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Review Article

A time-calibrated ‘Tree of Life’ of aquatic insects for knitting historical patterns of evolution and measuring extant phylogenetic biodiversity across the world

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ABSTRACT

The extent to which the sequence and timing of important events on Earth have influenced biological evolution through geological time is a matter of ongoing debate. In this context, the phylogenetic history of aquatic insects remains largely elusive, and our understanding of their chronology is fragmentary and incomplete at best. Here, after gathering a comprehensive data matrix of 3125 targeted rRNA and protein-coding gene sequences from nine independent gene portions, we built a well-supported time-calibrated phylogenetic tree comprising almost 1200 genera that represent a large proportion of extant families of dragonflies and damselflies (Odonata), mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera). We reviewed the main evolutionary and historical scenarios for each aquatic insect lineage as revealed by our best-scoring molecular tree topology, major ancient radiations, calibrated divergence estimates, and important events in geological history related to the spatial arrangement of land masses, continental drift, and mass extinctions. Molecular dating using the birth-death model of speciation, with a lognormal-relaxed model of sequence evolution informed by transcriptomic constraints, suggested that (i) dragonflies and damselflies first radiated approximately 220 million years (Ma) ago and most extant lineages thrived independently after the Triassic–Jurassic (Tr–J) extinction event; (ii) mayflies underwent bursts of diversification during the Cretaceous; (iii) ancestral divergence separating the stonefly suborders Arctoperlaria and Antarctoperlaria was consistent with geographical isolation after vicariant fragmentation and tectonic splitting of the supercontinent Pangaea around 170 Ma ago; and (iv) the most recent common ancestors of caddisflies extended back to the time of Pangaea, supporting the earliest offshoot of the ‘retreat-making’ Annulipalpia and a sister relationship between the predatory free-living Rhyacophilidae and Hydrobiosidae. Our ‘Tree of Life’ of aquatic insects also resolved shallow phylogenetic relationships related to key evolutionary innovations, such as the convergent evolution of exophytic oviposition in dragonflies or the Jurassic origins of the burrowing lifestyle in mayflies. In this study, we also illustrate how our

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time-calibrated phylogeny can help to integrate phylogenetic aspects in biogeographical and ecological research across the world. To do so, we used three empirical datasets of stream insects from subarctic Finland, north-eastern Spain, and southeastern Tibet as exemplary cases. These examples of application tested ecogeographical mechanisms related to (i) the responses of size structural resemblances to phylogenetic constraints, and patterns of (ii) phylogenetic relatedness and (iii) phylogenetic uniqueness along elevational and flow-intermittence gradients, respectively. We emphasise how specific details capturing different aspects of phylogenetic variation are dependent on the geological, geographical, and environmental contexts in different drainage basins. We finally highlight potential venues for future research, including evaluations of geographical patterns of phylogenetic diversity in space and time, evolution of ecological characters in relation to palaeoclimatic variation, and development of complementary algorithms for conservation prioritisation of evolutionarily valuable bioregions for aquatic insects. Overall, we hope that this work will stimulate multidisciplinary research efforts among different areas of the biogeosciences towards safeguarding the phylogenetic heritage of extant aquatic insects across the world.

1. Introduction

Understanding the timescale of biological evolution is best achieved in an integrated context with Earth's geological history (Benson et al., 2021; Liow et al., 2023). Important geological events have jointly shaped the characteristics of biotas and ecosystems seen in the present-day world. Tectonic pulses associated with mountain formation and continental drift often triggered prodigious consequences on organismal ancestral distributions and diversification (Ebach et al., 2003). For instance, tectonic splitting of the Mesozoic supercontinent Pangaea paved the way for evolutionary innovation in most terrestrial and freshwater vertebrate lineages, especially after Gondwana and Laurasia each began to break apart into smaller and increasingly more isolated land masses (Jordan et al., 2016; McIntyre et al., 2017). Occasional mass extinctions are also known to have had outsized effects on the tempo and mode of evolutionary change through the history of life (Jablonski, 2001; Bambach, 2006), creating long-ranging and contingent selective regimes during and in the aftermath of these events (Keller et al., 2003; Hull, 2015; Foster et al., 2023). Multiple lines of evidence seem therefore necessary to infer processes underlying the evolutionary history of extant biotas. One promising way is to combine phylogenetic reconstruction and molecular dating with geological and ecological evidence from different environmental and geographical settings.

Research priority should be given to unravel the chronology of evolution in freshwater systems. This is because (i) the pace of biodiversity loss in inland waters has disproportionately exceeded that in terrestrial and marine ecosystems (Harrison et al., 2018; Reid et al., 2019; Albert et al., 2021) and (ii) evolutionary and phylogenetic aspects of diversity have become headline indicators to guide freshwater monitoring and management under the auspices of the post-2020 Global Biodiversity Framework (GBF) of the Convention on Biological Diversity (CBD, 2022). Moreover, although inland waters cover less than 1% of Earth's total surface area, freshwater ecosystems have been estimated to harbour more than 9% of all animal species in our planet (Balian et al., 2008).

Aquatic insects are nearly cosmopolitan in freshwater ecosystems, occurring from the Tropics to the Arctic and inhabiting widely variable environmental conditions (Dijkstra et al., 2014a). They exploit a diverse array of microhabitats, provide ecosystem services that are vital for humans, and play key functional roles as primary consumers, predators, detritivores, and pollinators in both aquatic and terrestrial realms (Tachet et al., 2010; Lancaster and Downes, 2013; Noriega et al., 2018), contributing to energy flow in food webs and nutrient dynamics in lotic, lentic, and riparian ecosystems (Starr and Wallace, 2021; Fehlinger et al., 2022). Aquatic insects comprise species belonging to different taxonomic orders within the class Insecta. Their eggs and larvae are almost exclusively restricted to living in underwater environments, but many species move to terrestrial habitats as adults (Lancaster and Downes, 2013; Múrria et al., 2018), the most common of which are dragonflies and damselflies (Odonata), mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera). The biogeography,

ecology, and habitat preferences of these insects have been important components of fundamental research (e.g., Poff et al., 2006; Heino and Peckarsky, 2014; Sarremejane et al., 2020; Schmera et al., 2022), in part owing to their role as bioindicators of water quality, range-wide ecological niches, and relatively accessible taxonomy (CWA, 1972; WFD, 2000; Bilton et al., 2019). Unfortunately, the macroevolutionary patterns of these organisms remain largely enigmatic.

A comprehensive phylogeny of aquatic insects is not established thus far, and even an approximation of the global structure of their 'Tree of Life' has remained elusive (Lancaster and Downes, 2013; Dijkstra et al., 2014a; Múrria et al., 2018). The conventional view is that the ancestral forms of modern insects evolved on land and multiple orders began to occupy freshwater habitats (Wootton, 1988). When and how insects followed the successive transitions to novel environments is still debated (Maddrell, 1998; Dijkstra et al., 2014a), although major extensive radiations of extant aquatic insect orders have been recognised when early forms of mammals and non-avian dinosaurs started to diversify and dominate our planet during the Triassic (201–252 Mya) and Jurassic (145–201 Mya) periods (for details, see Lancaster and Downes, 2013). The timing and frequency of these evolutionary events varied geographically and across major aquatic insect lineages (Thomas et al., 2020; Kohli et al., 2021), a reason for which we need to consider phylogenetic reconstructions in the context of plate tectonics and continental drift at large spatial and temporal scales.

Early inferences about evolutionary relationships of aquatic insects were based primarily on conventional morphological information from mainly superficial external structures (e.g., McCafferty and Edmunds Jr., 1979; Zwick, 2000). Later, our understanding of the history of different taxonomic groups of aquatic insects has transitioned to molecular phylogenies based on sequence information from transcriptomes and nuclear and mitochondrial genomes (e.g., Zhou et al., 2016; Thomas et al., 2020; Kohli et al., 2021). For instance, a preliminary study of the aquatic insect 'Tree of Life' focused on deep ancestral splits only, integrated as part of a much larger clock-calibrated phylogeny of 143 well-classified freshwater macroinvertebrate families of Europe ranging from flatworms and hydrozoans to molluscs, crustaceans, and insects (Múrria et al., 2018). Over the years, a more practical and standard approach has been to address smaller taxonomic groups (e.g., major aquatic insect orders), with specialists working on their own discrete group (e.g., Ogden et al., 2009a on mayflies; Dijkstra et al., 2014b on damselflies; Carle et al., 2015 on dragonflies; Thomas et al., 2020 on caddisflies; Letsch et al., 2021 on stoneflies). However, a sense of urgency has recently been expressed about the pace of generating a comprehensive time-calibrated phylogeny of major lineages of aquatic insects for regional and global evaluations of phylogenetic diversity (García-Girón et al., 2023). Doing so would help to address shortfalls in our understanding of freshwater biodiversity patterns on Earth. More importantly, there is a primary reason to keep moving forward, as our ability to understand geographical gradients in aquatic insect biodiversity will hinge on addressing patterns of evolution and their relationships with the sequence and timing of important events in Earth's history.

In recent years, the evolutionary processes that generate and maintain biodiversity have been recognised as one of the most active areas of research in natural sciences (for details, see Velasco and Pinto-Ledeza, 2022). Such research underpins questions about where, why, and how local and regional biodiversity is distributed in space and time at large spatial scales (Heino, 2011; García-Girón et al., 2023). From an applied perspective, it follows that this research agenda is also pivotal for the phylogenetic gambit (sensu Tucker et al., 2019), which lies at the heart of nature conservation programs and suggests that prioritising evolutionarily valuable areas captures centres of biodiversity potentially important for sustaining diverse ecosystem functions (Vellend et al., 2011; Mazel et al., 2018; Owen et al., 2019; Tucker et al., 2019). Hence, inferring phylogenetic information for different lineages of aquatic insects that is both accurate and taxonomically comprehensive would be not only inherently interesting, but also a worthwhile objective. Incorporating phylogenetic information in spatial and temporal studies would also greatly improve our knowledge of the causes and consequences of phylogenetic diversity variation and guide future solutions aiming at the conservation of evolutionarily valuable areas across the world (García-Girón et al., 2023).

Here, we present an updated hypothesis on the phylogeny of key groups of aquatic insect lineages, i.e., dragonflies and damselflies, mayflies, stoneflies, and caddisflies. Our work builds on the advances made in previous papers that unravelled phylogenetic relationships and evolutionary events for specific groups of aquatic insects. We have generated a data matrix of 3125 targeted rRNA and protein-coding gene sequences from nine of the ‘standard’ toolbox genes for insect phylogenetic reconstructions (see below), comprising more than 6000 combined nucleotides. Our time-calibrated phylogenetic tree is relatively comprehensive, with molecular information of almost 1200 genera representing near-complete family-level coverage for the world’s stoneflies and caddisflies, as well as a large proportion of extant families of dragonflies, damselflies, and mayflies. Our working phylogenetic hypothesis is both relevant as a global overview and a snapshot of the evolutionary history within each major aquatic insect order, and it can be updated as our molecular estimates improve. For the moment, the generally well-supported topology of our phylogenetic estimates provides an integrated resource for researchers worldwide to bring new insights into our understanding of different aspects of aquatic insect evolution at different spatial and temporal scales and in different environmental and geographical settings.

Aquatic insect evolution is an enormous research field, and summarising everything known about these organisms is therefore far beyond the scope of our work. Rather than providing an exhaustive review on the macroevolution and biogeography of each aquatic insect lineage, our aim is simply to describe our phylogenetic reconstructions, illustrate potential and dated geological drivers behind major evolutionary events, and provide potential applications of combining this phylogeny with empirical field data on aquatic insects. To reach these goals, this paper is organised into the following sections. First, we describe materials and methods for phylogenetic inference. A general description of the best-scoring time-calibrated phylogenetic hypothesis is introduced in section two, while the third section provides a summary of major evolutionary and biogeographical scenarios for each aquatic insect lineage as revealed by tree topology, calibrated early divergence estimates, and important events in Earth’s geological history. In the fourth section, we illustrate how our time-calibrated phylogeny can be helpful when it comes to integrating phylogenetic aspects of biotic assemblages with three case studies of stream insects from different environmental and geographical contexts. Finally, we discuss potential limitations of our phylogenetic constructions, highlight potential avenues for future research, and propose possible lines of development and recommendations to improve our understanding of aquatic insect biodiversity across the world. Overall, we hope that the knowledge summarised in this paper will contribute to the emergence of new developments to assess and safeguard the phylogenetic heritage of extant

aquatic insects. We also hope that our work will benefit different research areas related to the multidisciplinary biogeosciences, including biogeography, limnology, macroevolution, and palaeoecology.

2. The path to phylogenetic reconstruction

2.1. DNA sequence data

DNA sequences for as many as possible genes and specimens of monophyletic and mostly aquatic lineages of dragonflies and damselflies, mayflies, stoneflies, and caddisflies were compiled from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). We aimed to target representative genus and family samples from each individual order for multiple gene portions with a wide taxonomic coverage. Hence, we avoided using genes for which there were only a few representative genera or families. In a few cases, when not all DNA sequence data were available for a single species, chimerical taxonomic units were assembled from several members of the same genus. In total, our resulting genetic dataset included molecular information of almost 1200 genera distributed among 127 recognised families and *incertae sedis* groups (Supplementary Information Appendix S1) for nine independent gene portions, i.e., mitochondrial protein-coding cytochrome *c* oxidase subunit I (COI), ribosomal subunits 12S rRNA, 16S rRNA, nuclear ribosomal subunits 18S rRNA, 28S rRNA, and protein-coding histone H3, elongation factor-1 α (EF-1 α), RNA polymerase II (RNA pol-II), and cadherin (CAD) (for details on GenBank accession nos. and outgroup taxa used to reconstruct phylogenetic affinities for each aquatic insect order, see Supplementary Information Appendix S1).

2.2. Phylogenetic analyses

Sequences were edited, assembled, and carefully checked for stop codons using AliView ver. 1.28 (Larsson, 2014). We aligned these sequences separately for each order in MAFFT ver. 7.182 (Kuraku et al., 2013; Katoh et al., 2019) using default (–auto) settings, subsequently removing divergent regions and ambiguously aligned positions with GBLOCKS ver. 0.91b (Castresana, 2000; Talavera and Castresana, 2007). We concatenated nucleotide alignments into a supermatrix in the phylip format using a custom Python script. Codon positions were assessed through a χ^2 -test for homogeneity of nucleotide state frequencies in PAUP* ver. 4.0a (Swofford, 2002). Results of these tests suggested that base compositional biases in third-codon positions had the potential to influence phylogenetic signals over time for protein-coding genes of most aquatic insect groups (Supplementary Information Appendix S2). Hence, to remove potential systematic errors in our phylogenetic analysis caused by nucleotide saturation and GC heterogeneity, purines (A or G) and pyrimidines (C or T) in third-codon positions were RY-coded (Phillips and Penny, 2003). RY-coded data were then retested for significance (Supplementary Information Appendix S2) and biased positions were finally excluded from the alignments (Thomas et al., 2013). The best-fitting partitioning schemes and corresponding substitution models of molecular evolution were selected with the Bayesian Information Criterion (BIC) in PARTITIONFINDER ver. 2.1.1 (Lanfear et al., 2017) for CentOS Linux ver. 7.7 (The CentOS Project; <https://www.centos.org/>) using the supercomputing resources managed by SCAYLE (Supercomputing Centre of Castilla y León; <https://www.scayle.es/>).

Two phylogenetic inference approaches were applied to reconstruct evolutionary relationships for each aquatic insect order using the CIPRES Science Gateway (Miller et al., 2010): (i) a maximum likelihood (ML) phylogeny obtained from RAxML ver. 8.2.12 (Stamatakis, 2014) under the general time-reversible (GTR) model of sequence evolution, with across-site rate variation modelled to a gamma distribution for each partition of the scheme selected by PARTITIONFINDER; and (ii) a Bayesian framework using MrBayes ver. 3.2.7 (Ronquist et al., 2012). For this latter approach, we defined the best-fitting partition scheme and corresponding models (see above) with two independent runs, each with

four chains and 10 million Markov chain Monte Carlo (MCMC) iterations, sampled every 1000th generation. The mixing and convergence of the MCMC chains were assessed by determining the effective sample size (ESS) values using Tracer ver. 1.7 (Rambaut et al., 2018). We further used the quartet sampling (QS) approach by Pease et al. (2018) to further investigate the consistency of phylogenetic support and the reliability of each terminal branch tip in our best-scoring phylogenetic trees by means of their quartet concordance factor (QC) and quartet fidelity (QF) scores, respectively. The QC score is an entropy-like measure that gives an estimate of how sampled quartet topologies agree with the putative phylogenetic tree, whereas the QF score reports the proportion of total replicates including the focal taxon that results in a concordant quartet topology (for details, see Pease et al., 2018).

Bayesian analyses closely resembled ML estimations and both approaches performed equally well (Supplementary Information Appendix S3–S6). Hence, we inferred our dated phylogeny using a fixed tree topology obtained from combining the individual best-scoring ML phylogenetic trees for each insect order in BEAST ver. 2.6.7 (Bouckaert et al., 2019), together with several isotomid saprophagous springtail representatives as outgroups (Collembola; Supplementary Information Appendix S7). Phylogenetic tree reconstructions for dragonflies and damselflies, mayflies, stoneflies, and caddisflies were combined into a single Newick file based on the well-supported backbone relationships of Misof et al. (2014) (for a similar approach, see Mitterboeck et al., 2016 and Wiemers et al., 2020). For consistency, despite the unsettled ambiguities of the tree topology and branching for the early winged insects (sensu Hovmöller et al., 2002), we chose to build upon the phylogenetic relationships among Odonata, Ephemeroptera, and the remaining Neoptera from Misof et al. (2014). We are following a justified reasoning here, not least because the ‘Palaeoptera hypothesis’ (Odonata + Ephemeroptera) has gained increased support from morphological similarities in the aquatic larvae and adult antennae, maxillae, and wings (e.g., Kulaková-Peck, 2008; Blanke et al., 2012), as well as from several molecular (e.g., Kjer et al., 2006; Thomas et al., 2013; Song et al., 2019) and phylogenomic analyses under different models of sequence evolution (e.g., Meusemann et al., 2010; von Reumont et al., 2012; Simon et al., 2018). However, given that the monophyly of extant palaeopterous insects remains a controversial issue of arthropod systematics (cf. Misof et al., 2007; Simon et al., 2009; Rutschmann et al., 2017), alternative approaches and phylogenetic comparative frameworks should be further explored in the future.

To facilitate convergence in our phylogenetic models, we applied the corresponding best-fitting partitioning schemes and substitution models selected by PARTITIONFINDER for sequences of all aquatic insect groups combined. Branch lengths were set to vary under an uncorrelated lognormal-relaxed molecular clock model, with the birth-death model of speciation, for a total of three independent chains of 100 million generations each, sampling every 5000th generation. The root (i.e., the split between Collembola and Insecta) and seven additional nodes were used as secondary calibration points for molecular dating based on an interpretation of Misof et al. (2014). Although some authors have expressed their concern regarding indirect (or secondary) calibration strategies (e.g., Schenk, 2016), several studies found very similar outcomes between primary and secondary calibrations (e.g., Sauquet et al., 2012; Powell et al., 2020). The incompleteness of the fossil record and the unequal distribution of calibration points along different portions of the supertree are both a limitation and a challenge in divergence time estimation analysis of phylogenetically diverse organismal groups (Barba-Montoya et al., 2017; dos Reis et al., 2018), such as aquatic insects (Lancaster and Downes, 2013; Karr and Clapham, 2015). Not surprisingly, indirect calibration strategies have been repeatedly used to estimate divergence times across the ‘Tree of Life’ (e.g., Hipsley and Müller, 2014; Chriki-Adeeb and Chriki, 2016; Albassatneh et al., 2020; Katz, 2020). Here, we followed a secondary calibration scheme and specified uniform distributions with hard upper and lower boundaries (Supplementary Information Appendix S8). All other priors were left as the default values set

by BEAUti (Bouckaert et al., 2019). Convergence and effective sampling were monitored using Tracer, with all ESS values above 100. Ten percent of the first generation of each chain was removed as burn-in and the maximum clade credibility tree was constructed with TreeAnnotator after combining the results of four independent chains using Log-Combiner (both programs available in BEAST).

3. An outline of the best-scoring phylogenetic hypothesis

The full concatenated alignment of conserved blocks comprised 3125 targeted gene sequences and consisted of 6038 nucleotide sites from nine independent gene fragments (see above). Our time-calibrated phylogeny for the entire combined rRNA and protein-coding sequences is shown in Fig. 1. This phylogeny represents near-complete family-level coverage for stoneflies (94% based on the Plecoptera Species File; DeWalt et al., 2022) and caddisflies (94% based on the Trichoptera World Checklist; Morse, 2011), and a large proportion of dragonfly and damselfly (76% based on the World Odonate List; Paulson et al., 2022), and mayfly families (67% based on the World Checklist of Freshwater Ephemeroptera; Barber-James et al., 2019). There are several ways to examine the details of the phylogenetic tree. The tree is available for download from the Zenodo repository (see Data Availability) as a Newick file, a format that can be easily viewed in freely available programs like R (R Development Core Team, 2021), Dendroscope (Huson and Scornavacca, 2012), and FigTree (Rambaut, 2018). Individual best-scoring phylogenetic trees for dragonflies and damselflies, mayflies, stoneflies, and caddisflies (Supplementary Information Appendix S3–S6, respectively) are also included in the Supplementary Materials, with bootstrap support values (BS) and Bayesian posterior probabilities (PP) given on each tree. Compared with the full phylogenetic tree, subordinal and familial relationships are easier to visualise in the appendix, and high-resolution versions of the figures are also available from Zenodo (see Data Availability).

We found that our tree topology was generally well supported by traditional measures of branch support (Supplementary Information Appendices S3–S6) and the quartet sampling routine (ca., 70% of all internodes with QC values above 0.2, denoting strong support; Bybee et al., 2021). There was a moderate concentration of relatively poor phylogenetic support among the highly polyphyletic arrangements of Setisura and Pisciforma (Ephemeroptera) (ca., 65% of all relatively low QC scores for mayflies; Supplementary Information Appendix S4), as well as among plenitentorian limnephiloids and families within Sericostomatoidea (Trichoptera) (ca., 70% of all relatively low QC scores for limnephiloid and leptoceroid lineages of the tube-case-making Integripalpia; Supplementary Information Appendix S6, Fig. S2). Interestingly, though, the relatively low skew in discordant frequencies (quartet differential, QD > 0.5) in these quartets suggests that no genuine biological conflict driven by introgressing gene flow or substitution rate heterogeneity is clearly favoured (Pease et al., 2018). In this regard, most of our molecular hypotheses during the quartet sampling were relatively well supported, with stoneflies, caddisflies, and narrow-winged damselflies showing the highest QF scores (representing 33%, 27%, and 20% of all study insect genera having a QF score ≥ 0.75 , respectively). Since some taxonomic groups in our phylogeny are still part of the controversy surrounding the evolution of insects (e.g., the position and relationships among the early branches of pterygotes) or have more observations to discuss than others (e.g., dragonflies and damselflies), we mostly focus on predominantly highly resolved clades and on areas and sections that are less known (e.g., mayflies and stoneflies). What follows is a broad descriptive presentation of some major evolutionary trends and implications underlying the phylogenetic information presented herein.

4. The new time-calibrated ‘Tree of Life’ of aquatic insects

4.1. Odonata (dragonflies and damselflies)

Our phylogenetic analyses (Fig. 1) recovered Zygoptera as a sister clade to Epiprocta, a monophyletic group produced by the synoptic work of Lohmann (1996) and the cladistic parsimony analysis of Rehn (2003) that comprises Anisozygoptera and Anisoptera. Anisozygoptera, represented in our set of samples by the Japanese and Himalayan endemic Epiophlebiidae, a family blending the robust synthorax of dragonflies with the petiolate wings that are zygopteran in shape, was supported as sister to Anisoptera with high support by all criteria (BS = 93, PP = 0.96, QC > 0.2). Consistent with recent branching from morphological features based on wing venation, skeletal morphology, larval, and copulatory characters to modern molecular data (e.g., Bybee et al., 2008, 2021; Dumont et al., 2010; Dijkstra et al., 2014b; Kim et al., 2014; Kohli et al., 2021), Zygoptera is unequivocally monophyletic (Fig. 1). This conclusion is supported by several synapomorphies demonstrated for almost two decades, the two most important being the widely separated eyes and the transverse head structure (Rehn, 2003). Wing vein characters and limited DNA and rRNA sequence studies, however, have provided conflicting conclusions on the structure of the Odonata tree (e.g., Bechly, 1996; Saux et al., 2003; Hasegawa and Kasuya, 2006). Interestingly, though, most of the studies supporting Fraser’s (1957) original views of damselfly paraphyly, placing leptomorphs as the sister clade to either Anisoptera or Anisoptera + Epiophlebioptera, suffer from lack of concatenated analysis, narrow systematic scope, and limited sampling of taxa (reviewed in Hovmöller, 2006), with rampant homoplasy in wing venation characters also obscuring phylogenetic relationships (Dijkstra et al., 2014b). The monophyly of Epiprocta is also broadly confirmed in our analysis (Fig. 1; Supplementary Information Appendix S3, Fig. S2), being supported by different synapomorphies, including a large and modified epiproct for grasping the females (Rehn, 2003).

No definitive timescale of dragonflies and damselflies has hitherto been published, and molecular dating of odonates is still in its infancy (e.g., Ballare and Ware, 2011). Nevertheless, the transcriptome-based evolutionary history of Kohli et al. (2021), the most ambitious study on divergence times of Odonata, provides a strong foundation for comparative evaluation. Findings presented herein are in substantial accordance with their phylogenetic reconstruction. For instance, our resulting topology is congruent with the hypothesis that crown dragonflies and damselflies first radiated during the Late Triassic (Fig. 1), approximately 220 million years (Ma) ago (Supplementary Information Appendix S9), further diverging during the Mesozoic after Gondwana and Laurasia each began to break apart. The age of damselflies is 192 Ma (171–209 Ma), whereas Anisoptera split from anisozygopterans around 206 Ma ago (174–225 Ma ago). Thus, not only have odonates survived the Triassic–Jurassic (Tr–J) extinction event, but most of the current evolutionary lineages have thrived independently since the Jurassic and the Cretaceous, some representing prime examples of radiation following the Cretaceous–Palaeogene (K–Pg) mass extinction (see below). A good example are the estimated divergence times for clubtail and higher libelluloid dragonflies (family Gomphidae and superfamily Libelluloidea, respectively), approximately 129 Ma ago (117–142 Ma ago) and 140 Ma ago (125–153 Ma ago), respectively (Supplementary Information Appendix S9). Both Gomphidae and Libelluloidea (here, families Synthemiidae, Corduliidae, Macromiidae, and Libellulidae) have evolved a reduced or vestigial ovipositor that does not require plant material for egg deposition, a strategy known as ‘exophytic oviposition’ (Tillyard, 1917; Corbet, 1999). However, neither ovipositor reduction nor exophytic oviposition lend support for a sister group relationship between Gomphidae and Libelluloidea (Supplementary Information Appendix S3, Fig. S2). Instead, the divergence estimates for both clubtail and higher libelluloid dragonflies in our study favour early intuitions that the evolution of this egg laying behaviour was

convergently derived (Misof et al., 2001; Carle et al., 2008), probably as a response to avoid increased predation levels from ancient frogs, fish, and crown birds during the Mesozoic (Ware et al., 2008; Kohli et al., 2021), or as an adaptation to exploit larval niche space in freshwaters (Carle, 2012; Carle et al., 2015).

Our reconstruction along the backbone of the phylogeny is remarkably congruent with previous morphological classifications and molecular evidence for this group of insects (e.g., Rehn, 2003; Bybee et al., 2008), albeit some specific branches still show relatively low support and conflicting patterns among families (see below), a situation that is typical when terminal branches are long compared to internal branches. Support for important clades, where relevant, is discussed in the following paragraphs. Although the focus of our study is not the resolution of lower-level phylogenetic structures, some of the systematic relationships presented here will remain in debate until the final topology of the Odonata ‘Tree of Life’ is completely settled.

The phylogeny of Zygoptera is only just starting to develop and there are many parts of the family tree that are yet considered unresolved. In line with some of the most recent studies (e.g., Bybee et al., 2008; Dumont et al., 2010; Dijkstra et al., 2014b; Kohli et al., 2021), a monophyletic Lestoidea was recovered (Supplementary Information Appendix S3, Fig. S1) as the earliest branching lineage in Zygoptera (BS = 93, PP = 0.98). These lestad-like family groups share distinctive characters of the head and secondary genitalia (Rehn, 2003), and include Hemiphlebiidae, Perilestidae, Synlestidae, and the more successful and cosmopolitan Lestidae. We lacked the first clade in our phylogenetic exercises, although Hemiphlebiidae has been found as the sister group of remaining leptomorphs in the past (e.g., Rehn, 2003; Dumont et al., 2010). Neither South African (e.g., *Chlorolestes*, *Echchlorolestes*) nor Australian (e.g., *Episynlestes*, *Synlestes*) synlestid damselflies are represented by reciprocally monophyletic assemblages, a finding that agrees with Dijkstra et al. (2013). Interestingly, Perilestidae and Synlestidae combined were found as paraphyletic, but their further relationships remain unstable considering the low statistical support (BS < 90, PP < 0.95) and QC scores (QC < 0.2) for this portion of the topology. We therefore argue that previous suggestions of transferring the African genus *Nubiolestes* from Perilestidae to Synlestidae should be used with caution (but see Dijkstra et al., 2014b). The family Platystictidae was recovered as monophyletic and the sister group to the remaining Zygoptera, subordinated to Lestoidea (Supplementary Information Appendix S3, Fig. S1). Its monophyly and early divergence is in line with previous molecular studies (e.g., Bybee et al., 2008, 2021; van Tol, 2009; Dumont et al., 2010; Dijkstra et al., 2014b; Kohli et al., 2021), and its relationship to Calopterygoidea + Coenagrionoidea is not surprising based on morphological features (for details, see Kohli et al., 2021). Our phylogenetic analysis recovered the forest-dwelling Neotropical genus *Palaemnema* as the sister taxa of the remaining platystictids (BS = 91, PP = 1.00), contrary to some previous analysis that retrieved the Asian *Sinostica* as an early-branching clade of the family (e.g., Dijkstra et al., 2014b). However, the tropical amphi-transpacific distribution of platystictids is more consistent with a western Gondwanan ancestor that dispersed into Asia and New Guinea through Africa during the Late Cretaceous, as originally hypothesised by van Tol (2009). Our estimated dates for the most common recent ancestor of Platystictidae (89–129 Ma) may agree with this evolutionary scenario, but additional Neotropical samples are still needed to test this hypothesis more fully.

The relationship of the Australasian family Isostictidae to other damselflies is still problematic, with this family being recovered as either sister to Coenagrionidae + Platycnemididae (e.g., Bybee et al., 2008, 2021; Carle et al., 2008), or as sister to Calopterygoidea (e.g., Dijkstra et al., 2014b). Our phylogenetic analyses (Supplementary Information Appendix S3, Fig. S1) are largely congruent with the classification of Dijkstra et al. (2014b) and recovered a monophyletic Isostictidae as sister to the broad-winged calopterygoids (BS = 93, PP = 0.96). However, we interpret the polyphyletic Coenagrionoidea as pending further verification, partly because only few informative

nucleotide sites (quartet informativeness, $QI = 0.24$; Pease et al., 2018) exist to inform this section of the topology (sensu Steel and Sanderson, 2010), as other researchers have suspected so far (Bybee et al., 2021). We are therefore reluctant to suggest alternative evolutionary histories, at least until the inclusion of additional molecular data supports or refutes our topological relationships (cf. Bybee et al., 2008, 2021; Carle et al., 2008). Coenagrionidae and Platycnemididae were both resolved as monophyletic ($BS = 90$, $PP = 1.00$), and our divergence estimates suggest a Cretaceous origin for their rapid radiation (Supplementary Information Appendix S9 and the individual Newick tree file therein), probably accompanied by a Mesozoic-to-Cenozoic ecological release of a similar magnitude to that hypothesised for calopterygids (Dumont et al., 2005). ‘Core’ and ‘ridge-faced’ coenagrionids were reasonably supported in our analysis ($QC > 0.2$), although many of the existing branches within the narrow-winged damselflies were still generally poorly resolved ($BS < 90$, $PP < 0.95$, $QC < 0.2$), probably as a result of the multiple radiation events experienced by these conspicuous insects (Sánchez-Herrera and Ware, 2012). The charismatic New World ‘protoneurids’ (e.g., *Amazona*, *Drepanoneura*, *Epipleoneura*, *Neoneura*, *Protoneura*, and *Psaironeura*) formed a well-supported clade falling outside the ‘ridge-faced’ complex (Supplementary Information Appendix S3, Fig. S1), a finding which is similar to the position found by Dijkstra et al. (2014b). Interestingly, the phylogeny presented is also among the first topologies to include high internode support ($BS = 90$, $PP = 0.95$) for the New World genus *Argia* as sister to the ‘core’ complex, a finding that is consistent with morphology but disagrees with recent targeted genomic analysis (Bybee et al., 2021).

The new phylogenetic reconstruction suggests that Calopterygoidea comprises a pectinate assemblage (Fig. 1; Supplementary Information Appendix S3, Fig. S1), mostly congruent with the recent molecular study of Bybee et al. (2021). Mutual relationships among calopterygoids have left mostly uncertain and are still subject to considerable debate (e.g., Carle et al., 2008; Dijkstra et al., 2014b; Bybee et al., 2021). The Chinese and Vietnamese Priscagrionidae (which includes the enigmatic genera *Priscagrion* and *Sinocnemis*) appeared highly supported as sister to the remaining Calopterygoidea + Coenagrionoidea ($BS = 99$, $PP = 0.95$). We also confirmed the close relationship between the Australian *Diphlebia* and *Lestoidea* (whose sister relationship was based on their similar venation ground plan and larvae morphology; Novelo-Gutiérrez, 1995) with the Asian family Euphaeidae (cf. Bybee et al., 2008, 2021; Carle et al., 2008; Dijkstra et al., 2014b) with reasonably good support ($BS = 90$, $PP = 0.98$). A sister group relationship between the Oriental *Philosina* and *Rhinagrion* was found (cf. Dijkstra et al., 2014b; Bybee et al., 2021), both resembling each other in the adult stage and sharing a clear nymphal apomorphy in their caudal gills (Kalkman et al., 2010). The South American *Heteragrion* and *Oxystigma* were recovered together with high support ($BS = 97$, $PP = 0.98$), although our topology (Supplementary Information Appendix S3, Fig. S1) slightly disagreed with the Bayesian analysis of Bybee et al. (2021) by excluding the Neotropical ‘megapods’ *Dimeragrion* and *Heteropodagrion* from a monophyletic Heteragrionidae, not least because the Amazon family Dicteriadidae and the Cuban endemic *Hypolestes* intermingled. The clade formed by the monotypic *Amanipodagrion* from the Tanzanian Usambara Mountains in tropical East Africa and the Oriental *Mesopodagrion* is not supported by our phylogenetic analysis either (cf. Bybee et al., 2021), a finding that is not surprising considering the clear morphological and behavioural differences between their respective families Amanipodagrionidae and Mesopodagrionidae. We also recovered the Central American family Thaumatoeuridae (including the two genera *Paraphlebia* and *Thaumatoeuria*) with the Malasian *Bornargiolestes* as its sister group (cf. Dijkstra et al., 2014b). However, *Bornargiolestes*, *Dimeragrion*, and *Heteropodagrion* were all considered *incertae sedis* (Supplementary Information Appendix S3, Fig. S1). The distinctive Oriental genus *Caliphaea* and the Thai endemic *Noguchiphaea*, each representing a monogeneric tribe, and the strictly New World hetaeriniids were confirmed as the sister groups of the remaining monophyletic Calopterygidae (cf. Rehn, 2003; Bybee

et al., 2008, 2021; Dijkstra et al., 2014b). Similar to the observation of Dijkstra et al. (2014b), the branching order (Supplementary Information Appendix S3, Fig. S1) revealed several geographically restricted tribes within Calopteryginae, i.e., the Afrotropical Saphoini (e.g., *Phaon*, *Sapho*), and the largely Oriental Vestalini (e.g., *Vestalis*), Calopterygini (e.g., *Matrona*, *Neurobasis*, with *Calopteryx* extending across most of the Holarctic realm), and Mnaisini (e.g., *Archineura*, *Echo*). Despite the shared use of conspicuous wing coloration patterns for communication (Kohli et al., 2021), Calopterygidae and Chlorocyphidae were not recovered as strictly sister taxa. Specifically, our topology (Supplementary Information Appendix S3, Fig. S1) did not support the Afrotropical Chlorocyphinae (e.g., *Africocypha*, *Chlorocypha*) as the sister group of all Asian chlorocyphid lineages, such as Bechly’s (1996) Rhinocyphinae (e.g., *Aristocypha*, *Rhinocypha*) and Libellaginae (e.g., *Libellago*, *Pachycypha*), as well as the clear-winged *Indocypha* and the Sulawesi *Disparocypha*. Instead, the Philippine endemic *Cyrano* stood apart from the remaining Asian radiations of chlorocyphid damselflies (Dijkstra et al., 2014b). Branch support values were, however, relatively low for some shallow branches ($BS < 90$, $PP < 0.95$, $QC < 0.2$) and a further examination of subfamilial relationships with an extended sampling scheme is still necessary.

The phylogenetic positions of dragonfly lineages within Aeshnoidea, Petaluroidea, Gomphoidea, and Cavilabiata (sensu Bechly, 1996) are still subject to considerable debate (e.g., Dumont et al., 2010; Ballare and Ware, 2011; Carle et al., 2015; Bybee et al., 2021; Kohli et al., 2021). Our analyses did not recover Gomphidae and Petaluridae as sister taxa (Supplementary Information Appendix S3, Fig. S2). Instead, clubtail dragonflies were found as sister to the clade containing Aeshnoidea (Austropetaliidae + Aeshnidae) and Petaluridae (cf. Bybee et al., 2008; Fleck et al., 2008; Fleck, 2011), with gomphoids and aeshnids diversifying around 130 Ma and 70 Ma ago, respectively (Supplementary Information Appendix S9). The results presented here clearly ($BS = 97$, $PP = 0.95$) did not support recent topologies that have recovered Austropetaliidae and the large and widespread family Aeshnidae as sisters to all other dragonflies (e.g., Bybee et al., 2008; Davis et al., 2011; Carle et al., 2015; Kohli et al., 2021; Suvorov et al., 2022). In contrast, our phylogenetic analyses (Supplementary Information Appendix S3, Fig. S2) placed Aeshnoidea, Petaluroidea, and Gomphoidea together in a monophyletic group that is hypothesised to share a putative synapomorphy, i.e., the fusion of sternites and postpleurites in larval abdominal segments (Fleck et al., 2008; Fleck, 2011). Whether Gomphidae is sister to libelluloid or petaltail dragonflies is of particular interest because it influences interpretations of the evolutionary scenarios behind exophytic oviposition behaviour (see above). Previous studies disagreed with the topological relationship proposed here (e.g., Bybee et al., 2008, 2021; Kohli et al., 2021; Suvorov et al., 2022), although recent four-cluster likelihood mapping and multispecies coalescent models from the large transcriptome dataset of Kohli et al. (2021) suggest that these branches are still conceivably difficult to resolve owing to ancient rapid radiation and incomplete lineage sorting. Indeed, the observed discordance levels of this internode in our analysis ($QD \sim 0.3$; Pease et al., 2018) agree with Suvorov et al. (2022) and indicate that additional underlying processes (e.g., ancestral introgression) might have also added noise to phylogenetic signals in this portion of the topology. Although the monophyly of aeshnoids, petaltails, and gomphoids appears well founded in our tree topology, constraining relationships with those obtained in previous research (e.g., Bybee et al., 2008, 2021; Dumont et al., 2010; Ware et al., 2014; Kohli et al., 2021) suggest that further analyses will be necessary to resolve these branches.

The diversity of aeshnid and clubtail dragonflies for which ribosomal, nuclear, and mitochondrial protein-coding data are available is still relatively limited for phylogenetic evaluation (Carle et al., 2015). Interestingly, though, our trees recovered concordant groupings for both families (Supplementary Information Appendix S3, Fig. S2). For instance, the subfamily Ictinogomphinae (including the tribe Hemi-gomphini) was placed as sister to the remaining clubtail dragonflies

(Carle et al., 2015) with strong support (BS = 93, PP = 1.00), whereas the morphologically and ecologically similar *Epiaeschna* (eastern North America), *Brachytron* (western Palaearctic), *Aeschnophlebia* (eastern Asia), and *Nasiaeschna* (eastern North America) formed a well-supported clade that appeared sister to most hawkers (cf. Dijkstra and Kalkman, 2012). On the other hand, our phylogenetic reconstructions supported that the relict family Petaluridae originated 140 Ma ago (Supplementary Information Appendix S9), although no strict reciprocally monophyletic assemblages representing their Laurasian and Gondwanan remnants were uncovered (cf. Ware et al., 2014): the Japanese and western North American *Tanypteryx* was found as sister to the eastern North American *Tachopteryx* and the Southern Hemisphere-distributed Petalurinae (Supplementary Information Appendix S3, Fig. S2), albeit internal branches showed relatively low support (BS < 90, PP < 0.95). Hence, we suggest accepting this interpretation with caution.

Our results consistently recovered Cavilabiata with a mid-Jurassic origin, around 165 Ma ago (Fig. 1; Supplementary Information Appendix S9). Following Carle et al. (2015), the extremely species-rich superfamily Libelluloidea is herein restricted to Synthemistidae, Macromiidae, Corduliidae, and Libellulidae. Chlorogomphidae and Cordulegastridae formed monophyletic groups with full support (BS = 98–99, PP = 1.00), both leading to libelluloids and being separated by relatively long internodes. This relationship has already been found in previous studies (e.g., Carle et al., 2015; Letsch et al., 2016; Bybee et al., 2021) and is founded on several shared nymph characters, including the dentition and shape of the labial mask. Libelluloidea was estimated to appear during the Early Cretaceous (140 Ma ago; Supplementary Information Appendix S9) and consisted of four monophyletic clades (QC > 0.2), with Synthemistidae (BS = 92, PP = 1.00) and Macromiidae (BS = 91, PP = 1.00) particularly well supported. Our age estimate for this group is relatively similar to that of Carle (1995), Sánchez-Herrera and Ware (2012), and the more recent work of Kohli et al. (2021), who originally supported the hypothesis of geographically isolated divergence after vicariant fragmentation and tectonic splitting of the supercontinent Pangaea for libelluloid dragonflies. For clarity, synthemistids are here tentatively regarded as equivalent to the informally called ‘GSI’ of Ware et al. (2007), including Synthemistidae (sensu Tillyard, 1917) and higher non-corduliine libelluloids (Fraser, 1954, 1957). In agreement with Ware et al. (2009), the Australian taxa *Cordulephya*, *Hesperocordulia*, and *Micromidia* were recovered in a clade, as well as the European *Oxygstra* and the Indomalayan *Idionyx*, but both synthemistid groups were recovered with relatively low bootstrap support and posterior probability (BS < 90, PP < 0.95). On the other hand, the Australasian *Archaeosynthemis*, *Choristhemis*, *Eusynthemis*, *Synthemopsis*, and *Synthemis* were found as a separate clade (cf. Letsch, 2007; Letsch et al., 2016; Bybee et al., 2021). Our divergence estimates (Supplementary Information Appendix S9 and the individual Newick tree file therein) agree with Ware et al. (2009) and suggest that these ‘GSI’ clades diverged during the Cretaceous approximately 70–110 Ma ago, which broadly corresponds with the rifting of Gondwana and the early separation of India and Madagascar from Australia, Antarctica, and New Zealand (McIntyre et al., 2017). Overall, our findings strongly support the idea of synthemistids as an expanded monophyletic lineage deserving further study and more extensive data sampling to fully resolve the arrangement of clades.

Our phylogenetic analysis (Supplementary Information Appendix S3, Fig. S2) revealed that corduliids are placed as the sister group of libellulid dragonflies, and macromiids as sister to the clade comprising Corduliidae + Libellulidae. *Macromia*, the North American *Didymops*, and the African *Phyllomacromia* were recovered in a fully supported (BS = 91, PP = 1.00) Macromiidae, a finding that is consistent with nearly all previous molecular analyses (e.g., Letsch, 2007; Letsch et al., 2016; Bybee et al., 2008, 2021; Dumont et al., 2010; Carle et al., 2015) since the pioneering morphological work of Gloyd (1959). Macromiids are a well-established monophylum justified by several nymphal and adult synapomorphies, such as the frontal horn between their eyes or a set of

secondary penile features, respectively (for details, see Bybee et al., 2021). As well as in Carle et al. (2015), *Hemicordulia* and *Procordulia* clearly belonged to Corduliidae, a finding that clearly refutes a familial status for Hemicorduliidae. Given our relatively limited sampling of corduliid dragonflies, we cannot address their complex evolutionary relationships further. There are also tenuous below-familial level relationships among groups of libellulids (cf. Carle et al., 2015), although we were available to confirm that the primarily Neotropical or southern Nearctic Dythemistinae appeared sister to the remaining Libellulidae (BS = 91, PP = 0.96). Compared to other dragonflies, libellulids are highly derived and present the signature of a rapid radiation around and shortly after the K–Pg boundary (Supplementary Information Appendix S9 and the individual Newick tree file therein), with relatively short internodes and long terminal branches (Supplementary Information Appendix S3, Fig. S2). Unfortunately, the monophyly and placement of each subfamily cannot evidently be discussed with our multilocus, target gene approach (for details, see Ware et al., 2008), at least until its final topology is completely settled. However, the timing of radiation for libellulids suggests that the evolution of this clade might have been related to the transformative cataclysmic event that laid the foundations for the charismatic lacustrine and fluvial faunas of today (García-Girón et al., 2022a), probably as a result of their expansion into newly vacated niches and subsequent exploitation of freshwater habitats in the aftermath of the K–Pg mass extinction (Carle, 2012; Carle et al., 2015).

4.2. Ephemeroptera (mayflies)

The results of our phylogenetic analyses strongly support a monophyletic Ephemeroptera with BS and PP values of 100 and 1.00, respectively (Supplementary Information Appendix S4). Quartet support for the monophyly of mayflies is also strong (QC > 0.2). This finding is not surprising since the monophyletic relationship of the mayflies is beyond question (reviewed by Monaghan and Sartori, 2009), as well as the establishment of their true earliest lineages during the Triassic (Fig. 1; Barber-James, 2010). The use of strict ribosomal and modern nuclear and mitochondrial protein-coding data in this group of insects is relatively recent (e.g., Ogden and Whiting, 2005; Sun et al., 2006; Ogden et al., 2009a). So far, the origins and evolution of certain lineages of mayflies have presented challenges for phylogenetic inference as a result of their ancient rapid radiations (reviewed in Sivaramakrishnan et al., 2011 and Misof et al., 2014). Classifications have been mostly morphological in nature and most phylogenetic schemes since the early 1970s have recognised major subordinal groupings and complex higher-level relationships under different names (e.g., Henning, 1966, 1979; Edmunds Jr., 1973; McCafferty and Edmunds Jr., 1976, 1979). The pioneering and widely followed classification of McCafferty and Edmunds Jr. (1979) constituted the basis for McCafferty (1991) putative phylogenetic relationships within and among suborders, where Schistonota was replaced by three different suborders, i.e., Pisciforma, Setisura, and Retracheata. Later modifications of this system (Wang and McCafferty, 1995; McCafferty, 1997, 2004; McCafferty and Wang, 2000) and the non-raking classifications of Kluge (1988, 2004) are roughly congruent (reviewed in Ogden and Whiting, 2005 and Barber-James et al., 2008), i.e., Pisciforma sensu McCafferty / Tridentisata sensu Kluge, Setisura, Furcatergalia [supported by presumed anatomical synapomorphies similar to McCafferty, 1991 original Retracheata], and Carapacea sensu McCafferty / Posteritorna sensu Kluge. However, phylogenetic relationships within and among suborders inferred from molecular data provided conflicting conclusions on the general structure of the mayfly ‘Tree of Life’ (e.g., Ogden and Whiting, 2005; O’Donnell and Jockusch, 2008; Ogden et al., 2009a; Miller et al., 2018), and positions for different clades remain largely unclarified. These inconsistencies between morphological and molecular data have been connected to the superficial external morphological similarities and the propensity for rampant homoplasy in mayfly characters in their nymphal and alate stages (Ogden and Whiting, 2005; Ogden et al.,

2009a). For reasons of consistency with previous research, the relationships presented here follow the most recent McCafferty hypothesis (McCafferty, 2004) and its subsequent modifications from Ogden and Whiting (2005).

In our phylogenetic analyses, the subordinal groups Pisciforma, Setisura, and Furcatergalia, the superfamilies Baetoidea, Ephemeroidea sensu McCafferty, and Siphonuroidea, and seven families (i.e., Ameletopsidae, Coloburiscidae, Ephemeridae, Leptohebiidae, Leptophlebiidae, Oniscigastridae, and Siphonuridae) were not supported as monophyletic (Supplementary Information Appendix S4). On the other hand, the infraorder Pannota, the superfamilies Caenoidea and Ephemerelloidea, and 11 mayfly families (i.e., Baetidae, Behningiidae, Caenidae, Ephemerellidae, Euthyplociidae, Heptageniidae, Metretopodidae, Nesameletidae, Oligoneuriidae, Potamanthidae, and Teloganodidae) were supported as monophyletic. Other families (e.g., Dipteromimidae, Ichthybotidae, Isonychiidae, Rallidentidae, and Siphlaenigmatidae) were monogeneric. However, as already noted by pioneering work of Ogden and Whiting (2005), some of these recognised families might be apomorphic genera of other families. More specifically, the putative Pisciforma and its two proposed superfamilies Baetoidea and Siphonuroidea (McCafferty, 2004) were not supported as being monophyletic because Baetidae was not supported as nesting within the other pisciform mayflies (Supplementary Information Appendix S4). This finding is consistent with Ogden and Whiting (2005) and Ogden et al. (2009a) and support the fishlike body form adapted for swimming as a plesiomorphic condition of mayfly ancestors. Still, our topology does not corroborate the endemic New Zealand *Siphlaenigma* as a lineage nesting with the other baetid taxa (cf. Staniczek, 1997). Instead, we found a relatively stable and well-supported relationship (BS = 91, PP = 0.97) between Baetidae and Oligoneuriidae. Our result of oligoneuriids being a close sister lineage to baetids remains enigmatic, as most previous morphological and molecular hypotheses commonly placed this family as sister to Furcatergalia (Ogden and Whiting, 2005; Ogden et al., 2009a) or closely related to an evolutionary sequence from primitive to more derived Setisura (Riek, 1973; McCafferty, 1990, 1991). The relatively long branch length of Baetidae might be playing a role to advocate for Baetidae + Oligoneuriidae. The low skew in discordant frequencies in this internode (QD > 0.5; Pease et al., 2018) and the moderately concordant quartet fidelity (QF) scores of oligoneuriid taxa ($0.53 \leq QF \leq 0.73$) indicate that conflicts due to a strongly biased biological process (e.g., secondary evolutionary history or ancestral introgression) cannot *alone* explain the phylogenetic signal detected here. The proportion of informative quartets in this portion of the topology (QI ~0.4; Pease et al., 2018), though, suggests that our molecular dataset still contains relatively low information to resolve the controversial phylogenetic placement of these groups. Interestingly, our old branching of oligoneuriids (Supplementary Information Appendix S4) and their calibrated early divergence estimates (Supplementary Information Appendix S9) during the Jurassic and Early Cretaceous (113–178 Ma) agree with the combined mitochondrial and nuclear dated phylogenies of Barber-James (2010), suggesting that the relationships between these clades may still be far from resolved.

As suggested by previous researchers (e.g., Ogden and Whiting, 2005), Nesameletidae (BS = 91, PP = 1.00) and Metretopodidae (BS = 91, PP = 0.99) were recovered with high phylogenetic support. The Japanese endemic *Dipteromimus* was not supported as nesting within Siphonuridae (cf. Ogden and Whiting, 2005; Ogden et al., 2009a), a finding that supports a familial status for Dipteromimidae. Another interesting outcome from our research is that Dipteromimidae and its sister groups may not have an origin as ancient as originally proposed by Ogden et al. (2009a). These authors extended back the early divergence of their ancestors to the time of Pangaea. Instead, our divergence estimates suggest that the majority of the cladogenesis in these assemblages occurred in Laurasia (Ameletidae, Siphonuridae, Dipteromimidae) and Gondwana (Ameletopsidae, Oniscigastridae) after the fragmentation of the continental shield, during the Late Cretaceous and after the K–Pg

boundary (Supplementary Information Appendix S9 and the individual Newick tree file therein). These inferences refuted Kluge et al. (1995) intuition that divided the superfamily Siphonuroidea into major family groups corresponding with differential diversification hotspots in each hemisphere. Despite several plesiomorphic characters in the hindwings and forewings, our topological reconstruction (Supplementary Information Appendix S4) corroborates the early findings from Ogden et al. (2009a) that the Southern Hemisphere-distributed Oniscigastridae is a paraphyletic assemblage (BS = 97, PP = 0.95) with an amphinotic distribution, i.e., the New Zealand *Oniscigaster*, the southern Neotropical *Siphonella*, and the southeastern Australian and Tasmanian *Tasmanophlebia*. The sequence of splitting within the also amphinotic Ameletopsidae in our phylogenetic exercises agrees with this latter pattern (cf. Edmunds Jr., 1972), i.e., the paraphyletic assemblage comprising the endemic Patagonian *Chiloporter* and *Chaquihua* branched off the New Zealand genus *Ameletopsis* approximately 30 Ma ago (Supplementary Information Appendix S9 and the individual Newick tree file therein), after the drowning of the Weddellian Isthmus that likely prevented faunal interchanges through the formerly contiguous Antarctica and the southern Andes (Reguero et al., 2014). Interestingly, our divergence estimate also lies close to the Eocene–Oligocene (E–Og) transition that marked the largest Cenozoic synchronous climate reorganisation and floral and faunal turnover event in present-day Patagonia (Selking et al., 2015) and Australasia (Hutchinson et al., 2021), although further studies are still needed to verify the correlation between this boundary and southern mayfly diversification (but see Bisconti et al., 2016 and Vuataz et al., 2016). On the other hand, the relationships among Coloburiscidae, Isonychiidae, Heptageniidae, and Oligoneuriidae (Supplementary Information Appendix S4) confirm that the concept of Setisura is highly polyphyletic (Fig. 1) and was originally mostly based on convergences. So far, our results also suggest that each heptagenioid line separated from their subordinal counterparts at different times during the Mesozoic (Supplementary Information Appendix S9 and the individual Newick tree file therein). Our topology also confirmed the inclusion of *Arthroplea* and *Pseudiron* within the other heptageniid taxa as proposed by previous studies (Edmunds Jr. and Traver, 1954; Ogden and Whiting, 2005).

Our data does not support the monophyly of Furcatergalia. The cosmopolitan and highly diversified Leptophlebiidae was found as non-monophyletic due to the position of the family outside the rest of Furcatergalia (cf. O'Donnell and Jockusch, 2008; Monjardim et al., 2020). In our reconstructions, Pannota was well supported as monophyletic (BS = 97, PP = 0.96) and was recovered as sister to the clade containing the burrowing mayflies (BS = 94, PP = 0.95), with the family Behningiidae nesting within Potamanthidae and rendering the remaining Fossoriae as monophyletic (cf. Ogden and Whiting, 2005; Ogden et al., 2009a; Miller et al., 2018). The families Euthyplociidae and Potamanthidae were also supported as monophyletic, whereas the position of the burrowing Ephemeridae did not support its monophyly, not least because the New Zealand endemic *Ichthybotus* intermingled (Supplementary Information Appendix S4). Interestingly, even if the more conservative approach of McCafferty (1991) is taken, Ephemeridae would resolve as a paraphyletic assemblage comprising the subfamilies Ephemerinae, Hexageniinae, and Ichthybotinae (cf. Miller et al., 2018). Within pannota groups, Caenidae was found as sister to a monophyletic Ephemerelloidea (BS = 97, PP = 0.96), contradicting Kluge's concept of Fimbriatotergeraliae (Ogden et al., 2009a). Within this superfamily, the monophyletic Ephemerellidae assembled in groups comparable to Ogden et al. (2009b) and was sister to all other clades with relatively high support (BS = 90, PP = 0.95), although further ephemerelloid relationships remained mostly unresolved as in previous morphological and mitogenome studies (Jacobus and McCafferty, 2006; Ogden et al., 2009b; Xu et al., 2020; Yu et al., 2021). These and some other higher-level relationships within Ephemeroptera (e.g., the polyphyletic nature of pisciform mayflies) are still problematic and a balance has not yet been reached (Sivaramakrishnan et al., 2011). Indeed, we recognise that

certain portions of our phylogeny were non-robust (e.g., the ambiguous positions within and between Heptagenioidea and Siphonuroidea) and conclusions about their relationships and radiation events are still difficult to confirm. For example, long-branch attraction caused by rapid evolutionary rates in early-diverging Ephemeroptera and methodological changes in sequence alignment and phylogenetic inference for nuclear and mitochondrial protein-coding genes may result in parameter perturbation and inconsistent ancient phylogenetic reconstructions among available studies (e.g., Rutschmann, 2015; Xu et al., 2020). Under these circumstances, the inclusion of an increased suite of available molecular data in our phylogenetic exercises and subsequent divergence estimates may help elucidate the conflicting evolutionary history of this lineage. Still, many of the familial and higher-level relationships recovered here are in line with most traditional morphological classifications (e.g., Kluge, 1988, 2004; Wang and McCafferty, 1995; McCafferty, 1997, 2004; McCafferty and Wang, 2000) and molecular evidence (e.g., Ogden and Whiting, 2005; O'Donnell and Jockusch, 2008; Ogden et al., 2009a; Miller et al., 2018).

Most interestingly, the chronology of mayfly diversification in our time-calibrated phylogeny (Fig. 1; Supplementary Information Appendix S9) appears to fit early intuitions worked out by Edmunds Jr. (1972, 1975) and others (e.g., Sinitshenkova, 1985; McCafferty, 1991; Brittain and Sartori, 2003) that there was once a burst of mayfly evolution during the Cretaceous (for a review, see Barber-James et al., 2008). Also, our phylogenetic topology and divergence estimates contribute to discussions around the role of the Gondwanan continental drift on mayfly biogeography, the mayfly gill to wing hypothesis (which assumes that highly muscled and movable gills evolved into wings), the evolution of mandibular tusks in burrowing clades, and the relationships between habitats and feeding behaviours (see below). For instance, the variable and derived phylogenetic position of mayflies with highly movable gills (e.g., *Choroterpes*, *Heptagenia*, *Macdunnia*, *Paraleptophlebia*, *Stenonema*), including the relatively derived branching of *Callibaetis* and *Centropitulum* within Baetidae (Supplementary Information Appendix S4), confirms early findings from Ogden and Whiting (2005) regarding the mayfly gill to wing origin hypothesis. We therefore suggest that movable gills may be a plastic and convergently derived character with multiple independent gains across the mayfly 'Tree of Life' to create water currents and thereby facilitate respiration in lentic systems with low levels of dissolved oxygen.

Considering their deduced low vagility, brief alate phase, and strict habitat constraints (Brittain, 1982), mayfly distributions and global diversification centres were assumed to reflect the sequence of continental drift, microplate disjunction, mountain uplifts, and oceanic transgressions (Edmunds Jr., 1972). Nevertheless, mayflies seem to disperse more than was intuitively obvious before the new millennium, and there is now evidence for bidirectional mainland-island and trans-marine dispersal in the Azores (Brinck and Scherer, 1961), the Canary Islands (Rutschmann et al., 2014), Madagascar (Monaghan et al., 2005; Vuataz et al., 2013), Madeira (Gattolliat et al., 2008), La Réunion (Gattolliat, 2004), the proto-Papuan archipelago (Cozzarolo et al., 2019), and the Seychelles (Perkins, 1933). In this regard, our divergence time estimates (Supplementary Information Appendix S9) and current distributions of Tricorythidae are consistent with the transport of the Oriental genus *Sparsorythus* (118–158 Ma ago) from eastern Gondwana to Asia after the drift of the Indian plate during the Early Cretaceous. The connection of East and West Gondwana is partially attested by the evolutionary relationships of leptophlebiid lineages in our phylogenetic reconstructions. Our topology (Supplementary Information Appendix S4) did not return Leptophlebiidae as a clade (see above). The deeper level placement of the clustered, Northern Hemisphere-distributed leptophlebiines and habrophlebiines outside the rest of Furcatergalia received weak support (BS < 90, PP < 0.95, QC < 0.2), but this paraphyly did not seem to result from secondary evolutionary history, introgression (QD ~ 0.6; Pease et al., 2018), or uncertainty associated to uninformative nucleotide sites (QI ~ 0.6; Pease et al., 2018). Instead, it

might have arisen caused by the use of different phylogenetic methods (Ogden and Whiting, 2005; Ogden et al., 2009a) and sets of gene fragments (e.g., O'Donnell and Jockusch, 2008; Monjardim et al., 2020) when compared to previous works, making us interpret this phylogenetic relationship as still unresolved. The estimated age for the origin of atalophlebiines was 140 Ma ago (120–159 Ma ago), during the initial fragmentation of East Gondwana (McIntyre et al., 2017). Divergence estimates for the *Choroterpes* group (sensu O'Donnell and Jockusch, 2008) support the affinities of atalophlebiines from southern Asia and the Indian Ocean Islands with African taxa (e.g., *Choroterpes*, *Choroterpides*) through trans-marine dispersal that postdated the early separation of India from mainland, whereas the New Zealand entomofauna from the pan-continental Palaeoaustral group (sensu O'Donnell and Jockusch, 2008) suggest that New Zealand atalophlebiines (*Zephlebia-Neozephlebia*; Supplementary Information Appendix S4) evolved mostly independently from their Southern Hemisphere-distributed counterparts during the last 80 Ma (Supplementary Information Appendix S9 and the individual Newick tree file therein). These findings support recent conclusions from Gatti et al. (2021) and suggest that, while it is possible to conjecture about colonisation and diversification only by a deduced sequence of continental movements, this does not really seem feasible across all mayfly lineages. Instead, evolutionary pathways in leptophlebiids were shaped by complex processes of overseas dispersal routes and vicariance. These processes are similar to what has been found for Tyrrhenian (Gattolliat et al., 2015) and Caucasian (Hrivniak et al., 2020) mayflies at the regional scale and for aquatic chironomid midges (Sæther and Ekrem, 2003) and gerromorphans globally (Møller-Andersen, 1982).

The position of potamanthids outside the rest of Ephemeroidea (Supplementary Information Appendix S4) contradicts McCafferty (2004) concept of Scaphodonta, which includes the tuskless Behningiidae (whose nymphs lack the tusks that protrude from the outer portion of the mandible) as sister to the tusked burrowers, as well as partly supports Kluge's concept of Fossoriae, with behningiids nesting within the burrowing mayflies (see above). These findings are mostly in agreement with recent anchored phylogenomic evidence (Miller et al., 2018) and early morphology-based hypotheses that placed the predatory Behningiidae as closely related to Potamanthidae (Edmunds Jr. and Traver, 1959). The monophyly of the burrowers is still an enigmatic issue after many years of entomological research (McCafferty, 1991, 2004; Kluge, 2004; Ogden and Whiting, 2005; Ogden et al., 2009b; Miller et al., 2018), although it proved to be strongly supported in our phylogenetic exercises (BS = 94, PP = 0.95). These relationships conflict with the proposal of Ogden and Whiting (2005) and Ogden et al. (2009b), who consistently recovered potamanthids as sister to the other burrowing mayflies and Pannota. These studies suggested either a single origin of the burrowing behaviour from a leptophlebiid-like ancestor eventually lost in the branch subtending the pannote groups, or two convergent origins of this lifestyle (both in Potamanthidae and Fossoriae), with a subsequent secondary loss of mandibular tusks and a retained semi-burrowing behaviour in behningiids. Rather, our phylogenetic reconstruction supports the notion that the burrowing lifestyle in mayflies emerged once during the Jurassic (Supplementary Information Appendix S9 and the individual Newick tree file therein) and that mandibular tusks were secondarily lost in the burrower lineage leading to the Behningiidae (Supplementary Information Appendix S4). The evolution of burrowing and the loss of mandibular tusks in behningiid nymphs have been explained as a morphological adaptation to preying in the sandy substrate (Keltner and McCafferty, 1986; Miller et al., 2018), an idea that was originally introduced by Edmunds Jr. (1972) to explain microhabitat relationships and feeding behaviours within Oniscigastriidae. Interestingly, the paraphyletic relationship and divergence estimates for this latter amphinotic lineage found in our phylogenetic reconstructions (see above) agrees with his pioneering views. Briefly, the benthic lifestyle and burrowing behaviour after maturity in the genus *Oniscigaster* (compared to the burrowing

behaviour of the Neotropical *Siphonella* and Australian *Tasmanophlebia* larvae only after hatching) might be an adaptation to the sandy bottoms of New Zealand streams after the separation of Zealandia from Australia and the rest of the original Gondwanan landmass approximately 70 Ma ago (Supplementary Information Appendix S9 and the individual Newick tree file therein).

4.3. Plecoptera (stoneflies)

Our phylogenetic analyses (Fig. 1) support the clear monophyly (BS = 99, PP = 1.00) of the stonefly suborders Arctoperlaria and Antarctoperlaria (Zwick, 1990). This finding is concordant with previous molecular evidence (e.g., McCulloch et al., 2016; Ding et al., 2019; South et al., 2021a; Letsch et al., 2021) and the currently most widely accepted classification system of Plecoptera based on morphological data (Zwick, 1973, 1980, 2000). This clear distinction at a molecular level is evidenced by very few derived character and behavioural expressions. For instance, arctoperlarians are united in Zwick's system by the single putative synapomorphy of drumming, a complex intersexual vibrational communication system (Zwick, 2000; Stewart and Sandberg, 2006). In contrast, antarctoperlarian stoneflies are united by two leg muscle characters and the presence of floriform chloride cells (Zwick, 2000). In general, our analyses returned relatively well-supported relationships for the resolution of previously contested hypotheses across the depth of the phylogeny (Supplementary Information Appendix S5) and the elucidation of novel evolutionary relationships among several clades at shallower levels (see below). Within Antarctoperlaria, the two recognised superfamilies Eusthenioidea (including Eustheniidae and Diamphipnoidea) and Gripopterygoidea (including Austroperlidae and Gripopterygidae) were supported as reciprocally monophyletic clades (BS = 90–97, PP = 1.00). The monophyly of Euholognatha and Systellognatha is also beyond doubt in our analyses (BS = 95, PP = 0.98). Interestingly, our results indicate that the evolutionary divergence between the two major stonefly suborders (the mainly Northern Hemisphere-distributed Arctoperlaria and the Southern Hemisphere-distributed Antarctoperlaria) dates at 170 Ma ago (163–176 Ma ago). This scenario may be consistent with independent, geographically isolated divergence after vicariant fragmentation and tectonic splitting of the supercontinent Pangaea in the Jurassic, with the majority of cladogenesis and subordinal diversification of most plecopteran families occurring during and after the Cretaceous (Fig. 1; Supplementary Information Appendix S9 and the individual Newick tree file therein). The dates presented here are considerably younger than estimates based on few genuine fossils (e.g., Béthoux et al., 2011; Cui et al., 2019). The analysis of Letsch et al. (2021) based on a transcriptomic and Sanger sequence dataset placed stem Plecoptera at a median age of 265 Ma, with confidence intervals extending back to 294 Ma. Nevertheless, since the maximum age of the closest fossil calibration point in Letsch et al. (2021) still lacks definitive character states shared by extant remnants of the order (sensu Béthoux et al., 2011), further analyses will be necessary to resolve this uncertainty. Previous divergence estimates based on mitogenomes (Ding et al., 2019) and combined mitochondrial and nuclear genes (McCulloch et al., 2016) resulted in divergence estimations similar to our chronology. Perhaps more interestingly, while the emergence times of stem stoneflies are older in Letsch et al. (2021) than in previous molecular analyses, the dates and relationships for subordinal groupings presented here (e.g., radiation of the families within Arctoperlaria and Arctoperlaria) seem palaeobiogeographically consistent and are not in a strict conflict with their confidence intervals (see below).

Within antarctoperlarian stoneflies, neither South American nor Australasian eustheniids and austroperlids are represented by reciprocally monophyletic assemblages, although there are several geographically restricted clades within Gripopterygidae (Supplementary Information Appendix S5). For instance, the only austroperlid genus in New Zealand (*Austroperla*) appears with a close affinity to a clade

containing both South American (e.g., *Klapopteryx*) and Australian (e.g., *Acruroperla*) genera, whereas the endemic *Zelandobius* and *Stenoperla* are placed sister to clades containing a set of South American gripopterygid (consistent with the subfamily Antarctoperlinae; McLellan, 1993) and eustheniid taxa, respectively. These findings are consistent with the pioneering molecular study of McCulloch et al. (2016). Indeed, these authors refuted the placement of Antarctoperlinae as part of Austroperlidae, originally suggested by Zwick (1973) based on morphological comparative analysis, and also found strong support for relatively young affinities of antarctoperlarian lineages from South America, Australia, New Zealand, and several subantarctic islands, as detected here. The majority of the cladogenesis within the polyphyletic assemblages of New Zealand Antarctoperlaria and their overseas sister taxa emerged after the Cretaceous (Supplementary Information Appendix S9 and the individual Newick tree file therein), thereby supporting the theory of circumpolar Cenozoic connections millions of years after the fragmentation of Gondwana (McLellan, 1975; McCulloch et al., 2016; cf. Letsch et al., 2021). Divergence estimates between Australian (e.g., *Eusthenia*, *Thaumatoperla*) and South American (*Neuroperla*, *Neuroperlopsis*) antarctoperlarians around 50 Ma ago in our phylogenetic analysis may also help explain some biogeographical disjunctions. In this case, through vicariant isolation after the rifting of Gondwana and the subsequent opening of the Drake Passage (Scher and Martin, 2006), long after the early separation of Africa, Madagascar, and India (McIntyre et al., 2017), where these stonefly lineages are unrecorded. These conclusions were originally introduced by McCulloch et al. (2016) and further supported by Kirkaldy (2020), although small discrepancies in molecular dating may arise potentially caused by the use of different algorithms and sets of gene fragments.

The evolutionary divergence between the largely Northern Hemisphere-distributed Euholognatha and Systellognatha was dated at 166 Ma ago (159–175 Ma ago), in the Late to mid-Jurassic, with high support (Supplementary Information Appendices S5 and S9). Within euholognathan stoneflies, our phylogenetic analyses recovered the traditionally recognised Leuctridae + Capniidae, which is consistent with the presence of similar morphological characters that facilitate sperm transfer (Illies, 1965; Zwick, 1973, 1980, 2000; Brodskiy, 1982; Nelson, 1984;). This phylogenetic affinity, however, differs from molecular results generated by mitochondrial genomic data (e.g., Chen and Du, 2018; Ding et al., 2019) and single-copy orthologous genes selected from transcriptomes (South et al., 2021a). For instance, Chen and Du (2018) and Ding et al. (2019) recovered Capniidae and Taeniopterygidae as sister taxa, although Chen and Du (2018) did not include Leuctridae in their analysis and the latter study received low branch support for Leuctridae as sister to the remaining Nemouroidea. On the other hand, the recent study of South et al. (2021a) for North American Plecoptera found placement of Leuctridae as inconclusive. Indeed, inconsistent topologies after four-cluster likelihood mapping led the authors to suggest that GC content variation at the third codon position introduced systematic biases in their analyses and compromised family-level relationships within the base of Nemouroidea. However, our results agreed with South et al. (2021a) when it comes to acknowledging the position of Megaleuctrinae within a monophyletic Leuctridae (Supplementary Information Appendix S5). This finding is also consistent with Zwick (2000) and Béthoux (2005), but it refutes Illies (1967) and Terry (2003) hypotheses that placed *Megaleuctra* in Notonemouridae and as a sister lineage to the remaining stoneflies, respectively. Our analyses also support the Asian endemic Scopuridae as a sister taxon to Taeniopterygidae (BS = 98, PP = 0.97), in agreement with the transcriptomic analysis of Letsch et al. (2021), although there are no clear morphological features uniting this group (Zwick, 2000). The need for more data and contradictory findings from previous studies (e.g., Zhao et al., 2020) make us interpret this phylogenetic affinity as pending further verification.

Contrary to the remaining Euholognatha, the family Notonemouridae is restricted to southern temperate regions (Illies, 1965; Zwick,

2000; Fochetti and Tierno de Figueroa, 2008), resembling the distribution of the antarctoperlarian lineages. Thus far, this classical Gondwanan distribution has been difficult to reconcile with phylogenetic theories of stonefly relationships. Here, Notonemouridae formed a monophyletic clade (BS = 92, PP = 1.00) and is not supported as a gradotaxon (cf. Zwick, 2000), with their most common recent ancestor extending back to the Cretaceous 110 Ma ago (95–125 Ma ago). The monophyly of notonemourids has repeatedly been questioned, with suggestions that Notonemouridae may be a polyphyletic or paraphyletic assembly representing multiple independent invasions of early Eurasian nemourid lines through Africa (e.g., Zwick, 1981, 1990, 2000; Fochetti and Tierno de Figueroa, 2008). However, southern notonemourids lack a set of synapomorphies found in Northern Hemisphere Nemouridae (Zwick, 2000). By incorporating more than 90% of recognised notonemourid genera, our tree topology and divergence estimates that postdate the separation of Africa and Australia (Supplementary Information Appendix S9 and the individual Newick tree file therein) agree with McCulloch et al. (2016) and the mitogenomic analysis of Ding et al. (2019), and do not support a phylogeographical scenario of Gondwanan vicariance (Illies, 1965; Zwick, 2000). Instead, the ancestors of Notonemouridae probably spread across ocean basins during the Cretaceous, after the breakup of Gondwana, most likely via the westerly winds surrounding Antarctica (McCulloch et al., 2016). This timing disagrees with Kirkaldy (2020), who found Notonemouridae to form an early, deep sister group to the remaining stoneflies. However, given that their topology largely differs from most recent phylogenetic analyses using mitochondrial and nuclear DNA data (McCulloch et al., 2016), and mitogenomes (Ding et al., 2019), their phylogenetic relationships and molecular dates are not considered further. Equally ambiguous was the origin and splitting of Notonemouridae shown by Letsch et al. (2021), who found persisting unstable relationships within Euholognatha using a constraint-based tree inference approach. However, the authors suggested that uncertainties driven by rapid radiation events and short internodes in euholognathan families hampered their phylogenetic estimates. In our study, however, no data on the genus *Madenemura*, the only stonefly genus present in Madagascar, was available, so its relationship with other notonemourids and their biogeography remains unsolved (Zwick, 2000, 2015).

Among systellognathan families, Styloperlidae, Peltoperlidae, Pteronarcyidae, Perlodidae, Chloroperlidae, and Perlidae were highly supported as monophyletic (BS = 94–98, PP = 0.98–1.00, QC > 0.2), with some of these lineages apparently diversifying post-Mesozoic (Supplementary Information Appendix S9 and the individual Newick tree file therein). This is consistent with the hypothesis that extant families and genera of Systellognatha evolved relatively recently (Zwick, 2000; McCulloch et al., 2016; Ding et al., 2019; but see Cui et al., 2016 and Letsch et al., 2021). Our phylogenetic analyses recovered the superfamily Pteronarcyzoidea as paraphyletic, with the Oriental Styloperlidae as sister group to the remaining Systellognatha (including Peltoperlidae; Supplementary Information Appendix S5), confirming the elevation of the subfamily Styloperlinae proposed by Uchida and Isobe (1989). Zwick (2000) originally summarized the phylogeny of Pteronarcyzoidea with an inner relationship as Pteronarcyidae + (Peltoperlidae + Styloperlidae), although subsequent morphological and molecular studies found inconsistent phylogenetic affinities (e.g., Thomas et al., 2000; Ding et al., 2019; South et al., 2021a; Letsch et al., 2021). Our combined mitochondrial and nuclear hypothesis of Styloperlidae as sister to the remaining Systellognatha (Supplementary Information Appendix S5) agrees with Terry and Whiting (2005) and Zhao et al. (2020), who also found that Perloidea rendered Pteronarcyzoidea paraphyletic. Perhaps more importantly, the phylogenetic relationship presented here is consistent with the geographical distributions of these families: Styloperlidae restricted to the Oriental region, Peltoperlidae in both the Holarctic and Oriental realms, and Pteronarcyidae mostly constrained to the Holarctic region. However, the non-monophyly and internal relationships of Pteronarcyzoidea are debatable and baseline comparisons

among different sequences and algorithms are worth additional study.

The superfamily Perloidea was a strongly supported (BS = 97, PP = 0.99) monophyletic assemblage with Perlidae as sister taxon to Perloidae + Chloroperlidae (Supplementary Information Appendix S5), which is consistent with traditional morphological (Illies, 1965) and molecular analyses (e.g., Chen et al., 2018; Wang et al., 2018, 2019; Letsch et al., 2021). The clade Perloidea has been acknowledged by many systematists based mainly on the occurrence of predatory larvae and the morphology of their mouthparts (Illies, 1965; Zwick, 1973, 1980, 2000; Brodskiy, 1982; Uchida and Isobe, 1989). However, relationships among these three families are yet not fully resolved and all alternative topologies have been proposed, i.e., Perlodidae + (Perlidae + Chloroperlidae) (Zwick, 1973; Brodskiy, 1982; Uchida and Isobe, 1989), Chloroperlidae + (Perlidae + Perlodidae) (Thomas et al., 2000), and a trichotomy (Nelson, 1984; Zwick, 2000). Our phylogenetic hypothesis of Perlidae + (Perlodidae + Chloroperlidae) is not only supported by previous analyses of mitogenomic and transcriptomic data (e.g., Chen et al., 2018; Wang et al., 2018, 2019; South et al., 2021a), but also by the presence of cuticular mandibular spinules in both perlodid (with the exception of the subfamily Isoperlinae, in which the absence would be secondary; Zwick, 2000) and chloroperlid larvae (Stewart and Stark, 2002). In the analysis of South et al. (2021a), Chloroperlidae rendered non-monophyletic due to the placement of the genus *Kathroperla*. South et al. (2021b) defined the family Kathroperlidae as being supported by combined phylogenomic and morphological evidence. This new family seems to be the sister taxon to the remaining Perloidea based on most recent molecular analyses made by these authors (South et al., 2021b). This result (i.e., *Kathroperla* as sister of Perloidea) is also congruent with findings from Terry (2003) and Letsch et al. (2021). Unfortunately, however, this new family was not included in our study.

4.4. Trichoptera (caddisflies)

The results of our phylogenetic analyses (Fig. 1; Supplementary Information Appendix S6) are very similar to original molecular hypotheses for caddisflies (Kjer et al., 2001, 2016; Morse et al., 2019; Thomas et al., 2020; Ge et al., 2023; Grigoropoulou et al., 2022). Our findings support a monophyletic Annulipalpia sister to the paraphyletic (BS = 94%, PP = 0.97) ‘spicipalpian’ families (i.e., Hydroptilidae, Ptilocolepidae, Glossosomatidae, Rhyacophilidae, and Hydrobiosidae), which were also closely related to the monophyletic tube-case-making Integripalpia (or Phryganides following Thomas et al., 2020) comprising Plenitentoria and Brevitentoria (Supplementary Information Appendix S6). Molecular dating analyses (Fig. 1; Supplementary Information Appendix S9) are broadly consistent with the timing of Thomas et al. (2020) and suggest that the most common recent ancestors of Annulipalpia (181–206 Ma ago) and ‘spicipalpian’ (202–215 Ma ago) probably extend back to the time of Pangaea. This dating is also congruent with records from the Siberian Early to mid-Jurassic sediments (e.g., Sukatcheva and Vassilenko, 2011), and with wing impressions and sclerite fossils of early-diverging Mesozoic Trichoptera (Ivanov and Sukatsheva, 2002), including the ancestor to all present-day caddisflies (Ansoorge, 2002). Our phylogeny conflicts with the findings of Malm et al. (2013) and is partially incongruent with the recent mitogenome study of Ge et al. (2023), who found the free-living Rhyacophilidae and the purse-case Hydroptilidae as the sister groups to all other caddisflies, respectively. However, Ge et al. (2023) recognised that they only sequenced a few specimens of hydroptilids in their study and that improved taxon sampling of mitochondrial genomes are still required to fully resolve this ambiguous phylogenetic affinity. Our findings instead supported the notion of Thomas et al. (2020) that the earliest offshoot of the cocoon-making rhyacophilids was probably an artefact of base composition biases, rather than a phylogenetic signal.

We recovered the filter net-makers Hydropsychidae as the sister taxon (BS = 98%, PP = 1.00) to the clade comprising Philopotamoidea + Psychomyioidea, a relationship that agrees with previous morphological

(Kjer et al., 2001) and molecular analyses (Kjer et al., 2016; Thomas et al., 2020; Ge et al., 2023). The monophyly of the purse-making Ptilocolepidae was not supported by our tree topology (Supplementary Information Appendix S6, Fig. S1), which corroborates recent studies (Holzenthal et al., 2007; Kjer et al., 2016; Thomas et al., 2020), although quartet sampling values (QC < 0.2, QF ≤ 0.5) and missing data make us interpret this paraphyly as unstable. The Greater Antilles endemic *Antillopsyche* and the more widely distributed *Pseudoneureclipsis* were found as sister (BS = 92, PP = 1.00) to Ecnomidae (cf. Johanson et al., 2012; Kjer et al., 2016), a finding that supports a familial status for Pseudoneureclipsidae. Interestingly, this relationship within Psychomyioidea was also confirmed by Ge et al. (2023) based on a unique apomorphy in the complex rearrangement patterns of their mitogenomes. Hydrobiosidae emerged as the sister group to the tube-case-making families of Integripalpia, a finding that obscures the Rhyacophilidae + Phryganides topology in recent phylogenies (e.g., Thomas et al., 2020). These previous studies, however, detected potentially conflicting phylogenetic signals in the relationships between the exclusively Northern Hemisphere-distributed rhyacophilids and the mostly Southern Hemisphere-distributed hydrobiosids, leaving the relative position of these two ‘spicipalpians’ families as an open-ended question (for details, see Ge et al., 2023). We found higher resolution and support values (BS = 90–94, QC > 0.2) for the Rhyacophilidae + (Hydrobiosidae + Phryganides) clade than previous hypotheses, and our molecular dating is also consistent with potentially rapid radiation of their crown lineages sometime after the split of Pangaea (e.g., Hydrobiosidae in Gondwana, around 130 Ma ago; Supplementary Information Appendix S9). However, it might be that the rapid ancient divergence followed by recent radiation of each family may have left little phylogenetic resolution in our combined set of rRNA and protein-coding genes as to confidently resolve this relationship. Hence, we still interpret this result as pending further verification.

In agreement with Malm et al. (2013) and Thomas et al. (2020), *Phryganopsyche* (Phryganopsychidae) was recovered as the sister taxon to the remaining plententorian caddisflies (BS = 92, PP = 1.00), confirming early discussions on several plesiomorphic characters in the larvae, pupae, and adult of these portable case-makers compared with other limnephilid branches (e.g., Wiggins and Gall, 1993). Our results (Supplementary Information Appendix S6, Fig. S2) also show relatively strong support (BS = 90, PP = 1.00) for Brachycentridae + Lepidostomatidae, although the association between Pisuliidae and Kokiriidae is not strongly supported (Thomas et al., 2020; but see Holzenthal et al., 2007 and Malm et al., 2013). Similarly unresolved are the relationships between Apatanidae and the Palearctic genus *Archithremma*, as well as low-level intrafamilial relationships of Limnephilidae. On the other hand, potential instability in the placement of Tasimiidae as a sister taxon to the remaining Brevitentoria may have affected the paraphyletic grade of several leptocecid families, although divergence dates between the southern Neotropical *Trichovespula* and the Australian *Tasimia* and *Tasiagma* (Supplementary Information Appendix S9 and the individual Newick tree file therein) mostly agree with Thomas et al. (2020) and is consistent with the split between South America and Australia (McIntyre et al., 2017). Stronger support regarding these evolutionary relationships awaits further analyses and the inclusion of additional data. Placements within Sericostomatoidea should also be considered only as partially resolved because of relatively less QC support. Compared to Johanson and Malm (2010) and Malm et al. (2013), we found no close relationship between Calocidae and Conoesucidae. Instead, these families were found in between Helicophidae, Hydrosalpingidae, and Parasericostomatidae (Supplementary Information Appendix S6, Fig. S2), which is a relatively similar finding compared to the branching order shown in Thomas et al. (2020). Perhaps more importantly, molecular dates in our phylogeny suggest that ancestral Phryganides (163–186 Ma) evolved on Pangaea, and that the more recent splits of its major subgroups correspond with differential diversification on each hemisphere (Supplementary Information

Appendix S9 and the individual Newick tree file therein).

5. Examples of application

The three case studies described below use datasets that have already been published and come from different environmental and geographical contexts. They were chosen to (i) illustrate potential applications of combining our time-calibrated phylogenetic tree with empirical field data on aquatic insects and stream environments, and (ii) emphasise the advantages of focusing on phylogenetic aspects of biotic assemblages. Yet, as these are examples only, we do not intend to draw detailed inferences about the drainage basins examined. These inferences have been made in previous studies (see below), whereas we here suggest ways to model phylogenetic aspects of biotic communities. All modelling exercises were done in the R environment (R Development Core Team, 2021) and a detailed list of packages and statistical routines are available from Zenodo (see Data Availability).

5.1. Example 1: Phylogenetic constraints on the size structure of subarctic stream insect assemblages

This first dataset has been used previously to study community-environment relationships and metacommunity dynamics in subarctic landscapes (Kärnä et al., 2015). A total of 55 stream riffles in near-pristine and pristine conditions from the Tenjoki drainage basin were sampled soon after the snowmelt in the first half of June 2012. This basin covers more than 16,000 km² across the northernmost areas of Finland and Norway, finally exiting in the Arctic Ocean. At each riffle site, a 3-min kick-net (net mesh size = 300 μm) pooled sample was taken, the pooled sample consisting of six 30-s subsamples divided among different microhabitats based on visual inspections of water velocity, depth, moss cover, and particle size. Several environmental variables related to both physical habitat (i.e., current velocity, m s⁻¹; mean depth, cm; channel width, m; moss cover, %; particle size classes, %; and estimated shading by riparian vegetation, %) and water chemistry (pH; conductivity, μS cm⁻¹; total nitrogen, μg l⁻¹; water colour, mg Pt l⁻¹; and iron concentration, μg l⁻¹) were measured at each riffle site (Supplementary Information Appendix S10, Table S1). Potential maximum dry weights (mg) for each taxon were obtained from length-weight relationships as published earlier in Pérez-Rocha et al. (2018) and García-Girón et al. (2022b). Detailed description of the study area as well as of the aquatic insect sampling, quantification of local environmental variables (Kärnä et al., 2015), and body size measurements can be found elsewhere (Pérez-Rocha et al., 2018; García-Girón et al., 2022b). Our main aim with this dataset was to assess whether the structure of aquatic insect assemblages (here, limited to include dragonflies, damselflies, mayflies, stoneflies, and caddisflies) based on body size distributions responded predictably to local environmental conditions, spatial factors, and phylogenetic constraints (Fig. 2). We anticipated that phylogenetic relatedness would be particularly important for resemblances in terms of body size structure (Pereira et al., 2021). However, a further prediction based on associations between species traits and habitat templates (Southwood, 1977) is that size structural resemblances would also result from the interplay between local environmental conditions and spatially modulated processes (Pérez-Rocha et al., 2018; García-Girón et al., 2022b).

To test these hypotheses, we used the size structural resemblance approach of De Cáceres et al. (2013), which is based on analysing community abundance profiles (CAPs). We first log-transformed the abundance values, i.e., the number of individuals, to reduce the effect of highly dominant species (Legendre and Legendre, 2012) and calculated CAPs after pooling all taxa (matrix Y^{STR}; for details, see De Cáceres et al., 2013). Following García-Girón et al. (2022b), we grouped stream insect genera into 0.1 log-unit bins spanning the total range of body sizes (mg) in the study area and log-transformed cumulative abundance values for further analyses (Supplementary Information Appendix S10, Fig. S1).

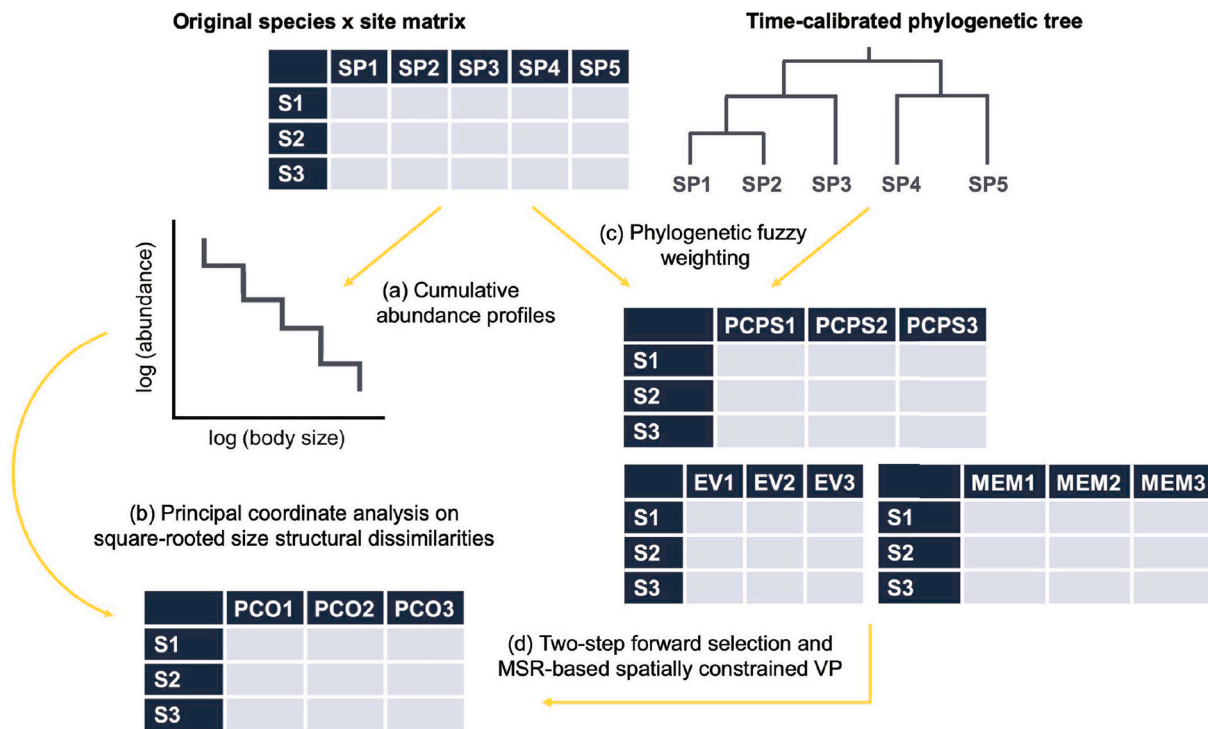


Fig. 2. Graphical scheme plotting the different steps of our modelling framework for the first case study in the Tenojoki drainage basin (Finland). In brief, we (a) determined cumulative abundance profiles (CAPs) using body size as the structural variable (De Cáceres et al., 2013) and (b) displayed a principal coordinate analysis (PCO) on the resulting square-rooted (Bray-Curtis) dissimilarity matrix accounting for between-site size structural differences. (c) Phylogenetic features of stream insect assemblages were addressed by the phylogenetic fuzzy-weighting method (Debastiani and Duarte, 2014). After running a two-step forward selection procedure (Blanchet et al., 2008; Borcard et al., 2018), (d) we finally applied a variation partitioning (VP) routine that uses Moran Spectral Randomisation (MSR) (Clappe et al., 2018) to address the influence of local environmental variables (EV), phylogenetic components (PCPS), and spatial factors (MEMs) on the size structure of stream insect assemblages.

We determined structural dissimilarities by calculating the percentage difference (Bray-Curtis) coefficient on matrix Y^{STR} , and the resulting square-rooted dissimilarity matrices were displayed using principal coordinate analysis (PCO). To correct for negative eigenvalues, we applied the Lingoes' method (Legendre and Legendre, 2012). As many orthogonal PCO eigenvectors were selected to maximise total adjusted R^2 (adj. R^2) values against a set of explanatory variables (Duarte et al., 2012) representing local environmental conditions, spatial features, and phylogenetic components. Local environmental variables were standardised to zero mean and unit standard deviation, whereas spatial predictors comprised an orthogonal set of spatial variables from Moran Eigenvector Maps (MEM). In this case, we used a distance truncation threshold that maximised the number of positive and significant spatial eigenvectors (Dray et al., 2006). From the new time-calibrated phylogenetic tree of aquatic insects, we addressed the phylogenetic structure of each stream site using the phylogenetic fuzzy-weighting method (PCPS) implemented by Debastiani and Duarte (2014). We used the scores along PCPS components to indicate the phylogenetic features of stream insect assemblages. Hence, the idea was that the greater the difference between PCPS scores, the greater the phylogenetic distance between the taxa that comprised the assemblages (for a similar approach, see García-Girón et al., 2020, 2021). In this example, we applied a variation partitioning (VP) routine that uses Moran Spectral Randomisation (MSR) as a constrained null model to adjust estimates for the potential biases of spurious spatial structures exhibited by explanatory variables (Clappe et al., 2018). This form of VP model includes an explicit evaluation of spatial autocorrelation in local environmental features and phylogenetic structures (i.e., the influence of local environmental variables and phylogenetic components dependent of space; Pereira et al., 2021). Before running the MSR-based VP routine, we evaluated collinearity among explanatory variables with bivariate

correlations and removed those with $r \geq 0.7$ or $r \leq -0.7$ (Dormann et al., 2013). We further applied forward selection with two stopping criteria (i.e., significance level α and global adj. R^2 ; Blanchet et al., 2008) to choose the most parsimonious subset of explanatory variables for the models (Borcard et al., 2018). Each forward selected model was finally evaluated with significance tests based on 999 replicates (Clappe et al., 2018).

The model combining selected environmental, pure spatial, and phylogenetic predictors (Supplementary Information Appendix S10, Table S2) explained more than 20% (adj. $R^2 = 0.22$) of body size variation of stream insect assemblages across the Tenojoki drainage basin (Table 1). The non-spatially structured local environmental conditions accounted for the largest fraction of variation in body size structure at the assemblage level (adj. $R^2 = 0.12$), whereas the phylogenetic component (after partitioning out the influence of spatial autocorrelation) accounted for 10% (adj. $R^2 = 0.10$) of the dissimilarities in the size structure of stream insect assemblages. This result confirms our first hypothesis that interpretation of resemblance measurements in terms of body size structure is not possible without considering evolutionary relationships of constituent lineages. Perhaps more interestingly, the significant components accounting for phylogenetic relatedness in our study area (Supplementary Information Appendix S10, Table S2) described relatively broad phylogenetic gradients related to deep nodes (sensu Debastiani and Duarte, 2014). This finding suggests that stabilising selection (sensu Diniz-Filho et al., 2007) may be a strong force operating on the body size structure of subarctic stream insect assemblages, as has been documented for birds, butterflies, and lemurs in terrestrial ecosystems (Peterson et al., 1999; Lehman, 2007). Although less important than pure local environmental effects and phylogenetic components, their spatially autocorrelated fractions also contributed significantly to body size dissimilarities, explaining around 2% and 5%

Table 1

Results from MSR-based spatially constrained variation partitioning for the body size structure of stream insect assemblages in the Tenjoki drainage basin (Finland). The explained variation by local environmental (E), spatial (MEM), and phylogenetic (PCPS) variables is based on adjusted R^2 (adj. R^2) after forward selection (Supplementary Information Appendix S10, Table S2). Shared contributions of local environmental conditions and phylogenetic features were estimated conditioning on spatial weighting matrices following Loewen et al. (2020) and García-Girón et al. (2022b).

Variable group	Variance explained (adj. R^2)	p -value
E	0.12	<0.01
E MEM	0.02	0.01
PCPS	0.10	<0.01
PCPS MEM	0.05	0.02
MEM	0.01	0.29
E \cap PCPS	0.18	<0.01
E \cap PCPS \cap MEM	0.22	<0.01

E, non-spatialised local environmental variation; E | MEM, spatially structured local environmental contribution; PCPS, non-spatialised phylogenetic variation; PCPS | MEM, spatially structured phylogenetic contribution; MEM, pure spatial variation; E \cap PCPS, non-spatialised joint environmental and phylogenetic variation; E \cap PCPS \cap MEM, joint environmental, spatial, and phylogenetic contribution.

of variation, respectively. On the other hand, the unique contribution of pure spatial variables was negligible (adj. $R^2 = 0.01$; p -value >0.05 ; Table 1), partly refuting our second hypothesis. Previous studies in the Tenjoki drainage basin have found little spatial structuring of stream macroinvertebrates based on taxonomic composition (Grönroos et al., 2013; Kärnä et al., 2015). Instead, these studies emphasised that species are filtered to occur at sites according to the prevailing environmental conditions. Our results suggest that, in addition to local environmental filtering, phylogenetic relatedness also affects body size structure of subarctic stream insect assemblages. In other words, although we found that local environmental features are key factors affecting dissimilarities in the size structure of stream insect assemblages, phylogenetic forces have a clear legacy in affecting body size variation in subarctic stream networks, and they should thus receive further research attention. This is because certain lineages occurring in similar sites in terms of environmental conditions are likely to have comparable body sizes (and possibly other traits). While this conclusion might seem intuitive, our results are particularly meaningful because (i) only few studies have explicitly focused on body size descriptors and associated resemblance measurements in freshwater systems (see García-Girón et al., 2022b for an example on stream macroinvertebrates in western Finland), (ii) we adjusted for the spurious contribution of spatial autocorrelation in our statistical models, and (iii) we tested the predictive role of phylogenetic components on the body size structure of biotic assemblages, which is uncommon in the literature. Overall, we confirmed that including phylogenetic relatedness in multivariate models (e.g., Múrria et al., 2018; Pereira et al., 2021) could be an important component for future endeavours to link variation in assemblage structure to resemblance measurements in terms of body size distributions. We encourage other researchers to use our phylogenetic tree in similar analyses on aquatic insect assemblages in other environmental and geographical settings across the world, provided that detailed and standardised information on insect assemblages, body sizes, and environmental variables can be obtained.

5.2. Example 2: Altitudinal patterns in phylogenetic and body size variation across the world's deepest canyon

The dataset used in this example was collected in the Yarlung Tsangbo Grand Canyon Region, southeastern Tibet (for details, see Li et al., 2019a, 2023). Field samplings were performed in October 2015 at 50 stream sites distributed along the Yarlung Tsangbo drainage basin, an area of 117,000 km² with an average elevation of 3100 m.a.s.l. and

elevation range between 550 and 7000 m.a.s.l. This drainage basin comprises complex landforms and distinct vegetation types that support highly unique aquatic habitats with characteristic alpine, temperate, and subtropical biotas (Chen et al., 2014). The streams located at high elevations in the Grand Canyon (ca., 2600–3600 m.a.s.l.) are extremely harsh environments and are fed mainly by snowmelt and glaciers, whereas the lower reaches (ca., 550–2600 m.a.s.l.) are more benign and experience humid tropical and subtropical climates, being replenished mostly by rainfall. Stream insects were sampled with a Surber sample (net mesh size = 300 μ m) using three quantitative subsamples from representative habitats at each riffle site. The dataset included local environmental variables (i.e., mean depth, cm; channel width, m; current velocity, m s⁻¹; particle size classes, %; water temperature, °C; conductivity, μ S cm⁻¹; dissolved oxygen, mg l⁻¹; and pH; Supplementary Information Appendix S11, Table S1) measured at each riffle site. For this illustrative example, we also calculated potential maximum dry weights (mg) as a measure of body size based on length-weight relationships (see above). This information was obtained from the literature (for details, see Supplementary Information Appendix S11, Table S2). Specifically, we explored the role of elevation in driving phylogenetic and body size variation of stream insects along one of the most remote and undisturbed freshwater ecosystems in the world, also known as the 'World's Third Pole'. We hypothesised that at high elevations, where species are adapted to cold waters (Cauvy-Fraunié and Dangles, 2019), the harsh environment would exert strong environmental filtering on Tibetan insect faunas, both in terms of phylogenetic relatedness (Graham et al., 2009) and size structure (Gardner et al., 2011). In other words, owing to the challenges posed by near-extreme environments in the high-elevation streams (Birrell et al., 2020), we expected that assemblages at higher elevations would be constrained in terms of body size variation (sensu James, 1970) and would mostly comprise close relatives (i.e., phylogenetic clustering). These factors would further lead to similar altitudinal patterns in phylogenetic and body size variation across the study area.

The standardised effect sizes (SES) of the abundance-weighted mean pairwise distances were calculated by shuffling the labels of the (cophenetic) phylogenetic and (Euclidean) body size distance matrices for 9999 iterations (Webb et al., 2008). Positive SES values indicated phylogenetic or body size overdispersion (i.e., taxa being less closely related or less similar in their body sizes than expected by null models; Swenson, 2014), whereas negative SES values indicated phylogenetic or body size clustering (i.e., taxa being more closely related or more similar in their body sizes than expected by null models; Swenson, 2014). The square roots of the phylogenetic and body size site-by-site dissimilarities were further synthesised into PCO and the overall degree of correspondence between the resulting axes was tested with Procrustes rotation analysis (Gower, 1971) and the permutation-based PROcrustean randomisation TEST (PROTEST) (Jackson, 1995). See Fig. 3 for the main steps of our statistical approach.

Most SES values of the phylogenetic and body size dissimilarities in the Yarlung Tsangbo Grand Canyon Region were neither significantly lower nor higher compared with the null expectations (p -values >0.05 ; Fig. 4). The PCO axes of the phylogenetic and body size dissimilarities converged to a highly similar configuration according to the Procrustes rotation analysis ($m^2_{12} = 0.26$; p -value <0.01), which further supports a similar ecological interpretation for altitudinal patterns in phylogenetic and body size variation. Our hypothesis that the most environmentally extreme ecosystems in the uppermost streams of the Grand Canyon area would exert strong abiotic filtering on body size and phylogenetic relatedness of Tibetan insect assemblages was therefore mostly rejected. These patterns also appear to run counter to what could be expected based on theory (e.g., decreasing differences in body size among assemblages at increasingly higher elevations), re-emphasising that ecological rules are more like general trends than universal natural laws (Lomolino et al., 2006). This finding is not surprising, as it is understandable that a hypothesis originally developed to account for body

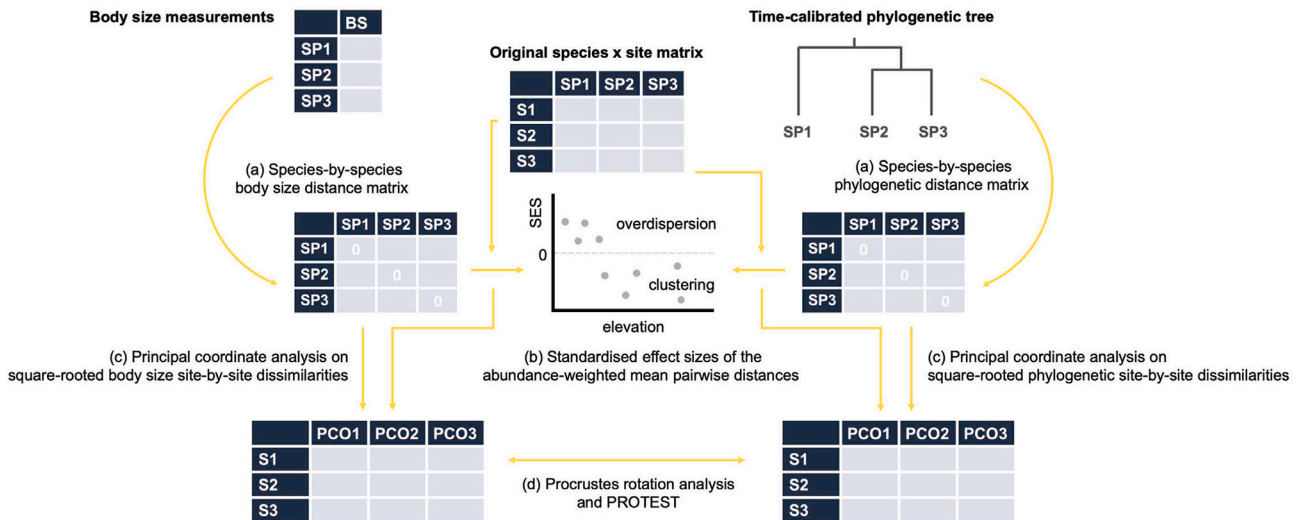


Fig. 3. Statistical workflow for the second case study assessing altitudinal gradients in phylogenetic and body size variation across the Yarlung Tsangbo drainage basin (Tibet). First, (a) we computed the species-by-species (Euclidean) body size and (cophenetic) phylogenetic distance matrices. (b) The standardised effect sizes (SES) were then calculated after 9999 iterations to identify patterns of phylogenetic or body size overdispersion and clustering (sensu Swenson, 2014). (c) We synthesised the square roots of the phylogenetic and body size site-by-site dissimilarity matrices into principal coordinate analysis and (d) evaluated their overall degree of correspondence with Procrustes rotation analysis (Gower, 1971) and permutation-based PROcrustean randomisation TEST (PROTEST) (Jackson, 1995).

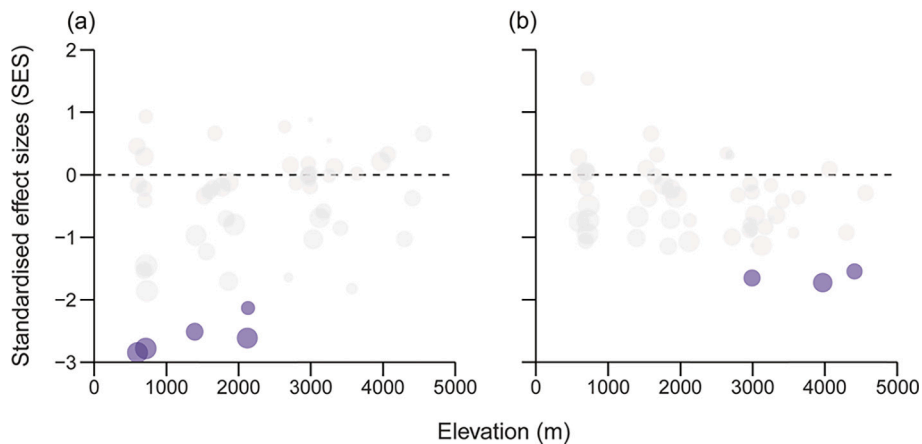


Fig. 4. Standardised effect sizes (SES) of the (a) phylogenetic and (b) body size structure of stream insect assemblages in relation to elevation across the Yarlung Tsangbo Grand Canyon Region, southeastern Tibet. The size of the circles is proportional to the number of taxa in the assemblage. Grey and dark purple colour indicates mostly random and significantly clustered patterns, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

size variation in birds and mammals did not apply to ectothermic organisms (e.g., Zeuss et al., 2017; Horne et al., 2018). Effects of sampling time or size-filtering biases cannot be invoked as an explanation for the body size distribution patterns we found here. This is because most aquatic insects (including dragonflies, mayflies, stoneflies, and caddisflies) across the Yarlung Tsangbo drainage basin were close to their final larval stage and attained their largest body sizes in October (Li et al., 2022).

Some phylogenetically clustered assemblages were marginally located in the lowlands (Fig. 4), independent of the most physiologically challenging environmental conditions at higher elevations (i.e., too cold, turbulent, fast-flowing water, low oxygen availability, and unstable channel). The finding of increasing procrustean residuals at higher elevation ($r_s = 0.46$; p -value < 0.01 ; Fig. 5) is also consistent with the existence of a few clustered communities in terms of body size across the glacier-fed and near-pristine streams of the most remote upper canyon (Fig. 4). The superimposition ordination space summarising body size and phylogenetic axes mimicked major gradients in water temperature,

water chemistry, and substrate types (Fig. 5). Although this finding agrees with previous studies of stream macroinvertebrates conducted in the heterogeneous mountain belts of the Yarlung Tsangbo drainage basin (Li et al., 2019b, 2023), the high degrees of randomness detected in the study area suggests that these environmental gradients are unlikely to explain the phylogenetic and body size patterns we found here. Instead, the absence of evidence for pronounced altitudinal patterns in phylogenetic and body size clustering indicates (i) potential idiosyncratic or convergent eco-evolutionary patterns in Tibetan stream insects such that evolutionarily distinct clades share similar body sizes (Kraft et al., 2015) and (ii) lack of short-term diversification among closely related co-occurring taxa. This may result from the relatively short geological age of the world's deepest canyon (Ge et al., 2021) and the frequent flooding disturbances induced by glacial ablation that result in random extinction and recolonisation dynamics at the level of local stream communities (Li et al., 2019b). These results therefore suggest that the understanding of phylogenetic and body size variation of aquatic insect assemblages along elevational gradients is a complex

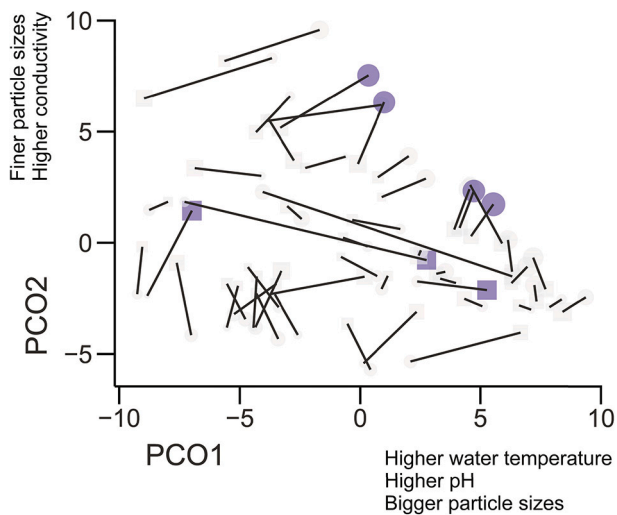


Fig. 5. Procrustean superimposition rotation generated from the ordination results of phylogeny-based and body size-based principal coordinate analysis (PCO) for stream insect assemblages of the Yarlung Tsangpo Grand Canyon Region, southeastern Tibet. Circles and squares represent PCO scores from phylogeny-based and body size-based ordinations, respectively. The distance between the two (i.e., black arrows) is the Procrustean residual. Most important environmental gradients based on correlations with orthogonal PCO axes are also projected. The size of the circles and squares is proportional to their absolute standardised effect sizes (Fig. 4). Grey and dark purple colour indicates mostly random and significantly clustered patterns, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

issue, implying that the underlying mechanisms involved may also be multifaceted.

5.3. Example 3: Contribution of perennial and temporary streams to phylogenetic and environmental uniqueness across seasons

This third dataset has been used to investigate the role of hydrological conditions and spatial connectivity on stream macroinvertebrate communities across small Mediterranean stream networks located in the Sant Llorenç del Munt i l'Obac Natural Park, northeastern Spain (Pineda-Morante et al., 2022). The study area has a Mediterranean climate with irregular rainfall. Seasonal observations of aquatic insects were made at 30 stream sites that covered a wide hydrological gradient. Flow intermittence can be described using different criteria (e.g., Datry et al., 2017), but we used the aquatic states of Gallart et al. (2012) to update and synthesise the existing classification of mesohabitats based on their hydrological conditions (i.e., perennial vs. temporary). Most temporary streams in the study area dry up during the summer, either forming disconnected pools or completely dry riverbeds, whereas perennial streams maintain very low flows replenished by water discharges from karstic aquifers (for details, see Pineda-Morante et al., 2022). Insects were collected seasonally in winter (January), spring (May), summer (July), and autumn (November) of 2019 with a kick-net (net mesh size = 250 μm) method covering all different microhabitats along a 100-m stream site. Physico-chemical parameters were measured at each sampling campaign and included pH, dissolved oxygen (mg l^{-1}), temperature ($^{\circ}\text{C}$), electrical conductivity ($\mu\text{S cm}^{-1}$), specific conductivity ($\mu\text{S cm}^{-1}$), and total dissolved solids (mg l^{-1} ; Supplementary Information Appendix S12, Table S1). In this example, we seek to disentangle the role of flow intermittence in shaping seasonal changes in a site's ecological uniqueness based on its phylogenetic (Shooner et al., 2018) and environmental (Castro et al., 2019) features. We addressed the following questions: (i) Do perennial and temporary stream sites contribute equally to phylogenetic and environmental uniqueness across

seasons? and (ii) How does phylogenetic and environmental uniqueness correspond to each other across seasons? By responding to these questions, it is possible to test the congruence between different uniqueness measures (e.g., Heino et al., 2022) and test if perennial and temporary streams play complementary roles in supporting phylogenetically and environmentally most unique sites through time (e.g., Rogosch and Olden, 2019). Our hypotheses were that (i) temporary stream sites would be most likely to show high phylogenetic and environmental uniqueness (Valente-Neto et al., 2020); (ii) relative contributions of perennial and temporary stream sites would be invariant across seasons (Rogosch and Olden, 2019); and (iii) relationships between phylogenetic and environmental uniqueness would be generally strong (cf. Castro et al., 2019; Heino et al., 2022).

In brief, by applying the original local contribution to beta diversity (LCBD) approach of Legendre and De Cáceres (2013), we calculated two measures describing the ecological uniqueness of each stream site, i.e., a site's contribution to phylogenetic beta diversity (LCBD- p) and local contribution to environmental heterogeneity (LCEH). Although this approach was originally developed for taxonomic site-by-species data, it can easily be applied in studies on the phylogenetic features of assemblages (e.g., Shoener et al., 2018) and abiotic environmental conditions (e.g., Castro et al., 2019). For environmental data, we followed Castro et al. (2019) and Heino et al. (2022) and calculated LCEH values from standardised (i.e., each environmental variable was standardised to zero mean and unit standard deviation) between-site Euclidean distances. For the phylogenetically explicit measures, the site-by-species community matrix was used along with our time-calibrated phylogeny to compute a phylogenetic community distance matrix and the resulting site-by-site dissimilarity matrix was then used to calculate LCBD- p for each stream site (for details, see Shoener et al., 2018). We stratified all phylogenetic and environmental models by season (i.e., winter, spring, summer, autumn) and partitioned the Jaccard index of dissimilarities into its constituent components (sensu Carvalho et al., 2013) to see if patterns of phylogenetic uniqueness across perennial and temporary streams were driven by their relationships to species replacements and richness differences (Hill et al., 2021). Finally, we tested for relationships between LCBD- p and LCEH using Spearman's rank correlations (for a workflow of the statistical framework used, see Fig. 6).

Flow intermittence had no significant effects on phylogenetic uniqueness (Fig. 7a) and environmental uniqueness (Fig. 7b) across the study Mediterranean stream networks. We expected an increase in LCBD- p and LCEH among temporary streams because the marked spatial and temporal gradients in stability, periodic resetting of stochastic successional trajectories when flow resumes, and greater dispersal limitation associated to intermittently flowing sites would result in increased ecological uniqueness within a drainage basin (Sarremejane et al., 2017; Rogosch and Olden, 2019; Crobot et al., 2020, 2021). Contrary to this prediction, temporary streams did not contribute disproportionately to either LCBD- p or LCEH (Table 2). On the other hand, we were able to confirm our hypothesis that the contributions of perennial and temporary sites to phylogenetic and environmental uniqueness were mostly invariant across seasons (Fig. 7). In other words, contributions of temporary sites to phylogenetic and environmental uniqueness remained relatively stable through time. This partially contradicts previous findings suggesting that macroinvertebrate assemblage composition within temporary streams is more variable than among perennial streams (Clarke et al., 2010). Controversy thus remains on whether temporary streams are more (e.g., Sánchez-Montoya et al., 2020; Valente-Neto et al., 2020) or less (e.g., Warfe et al., 2014; Soria et al., 2017) diverse and ecologically unique than perennial streams. Further analyses based on the additive components of LCBD- p showed no significant effect of hydrological harshness on species replacements and richness differences, resembling fluctuations over time compared to the overall phylogenetic uniqueness component (Supplementary Information Appendix S12, Fig. S1 and Table S2). Similar to what has been found for fish beta diversity in dryland rivers of the North American

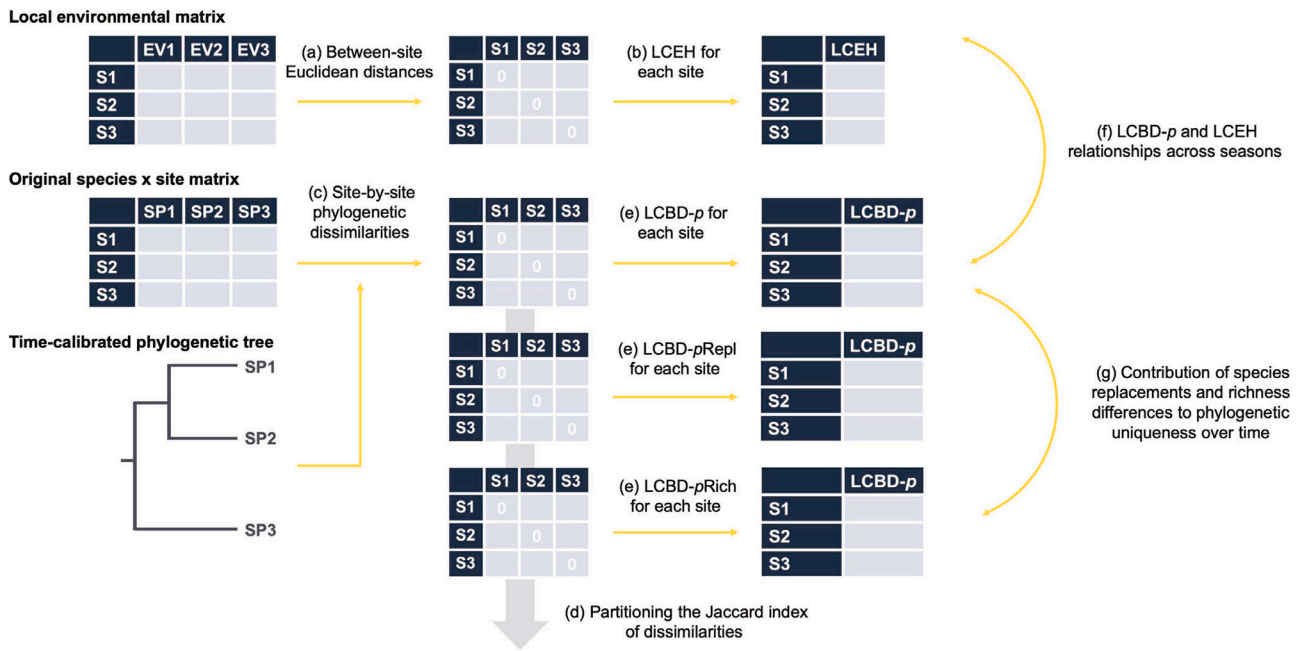


Fig. 6. Flow chart of the statistical analysis used to calculate phylogenetic and environmental uniqueness in the third case study. (a) For standardised environmental data, we calculated between-site Euclidean distances and (b) obtained local contributions to environmental heterogeneity (LCEH) (Castro et al., 2019; Heino et al., 2022). For phylogenetic measures, (c) our time-calibrated phylogeny was used along with the site-by-species community matrix to compute the phylogenetic community distance matrix. (d) We also partitioned the Jaccard index of dissimilarities into species replacement (Repl) and richness difference (Rich) components (sensu Carvalho et al., 2013). (e) The resulting site-by-site dissimilarity matrices were then used to calculate the local contributions to phylogenetic beta diversity (sensu Shooner et al., 2018). Finally, (f) we tested for relationships between LCBD-*p* and LCEH using Spearman’s rank correlations across seasons and (g) checked if patterns of phylogenetic uniqueness were driven by species replacements or richness differences in streams covering a wide hydrological gradient in north-eastern Spain.

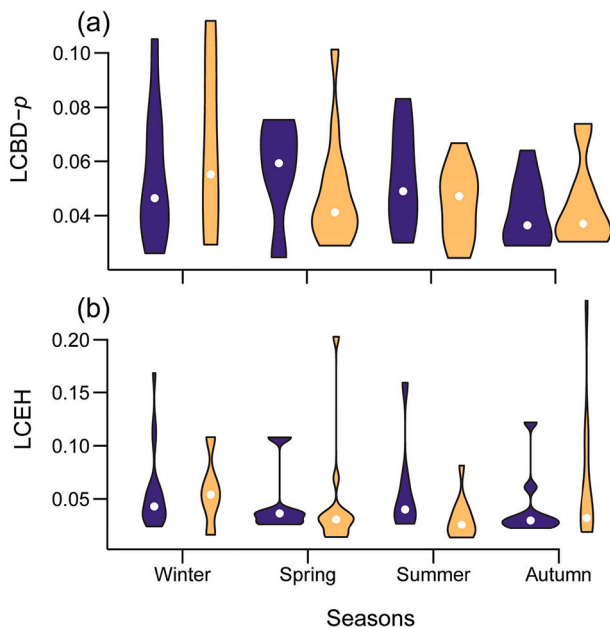


Fig. 7. Violin plots showing seasonal variation in (a) phylogenetic uniqueness (LCBD-*p*) of insect assemblages and (b) local contribution to environmental heterogeneity (LCEH) across a set of perennial (dark purple) and temporary (light orange) Mediterranean stream sites. White points within the violin plots represent the median. Mann–Whitney *U* tests showed no significant differences (*p*-values >0.05) in LCBD-*p* or LCEH values between perennial and temporary streams across seasons (Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Results of Mann–Whitney *U* tests showing differences in phylogenetic uniqueness (LCBD-*p*) of insect assemblages and environmental uniqueness (LCEH) between perennial and temporary stream sites across seasons. Numbers are Mann–Whitney statistic (*W*-values) and *p*-values (the latter in parenthesis).

Seasons	LCBD- <i>p</i>	LCEH
Winter	55 (0.63)	25 (0.12)
Spring	22 (0.34)	26 (0.57)
Summer	20 (0.10)	46 (0.54)
Autumn	40 (0.50)	25 (0.07)

Southwest (Rogosch and Olden, 2019), species replacement contributed more than differences in species richness across perennial and non-perennial stream sites. This finding suggests that local extirpations have been rather stochastic and not so strong in harsher temporary stream sites (Ruhf et al., 2017), which may still harbour some phylogenetically distantly related rheophilic taxa.

We also evaluated whether more phylogenetically unique insect assemblages were found in more unique abiotic environments. Contrary to our hypothesis, LCBD-*p* seemed virtually independent of LCEH across seasons ($r_s = 0.18–0.32$; *p*-values = 0.20–0.40). Only in autumn phylogenetic uniqueness and environmental uniqueness showed a positive relationship ($r_s = 0.56$; *p*-value = 0.01). Although higher phylogenetic uniqueness is expected in stream sites with most unique environmental conditions, especially in naturally complex stream networks with different hydrological regimes (Datry et al., 2017), different facets of biotic uniqueness have usually been found to be weakly correlated with LCEH values across different organismal groups and geographical contexts (e.g., Heino et al., 2022; Snåre et al., 2024). The apparent lack of relationship between LCBD-*p* and LCEH may be explained by the small length of main environmental gradients across the study stream networks and the role of spatial connectivity in

structuring aquatic invertebrate assemblages (Cañedo-Argüelles et al., 2020), with recent evidence suggesting that dispersal limitation may be operating in the study area (Pineda-Morante et al., 2022). These previous observations, combined with the present results reported here, warrant further investigation. This is because empirical evidence on eco-evolutionary responses to flow intermittence remains extremely limited (for a review, see Rolls et al., 2018). The scarcity of such studies is unfortunate because identifying spatio-temporal variation of stream sites that disproportionately contribute to regional phylogenetic history is important for conservation planning and prioritisation (Tucker et al., 2019). By combining the time-calibrated phylogeny that we present here with site-specific contributions to phylogenetic dissimilarities over time across drainage basins with different hydrological regimes, we expect that future empirical studies will shed some light on this research topic.

6. Concluding remarks and future perspectives

Mapping the evolutionary courses and pathways of aquatic insects is a lofty goal. Although it has recently been possible to imagine the articulation of a ‘Tree of Life’ for extant birds (e.g., Prum et al., 2015) and mammals (e.g., Upham et al., 2019), the scale and complexity of this endeavour for insects is far greater. With a new dataset of unanticipated taxonomic coverage comprising almost 1200 genera of dragonflies, damselflies, mayflies, stoneflies and caddisflies, we presented the largest time-calibrated molecular phylogeny of these key groups of aquatic insects to date. Even if our working phylogenetic hypothesis represents only a fraction of the enormous history and diversification of aquatic insects, we believe that the backbone of a relatively comprehensive ‘Tree of Life’ for these lineages is now in place. Several portions of the topology and associated divergence time estimates have enabled a re-evaluation of potential dated geological drivers behind major evolutionary scenarios that occurred dozens to hundreds of millions of years ago. Examples are the chronology of divergence during and after the tectonic splitting of the supercontinent Pangaea and the identification of rapid radiation events around and shortly after the end-Cretaceous mass extinction. In consequence, our time-calibrated phylogenetic tree contains a great deal of potentially useful information for biogeosciences. For instance, researchers working with a certain aquatic insect lineage can obtain hypothesised sister taxa that might work as outgroups in phylogenetic evaluations.

Still, the phylogenetic tree we presented is likely to contain several weaknesses. First, some taxa (e.g., inadequately known endemic genera and species) will continue to be poorly characterised at the molecular level for the foreseeable future, even despite significant recent progress in sequencing efforts (Hotaling et al., 2020). Second, inaccuracies can arise from the contamination, misclassification, and misidentification errors that are frequent in large, published databases, such as GenBank. Third, our best-scoring phylogenetic hypothesis is subject to the inherent limitations of the targeted rRNA and protein-coding genetic data itself. Fourth, as with other calibration strategies of divergence time estimation, such as geological and biogeographical events or the fossil record, the use of secondary calibration has its own disadvantages and suffers from various potential sources of errors (for details, see Schenk, 2016). The chronology of divergence in our best-scoring phylogenetic hypothesis must be viewed in the light of the information that was used to build the tree, and uncertainty associated with phylogenetic estimates must be considered in further explorations through different molecular comparative frameworks. For example, divergence dates from previous multi-gene, phylogenomic, and transcriptomic assessments (e.g., Thomas et al., 2020; Kohli et al., 2021; Letsch et al., 2021) could be used to constrain our resulting phylogenetic estimates (e.g., dos Reis et al., 2018) and minimize the uncertainty associated with the age of several insect lineages.

These potential sources of inaccuracy can be overcome as our understanding improves (e.g., by correcting or reclassifying voucher specimens), but most of these previous problems should have little effect

on evolutionary trends in most studied insect groups (Zhou et al., 2016). In any case, using the continuous range of values obtained from quantitative branch lengths of our molecular tree should improve our phylogenetic diversity estimates compared to the conventional strategy of considering Linnaean taxonomic classification as a surrogate for phylogenetic relatedness in freshwater studies (Heino and Tolonen, 2017; Alahuhta et al., 2019). In addition, one can use different methods to address phylogenetic uncertainties while developing these studies (Huelsenbeck et al., 2000; Rangel et al., 2015). However, because regional and continental checklists each include hundreds of taxa, some of which will certainly not be included in our targeted rRNA and protein-coding data, users of our time-calibrated phylogenetic tree are encouraged to use existing software tools that are able to graft missing genera to a backbone supertree. These serial of user-friendly and flexible packages (e.g., Webb and Donoghue, 2005; Jin and Qian, 2023; Li, 2023) have demonstrated to give reliable and nearly identical results to those generated exclusively by sequence data across ecosystem types and organismal groups (e.g., Li et al., 2019b; Nakamura et al., 2021; Qian and Jin, 2021). We therefore believe that our phylogenetic tree will be useful to respond to a broad palette of research questions at the interfaces of biogeography, limnology, macroevolution, and palaeoecology.

Examples from individual case studies have been shown in this paper for different situations and provide compelling rationale for more integrated assessments of phylogenetic aspects of aquatic insect assemblages. For instance, the effects of contemporary spatial location and environmental conditions on compositional variation of aquatic insect assemblages have been studied at scales ranging from single streams (e.g., Heino et al., 2013) to entire continents (e.g., Bini et al., 2014). However, researchers are nowadays more interested in different facets of biotic communities beyond traditional taxonomic measures, including taxonomic alpha, beta, and gamma components of diversity (sensu Whittaker, 1960). By allowing the implementation of the phylogenetic information presented here, we are opening the door to the quantification of geographical gradients in phylogenetic diversity of aquatic insect assemblages and begin searching for current hotspots across the world. As any research questions that use species richness as a surrogate for biodiversity, metrics of phylogenetic diversity could also be regressed against a set of local environmental (e.g., substratum particle size), catchment-level (e.g., hydrology), and historical (e.g., glaciations) variables to unravel the mechanisms underlying phylogenetic diversity and composition of aquatic insect assemblages at broad spatial extents. This approach has recently been highlighted as an important venue for future work (García-Girón et al., 2023).

A promising course of action is to study diversification rates and integrate these with key evolutionary innovations in aquatic insect groups (Dijkstra et al., 2014a), such as oviposition behaviour (Heino and Peckarsky, 2014). There is also tremendous potential for studying the evolution of ecological characters and morphological divergence by integrating our time-calibrated phylogenetic tree with the considerable amount of natural history observations on functional traits of aquatic insects in general (e.g., Schmidt-Kloiber and Hering, 2015) or dispersal-related traits in particular (e.g., Sarremejane et al., 2020). As an illustration, highlighting the impact of palaeoclimatic changes (e.g., Scotese et al., 2021) on phylogenetically informed trajectories of body size evolution may benefit theoretical discussions around how general rules attempted to summarise the biology, morphology, and physiology of mammals, birds (e.g., Clavel and Morlon, 2017), and ectothermic vertebrates (e.g., Troyer et al., 2022) also work for insects. On the other hand, since closely related taxa are more likely to share similar traits and consequently resource requirements (Elton, 1946), the phylogenetic tree presented in this paper could further be used to infer similarities among taxa that may affect outcomes of interspecific competition within and across biotic assemblages (Strydom et al., 2022).

A foremost research priority is to use aquatic insect distributions as the basis of meaningful geographical regionalisation across the world,

especially now that freshwater ecoregions (Abell et al., 2008) have not been found to fit organismal groups other than fish (for freshwater plants, see Alahuhta and García-Girón, 2022). Testing regionalisation patterns needs a spatially explicit approach at the grid-cell or sub-drainage basin level. Luckily, however, open-access databases (e.g., GBIF) coupled with the recent publication of worldwide records of aquatic insects (Grigoropoulou et al., 2023) may provide a preliminary opportunity to produce global regionalisation based on aquatic insect distributions. In combination with our comprehensive time-calibrated phylogenetic tree, delineating bioregions based on phylogenetic relatedness seems highly promising to understand evolutionary distinctiveness across spatial scales and infer the origins and generation of aquatic insect biodiversity gradients between Antarctica and the Arctic regions of the Northern Hemisphere (e.g., Daru et al., 2020). Once these data sources are mobilised, we can also expect to see key empirical advances in utilising systematic complementary frameworks for conservation prioritisation (e.g., Strecker et al., 2011) that move away from using traditional measures of species numbers for sustaining evolutionarily valuable areas in insect conservation programs (Cadotte and Tucker, 2018). For example, bioregions tailored to accommodate patterns of phylogenetic relatedness can be used with Key Biodiversity Areas (KBA) and heuristic spatial zoning algorithms (e.g., Moilanen et al., 2009) to define priorities as conservation foci for aquatic insect faunas. The phylogeny we presented here is also highly suitable for this type of research.

The global ‘Tree of Life’ of aquatic insects will always be a work in progress. Our concentrated efforts contribute to synthesising macro-evolutionary scenarios for a relatively large proportion of extant genera of dragonflies, damselflies, mayflies, stoneflies, and caddisflies. We have also shown how this time-calibrated phylogenetic tree can be applied in empirical data analysis to help understand phylogenetic aspects of biotic assemblages in different geographical and environmental settings. These approaches are necessary for drawing major events of aquatic insect evolution and may become a part of the standard toolkit to improve estimates of where, why, and how aquatic insect biodiversity is distributed in space and time. Overall, we hope that this updated phylogenetic hypothesis and discussions therein will stimulate new research aimed at assessing and safeguarding the phylogenetic heritage of aquatic insects and the future mobilisation of existing and yet unexplored data sources across the world.

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

High-resolution versions of the figures, dated phylogenetic tree in Newick format, and list of statistical routines for the three empirical studies are available on Zenodo (<https://doi.org/10.5281/zenodo.10254837>). The raw biological and environmental datasets used in the exemplary cases can be obtained from their primary sources (i.e., Kärnä et al., 2015; Pineda-Morante et al., 2022; Li et al., 2023).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.earscirev.2024.104767>.

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