., in preparation) for two additional specimens (*T. thoreyi*, and an additional *P. ingentissima*), as funding constraints did not allow for the full probeset sequencing for specimens.

2.2. Sequence assembly and analysis

Read trimming and assembly followed established protocols (Breinholt et al., 2018; Goodman et al., 2023). Adapters were trimmed from raw reads with fastp (Tang and Wong, 2001), and trimmed reads quality assessed with multiQC (Ewels et al., 2016). Loci were then assembled using an iterative baited assembly with SPAdes (Breinholt et al., 2018; Prjibelski et al., 2020). Orthology screening was conducted by excluding loci with multiple BLAST (v2.12.0 +) hits in the *T. hageni* reference genome (Tolman et al., 2023a), and those without reciprocal best hits between query and reference sequences using parameters previously specified in Bybee et al. (2021) and Goodman et al. (2023). As missing data and mis-assembly can heavily bias phylogenies estimated with this pipeline (Goodman et al., 2023), only specimens with more than 500,000 or 100,000 reads (for the two probe sets, 1300 loci and 90 loci subset, respectively) were retained.

In addition to the newly sequenced species here, we utilized existing genomic data for Petaluridae including the draft genomes of *Uropetala carovei* (Tolman et al., 2023b) and *Tanypteryx hageni* (Tolman et al., 2023a), and sequenced transcriptomes of *Tanypteryx pryeri* and *Phenes raptor* (Suvorov et al., 2021). Because Gomphidae is likely sister to Petaluridae (Bybee et al., 2022; Kohli et al., 2021) we also included publicly available transcriptomes (Suvorov et al., 2021) of the gomphids *Ictinogomphus pertinax*, *Stylurus spiniceps,* and *Phanogomphus spicatus* as outgroup taxa in addition to *Mattigomphus pinratani* which was sequenced using the 1300 loci dataset (supplementary table 1). We identified the probe regions in the assembly of *U. carovei* and transcriptome assemblies with BLASTn (Camacho et al., 2009), with the parameters "-task megablast –outfmt '6 qseqid sseqid evalue pident length sseq' –num_threads 16 –evalue 1e-25." We then selected the best-hit by selecting the hit with the longest length, and then highest percent identity. The probeset for *T. hageni* had previously been extracted from the genome assembly (Goodman et al., 2023).

2.3. Species tree estimation

We generated a multiple sequence alignment for each homologous locus by aligning the nucleotide sequence of probe regions with the MAFFT-linsi algorithm from MAFFT v.7.465 for increased accuracy (Katoh and Standley, 2013). We individually inspected each locus alignment in AliView (Larsson, 2014), where we checked the reverse-complement of spurious alignments, and removed sequences that failed to align, if necessary. We concatenated the resulting alignments with FASconCAT v1.11(Kück and Meusemann, 2010), resulting in a supermatrix of 252,867 sites. We then selected an optimal partitioning scheme using the relaxed clustering algorithm with the substitution model fixed to GTR+G in each partition in IQtree v.2.1.3 (Minh et al., 2020). We then selected a model for each partition using ModelFinder (–m MFP option in IQtree) (Kalyaanamoorthy et al., 2017). Finally, we estimated a maximum likelihood species tree with 1,000 ultrafast bootstrap replicates in IQtree v.2.1.3 with the concatenated alignment (Minh et al., 2020), and estimated an additional 50 maximum likelihood trees, 25 with a random starting tree and 25 with a neighbor-joining starting tree, to check for model convergence. Additionally, to test the stability of the tree, as ML methods can be inconsistent under certain evolutionary scenarios (Roch and Steel, 2015), we estimated a putative species tree using weighted-ASTRAL (Liu & Warnow, 2023), a method that is statistically consistent under multispecies coalescent framework (Liu & Warnow, 2023. To that end, we inferred ML locus trees in IQtree (Minh et al., 2020) using the option "-m test" for model selection, and 1000 ultrafast bootstrap replicates and then using these locus-based trees we estimated a species tree in weighted-ASTRAL using the command astral-hybrid, with default parameters.

2.4. Incorporating Petalura pulcherrima

Because no genomic-level data is available for *Petalura pulcherrima* we utilized an alignment of 5 mitochondrial genes and 3 nuclear genes for all of the *Petalura* except *P. litorea* (Ware et al., 2014a) as input for UsHer (v0.6.2)(Turakhia et al., 2021), along with the estimated ML tree, to estimate the placement of this taxon. We also considered the divergence between the *Petalura* in the 8 gene alignment, and the average divergence between each individual pair from the AHE probeset for comparison. We did not include *P. pulcherrima* in further analyses.

2.5. Resolving contentious relationships

The concatenated species and coalescent trees both recovered low support for the relationships between the genera *Phenes, Uropetala, and Petalura* in the Gondwanan clade, and the placement of *Petalura hesperia* within *Petalura.* Because a handful of genes can be drivers of so-called "contentious" phylogenetic relationships (Shen et al., 2017), we calculated gene likelihood scores for alternative topologies for each of the loci, with the models identified by IQtree (Minh et al., 2020). We tested all possible relationships of *Petalura, Uropetala* and *Phenes*, and the placement of *P. hesperia* as sister to all other *Petalura*, *P. ingentissima,* or *P. gigantea* and *P. litorea.* For both of these nodes in the Gondwanan clade we estimated the branch lengths of the phylogeny in IQtree (Minh et al., 2020), using the previously identified partitions and models. We then calculated the likelihood of the alternative topologies for each gene using IQtree (Minh et al., 2020). To further test the reliability of the species tree, we estimated quartet concordance by calculating quartet likelihoods of the possible sister taxa in the Gondwanan clade with IQtree (Minh et al., 2020), and quartet concordance, quartet differential, quartet informativeness, and quartet fidelity with Quartet Sampling (v1.3.1.b) (Pease et al., 2018). We also used the likelihood mapping function in IQtree (Minh et al., 2020) to test the possible topologies of the Gondwanan clade.

Using the afex package in R (Singmann et al., 2023), we employed multinomial logistic regression to determine if the mean substitution rate and chromosomal placement of a given locus, as well as potential interactions among these factors were related to whether a gene supported a certain topology in the Gondwanan polytomy. We estimated the relative mean nucleotide substitution rate for each gene with IQtree (Minh et al., 2020) as a proxy for the locus-specific evolutionary rate. We identified chromosomal placement for each locus by scaffolding the contig-level genome assembly of *U. carovei* against the chromosome length assembly of *T. hageni* with progressive cactus (Armstrong et al., 2020) and Ragout (Kolmogorov et al., 2014; Muffato et al., 2023). As there is very little movement of genes between chromosomes across Odonata (Tolman et al. 2023b), it is likely that chromosome positions for our AHE probeset are consistent across the family, however this cannot be confirmed until chromosome length genome assemblies are available for all species. Model assumptions were checked by identifying a linear relationship between predictor and modeled outcome (logit), and p-values were calculated using likelihood ratio tests without a correction. A similar process was used to investigate the relationship between mean substitution rate and support for *P. hesperia* as sister to all other *Petalura*, *P. ingentissima,* or (*P. gigantea* + *P. litorea*). Genes on microchromosomes and those that did not clearly support clade placement were also not used in the analysis.

2.6. Morphology of the secondary genitalia

To evaluate morphological synapomorphies which might support the recovered intergeneric relationships, we dissected the male-secondary genitalia. The penes were mounted on aluminum stubs and coated with gold palladium using a Cressington 108E sputter coater. Specimens were then imaged using an S-7400 Hitachi scanning electron microscope. Penes structures have been demonstrated to vary at the generic level allowing differentiation among genera in Cavilabiata (May 1997), although studies of the family Libellulidae have found limited utility for among-species comparison (Kennedy, 1922; Miller, 1991; Ware, 2008). This is the first such attempt to compare these structures in Petaluridae.

2.7. Time calibrated phylogeny and biogeographical analysis

To calibrate our model, we needed to phylogenetically assess the known putative Petalurida fossils. To do so we generated a character matrix of previously described wing characters (based on Nel et al. (1998) and Ware et al. (2007)) for all extant Petaluridae, and fossils that have putatively been identified as Petalurida (supplementary table 2). In this work we aimed to provide phylogenetic justification for crown and stem Petalurida fossils for this and future divergence time estimation analyses, removing doubt sparked by past debates about the phylogenetic status of these taxa (Fleck, 2011; Kohli et al., 2021, 2016; Martill et al., 2007; Nel et al., 1998; Ware et al., 2014a). We also included four extant Gomphidae, and the fossil Gomphidae *Cratolindenia knuepfae* (Bechly, 2000), and *Gunterbechlya pumilio* (Huang et al., 2019), as well as *Proterogomphus renatae* (Bechly et al., 1998). Our analysis was hampered by the fragmentary nature of many known Petalurida fossils. *Argentinopetala archangelski* was recovered as sister to *Phenes*, but all other fossils were recovered outside of crown Petaluridae suggesting they would not be appropriate to use in divergence time estimates for this group. *A. archangelski* has previously been suggested to be the only crown fossil Petaluridae (F. Petrulevicius, 2003), and was used to calibrate Petaluridae in broader phylogenies of Odonata (Suvorov et al., 2021). Our analysis confirmed this placement; hence we used the minimum age of this fossil (113 ma) as a minimum age constraint on the age of the Gondwanan clade. We set a maximum age for all nodes at 199 Ma, the age of the oldest crown Epiprocta fossil (*Liassophlebia* sp.) and set a minimum root node age based on the minimum age of *P. renatae* (145 Ma), both fossils have extensive justifications set forth by Kohli et. al. (Kohli et al., 2021). Although using *Liassophlebia* sp. as a maximum age constraint is the best practice for this node according to existing literature (Kohli et al., 2021), we recognize that future fossil discovery could merit an increased root age. To determine how an increased root age influences divergence times in the Petaluridae, we also tested a highly relaxed upper age constraint of 419 Ma, representing the beginning of the Devonian. We then reconstructed four divergence time estimates using both Calibrated Yule and Birth Death Models with upper age constraints of 199 and 419 Ma in BEAST2 (Bouckaert et al., 2014; Höhna et al., 2016) using our concatenated sequence alignment with a GTR+G4 substitution model. In all models, we constrained *Uropetala* and *Petalura* as sister genera in the Gondwanan clade, and *P. hesperia* as sister to *P. ingentissima* in *Petalura*, with all other parameters being unconstrained.

As the birth–death and Calibrated Yule phylogeny converged, we then used a Calibrated Yule phylogeny with an upper age constraint of 199 Ma to conduct a biogeographical analysis in BioGeoBEARS (Matzke, 2018). To reflect the continental drift that has occurred throughout the evolution of Petaluridae, we utilized a time stratified area adjacency matrix, allowing a maximum size range of 3 (Table 1).

We tested DEC, DEC+J, DIVALIKE, DIVALIKE+J, BAYAREALIKE, and BAYAREALIKE+J parameters and selected the best model with AIC weights. Although we acknowledge the ongoing debate surrounding the use of the "jump" parameters (Matzke, 2014; Ree and Sanmartin, 2018; Matzke, 2022), this parameter was not retained in either of the two best fitting models, so we do not consider its utility or influence upon this system any further.

Table 1

Time stratified area-adjacency matrix used in Biogeography analysis between Western North America (WNA), Eastern North America (ENA), South America (SA), Japan (JA), Eastern Australia (EA), Western Australia (WA), and New Zealand,

Time period	Adjacent states
0–30 Ma	WNA+ENA+SA+JA, EA+WA, NZ
30–70 Ma	WNA+ENA+SA, EA+WA, JA, NZ
70–112 Ma	WNA+JA, EA+WA+SA+NZ, ENA
112–160 Ma	WNA+ENA+JA+SA+EA+WA+NZ

3. Results

The maximum likelihood phylogeny yielded 100 % ultrafast bootstrap support for each genus and the two major clades (Laurasian and Gondwanan) (Fig. 2A), and all tested maximum likelihood trees had 100 % concordance in tree topology. However, the relationships among the Gondwanan genera *Petalura*, *Uropetala*, and *Phenes*, as well as the placement of *P. hesperia* within *Petalura*, lacked robust support (Fig. 2A). *Petalura pulcherrima* was recovered as sister to *Petalura ingentissima* (Fig. 2A). The percent divergence between the *Petalura* (Ware et al., 2014a) ranged from 2.66 % between *P. gigantea* and *P. litorea* to 22.49 % between *P. litorea* and *P. ingentissima* (supplementary table 3). Although it was placed next to *P. ingentissima* in the UsHER parsimony analysis, *P. pulcherrima* shared the least sequence divergence with *P. gigantea* (8.76 %) (supplementary table 3). The sequence divergence of the Sanger alignment (Ware et al. 2014) was much higher than any pair of individuals from the AHE alignment (supplementary table 4), including between Petaluridae and Gomphidae. The weighted-ASTRAL species tree recovered Gondwanan and Laurasian clades, but placed *Phenes* and *Uropetala* as sister, and *P. hesperia* as sister to *P. gigantea* + *P. litorea*, both with low support (supplementary Fig. 1).

The monophyly of the Gondwanan and Laurasian clades was also strongly supported when assessed with quartet concordance (Fig. 2A). The recovery of *Uropetala* and *Phenes* as sister to each other was supported by a small majority of quartets (QC=0.022), with the other two topologies being supported by a minority of the quartets used (Fig. 2A-B), although one alternative topology was not supported more than another (QF=0.92) (Pease et al., 2018). The maximum likelihood topology of *P. hesperia* as sister to *P. gigantea* and *P. litorea* was not supported in most sampled quartets (QC=-0.056) (Fig. 2A). Instead, a weak majority (<60 %) of quartets supported the placement of *P. hesperia* as sister to the rest of *Petalura* (QC=0.057) (Fig. 2B). Likelihood mapping results offered weak support for *Petalura* and *Phenes* as sisters, with *Petalura* and *Uropetala* as sisters still receiving sizeable support (>33 %) (Fig. 2C).

3.1. Gene tree support

Analysis of gene tree likelihoods revealed varying levels of support for different topologies within the Gondwanan clade and within *Petalura* (Fig. 3A-F). *Phenes* received broader support as sister to the other two genera within the Gondwanan clade compared to *Petalura* or *Uropetala*. The recovery of *P. hesperia* as sister to *P. ingentissima* garnered more support than alternative topologies. Most gene trees were concordant with both of the prevailing hypotheses (Fig. 3G, H).

Results from the multinomial model indicated a significant interaction between mean substitution rate and chromosome on support for placement of *Phenes, Uropetala,* or *Petalura* as sister to the other Gondwanan taxa ($\chi^2_{18} = 33.9$, $p = 0.013$), so the impact of mean substitution rate was analyzed separately for each chromosome. Mean substitution rate only impacted tree support for genes in chromosome 5, where genes with higher substitution rates favored *Phenes*, and the sex chromosome, where faster evolving genes favored *Petalura* over *Phenes*. Mean substitution rate did not impact the support of genes for placement of *P. hesperia,* though faster evolving genes did tend to support *P. hesperia* as sister to *P. ingentissima* compared to sister of *P.gigantea* and *litorea* (Fig. 4A).

3.2. Analysis of secondary genitalia

We identified two noteworthy traits in the SEM scans of the male secondary genitalia of the imaged Petaluridae. The first trait is a barb below the junction of the second and third sections. This barb is pronounced and angled away from the shaft in *Tachopteryx*, *Tanypteryx* and *Uropetala* (with *Uropetala* displaying a truncated process near the barb), and short and angled towards the body of the shaft in *Petalura* and *Phenes.* The second trait is the horns on the dorsal side of the third segment, with each genus displaying a unique phenotype. These horns are elongate and thin in *Uropetala*, of an intermediate length and heavy thickness in *Petalura,* barely present in *Phenes*, and short and truncated in *Tanypteryx* and *Tachopteryx*, with plates under the horns in *Tanypteryx*.

The maximum likelihood tree based on 38 wing characters recovered *A. archangelski* as the only crown Petaluridae fossil, placed sister to *P. raptor* (supplementary Fig. 2). This confirmed prior hypotheses that *A. archangelski* is the only crown fossil Petaluridae (F. Petrulevicius, 2003). Due to its placement as sister to *P. raptor* (which is further supported by the geographic proximity of *A. archangelski* and *P. raptor*) we used this fossil as a minimum age calibration point for the Gondwanan clade of Petaluridae.

3.3. Root age and model impacts

The tested models showed generally adequate mixing, with high Effective Sample Size (ESS) (>190) for the ages of the root and crown Petaluridae, and for the posterior and tree likelihood (supplementary table 5). The estimated age of the MRCA of crown Petaluridae was ∼ 160 Ma when an upper root bound of 199 Ma was used, and ∼ 199 Ma with the maximum root age bound at 419 Ma. The former estimate is congruent with previous findings of a late Jurassic origin and diversification of the family (Ware et al., 2014a). We consider the analysis we ran using a calibrated Yule model (Fig. 6) with an upper root age constraint set at 199 Ma in further analyses, as the results are largely indistinguishable for the Birth Death Model (supplementary Fig. 3). In this analysis, the mean divergence times between extant species were estimated to be between 6 and 120 million years before present (Fig. 6).

The DIVALIKE model was selected as the best fitting model with AICc-weights (0.42) (Supplementary figure 4) followed by DEC (0.28) (Fig. 7)(Table 2). Both models support the hypothesis that the Laurasian taxa migrated to Eastern North America and Japan, while *Phenes*, *Uropetala*, and *Petalura* diverged with the separation of Gondwana, with all Gondwanan remnants as highly plausible ancestral states in this clade (Fig. 7). The ancestral geographic state of *Petalura* is most likely in Eastern Australia (Fig. 7), but it is possible that the range of *Petalura* once extended well beyond where they are found today, so the true ancestral state could be missing from this analysis.

4. Discussion

4.1. Towards a fully resolved phylogeny of Petaluridae

The maximum likelihood tree recovered a strongly supported Gondwanan clade comprising *Phenes*, *Petalura*, and *Uropetala*, as well as a Laurasian clade consisting of *Tachopteryx* and *Tanypteryx* with high support (Fig. 2A). Despite this, the relationships within the Gondwanan clade remain unresolved, as indicated by the low bootstrap support for sister genera within this clade and the uncertain placement of *Petalura hesperia* (Fig. 2A). The maximum likelihood tree recovered *Uropetala* and *Petalura* as sister taxa, contrary to previous work which placed *Phenes* and *Uropetala* as sister (Ware et al., 2014a), although both analyses had low ultra-fast bootstrap support (Fig. 2A). Additionally, the placement of *Petalura hesperia*, the only species of *Petalura* in Western Australia, is recovered with low support in the maximum likelihood tree (Fig. 2A).

Further analysis of individual gene trees and quartets proved useful in resolving these polytomies. The placement of *Uropetala* and *Petalura* as reciprocally monophyletic was supported by a strong majority of gene trees (Fig. 4: A-C), and a weak majority of quartets (Fig. 2A, note that, in the metric developed by Pease et al. (Pease et al., 2018) and used here, a QC>0 indicated a majority of quartets support the topology, and a QC of 1 was supported by all tested quartets), although likelihood mapping was inconclusive (Fig. 2C). The placement of *Petalura hesperia* as sister to

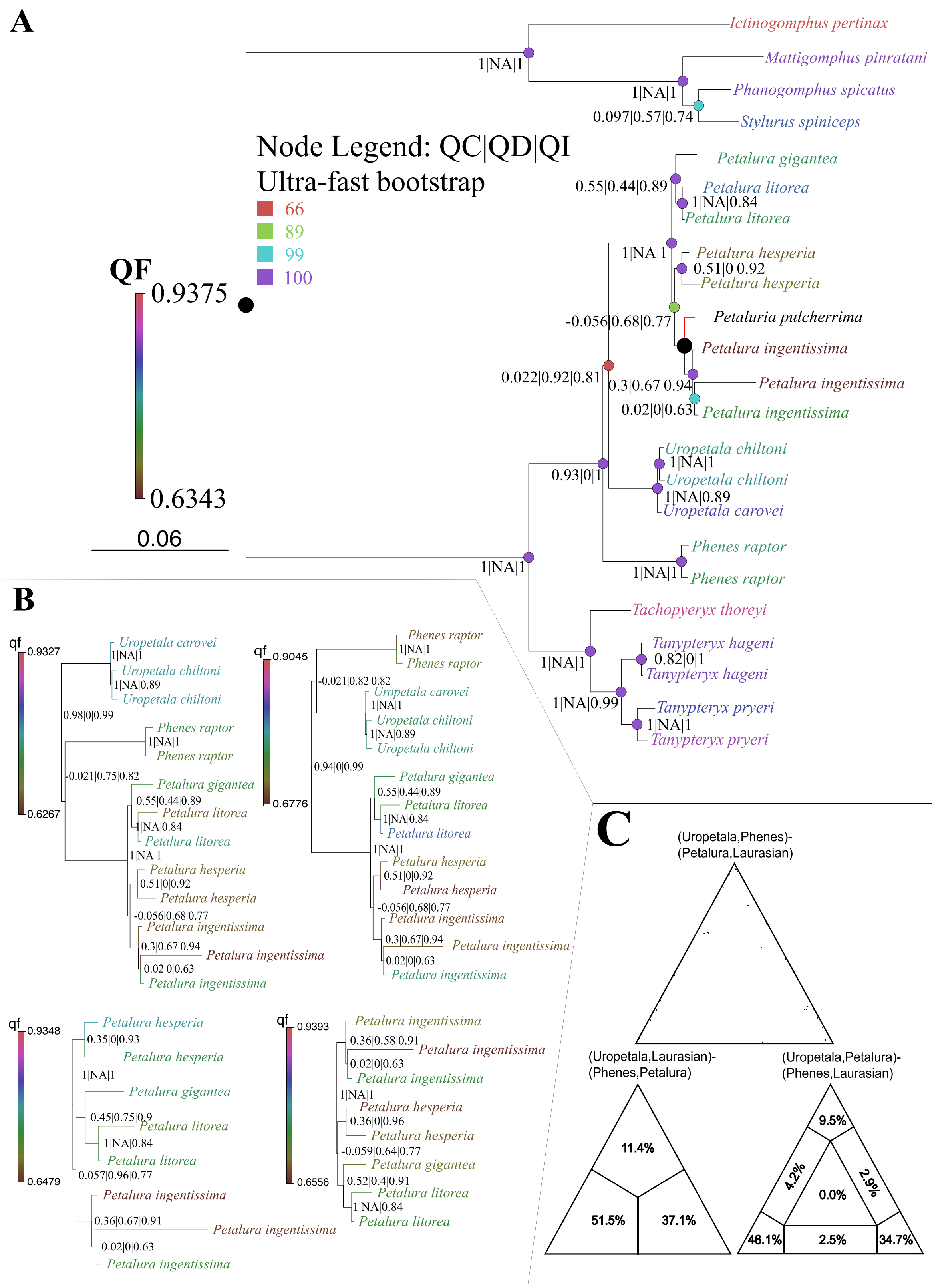

Fig. 2. Maximum Likelihood Tree Reconstruction of Petaluridae. Maximum likelihood tree reconstruction of Petaluridae with four Gomphidae as outgroups calculated with IQtree (Minh et al., 2020). (A) Maximum Likelihood Phylogeny annotated with Quartet Concordance (QC), Quartet Discordance (QD), and Quartet Informativeness (QI) at each node, and with each tip colored by Quartet Fidelity (QF). Support from 1000 ultrafast bootstrap replicates is shown at each node. *P. pulcherrima* (red branch) was incorporated into the tree with UShER (Turakhia et al., 2021), and does not have any influence upon the tree topology or ultrafast bootstrap scores. (B) Alternative topologies between Gondwanan genera and within *Petalura* annotated with QC, QD, QI, and QF. (C) Likelihood mapping of the possible sister taxa in the Gondwanan clade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

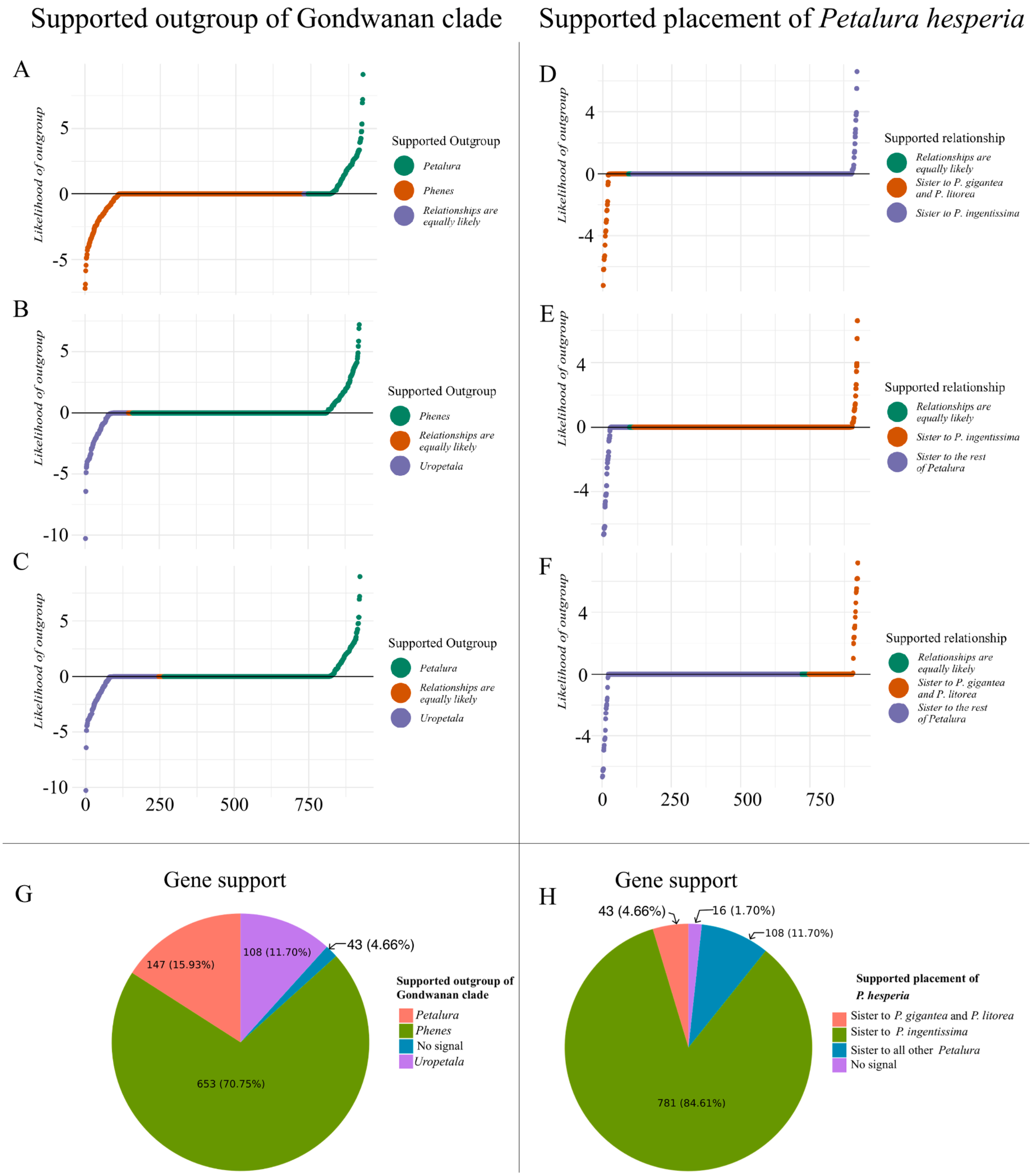

Fig. 3. Support for possible Gondwanan topologies. (A-C) Comparisons of gene tree support for the possible topologies of the Gondwanan clade and for (E-F) the placement of *Petalura hesperia* within the genus *Petalura*. The proportion of genes supporting the possible placements of *P. hesperia* (G) within *Petalura*, and (H) between the genera of the Gondwanan clade. Of the possible topologies, *Phenes* is the most well supported sister to the reciprocally monophyletic *Uropetala* and *Petalura*, and *P. hesperia* is most well supported as sister to *P. ingentissima*.

Petalura ingentissima (although *P. ingentissima* is most likely truly sister to *P. pulcherrima* based upon the grafting of *P. pulcherrima* into the tree, so we assume *P. hesperia* is sister to these two taxa in this topology) was supported by a majority of gene trees (Fig. 3B). However, the placement of *P. hesperia* as sister to the remaining *Petalura* was supported by a weak majority of quartets (QC=.056), contrary to these gene tree likelihoods (Fig. 3B).

The difficulty in building predictive models for the possible topology a gene supported highlighted the overall difficulty of resolving these relationships. In the Gondwanan clade, more quickly evolving genes were more likely to support alternative topologies from the maximum likelihood phylogeny on the fifth chromosome (Fig. 4B). The QD value of the maximum likelihood topology (0.92) did not suggest that one of the alternative topologies was more well supported than the other, consistent with incomplete lineage sorting (ILS) and other random processes. It has been demonstrated that introgression can influence deep nodes in Odonata (Suvorov et al., 2021). Further testing using whole genome alignments and population genomics should be undertaken to rule out this hypothesis.

Given the concordance of the maximum likelihood phylogeny, gene tree likelihoods, and quartet sampling, we cautiously concluded that *Petalura* and *Uropetala* can be considered as sister genera in the Gondwanan clade. Further clarification could be provided by the generation of high quality genomes from *Phenes* and *Petalura*, allowing for a tree to

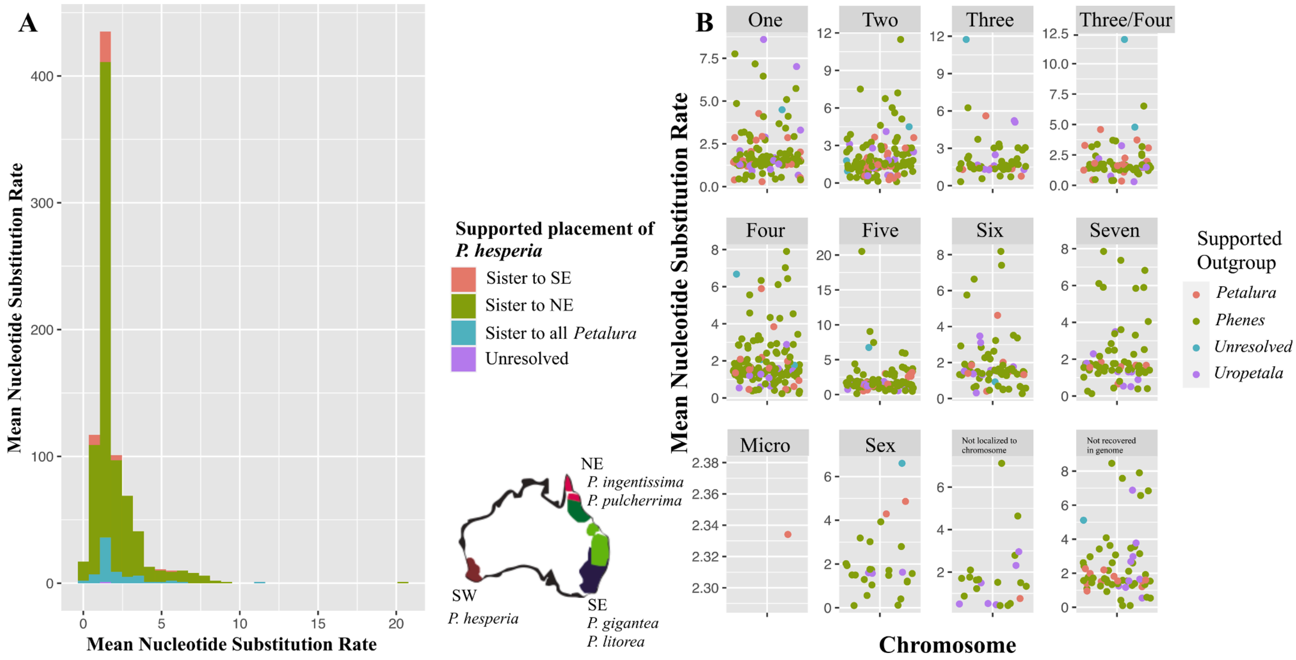

Fig. 4. Gene support for discordant topologies. (A) Gene support for the placement of *Petalura hesperia* within *Petalura* by mean nucleotide substitution rate. (B) Gene support for the placement of *Phenes*, *Petalura*, or *Uropetala* as sister to the remainder of the Gondwanan clade by chromosome and mean nucleotide substitution rate.

be built using micro-synteny, a method which is generally more robust to introgression and incomplete lineage sorting than the sequencing based methods used here, and could be a more appropriate method for identifying the "main'' tree (Zhao et al., 2021). *Petalura* is not as easily resolved. While it could be expected for *P. hesperia* to be recovered cleanly as sister to the rest of the *Petalura*, having been separated by the formation of the Nullarbor plain 13–14 Ma as with many other Australian taxa (Ansari et al., 2019, Crisp and Cook, 2007), its position is not cleanly recovered in our analysis. It is quite possible that other species in western and central Australia have gone extinct with desertification, and *P. hesperia* is the only remaining relict, or that historically broader distributions through central Australia facilitated gene flow between other *Petalura* and *P. hesperia.* Another possibility is the influence of Antarctica. Species of Petaluridae likely existed in Antarctica given the current distribution of the Gondwanan species; eastern and western Australia may have separated from Antarctica at different rates, allowing for more dispersal between Antarctica and western Australia in more recent periods. It will likely require population genomics to understand the dynamics of *Petalura* more clearly.

It has been suggested that *P. pulcherrima* should be considered a junior synonym of *P. ingentissima* (Mitchell et al., 2021).We do not believe that our results provided sufficient evidence to synonymize *P. pulcherrima*. Indeed, we were unable to resolve the relationships between the species within *Petalura* using our AHE dataset, so any conclusions regarding this genus with a more limited Sanger dataset are premature. A previous molecular phylogeny also concluded that these are two separate species (Ware et al., 2014a) and a modified version of this dataset was used by Mitchell et al. (2021) to synonymize the species. Furthermore, this dataset showed much higher divergence than the AHE probe set (supplementary tables 3-4) and could be a biased data source. The Australian government currently considers *P. pulcherrima* to be an endangered species (Pintor et al., 2019), so delimitations have major conservation implications. Given these important ramifications, we do not believe it would be appropriate to synonymize these two species at this time with the Sanger dataset used to incorporate *P. pulcherrima* into our tree, and in previous phylogenies of *Petalura* (Mitchell et al., 2021; Ware et al., 2014a). Given concerns of declining insect diversity (Hallmann et al., 2017; Wagner et al., 2021), we recommend that future work use whole genome resequencing for multiple individuals from both *P. ingentissima* and *P. pulcherrima* to better understand the dynamics between these two species before they are formally synonymized.

4.2. The evolution of secondary genitalia

There are clear morphological differences within the secondary genitalia of family members that did not reflect the phylogeny (Fig. 5). Except for *Uropetala*, the curvature of the barb on the shaft appeared to be conserved within both the Gondwanan and Laurasian clades, while the horns are variable. It has previously been hypothesized that cryptic species may exist in this family (Turner, 1970). Alongside population genomic data, the variability of the genitalia (e.g., horns and barb) could be useful features in identifying potentially cryptic species in the family. Although the discordance between the tree topology and the morphological variation of secondary genitalia suggested that sexual selection has strongly influenced these features, there is currently little understanding of the function of the identified divergent features in the secondary genitalia. More sampling of multiple individuals from each species will be needed to quantify the diversity of the secondary genitalia more fully. Further work imaging the reproductive structures of females, and the imaging of pairs in copula will be key to understanding the function of these genital structures. As these structures are recovered from each species in the family, it also will be possible to test for ancestral states more thoroughly.

4.3. Divergence time estimation and biogeographical implications

Our tree of the putative Petalurida (supplementary Fig. 2) confirmed the suggestion by F. Petrulevicius (2003) that *A. archangelski* is the only crown fossil Petaluridae and supports its use for calibrating the age of Petaluridae (Suvorov et al., 2021). We did recover a clade of stem Petaluridae, however this clade did not contain all proposed Petalurida, nor were the Petalurida grouped according to previous hypotheses (supplementary Fig.2). This shows that the fossils currently included in the clade Petalurida are in need of revision.

Model choice had little impact on the estimated ages of these dragonflies but the root age bound did cause the family age to increase by ~

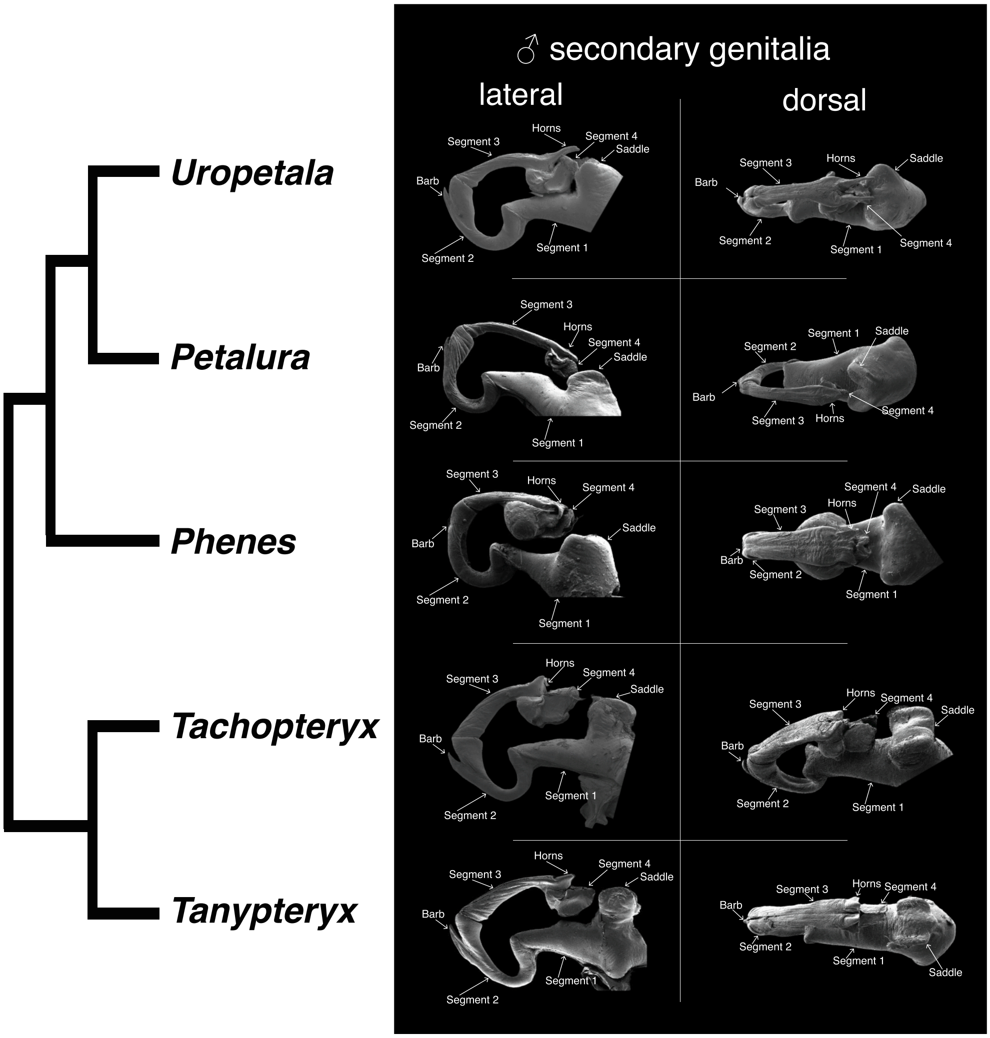

Fig. 5. Secondary genitalia of the genera of Petaluridae: Lateral and dorsal SEM scans of the secondary genitalia of Petaluridae, including detailed structures of a barb on the shift, and horns on the tip of the structures.

30 Ma (supplementary Fig. 3). Despite this difference in root age, the ages of the genera have overlapping confidence intervals, and are not as dramatically impacted (supplementary Fig. 3). This demonstrated that a careful consideration of root age maxima during analyses is needed when considering deep divergences such as in the Petaluridae, as has been previously suggested (Budd and Mann, 2020).

The mean divergence times recovered here were generally comparable to the most recent dated phylogeny published with a much smaller molecular dataset (Ware et al., 2014a), and our current recovered dates still fell within the confidence intervals of that earlier work. The recovered divergence times continued to demonstrate that the lineages within Petaluridae have persisted for remarkable amounts of time with little diversification, especially considering the highly specialized habitat requirements across this family (Ware et al., 2014a). Divergence patterns appeared to be largely correlated with geographical separation. The Laurasia/Gondwana split occurred as North and South America became more distant in the late Jurassic. The split between *Phenes*, *Uropetala* and *Petalura* (113 Ma) was likely mediated through Antarctica, which connected South America, Australia, and New Zealand. It is likely that Antarctica once harbored now extinct lineages in the Gondwanan clade and may have even mediated secondary contact between the genera. Gondwana may have once harbored a large diversity of Petaluridae around the time divergences in this clade occurred. Indeed fossilized burrows have been discovered in Antarctica (Fitzgerald and Barrett, 1986) and Patagonia (Genise et al., 2020) that could have harbored Petaluridae. Any of these occurrences could be a reason for the difficulty in resolving the relationships between *Uropetala*, *Petalura*, and *Phenes*.

The split between *Petalura* and *Uropetala* occurred (110 Ma) prior to the separation of New Zealand from Gondwana (~80 Ma), suggesting that *Uropetala* has been isolated on the sub-continent ever since this separation occurred. It has been suggested that New Zealand was inundated approximately 22 Ma (Wallis and Trewick, 2009). Our results confirm that *Uropetala* are among a growing list of species including Onychophora (velvet worms) (Murienne et al., 2014), earthworms (Buckley et al., 2011), stoneflies (Sroka and Prokop, 2023), and mite harvestmen (Baker et al., 2020), that likely drifted with New Zealand as it rifted from Gondwana, suggesting that the hypothesis of widespread inundation of Zealandia may need to be refined. The divergence between the montaine *U. chiltoni* and more lowland *U. carovei* (~6 Ma) is remarkably close to the period within which the Southern alps began to form (~5 Ma) (Tippett and Kamp, 1995). Given this correlation, it is possible that the uplifted mountain range provided new habitat and fostered this speciation event.

Previous work has hypothesized that the divergence of *Tachopteryx* and *Tanypteryx* could have been driven by the development of inland seas in North America (Ware et al., 2014a). Our recovered divergence between the genera (61 ma) occurred long after the inland sea had dissipated. However, early uplift of the Rocky Mountains (Bird, 1988) and the development of the Cretaceous Seaway (Ware et al., 2014) both

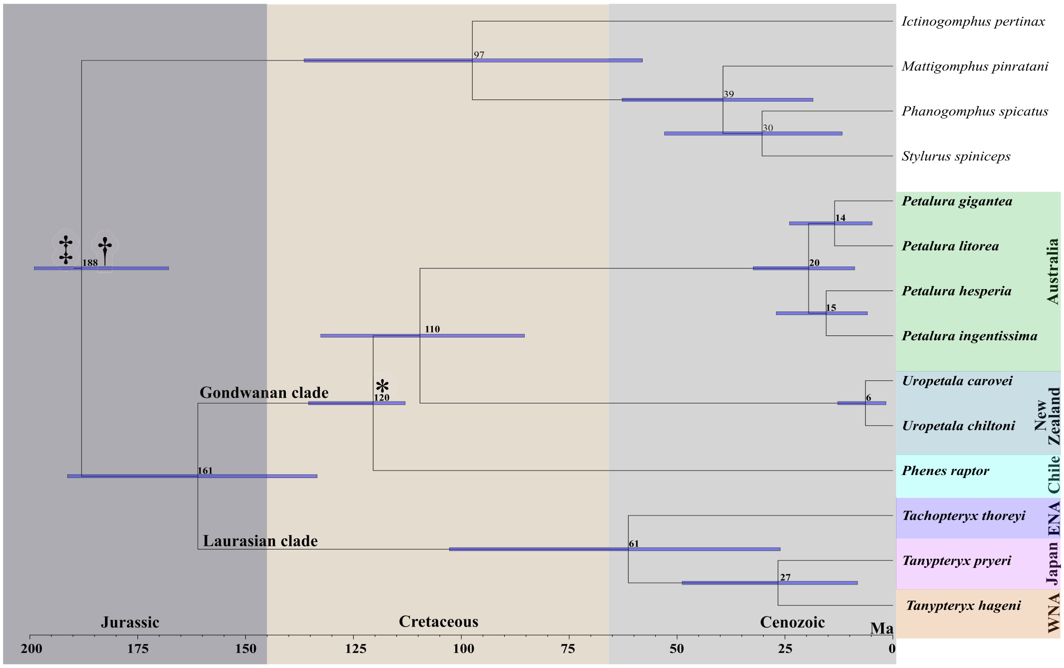

Fig. 6. Dated Phylogeny of Petaluridae. Dated phylogeny of Petaluridae generated with a calibrated Yule model in Beast2 (Bouckaert et al., 2014). The Gondwanan clade (*Phenes*, *Uropetala* and *Petalura*) was calibrated with *A. archangelski** (F. Petrulevicius, 2003). The minimum root age was calibrated with *Proterogomphus renatae*†, and the maximum root age was constrained at 199 Ma, according to the age of *Liassophlebia* sp.‡ Justifications for fossil placements have previously been set forth by Kohli et al. (Kohli et al., 2021).

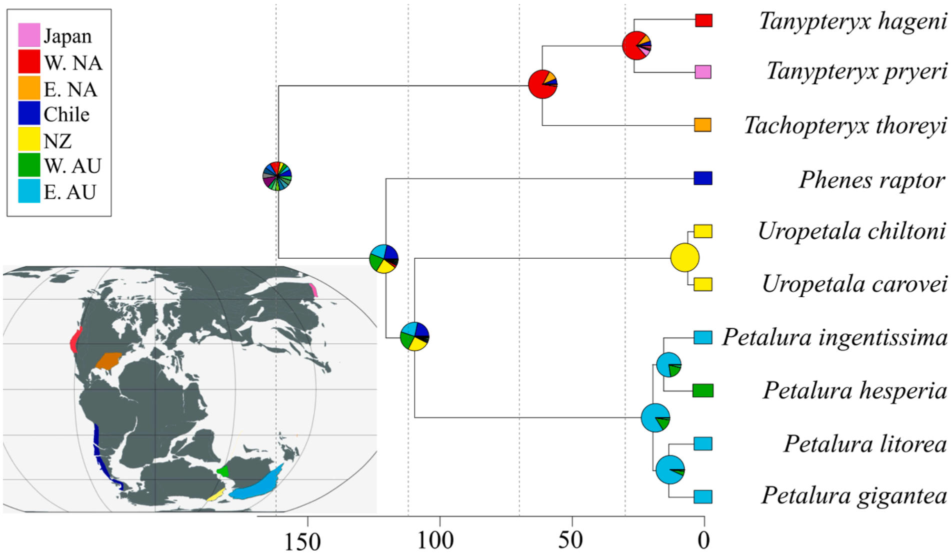

Fig. 7. Biogeography of Petaluridae. Fig. 7: A biogeographical analysis of Petaluridae, using the DEC model. The Gondwanan clade (*Uropetala*, *Phenes* and *Petalura*) most likely originated in Eastern Australia, while the Laurasian clade most likely originated in North America. DIVALIKE (supplementary Fig. 4) was selected as the best fitting model with AICc-weights, but the assumption of diversification followed by geographic separation may not fit the patterns observed in Petaluridae.

Table 2
BIOGEOBEARS model comparison. Table 2: Comparison of tested biogeobears models.

	LnL	numparams	d	e	j	AICc	AICc_wt
DEC	−10.70	2	0.04	0.76	0	27.12	0.28
DEC+J	−10.21	4	0.08	2.32	0<.01	36.42	0<.01
DIVALIKE	−10.31	2	0.08	1.81	0	26.31	0.42
DIVALIKE+J	−10.01	3	0.09	5.00	0<.01	30.01	0.07
BAYAREALIKE	−11.26	2	0.03	0.35	0	28.24	0.16
BAYAREALIKE+J	−10.01	3	0.09	5.00	0<.01	30.01	0.07

fall within the confidence interval, and both remain plausible causes for the separation of these genera. The divergence of *T. hageni* and *T. pryeri* (27 Ma) was likely mediated by a non-glacial land bridge between North America and Asia. The historical opening and closing of Beringia has driven species diversity and speciation in birds (Winker et al., 2023), flies (Geml et al., 2006), small mammals (Hope et al., 2013; Wiens et al., 2022, 2022), and tapeworms (Haas et al., 2020). In each of the aforementioned taxa, separation and secondary contact across Beringia occurred in so called “pulses” with glacial cycles, and it is possible that Beringia once harbored now-extinct lineages of *Tanypteryx* and mediated secondary contact between *T. hageni* and *T. pryeri*.

5. Conclusions

This work adds considerable depth to the story of Petaluridae and provides a potential model for evolutionary research on other families of so-called “living fossils” with diversity scattered across continents. Divergence time and biogeographical analyses demonstrated that the separation of Gondwana and Laurasia, and the eventual breakup of Gondwana into Zealandia, Australia, and South America were the driving forces of ancient speciation events in this family. On the other hand, these analyses may demonstrate that, in the more recent (10–60 ma) past, extinction may have been a major force shaping the current distribution of this family. *Tanypteryx* and *T. thoreyi* were likely separated by the loss of connective populations in North America (either through the development of the Cretaceous Seaway or the Rocky Mountains), while the loss of a connecting population in Beringia between *T. hageni* and *T. pryeri* and a possible extinction of *Petalura* from most of mainland Australia seem to have driven speciation in *Tanypteryx* and *Petalura* respectively.

While this work represents the most comprehensive analysis of the family Petaluridae to

date, it is not without limitations, as we still do not have full genomic data for all species. The sequencing of whole genomes from each species in this family will lead to the identification of genomic adaptations to the differing and shared environmental pressures Petaluridae face. Furthermore, a micro-synteny based phylogeny using genome assemblies from each species in this family would reduce noise from ILS and introgression, and provide an even more in-depth estimate of the “main” species tree (Zhao et al., 2021). A whole genome alignment with all of Petaluridae, alongside comprehensive population genomics sampling, would allow for more thorough tests of ancient introgression (Hibbins and Hahn, 2022). Beyond molecular work, the discovery of more Petaluridae fossils (especially from Antarctica and other Gondwanan remnants), along with further morphological work to refine fossil classification would provide further clarification of diversification patterns, and more accurate divergence time estimation. While Petaluridae have survived numerous mass extinctions, it is not clear that they will survive the current one, as several species are already regarded as threatened or endangered due to habitat loss (Pintor et al., 2019; Tolman et al., 2023a). Through intensive study of these living fossils, it will be possible to ensure that they persist into the future.

CRediT authorship contribution statement

Ethan R. Tolman: . **Christopher D. Beatty:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Manpreet K. Kohli:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **John Abbott:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Seth M. Bybee:** Writing – review & editing, Visualization, Resources, Project administration, Funding acquisition, Conceptualization. **Paul B. Frandsen:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **J. Stephen Gosnell:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Robert Guralnick:** Writing – review & editing, Funding acquisition, Conceptualization. **V.J. Kalkman:** Writing – review & editing, Funding acquisition, Conceptualization. **Lacie G. Newton:** Writing – review & editing, Methodology, Conceptualization. **Anton Suvorov:** Writing – review & editing. **Jessica L. Ware:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Links to data are set forth in manuscript.

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Data statement.

Our concatenated and gene tree alignments, character matrix, and rmarkdown file containing statistical analysis are available on github (https://github.com/e-tolman/molecular_phylogeny_of_petaluridae). The raw reads have been uploaded to the sequencing read archive (BioProjectID: PRJNA1098640).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108185.

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