

Short Communication

# Divergence dates of libelluloid dragonflies (Odonata: Anisoptera) estimated from rRNA using paired-site substitution models

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## 1. Introduction

Molecular dating methods have provided valuable insights into the rates and time-scales of evolution in a variety of organisms. All of these methods make a number of assumptions about the evolutionary process that can have a profound influence on the resulting date estimates. Accordingly, model selection is an important component not only of topological reconstruction but also of divergence time estimation; poorly chosen models can lead to errors in date estimates (e.g., Sullivan and Swofford, 1997). There has been a consistent focus on the assumption of a molecular clock, with substantial variation in substitution rates having been observed in a range of taxonomic groups (Bromham and Penny, 2003). This prompted the development of sophisticated methods for dealing with rate heterogeneity among lineages (e.g., Thorne et al., 1998; Sanderson, 2002; Drummond et al., 2006).

The impact of substitution model selection on date estimates has received considerably less attention. The potential effect of model selection is perhaps most pronounced in the analysis of rDNA molecules, the rRNA products of which are subject to distinctive substitution patterns on account of secondary structural constraints (Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993). Ribosomal RNA (rRNA) folds on itself, creating a complex secondary structure maintained by bonded nucleotide pairs, which is important for ribosome function (Noller, 1984). In these stem regions, a substitution at one site is often accompanied by a compensatory substitution at its complement in order to prevent disruption to the secondary structure of the molecule (Smith et al., 2004; Tillier and Collins,

1998). For example, in an analysis of eubacterial rRNA sequences, Savill et al. (2001) found that the rate of double substitutions was significantly non-zero.

The correlated mode of molecular evolution in RNA stems violates one of the fundamental assumptions of standard nucleotide substitution models, that of independence among sites (Wheeler and Honeycutt, 1988; Dixon and Hillis, 1993). In such cases, it is more appropriate to analyse the alignment using a paired-site model that treats the nucleotide and its complement as a single character (Jow et al., 2002). The RNA loop regions, which are unpaired, can be suitably analyzed using standard substitution models. This fundamental difference in evolutionary modes also argues for data partitioning in the phylogenetic analysis of rRNA molecules based on secondary structure. These concerns have received scant attention in divergence dating studies despite the widespread use of rRNA as a phylogenetic marker: among 14 studies that used rRNA data to estimate divergence times, only one used a paired-sites model (Table 1).

A number of paired-sites substitution models are available for the phylogenetic analysis of RNA sequences. The earliest models treated all 16 possible pairs that can be formed with four nucleotides as distinct states, producing a time-reversible  $16 \times 16$  rate matrix (Schöniger and von Haeseler, 1994; Muse, 1995; Rzhetsky, 1995). This may be unnecessarily complex, since only matching pairs (the four Watson–Crick pairs, along with G–U and U–G pairs) are frequent in the helix regions of ribosomal molecules (Savill et al., 2001). Subsequently, less general models have been developed. Seven-state models, such as that described by Tillier and Collins (1998), consider only the six matching pairs and group the remaining 10 mismatch pairings into a single state (Higgs, 2000). These have been shown to fit the data as well as 16-state models (Savill et al., 2001).

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Table 1

Fourteen divergence dating studies using RNA data sets, found using database searches of CAB abstracts, Google Scholar, Pubmed, and *Systematic Biology* with the search terms ‘phylogeny’, ‘dating’, ‘r8s’, ‘divergence estimation’, ‘paired sites’, and ‘doublet’

Reference <sup>a</sup>	Data set	Gene	Substitution model
Baker et al. (2005)	Moa	12S, <i>tRNA<sub>Lys</sub></i>	GTR+I+G
Bossuyt et al. (2006)	Frogs (Ranidae)	12S, 16S, <i>tRNA<sub>Val</sub></i>	GTR+I+G
Brammer and Dohlen (2007)	Flies (Stratiomyidae)	28S	GTR+I+G
Crayn et al. (2006)	Trees (Elaeocarpaceae)	<i>trnL-trnF</i> , ITS	GTR+G
Dumont et al. (2005)	Dragonflies (Calopterygoidea)	ITS1, ITS2, 18S, 28S	GTR+I+G
Gómez-Zurita et al. (2007)	Leaf beetles (Chrysomelidae)	28S, 18S, 16S	F84-C8
Jansen et al. (2006)	Fish (Clariidae)	ITS1, ITS2, 18S, 28S	GTR+G
Near et al. (2005)	Fish (Centrarchidae)	16S	Paired- and unpaired-sites were partitioned, but a paired-sites model was not used
Pereira and Baker (2006)	Birds	12S, 16S, 22 mt tRNAs	HKY+G
Perez-Losada et al. (2004)	Barnacles (Thoracica)	12S, 16S, 18S, 28S	HKY+G
Wiegmann et al. (2003)	Flies (Brachycera)	28S	HKY+G*
Winterton et al. (2007)	Flies (Acroceridae)	16S, 28S	GTR+I+G
Williams et al. (2003)	Periwinkles (Gastropoda)	12S, 18S	GTR+I+G (28S+12S), SYM+I+G (18S), GTR+G (12S)
Zhang et al. (2006)	Salamanders (Hynobiidae)	12S, 16S, <i>tRNA<sub>Val</sub></i>	?

<sup>a</sup> Baker et al. (2005) *Proc. Natl. Acad. Sci. USA*, **102**: 8257–8262; Bossuyt et al. (2006) *Syst. Biol.*, **55**: 579–594; Brammer and Dohlen (2007) *Mol. Phylogenet. Evol.*, **43**: 660–673; Crayn et al. (2006) *Am. J. Bot.*, **93**: 1328–1342; Dumont et al. (2005) *Syst. Biol.*, **54**: 347–362; Gómez-Zurita et al. (2007) *PLoS ONE*, **2**: e360; Jansen et al. (2006) *Mol. Phylogenet. Evol.*, **38**: 65–78; Near et al. (2005) *Evolution*, **59**: 1768–1782; Pereira and Baker (2006) *Mol. Phylogenet. Evol.*, **38**: 499–509; Perez-Losada et al. (2004) *Syst. Biol.*, **53**: 244–264; Wiegmann et al. (2003) *Syst. Biol.*, **52**: 745–756; Winterton et al. (2007) *Mol. Phylogenet. Evol.*, **43**: 808–832; Williams et al. (2003) *Mol. Phylogenet. Evol.*, **28**(1): 60–86; Zhang et al. (2006) *Proc. Natl. Acad. Sci. USA*, **103**: 7360–7365.

In this study, the effect of using a paired-sites model in divergence date estimation is investigated using a libelluloid dragonfly rRNA data set. Libelluloid dragonflies are highly specialized, widely collected, and often brightly colored members of the most recently derived part of the Anisopteran lineage. Libelluloidea comprises seven major groups (six families: Cordulegastridae, Neopetaliidae, Chlorogomphidae, Macromiidae, Corduliidae, and Libellulidae; and one monophyletic group, the GSI *sensu* Ware et al. (2007), of undetermined taxonomic status; the “higher Libelluloidea” comprise GSI, Macromiidae, Corduliidae, Libellulidae). In comparison with other dragonflies, libelluloids are considered highly derived, primarily because they possess a reduced ovipositor that allows for endophytic oviposition, a probably convergent trait shared with Gomphidae (Carle, 1995), and unique larval labial modifications. The earliest libelluloid fossils are not older than the mid-Cretaceous, roughly 120–130 Myr (e.g., Jarzembowski and Nel, 1996).

Date estimates from two relaxed-clock analyses are presented, one in which the tree is obtained using a paired-sites model for RNA stem regions, and one in which the tree is obtained using standard unpaired-sites models for all data partitions. We investigate the differences between the results of these two analyses in the context of non-independent evolution at stem sites, and examine how interpretations of biogeographical and morphological evolution are affected by differing date estimates.

## 2. Materials and methods

### 2.1. Data set

The rRNA sequences used in the present study were a subset of those analyzed by Ware et al. (2007), with gene

fragments from the nuclear large subunit (28S; D2, D3, D7) and the mitochondrial large subunit (16S) (Supplementary material). Sequences were aligned manually using the structural methods described in Kjer (1995). Ambiguously aligned regions were excluded from the data set, yielding a final alignment of 1763 bp from 32 taxa. The alignment was divided into four partitions (28S stem, 28S loop, 16S stem, and 16S loop). Our alignments are available on the Kjer lab website ([www.rci.rutgers.edu/~entomology/kjer](http://www.rci.rutgers.edu/~entomology/kjer)).

### 2.2. Model selection

Substitution models were selected for each loop partition by comparison of Akaike Information Criterion (AIC) scores. There is no immediately apparent method for comparing the fit of non-nested paired and unpaired models for the RNA stem partition, because the most commonly used methods for model selection are not readily able to compare models with different state spaces. Consequently, analyses were performed using paired- and unpaired-sites models, and the results were compared.

### 2.3. Phylogenetic analysis

Bayesian phylogenetic analyses were performed using the software PHASE (Jow et al., 2002). Two separate analyses were performed: (i) the AIC-selected substitution model was assumed for each partition; (ii) AIC-selected substitution models were assumed for non-stem partitions, but the RNA7A model was assumed for stem partitions. This model was chosen because it is a realistic simplification of the full 16-state model (Savill et al., 2001; Jow et al., 2002). The simpler RNA6A

model was not used because inspection of the alignment revealed that some paired stem sites did not contain matching pairs. Posterior distributions were approximated by Markov chain Monte Carlo (MCMC) sampling, with samples drawn every 500 steps over a total of 10,000,000 steps following a discarded burn-in of 1,000,000 steps. Convergence to the stationary distribution and acceptable mixing were checked by inspection of MCMC traces. Consensus topologies and optimal branch lengths were computed using maximum likelihood in *PHASE*. The resulting trees were used as fixed input trees for subsequent divergence dating analysis.

Following rejection of a molecular clock using a likelihood ratio test ( $p = 0.008$ ), divergence dates were estimated in a relaxed-clock framework by the program *r8s* (Sanderson, 2003) using penalized likelihood, a method that applies a penalty against large rate changes between neighboring branches. The magnitude of the penalty is dictated by a smoothing parameter, the value of which was optimized by cross-validation analysis. A logarithmic penalty was used on account of its similarity to the autocorrelated relaxed-clock models used in Bayesian phylogenetic methods (e.g., Thorne and Kishino, 2002). Dates and substitution rates were estimated using the truncated Newton algorithm with 10 independent starts.

#### 2.4. Calibration using fossils

We used fossil-based estimates of divergence times between Chlorogomphidae, Macromiidae, Corduliidae, Libellulidae, and the Libelluloidea to calibrate the divergence dating analysis (Supplementary material). Due to the difficulty in acquiring confident genus-level identifications of dragonfly fossils, age bounds were for family-level limits only.

#### 2.5. Simulations

Analyses of simulated data were performed in order to investigate the effect of ignoring the paired nature of stem regions. Using parameter values and the tree estimated from the stem region of 28S rRNA, 50 data sets were generated by simulation under a paired-sites model, with substitution parameters and sequence length (532 bp) matching those estimated from the real data set. A strict molecular clock was assumed.

Each of the 50 alignments was analyzed with paired- and unpaired-sites models in *PHASE*, with the topology fixed. In each case, the consensus tree (with average branch lengths) was used as the fixed input tree for divergence time estimation using *r8s*. The Langley–Fitch algorithm was used, which assumes a strict molecular clock. Paired *t*-tests were performed to investigate differences in divergence times inferred under paired- and unpaired-sites models.

### 3. Results

#### 3.1. Model selection

Inspection of likelihoods reveals that the best tree under the paired-sites model, with branch lengths optimized using maximum likelihood, is 712 log-likelihood units more likely than the best tree under the unpaired-sites model. This result, along with the known paired nature of RNA stem sites, can be taken as at least a qualitative indication that the paired-sites model is preferred. Nevertheless, a more formal model selection framework is desirable for future studies.

#### 3.2. Topology

Two taxa with zero-length terminal branches were removed from the paired-sites analysis, because the log penalty algorithm is unable to handle zero values. Removal of these same taxa from the unpaired-sites analysis did not substantially affect the resulting estimates.

As in previous studies, analyses using both paired- and unpaired-sites models strongly supported the monophyly of Corduliidae (Fig. 1). The two topologies differ in their placement of Corduliidae and Macromiidae, whose mutual relationships with Libellulidae were unresolved in a previous analysis by Ware et al. (2007) and have been the subject of considerable debate (see Ware et al., 2007 for a detailed review). There were two main differences between the phylogenetic reconstructions produced using the different substitution models: the placement of the libellulid taxa *Urothemis* and *Perithemis* (Fig. 1). The unpaired-sites model yielded trees in which *Perithemis* was closely related to *Pantala*. By contrast, the analysis using a paired-sites substitution model placed *Perithemis* at the base of Libellulidae, as sister taxon to all other libellulids. *Urothemis* was placed as sister to the remaining Libellulidae by the analysis using an unpaired-sites model, although with low support. The analysis using a paired-sites model reconstructed an alternate topology, with *Urothemis* nested well within the Libellulidae, as sister to the {*Libellula* + *Ladona* + *Plathemis* + *Pantala*} clade, again with extremely low support. The position of *Perithemis* was also variable in the analyses of Ware et al. (2007). *Urothemis*, however, was consistently recovered in a basal position within Libellulidae in all analyses by Ware et al. (2007).

We acknowledge that topology changes may also affect divergence estimates. In addition, there might be a link between loss of phylogenetic signal observed and the variation in divergence time estimates. Future research should explore this, and evaluate taxa within Libelluloidea whose position is variable with ingroup and model selection, such as *Perithemis*. Studies using these taxa should treat their phylogenetic position with caution.

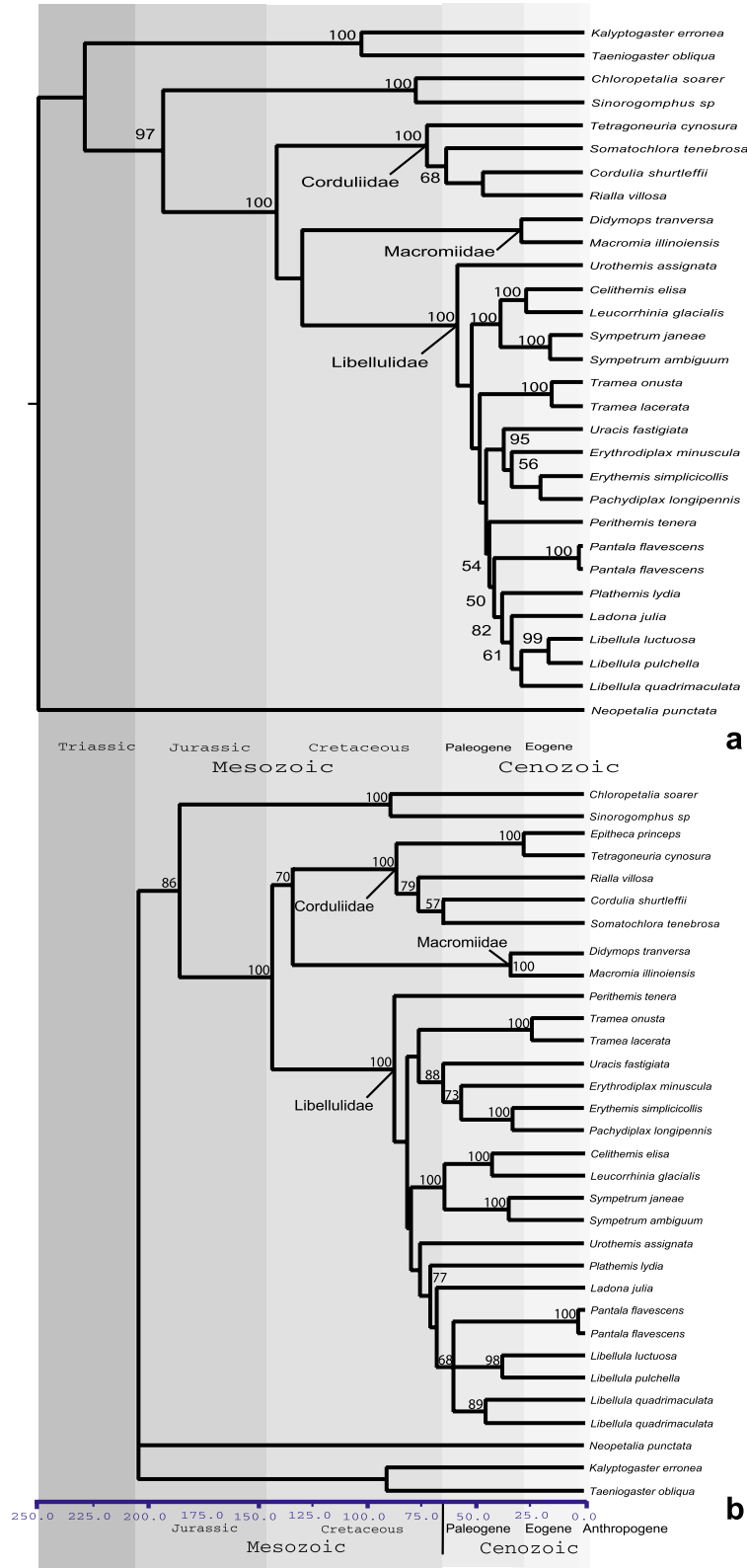


Fig. 1. Results of r8s analyses using an unpaired-sites model (a) and a paired-sites model (b).

### 3.3. Divergence time estimates

For most nodes, divergence time estimation from the paired-sites tree yielded more recent dates than those from

the unpaired-sites tree (Table 2). The estimates for the time to the most recent common ancestor (TMRCA) of (i) Libellulidae, (ii) Corduliidae, and (iii) *Cordulia* + *Somatochlora*, however, were younger in the unpaired-sites analysis. For

the *Cordulia* + *Somatochlora* node, the unpaired-sites model suggested shorter branch lengths and a lower average substitution rate (unpaired-sites:  $6.04 \times 10^{-4}$  subs/site/Myr; paired-sites:  $6.04 \times 10^{-3}$  subs/site/Myr) than the paired-sites analysis. Similar patterns were observed for the Libellulidae and Corduliidae nodes (unpaired-sites: Corduliidae  $5.17 \times 10^{-4}$  subs/site/Myr, Libellulidae  $5.02 \times 10^{-4}$  subs/site/Myr; paired-sites: Corduliidae  $1.22 \times 10^{-3}$  subs/site/Myr, Libellulidae  $1.13 \times 10^{-3}$  subs/site/Myr). This resulted in older date estimates in the analysis using the paired-sites model.

In several cases, the differences in date estimates resulted in the nodal age being assigned to a different evolutionary time period (Table 2). For example, the unpaired-sites dating analysis suggested a Permian/Triassic age for the root of the tree, while the paired-sites model dating analysis suggest a younger, Jurassic age (Table 2). The paired-sites model dating analysis suggests a Cretaceous age for the most recent common ancestor of Libellulidae, while the unpaired-sites model analysis suggests a younger, Cenozoic age.

### 3.4. Simulations

There were significant differences between the divergence times inferred under paired- and unpaired-sites models. For all major nodes (interfamilial relationships), the estimated ages were within one standard deviation of the true (simulation) ages. Age estimates made under the unpaired-sites model were older than those made under the paired-sites model, although to varying degrees (paired *t*-tests, *p*-values ranging from  $9.36 \times 10^{-5}$  to 0.065); this result is consistent with those obtained from the real data set.

In the trees inferred using *PHASE*, with branch lengths measured in substitutions per site, the ratio of internal to external branch lengths was significantly different (paired *t*-test,  $p = 2.96 \times 10^{-9}$ ) between paired- and unpaired-sites

models (Fig. 2). This suggests that the inappropriate use of an unpaired-site model does not simply alter the total tree length, as would be expected if the model produces a consistent bias among branches.

## 4. Discussions

Our analyses yielded strong preference for a paired-sites model over an unpaired-sites model for the analysis of rRNA sequences from libelluloid dragonflies. Based on comparison of likelihood scores from the two analyses, along with strong support in the literature for the paired-sites model in studies of rRNA (Savill et al., 2001; Jow et al., 2002; Hoyle and Higgs, 2003; Hudelot et al., 2003), and from our simulation studies, we consider divergence times obtained using the paired-sites model to be better estimates than those obtained using the unpaired-sites model. This suggests that use of the latter has led to erroneous estimates of the ages of several nodes in the tree: the age of the root of the tree, the TMRCA of Chlorogomphidae + Libelluloidea, and the TMRCA of Corduliidae + Macromiidae were overestimated; whereas the TMRCA of Libellulidae, Corduliidae, and Macromiidae were underestimated. Although it may be difficult to objectively assess which date estimates are unreasonable, the age of 249 Myr for the root of the unpaired-sites tree is surprisingly old, and close to the upper calibration limit of 250 Myr.

### 4.1. Do differences in date estimates really matter?

In many cases, the dates obtained by analyses using paired- and unpaired-sites treatments did not differ greatly. Consequently, the impact on evolutionary interpretations based on these would not be substantial. For Libelluloidea, however, two important nodes of interest were inconsistent between models: the age of the root of the tree and the

Table 2  
Divergence times and substitution rates estimated using the paired- and unpaired-sites models

Parameter	Estimate	
	Paired-sites model	Unpaired-sites model
Divergence times		
Root	205.7 Myr	249.1 Myr
Macromiidae	34.2 Myr	28.2 Myr
Epitheca – Tetragoneuria	28.5 Myr	N/A
Cordulia – Somatochlora	65.7 Myr	62.3 Myr
Corduliidae	87.1 Myr	71.6 Myr
Corduliidae – Macromiidae	134.2 Myr	140.1 Myr
Libellulidae	87.6 Myr	57.7 Myr
Libellulidae + Macromiidae + Corduliidae	144.0 Myr	N/A
Chloropetaliidae + Sinorogomphidae + ingroup	186.3 Myr	192.4 Myr
Substitution rate		
Mean	$7.95 \times 10^{-4}$ subs/site/Myr	$5.81 \times 10^{-4}$ subs/site/Myr
Minimum	$3.67 \times 10^{-5}$ subs/site/Myr	$7.49 \times 10^{-5}$ subs/site/Myr
Maximum	$1.85 \times 10^{-3}$ subs/site/Myr	$1.28 \times 10^{-3}$ subs/site/Myr

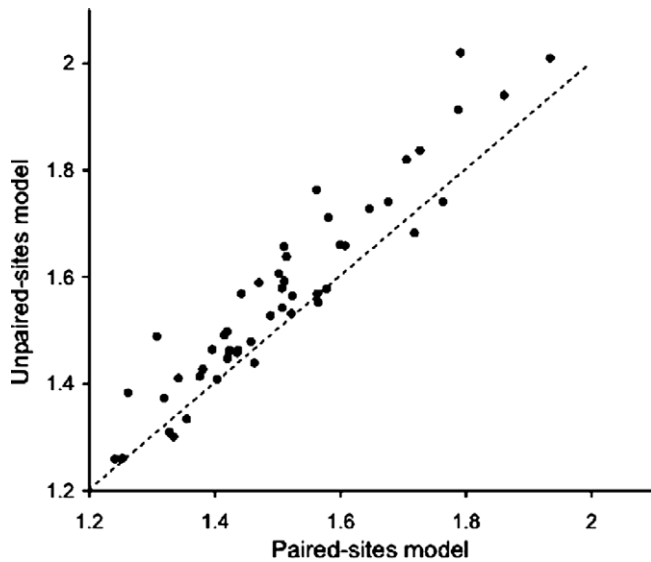


Fig. 2. Results of simulation study: a plot of the ratios of internal to external branch lengths from analyses of 50 independent data sets using unpaired- and paired-sites models.

divergence date for the most speciose, heterogeneous, and widely studied libelluloid family, Libellulidae.

In the case of the root of the tree, the unpaired-sites analysis returned a surprisingly early date at the Permian/Triassic boundary instead of being in the Jurassic as suggested under the paired-sites model. The difference in these dates would affect assumptions of the geographical positions of continents, air temperature, sea level, and the biodiversity that would have been present when this node of interest diverged. If the Libelluloidea had diverged at the Permian/Triassic boundary, it would have been during a period where only one large continental landmass, Pangaea, existed (in what is considered a period of hot dry climate; e.g., Parish, 1993). This would have just followed one of the largest extinctions of insects in evolutionary history (e.g., Erwin, 2006). This differs dramatically from the conditions that the Libelluloidea would have encountered had they diverged 50 million years later, in the early Jurassic, as the paired-sites tree model analysis suggests. At this younger date, Pangaea would have already begun to break apart, with an associated increase in the amount of inland water, a decrease in overall temperature, and an increase in humidity levels on the continents (e.g., Hallam, 1993). Our understanding of the biogeographical history of Libelluloidea is strongly dependent on correct assumptions about continental positions. Did the Libelluloidea diverge when there was a single continental land mass after which vicariance events involved in the break up of Pangaea led to speciation? Alternatively, did the Libelluloidea diverge during the early Jurassic in Gondwana, for example, and then disperse to other continents?

Our understanding of character evolution in Libelluloidea is also strongly affected by incorrect date estimation. For example, Libelluloidea have in common a reduction in their ovipositor, convergently shared with Gomphidae

(Carle, 1995). In libelluloids the ovipositor is modified for exophytic oviposition (i.e. not requiring plant tissue for egg deposition; Tillyard, 1917; Carle, 1995; see Ware et al., 2007 for figures). In the higher libelluloid taxa studied here (Macromiidae, Corduliidae and Libellulidae), the first processes are reduced to small flaps and the other structures are apparently absent except for the probable vestige of the styli emerging directly from the ninth sternite (Tillyard, 1917). The divergence estimates for Libelluloidea influence our hypotheses about the evolutionary process of this reduction. If the unpaired-sites model is applied, and we assume that Libelluloidea diverged 249 Myr ago, we might favor a hypothesis proposing ovipositor reduction and exophytic oviposition in response to limited freshwater niche space during the Triassic. Using information from the paired-sites model analysis, however, which suggests a divergence age of 205 Myr ago, we might suppose that ovipositor reduction occurred in response to an increase in the number of predators at the oviposition site. Exophytic oviposition is generally faster than endophytic oviposition, which may reduce the number of predators encountered (e.g., Corbet, 1999). Fish, frogs, and birds impose a strong predation threat on ovipositing females, particularly those that do so endophytically (Corbet, 1999). Exophytic oviposition may have evolved as a response to the diversification of these predators (modern birds diverged during the Cretaceous, Brown et al., 2007; Neobatrachid frogs diverged during the Jurassic, Roelants et al., 2007). Certainly there are numerous other hypotheses that could be supposed for the evolution of dragonfly genitalia, but without realistic dating estimates it will be hard to evaluate them effectively.

## 5. Conclusions

The results of our analyses here, coupled with the general desirability of utilizing evolutionary models that are biologically realistic, suggest that it is very important to take stem pairing into account during analyses of rRNA data sets. The impact of using paired-sites substitution models on divergence time estimates is not easily predictable, particularly when explicit models of among-lineage rate heterogeneity are used in conjunction with partitioned analyses of complex data. Paired-sites models apparently do not lead to uniformly lower dating estimates, although it is necessary to investigate a wider range of data sets before further inferences can be made. Accordingly, it is prudent to assess the effects of model selection on resulting date estimates, as well as on the consequent ecological and biogeographic interpretations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2007.10.008](https://doi.org/10.1016/j.ympev.2007.10.008).

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