

Not going with the flow: a comprehensive time-calibrated phylogeny of dragonflies (Anisoptera: Odonata: Insecta) provides evidence for the role of lentic habitats on diversification

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Abstract

Ecological diversification of aquatic insects has long been suspected to have been driven by differences in freshwater habitats, which can be classified into flowing (lotic) waters and standing (lentic) waters. The contrasting characteristics of lotic and lentic freshwater systems imply different ecological constraints on their inhabitants. The ephemeral and discontinuous character of most lentic water bodies may encourage dispersal by lentic species in turn reducing geographical isolation among populations. Hence, speciation probability would be lower in lentic species. Here, we assess the impact of habitat use on diversification patterns in dragonflies (Anisoptera: Odonata). Based on the eight nuclear and mitochondrial genes, we inferred species diversification with a model-based evolutionary framework, to account for rate variation through time and among lineages and to estimate the impact of larval habitat on the potentially nonrandom diversification among anisopteran groups. Ancestral state reconstruction revealed lotic fresh water systems as their original primary habitat, while lentic waters have been colonized independently in Aeshnidae, Corduliidae and Libellulidae. Furthermore, our results indicate a positive correlation of speciation and lentic habitat colonization by dragonflies: speciation rates increased in lentic Aeshnidae and Libellulidae, whereas they remain mostly uniform among lotic groups. This contradicts the hypothesis of inherently lower speciation in lentic groups and suggests species with larger ranges are more likely to diversify, perhaps due to higher probability of larger areas being dissected by geographical barriers. Furthermore, larger range sizes may comprise more habitat types, which could also promote speciation by providing additional niches, allowing the coexistence of emerging species.

Keywords: climate change, divergence time estimation, dragonflies, freshwater ecology, phylogeny, speciation rates

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Introduction

Ecological diversification of aquatic insects has long been suspected to have been driven by differences in freshwater habitats, which can be classified into two different groups: (i) flowing (lotic) waters (e.g. springs, small streams, rivers, seepages) and (ii) standing (lentic)

water bodies (e.g. swamps, ditches, pools, lakes). The contrasting spatial and temporal characteristics of lotic and lentic freshwater systems imply different ecological constraints on their inhabitants (Southwood 1977). The ephemeral and discontinuous character of most lentic water bodies may make dispersal more important for lentic species, whereas species living in more stable lotic habitats are suggested to be less dispersive (Ribera & Vogler 2000). Previous works, especially on insects (mainly beetles and dragonflies), suggest higher

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dispersal activity and larger geographical ranges in lentic representatives (e.g. Ribera *et al.* 2001, 2003; Monaghan *et al.* 2005; Hof *et al.* 2006; Grewe *et al.* 2013), as well as a better ability of lentic species to track environmental changes than lotic taxa (Hof *et al.* 2012). High dispersal ability, which does not necessarily translate into larger geographical ranges, may allow species to maintain populations over large ranges. This in turn may reduce geographical isolation between populations leading to lower interpopulation genetic variability. In this scenario, the probability for allopatric or peripatric speciation should be higher in lotic species, whose limited dispersal abilities might be seen as an isolation mechanism ('habitat-stability-hypothesis', Ribera 2008; Dijkstra *et al.* 2014). Additionally, the smaller geographical ranges of lotic species should also increase the probability of extinction with both affects leading to a higher evolutionary turnover (Ribera 2008). Although lentic species have been suggested to have lower genetic structure among populations in several groups of freshwater invertebrates (Marten *et al.* 2006; Abellán *et al.* 2009), a single formal approach to compare habitat and diversification between two beetle genera was equivocal and did not show a significantly higher diversification in the lotic genus (Ribera *et al.* 2001). However, the applied methodological framework as well as taxonomic sampling might have been insufficient to reveal potential differences in diversification rates among lentic taxa. Newer methods allow for the explicit testing of specific ecological traits as driving forces for differential diversification among clades, by implementing the inference of speciation rates and the evolution of discrete and continuous traits in a phylogenetic framework (see Pyron & Burbrink 2013; Morlon 2014; Ng & Smith 2014).

Odonata are an ideal model organism for studies in ecology and evolutionary biology in insect orders, as species numbers are relatively small (~6000) and knowledge of their taxonomy, biogeographic distribution and especially habitat use is comprehensive (e.g. Kalkman *et al.* 2008; Córdoba-Aguilar 2009; Damm *et al.* 2010; Dijkstra *et al.* 2014). In general, species richness differs considerably among anisopteran families. For example, clubtails (Gomphidae) and skimmers (Libellulidae) comprise the most diverse groups with 980 and 1037 species respectively, followed by the hawkers (Aeshnidae) with 456 species. In contrast, petaltails (Petaluridae) and austropetaliids (Austropetaliidae) each include only ten and eleven species respectively, and the South American species *Neopetalia punctata* is addressed as the single representative of the family Neopetaliidae, clearly distinguished from other families (Dijkstra *et al.* 2013). However, species richness cannot directly be correlated with habitat choice, as the clubtails and cruisers

(Macromiidae, 125 species) actually occupy lotic habitats, and both families have species numbers that are comparable to skimmers and emeralds (Corduliidae, 154 species), respectively. Studies on the potential causes of heterogeneous species richness among anisopteran families are scarce, but sister group comparisons indicate that sexual selection, based on differences in wing and body coloration between the sexes, may drive group speciation (Misof 2002). However, sexual dimorphism can be found among most anisopteran families, and it is therefore difficult to explain species richness by sexual selection alone.

In this study, we apply a comprehensive phylogenetic approach to assess the impact of habitat use on diversification patterns in the insect suborder of dragonflies (Anisoptera, Odonata). Based on a molecular data set, comprising eight independent gene fragments representing all putative dragonfly families, we reconstructed phylogenetic relationships and estimated divergence times of anisopteran families. To infer species diversification, we relied on model-based evolutionary frameworks, to account for rate variation through time and among lineages (Rabosky 2014) and to estimate the impact of lentic or lotic habitat choice on the potentially nonrandom diversification among anisopteran groups (Maddison *et al.* 2007; FitzJohn *et al.* 2009).

Materials and methods

Taxon and marker sampling

The total molecular data set comprised 522 ingroup species, representing 17% of the currently recognized anisopteran species diversity, 60% of the proposed genera (cf. Dijkstra *et al.* 2013) and all eleven families. An overview of the sampling fraction for each family is given in Table 1. As outgroups, we included seven zygopteran species and the anisozygopteran species *Epio-phlebia superstes*. The data set includes mitochondrial (mt) and nuclear (nuc) sequences. In terms of nuclear gene fragments, we included almost entire sequences of 28S rRNA (nuc rRNA, 5428 nucleotides), as well as the complete 18S rRNA, and the protein coding elongation factor 1-alpha (EF1a, 702 nucleotides) and histone 3 (H3, 327 nucleotides). In terms of the mitochondrial gene fragments, we included a major fragment of the 12S rRNA, the complete mt tRNA valine, the (nearly) complete mt 16S rRNA (mt rRNA, 1495 nucleotides) and the protein coding cytochrome c oxidase I (COI, 735 nucleotides), and cytochrome c oxidase II (COII, 687 nucleotides). The 18S rRNA and most sequences of protein coding genes were retrieved from NCBI GenBank. To increase the matrix completion, we additionally sequenced EF1a and COI gene fragments for 45

dragonfly species. Tissue samples of newly sequenced specimens are available at Zoologisches Forschungsmuseum A. Koenig (ZFMK) in Bonn, Germany, and Department of Biological Sciences, Rutgers University in New Jersey, USA. Laboratory protocols, as well as the data set compilation workflow, can be found in Appendix S1 (Supporting information). The taxon sampling with the corresponding GenBank accession numbers is provided in Appendix S2 (Table S3, Supporting information).

Phylogenetic reconstruction and divergence time estimation

For computational efficiency, we exclusively relied on maximum-likelihood (ML) approaches to explore both phylogenetic relationships and divergence times of Anisoptera. Phylogenetic tree reconstructions were conducted with a parallelized version of RAXML v8.1.11 (Stamatakis 2014) in the CIPRES Science Gateway (Miller *et al.* 2015). To reduce the risk of being trapped in a local optimum, rapid bootstrapping and search for the best likelihood tree were performed in two steps. The inference of the best likelihood tree was conducted 50 times with different random starting trees, obtained via maximum parsimony and with a final optimization of the best trees' branch lengths and parameters. Subsequently, 100 bootstrap replicates were conducted. All ML analyses were calculated with the GTRGAMMA model, the general time-reversible model with gamma distribution of rate heterogeneity among sites (Yang 1994; Stamatakis 2006). Model parameters were

estimated independently for the 11 data partitions (for details on the modelling scheme, please refer to Appendix S1 (Table S1, Supporting information). The resulting best likelihood tree served as input phylogram for the subsequent age estimation analyses.

For the latter, we used the penalized likelihood (PL) approach, introduced by Sanderson (2002) and implemented in the program TREEPL v1.0 (Smith & O'Meara 2012). This is a modified and speed-enhanced version of the program R8S (Sanderson 2003), using stochastic optimization and hill-climbing gradient-based methods, but it considers rates to be auto-correlated (although, see the following for a discussion of autocorrelation of rates in divergence time estimation: Lepage *et al.* 2007; Rehm *et al.* 2011). We chose to implement this software for our data set because preliminary attempts to run a Bayesian Markov chain Monte Carlo analysis in BEAST v1.8.1. (Drummond & Rambaut 2007; Drummond *et al.* 2012) consistently did not reach convergence after several hundred million generations. The PL algorithm calculates evolutionary rates and divergence times on a given tree and a set of fossil calibrations. It further accounts for the amount of among-branch rate heterogeneity, using a so-called smoothing parameter (Sanderson 2002). The latter was empirically determined using cross-validation procedure with seven values, ranging from 0.00001 to 100. A χ^2 test found 0.0001 to be the optimal smoothing value, indicating generally non-clock-like rates in tree. For the implementation of fossil calibration points, PL approaches either need a defined fixed age of a node, or a minimum and/or a maximum age constraint on a node. Furthermore, a hard maxi-

Table 1 Taxon sampling

Family	Species no.	Sample no.	Ratio	Genera no.	Sample no.	Ratio
Austropetaliidae	11	6	0.55	7	6	0.86
Aeshnidae	456	49	0.11	51	19	0.37
Petaluridae	10	6	0.60	5	5	1.00
Gomphidae	980	81	0.08	87	36	0.41
Neopetaliidae	1	1	1.00	1	1	1.00
Chlorogomphidae	47	5	0.11	3	3	1.00
Cordulegastridae	46	16	0.35	11	10	0.91
Synthemistidae <i>s.l.</i>	144	24	0.17	28	17	0.61
Macromiidae	125	20	0.16	4	4	1.00
Corduliidae	154	40	0.26	20	11	0.55
Libellulidae	1037	274	0.26	142	97	0.68
Total	3011	522	0.17	359	215	0.60

Species no. and genera no. refer to the estimated total numbers of anisopteran taxa, according to Dijkstra *et al.* (2013). Sample no. refers to the number of species and genera, respectively, which were included in our study. Synthemistidae *s.l.* include all genera and species defined as *incertae sedis* by Dijkstra *et al.* (2013), as these were monophyletic in our study and previous works on dragonfly relationships (Ware *et al.* 2007; Carle *et al.* 2015).

imum for the root of the tree is needed. For defining reasonable calibration points, we relied on recent studies on insect dating. The maximum age of the root node (Odonata) was set to 287 Ma. This is the upper bound of the 95% confidence interval for Odonata, found by Misof *et al.* (2014). This is further in concordance with ages found by another molecular study on palaeopteran divergence times (Thomas *et al.* 2013). We further applied minimum age constraints on nine deep nodes (e.g. families), as well as on several genera, using fossil calibrations. Deep node calibration fossils for age estimation in Odonata at the family level were recently evaluated by Kohli *et al.* (2016), according to the recommendations of Parham *et al.* (2012) for the best practices for justifying fossil calibrations (Table 2). We chose not to use additional fossils identified to extant genera, as we could not confirm their placement as crown taxa within these genera. As penalized likelihood does not automatically provide confidence intervals, we conducted another 100 bootstrap replicates with fixed best tree topology inferred with RAXML and used these as input for further TREEPL analyses. The resulting chronograms were summarized with TREEANNOTATOR v1.7.5 (Drummond *et al.* 2012), and corresponding confidence intervals were applied to the original chronogram. We deliberately refrained from using RNA models for the nuc and mt RNA partitions of our data set. Our previous studies on their effect on anisopteran phylogeny were equivocal (Ware *et al.* 2008; Letsch & Kjer 2011), but as test runs in the current study result in the fail of specific RNA models to find well-supported groups, especially Gomphidae or Aeshnidae, we decided to rely on classical DNA models for tree reconstruction.

Correlation of trait evolution and species diversification

Data for habitat use were obtained from either the IUCN database (<http://iucnredlist.org>) or specific taxonomic literature (cf. Appendix S2—Table S4, Supporting infor-

mation). As dragonflies are one of the best-researched insect groups relative to species numbers and habitat use (Clausnitzer *et al.* 2009), we were able to consider ecological information for all included species.

The impact of differential habitat use on speciation and extinction among anisopteran groups was estimated with Binary State Speciation and Extinction (BiSSE: Maddison *et al.* 2007), implemented in the DIVERSITREE v0.9-7 package (FitzJohn 2012) of the R statistical software (R Development Core Team 2014). For our approach, we first calculated an initial model in which all six rate parameters were free to vary and subsequently compared this model with several submodels in which we constrained the following parameters being equal: (i) speciation, (ii) extinction, (iii) state transition, (iv) speciation + extinction, (v) speciation + extinction + state transition, (vi) speciation + transition and (vii) extinction + transition. Each model's support was estimated with Akaike weights (AICw), which evaluate the probability of a given model among a set of candidate models (Burnham & Anderson 2002). For the best-fit BiSSE model, we estimated parameter uncertainty using Markov chain Monte Carlo algorithm with 10 000 generations and an exponential prior with rate $1/2r$ (r = character independent diversification rate). After discarding the first 1000 generations as burnin, we assessed variation in speciation (λ) and extinction (μ) rates and also calculated net diversification rates ($r = \lambda - \mu$). We further estimated the impact of phylogenetic and dating uncertainty by (i) using the 100 dated bootstrap trees with a fixed topology and (ii) the 100 bootstrap trees calculated without a fixed topology to assess node support in the tree reconstruction. For a detailed description of this approach, please refer to Appendix S1 (Fig. S1, Supporting information).

Differing rates in speciation, extinction and state transition are known to violate assumptions of rate-homogeneous models, which are frequently used in macro-evolutionary studies to perform ancestral charac-

Table 2 Fossils used for calibrating the anisopteran phylogeny, with minimal ages assigned to the according clades

#	Fossil	Clade	Age (Ma)	Formation	Fossil reference
A	<i>Mersituria ludmilae</i>	Zygoptera	152.2	Doronino Formation, Late Jurassic	Vasilenko (2005)
B	<i>Liassophlebia</i> spec.	Epiprocta	199.3	Bayreuth Formation, Late Jurassic	Bechly (1999)
C	<i>Sinacymatophlebia mongolica</i>	Anisoptera	168	Jiulongshan Formation, Mid-Jurassic	Nel & Huang (2009)
D	<i>Gomphaeschna inferna</i>	Aeshnidae	139.8	Zara Formation, Early Cretaceous	Priytkina (1977)
E	<i>Proterogomphus reateae</i>	Gomphidae	150	Solnhofen limestones, Late Jurassic	Bechly (1998)
F	<i>Juralibellula ningchengensis</i>	Cavilabiata	168	Jiulongshan Formation, Mid-Jurassic	Huang & Nel (2007)
G	<i>Epophthalmia biordinata</i>	Macromiidae	15.5	Latah Formation, Miocene	Lewis (1969)
H	<i>Croatocordulia platyptera</i>	Corduliidae	12.7	Radoboj limestone, Croatia, Miocene	Charpentier (1843)
I	<i>Tauriphila cerestensis</i>	Libellulidae	29.2	Vaucluse, France. Early Oligocene	Nel & Paicheler (1993)

Ma, million years ago.

ter state reconstruction (ASR) in a maximum-likelihood framework (Goldberg & Igić 2008). We therefore employed marginal ancestral state estimates of different larval habitat use also from the BiSSE models described above, which allows to incorporate the effects of differing model parameters on the ASR. To avoid bias caused by incomplete taxon sampling, we account for missing taxa by specifying the percentage of species that have been sampled (FitzJohn *et al.* 2009).

However, BiSSE can be biased by specific characteristics of the underlying methodology. First of all, the performance of BiSSE depends strongly on sample size (i.e. the number of included terminals) and the distribution of character states in the data set (Davis *et al.* 2013). While these primary requirements (>300 terminal taxa and >10% sampling per state) are fulfilled in our data set, specific pattern in the data and/or tree may further mislead BiSSE results. It has been proposed that a single diversification rate shift in a tree can suggest a strong correlation between character states and diversification, although the shift is independent from the traits under scrutiny (Maddison & FitzJohn 2015; Rabosky & Goldberg 2015). To reveal individual clades with deviating speciation rates, we therefore additionally calculated clade-specific speciation rate changes in our phylogenetic tree. For this purpose, we applied a Bayesian approach where rate-shift configurations are considered in proportion to their posterior probability. This approach is implemented in the software BAMM v2.5.0 (Bayesian analysis of macroevolutionary mixtures: Rabosky *et al.* 2013, 2014a; Shi & Rabosky 2015). BAMM uses 'reversible jump' Markov chain Monte Carlo (rjMCMC) to account for rate variation through time and among lineages (Rabosky 2014).

BAMM was applied on the TREEPL chronogram, and MCMC analyses were run with eight chains for 10 000 000 generations, sampling every 10 000 generations. Effective sample sizes of both the log-likelihood and the number of shift events, as well as post-run analysis and result visualization, were conducted with the 'R' package BAMMTOOLS v2.1.0 (Rabosky *et al.* 2014b). Additionally, the prior distributions on speciation (λ) and extinction (μ) rates were estimated in BAMMTOOLS with the 'setBAMMprior' command ($\lambda_{\text{initPrior}} = 6.454$, $\lambda_{\text{shiftPrior}} = 0.005$, $\mu_{\text{initPrior}} = 6.454$). To analytically account for the

assumed nonrandom sampling of our data set, we directly identify a backbone sampling fraction, as well as the sampling fraction of each dragonfly family, by specifying the percentage of species that have been sampled in each anisopteran family (cf. Table 1). To explore the impact of the sampling scheme on our results, we also conducted additional BAMM analyses with (i) a taxon sampling based of genera numbers (cf. Table 1), (ii) an overall sampling fraction by specifying the percentage of all dragonfly species that have been sampled and (iii) a total sampling fraction, assuming an complete taxon sampling. Speciation rate variation among the clades of our anisopteran tree was evaluated with the following approaches in BAMMTOOLS: (i) the mean diversification rates at any point along every branch of the tree were displayed by a so-called phylo-rate plot, which indicates distinct speciation rates by mapping colours to rates on all branches, (ii) the best overall shift configuration was shown by the maximum shift credibility (MSC) configuration, which maximizes the marginal probability of rate shifts along individual branches and (iii) speciation rates of the individual families were extracted by rate-through-time analyses.

Results

In the following, we present and discuss the results of trait evolution and differential speciation among dragonfly families. Details on the phylogeny and divergence times in dragonflies are provided in Appendices S1 and S3—Figs S2 and S3 (Supporting information).

Ancestral state and diversification rate analyses (BiSSE)

Ancestral state reconstruction analyses show that ancestral anisopteran most likely used lotic habitats and that there were three independent shifts to lentic habitats throughout the history of dragonflies (Fig. 1A), that is in Aeshnidae, Corduliidae and Libellulidae. For Libellulidae, the ancestors are proposed to have colonized lentic habitats ($P = 0.99$), whereas for the other principally lentic groups (Aeshnidae and Corduliidae), ASR analyses propose ancestral lotic habitats with a probability of 0.81 and 0.66, respectively. In Aeshnidae, there are at least two changes from lotic to lentic habitats, in

Fig. 1 Results of the BiSSE state-dependent diversification (SSE) and ancestral state reconstruction (ASR) analyses. (A) SSE results: Bayesian parameter estimates from best-fit BiSSE model, showing differences in speciation, extinction and net diversification rates between lentic and lotic species. The 95% credibility intervals are shaded and indicated by horizontal bars along the x-axis. (B) ASR results: Chronogram based on maximum-likelihood tree inference in RAxML and the subsequent estimation of divergence ages of 522 anisopteran species in TreePL. Pie charts show estimated percentage of larval habitat use for the accordant node. Blue indicates lotic habitat, and yellow indicates lentic habitat.

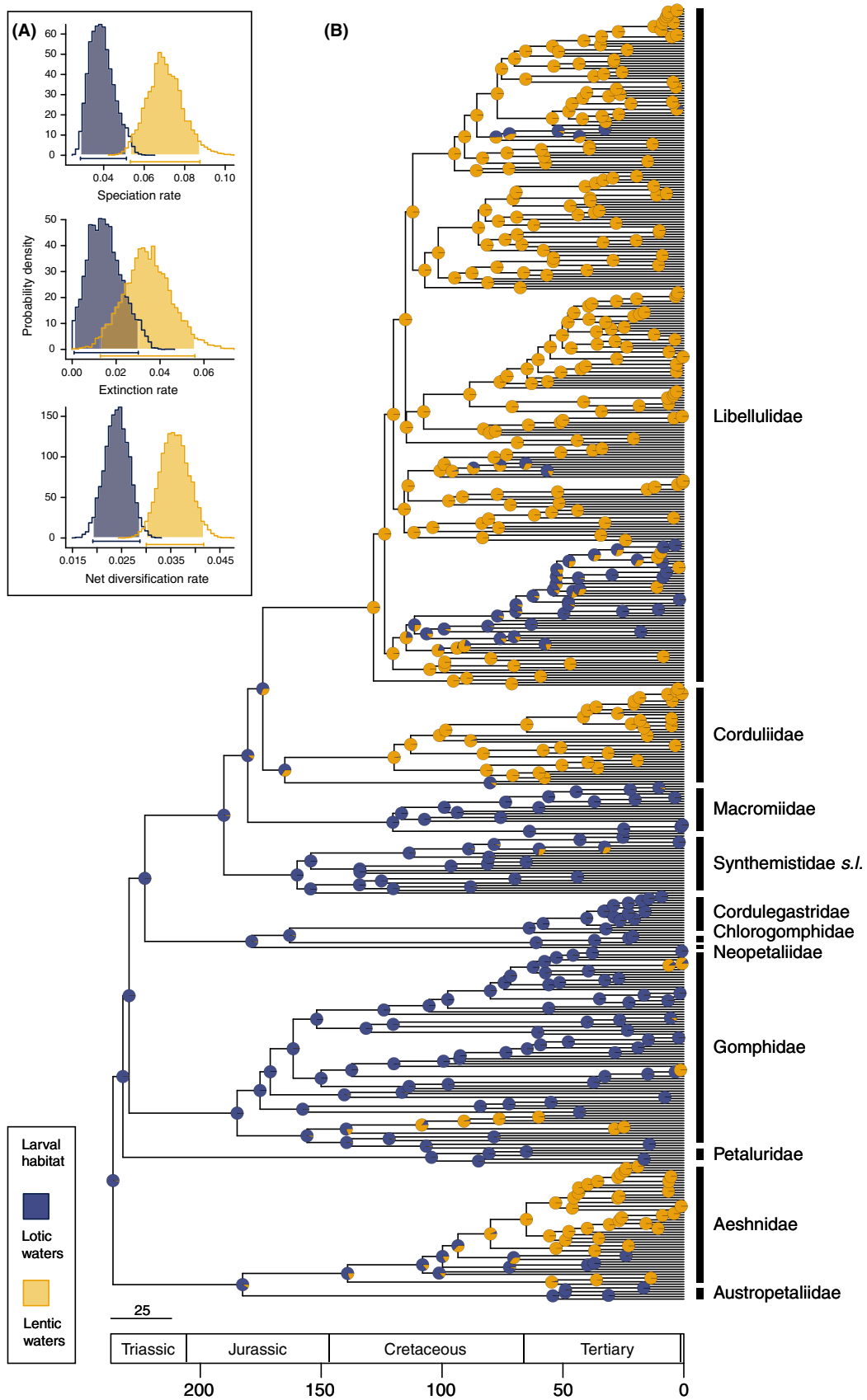


Table 3 Comparison of likelihoods for alternative models constructed in BiSSE

Model	df	λ_0	λ_1	μ_0	μ_1	q01	q10	lnL	AIC	Δ AIC	AICw
Full	6	0.0386	0.0701	0.0150	0.0347	0.0011	0.0021	-2736.1	5484.3	0.8	0.25
Equal λ	5	0.0553	—	0.0354	0.0159	0.0007	0.0029	-2739.9	5489.8	6.3	0.02
Equal μ	5	0.0450	0.0615	0.0234	—	0.0009	0.0025	-2737.0	5483.9	0.4	0.30
Equal q	5	0.0371	0.0735	0.0122	0.0394	0.0016	—	-2736.7	5483.5	0	0.37
Mk2	4	0.0623	—	0.0348	0.0348	0.0017	0.0013	-2757.1	5522.1	38.6	0.00
Mk1	3	0.0618	—	0.0342	—	0.0015	—	-2757.4	5520.7	37.2	0.00
Equal $\lambda + q$	4	0.0570	—	0.0353	0.0203	0.0014	—	-2743.8	5495.5	12.0	0.00
Equal $\mu + q$	4	0.0464	0.0610	0.0237	—	0.0015	—	-2739.5	5487.0	3.5	0.06

Abbreviations: 0, lotic; 1, lentic; λ , speciation rate parameters; μ , extinction rate parameters; q, transition rate parameters; lnL, log likelihood of model. AIC, Akaike information criterion; Δ AIC, difference between the current AIC score and the model with the best AIC score (bold); AICw, Akaike weights: probability of each model being the true model, best model in bold.

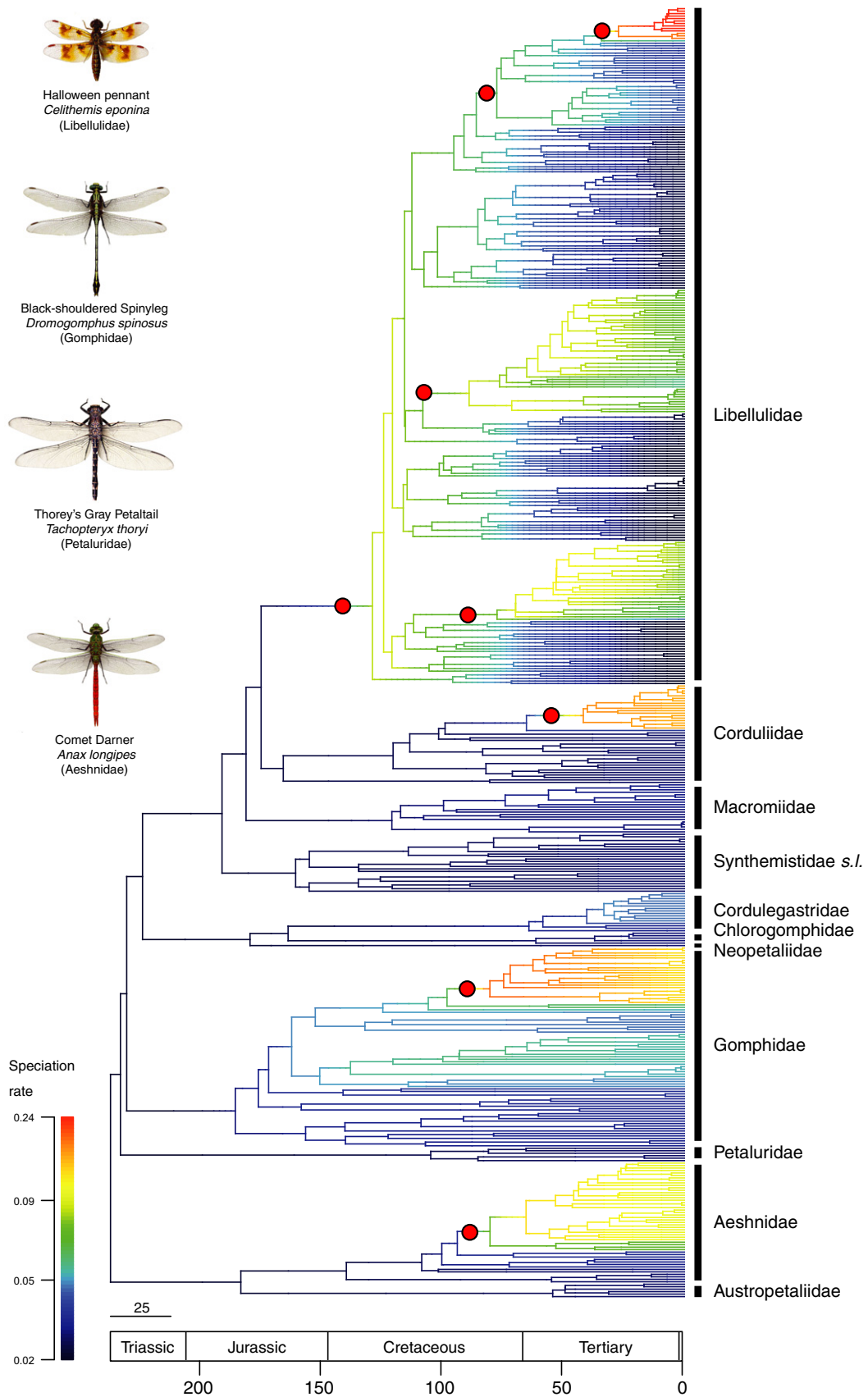
the basal subfamily Gomphaeshninae and in the derived subfamily Aeshninae, with intermediate groups remaining in lotic habitats. In Corduliidae, the first splitting taxa *Aeschnosoma* + *Penthathemis* are generally considered to be lotic, whereas the ancestor of all other corduliids switched to lentic habitats. In addition to these ancient shifts, there were several additional colonizations back into to either lotic or lentic habitats in several larger families, that is Libellulidae, Gomphidae and Synthemistidae *s.l.* However, all families can be generally assigned to one of these two habitat classes. In this context, the subsequent BiSSE analyses further indicate a correlation of shifts to lentic habitats and increased speciation rates. The best-fit BiSSE model was model (iv) with unequal speciation (λ) and extinction (μ) rates, but equal transition rates (Table 3). While this best-fit model did not have a dominant weight (AICw = 0.37), the combined AICw of all models with two independent parameters for speciation rates in lentic vs. lotic groups ($\lambda_0 \neq \lambda_1$) was 0.98 (Burnham & Anderson 2001). The results of the Bayesian MCMC analyses based on the best-fit BiSSE model and the original tree also indicate a higher speciation and net diversification rate (the difference between speciation and extinction rates) in lentic species (Fig. 1B). This was confirmed in both Bayesian MCMC analyses based on the bootstrap trees, where speciation and net diversification rates are also generally higher in lentic species (Fig. S1b,c, Supporting information). Nevertheless, there is at least a slight overlap of the 95% confidence intervals, indicating that these differences are frequent, but not significant. Higher extinction rates are also esti-

mated for lentic species, but these show a considerable overlap of the 95% confidence intervals.

Diversification rate-shift analysis (BAMM)

Markov chain Monte Carlo runs of the BAMM analysis converged quickly and excluding 1 000 000 generations as burnin proved to be sufficient. The effective sample size of the log likelihood and the number of shift events were 266 and 485, respectively, thus both higher than the threshold of 200. The BAMM phylorate plot of speciation rate, as well as the maximum shift credibility score (Fig. 2), displays considerable rate heterogeneity across the anisopteran tree. Increased speciation is indicated at the base of the Libellulidae, within Aeshnidae at the base of Aeshninae, and within the Gomphidae at the base of the Gomphinae. Additionally, higher speciation rates are inferred in the libellulid genera *Trithemis*, *Sympetrum*, *Libellula* and *Sympetrum* + *Orthetrum*, as well as in the corduliid genus *Somatochlora*. Application of different sampling schemes in the additional analyses provides slightly different results; however, all approaches show increased speciation rates in the lentic groups Libellulidae, Aeshnidae and Corduliidae (Appendix S3—Fig. S4, Supporting information). The rate-through-time plots (Fig. 3), which display the change of speciation, extinction and net diversification rate over time in the different anisopteran families, also indicate an increased speciation in Aeshnidae, Corduliidae and Libellulidae, and—to a lesser extent—in Gomphidae. However, net diversification rate is not increased in lotic Gomphidae, but in all lentic families. In Libelluli-

Fig. 2 Results of the evolutionary rate analysis in BAMM. Phylorate plots of anisopteran families. The colours indicate relative speciation rates on each branch on the TreePL chronogram. The warmer (red) colours indicate faster rates than cooler (blue) colours. Red circles on the branches indicate the positions of regime shifts in the maximum shift credibility (MSC) configuration. Pictures of dragonflies by Forest Mitchell, Texas A&M University.



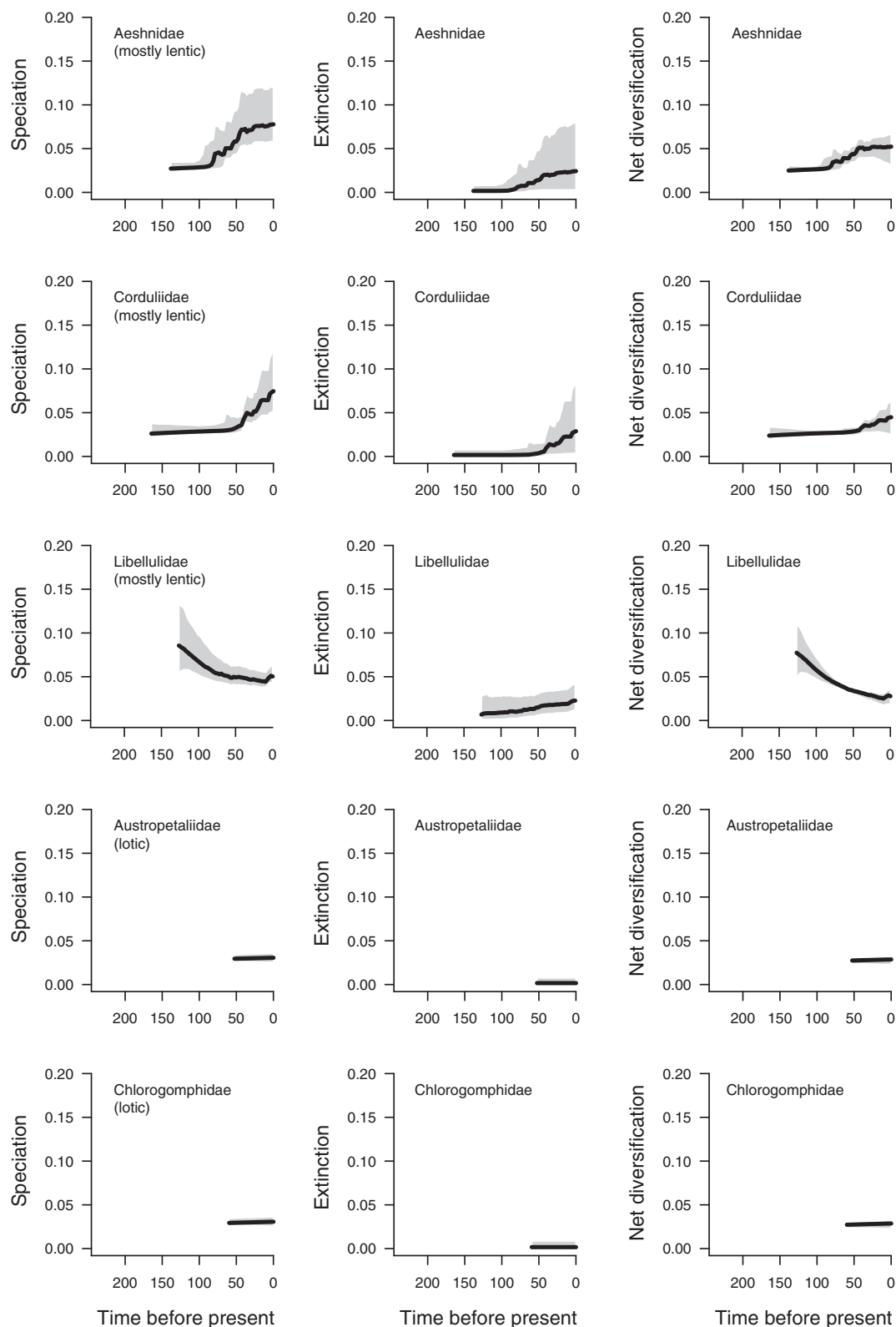


Fig. 3 Diagrams show results of the speciation, extinction and net diversification rate over time estimations in all anisopteran families, except Neopetaliidae, which include only one species. Rates of the individual families were extracted by the rate-through-time approach with BAMMTOOLS. Grey polygon denotes the 10% through 90% Bayesian credible regions on the distribution of rates, calculated in BAMM.

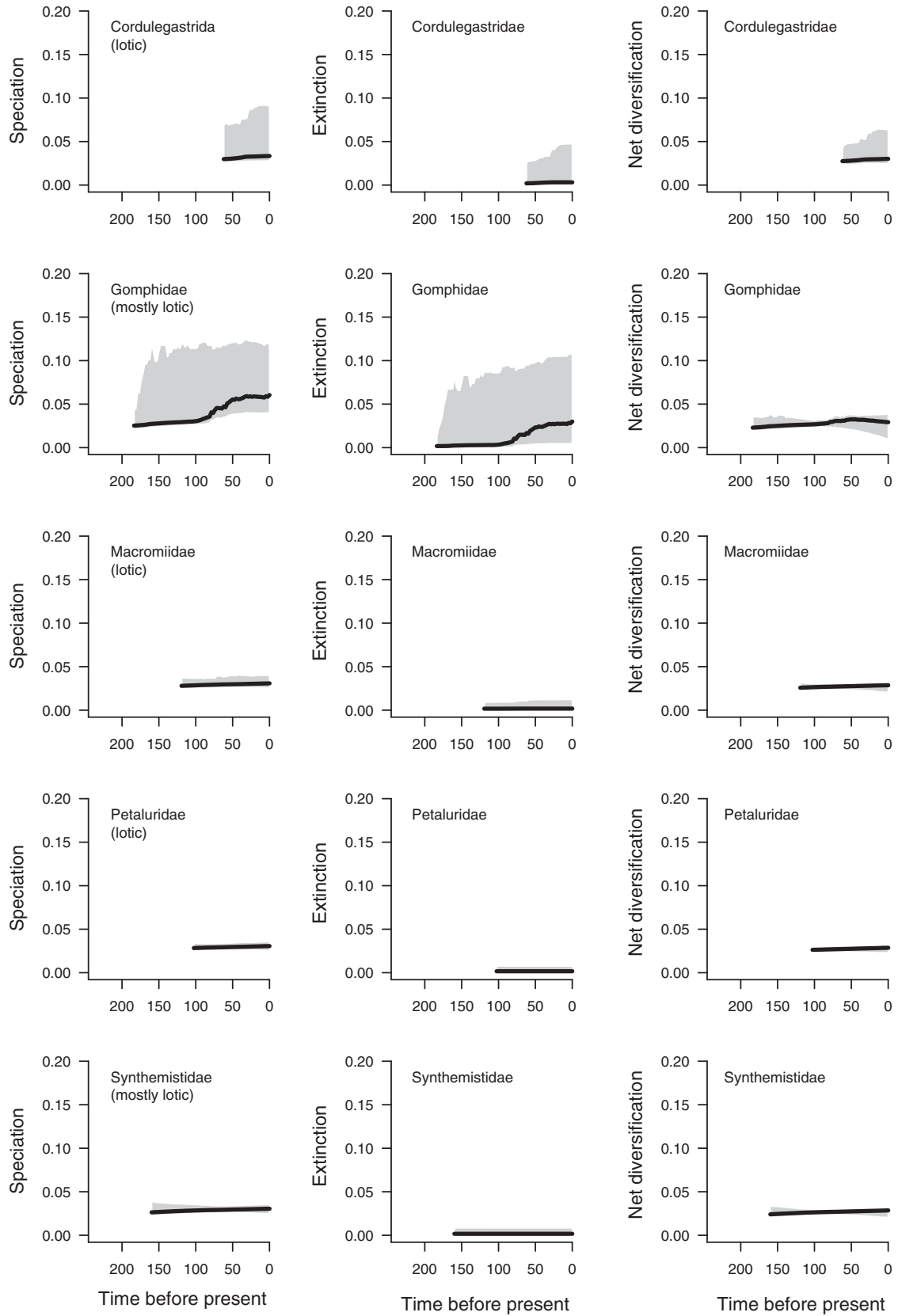


Fig. 3 Continued.

dae, speciation and net diversification rates considerably decrease over time, but start considerably above the average anisopteran rate level, indicating an ancient rapid radiation pattern. In contrast, all other families with elevated speciation and net diversification rates start to evolve with an average speciation rate, but this increased over time. In summary, six of eight rate shifts identified in BAMM are associated with lentic habitat use. The basal shifts in Libellulidae and Aeshnidae furthermore coincide with a shift from lotic to lentic habitats.

Discussion

Given the results and experiences of the present study, the combined application of both the BiSSE and BAMM methods is generally worth to consider for testing trait-dependent macro-evolutionary diversification and speciation in phylogenetic trees. As outlined in section 2.3, BiSSE and its related approaches (c.f. FitzJohn 2012), which simultaneously model character change and its effects on the diversification depicted in the phylogeny, may be prone to type I errors and thus may infer statistically significant associations of neutral traits and speciation rate (Rabosky & Goldberg 2015). However, in this context, BAMM can be used as a cross-validation to locate shifts in speciation and/or extinction rates in specific clades of the phylogenetic target tree. This further allows to compare identified shifts with the pattern of traits under scrutiny and evaluate, if shifts inferred by the BAMM models are congruent to potential trait-dependent diversification, proposed by SSE models.

Here, the results of both the BiSSE and BAMM methods evidence a higher diversification in anisopteran families, which colonized lentic freshwater habitats than in those inhabiting lotic waters. This contradicts one of the predictions of the 'habitat-stability hypothesis' (Ribera 2008). Originally based on geographical data indicating smaller ranges for lotic species, the 'habitat-stability-hypothesis' proposes a generally higher diversification in insect groups inhabiting lotic waters (Ribera *et al.* 2001; Ribera 2008). Additional evidence for a higher population genetic structure in lotic species has also supported this hypothesis that the limited dispersal abilities of lotic taxa act as a kind of isolation mechanism (Marten *et al.* 2006; Abellán *et al.* 2009). The results of our study are in stark contract with this hypothesis and rather support the alternative macroevolutionary scenario that larger range sizes promote speciation. This idea was already suggested by Darwin (1859) and Rosenzweig (1995) postulated that species with larger ranges are more likely to speciate, simply by a higher probability of larger areas being dissected by geographical barriers. Additionally, it has also been proposed that larger range sizes comprise more habitat (sub-) types (e.g. Price *et al.*

2011), which allows for more species distributed across a larger number of niches. However, niche filling depends on niche breadth (e.g. Gallagher *et al.* 2015), and it is unclear whether odonate lentic or lotic species show a pattern in terms of their niche breadth. Indeed, *Enochrus bicolor* water beetles shifting from lotic to lentic habitats did not show an increase in niche breadth (Arribas *et al.* 2012), but the fundamental and realized niches of odonate larvae are not yet well known for many taxa. Generally, in the case of freshwater invertebrates, larger range sizes would mean for example different types of lentic water bodies, such as marshes, swamps, bogs, small and larger lakes which can be further partitioned into deeper and shallower water, vegetated banks and open water, rocky vs. sandy substratum and so on. This could also promote speciation by providing additional niches, which would allow the coexistence of emerging species. In this context, the BAMM analysis allows interpreting speciation rate shifts correlated with paleo-geographical and paleo-environmental changes over time in the past. Divergence time estimations indicate the independent appearance of lentic groups is located not before the Early Cretaceous in Libellulidae (95–150 Ma) and Corduliidae (96–139 Ma). At that time, the break-up of the Gondwana supercontinent formed in the Jurassic was already underway. This ongoing break-up was correlated with deep changes in earth climate, as the interior of huge landmass Gondwana had a hot and arid climate, whereas the continental drift of the Late Jurassic and Early Cretaceous lead to a more humid and warm climate with increased sea level and precipitation (see Chaboureaud *et al.* 2014; Cui & Kump 2014). If this translated into an increased amount of freshwater habitats, lentic species with larger ranges would be able to faster occupy these new habitats and speciate more rapidly, they are proposed to have a higher ability to track changes in the environment (Hof *et al.* 2012). Indeed, if lentic species have more niche breadth, perhaps that may affect extinction rates (Gallagher *et al.* 2015) and be reflected in the diversification rates we infer here.

Additionally, studies on fossil anisopteran groups indicate a remarkable turnover in the mid-Cretaceous: Aeshnidiidae and Stenophlebiidae flourish in the Late Jurassic and the Early Cretaceous but probably became extinct in the Cenomanian (Carle & Wighton, 1990; Zhang 1999; Fleck *et al.* 2003; Fleck & Nel 2003). In particular, Aeshnidiidae are relatively well known by their adult and larval fossil record, and because of their large size and distribution, they have probably been dominant insect predators and considerable competitors for other anisopteran groups. Thus, the general turnover in the mid-Cretaceous could be correlated with their extinction, leading to an enhanced niche-building in libellulid dragonflies (Nel *et al.* 2015).

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H.L. designed the study, conducted the phylogenetic and statistical analyses and wrote the manuscript; B.G. performed all laboratory work; H.L. and J.L.W. conducted divergence time analyses; all authors contributed to the improvement of the manuscript.

Data accessibility

DNA sequences: GenBank accessions numbers are provided as online Supporting Information. Alignments

and tree files have been deposited in the Dryad repository: <http://dx.doi.org/10.5061/dryad.f3d4f>

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Laboratory protocols, additional comments on the anisopteran tree reconstruction and the results of the BiSSE Bayesian MCMC analyses.

Appendix S2 Taxon sampling, including GenBank accession numbers and habitat information for all included species.

Appendix S3 Detailed results of the maximum likelihood tree reconstructions (Fig. S2) and divergence time estimations (Fig. S3), as well as of the additional BAMM analyses (Fig. S4).

Table S1 PartitionFinder output: subset schemes and models.

Table S2 Results of the divergence time estimations, including mean ages and upper and lower bounds of the highest posterior density (HPD). Results of the BiSSE ancestral state analyses (ASR).

Table S3 Taxon sampling including NCBI genbank accession numbers.

Table S4 Habitat (and codes) for all included species.

Fig. S1 Results of the Bayesian MCMC analyses in BiSSE.

Fig. S2 Tree representing the optimal bifurcating solution under maximum likelihood computed with RAxML and based on the partitioned data set.

Fig. S3 Chronogram based on the maximum likelihood inference in RAxML and a subsequent estimation of divergence ages in TreePL.

Fig. S4 Phylorate plots and maximum shift credibility (MSC) configuration (red circles) of the additional BAMM analyses to explore the potential impact of non-random taxon sampling on the estimations of differential speciation among dragonfly families.