

## ORIGINAL ARTICLE

# Phylogenetic analysis of the insect order Odonata using 28S and 16S rDNA sequences: a comparison between data sets with different evolutionary rates

Eisuke HASEGAWA<sup>1</sup> and Eiiti KASUYA<sup>2</sup><sup>1</sup>Laboratory of Animal Ecology, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Sapporo and <sup>2</sup>Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka, Japan

## Abstract

Molecular phylogenetic analyses were conducted for the insect order Odonata with a focus on testing the effectiveness of a slowly evolving gene to resolve deep branching and also to examine: (i) the monophyly of damselflies (the suborder Zygoptera); and (ii) the phylogenetic position of the relict dragonfly *Epiophlebia superstes*. Two independent molecular sources were used to reconstruct phylogeny: the 16S rRNA gene on the mitochondrial genome and the 28S rRNA gene on the nuclear genome. A comparison of the sequences showed that the obtained 28S rDNA sequences have evolved at a much slower rate than the 16S rDNA, and that the former is better than the latter for resolving deep branching in the Odonata. Both molecular sources indicated that the Zygoptera are paraphyletic, and when a reasonable weighting for among-site rate variation was enforced for the 16S rDNA data set, *E. superstes* was placed between the two remaining major suborders, namely, Zygoptera and Anisoptera (dragonflies). Character reconstruction analysis suggests that multiple hits at the rapidly evolving sites in the 16S rDNA degenerated the phylogenetic signals of the data set.

**Key words:** damselfly, dragonfly, molecular phylogeny.

## INTRODUCTION

In the insect order Odonata, three extant suborders, namely Zygoptera (damselflies), Anisoptera (dragonflies) and Anisozygoptera, are recognized. The suborder Anisozygoptera consists of a single extant genus, *Epiophlebia*. The two described species of *Epiophlebia* have characteristics that are intermediate between those of Zygoptera and Anisoptera in terms of morphology and behavior (Rueppell & Hilfert 1993). Phylogenetic relationships within Odonata have been recently studied based on molecular (Polhemus 1997; Chippindale *et al.* 1999; Kambhampati & Cherton 1999; Misof *et al.*

2000; Artiss *et al.* 2001; Weekers *et al.* 2001; Jordan *et al.* 2003; Misof & Fleck 2003; Saux *et al.* 2003; Kjer 2004) as well as morphological (May 2002; Rehn 2003) data. However, the majority of these studies dealt with a restricted group of Odonata, and several scientific issues remain unclear. One of the two major issues is the monophyly of the Zygoptera and Anisoptera, and the other is the phylogenetic position of the Anisozygoptera. For the Zygoptera, several studies have revealed the paraphyletic nature of this suborder based on morphological (Bechly 1996; Trueman 1996) or molecular (Saux *et al.* 2003) data, but Rehn (2003) asserted the monophyly of the Zygoptera by means of a detailed re-analysis of skeletal morphology and wing venation. Misof *et al.* (2001) attempted to resolve the phylogeny of the Odonata by using 16S and 12S rDNA coded on the mitochondrial genome; however, the evolutionary rates of these regions were too fast to resolve deep branching within this order. Misof and Fleck (2003) analyzed the phylogenetic signal of the secondary struc-

Correspondence: Eisuke Hasegawa, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, 060-8587 Japan.  
Email: ehase@res.agr.hokudai.ac.jp

Received 28 September 2004; accepted 12 September 2005.

ture of 16S rRNA, but they too were unsuccessful. Information from the 18S rDNA on the nuclear genome, as used by Kjer (2004), suggests that the Zygoptera are monophyletic and that the Anisoptera are paraphyletic; however, Kjer noted that the published results differed from his unpublished data based on information from 28S rDNA and EF-1 $\alpha$ . It is thus reasonable to state that the monophyly of the two major suborders remains unconfirmed.

The phylogenetic position of the Anisozygoptera is another ambiguous point in the phylogeny of the Odonata. Several studies have suggested that *Epiophlebia* is the sister taxon of the entire Anisoptera (Pfau 1991; Rueppell & Hilfert 1993; Bechly 1996; Trueman 1996); however, based on wing characters, Nel *et al.* (1993) suggested that it is actually a relict of the early branching of the Anisozygoptera from the other two suborders. A phylogenetic analysis of the entire Insecta using 18S rDNA supports the viewpoint of Nel *et al.* (Kjer 2004); however, Kjer noted that the sequence diversity of this gene was very low in the Odonata, suggesting that his result may not be entirely reliable. Lohmann (1996) provided morphological evidence for the non-monophyly of the entire Anisozygoptera (including fossil taxa) and proposed the transfer of anisozygopteran species into the Anisoptera. The 16S and 12S rDNA data of Misof *et al.* (2001) suggested that *Epiophlebia superstes* was genetically closer to anisopteran than to zygopteran species, but could not clarify the exact position of *E. superstes* because of the decay in phylogenetic signals in these genes due to their rapid evolutionary rates. It can be concluded that the identification of genes with suitable evolutionary rates for resolution of deep branching is very important in phylogenetic studies of the Odonata, although molecular data can be applied only to extant taxa.

In the present study, we carried out phylogenetic analyses within the entire Odonata by using the 28S rRNA gene coded on the nuclear genome, in addition to the 16S rRNA gene coded on the mitochondrial genome. Nuclear DNA is known to have slower substitution rates than mitochondrial DNA (Brown *et al.* 1979, 1982). In addition, the two genes can be regarded as independent sources in phylogenetic analyses because each genome is duplicated by a different system (i.e. in the mitochondrion or in the nucleus). Transmission systems also differ between the two genes: the mitochondrial genome is transmitted maternally, whereas the nuclear genome is inherited in a Mendelian manner. Thus, genes on each genome can be regarded to be qualitatively different.

Multiple independent sources are valuable in phylogenetic studies because when the phylogenetic relationships from multiple sources are congruent with each other, the obtained relationship is strongly supported. Even in cases where multiple sources show major incongruency, the reliability of each hypothesis can be checked by examining the characteristics of the source sequences, such as differences in substitution rates, the existence of saturation, or the value of the transition/transversion ratio.

For the treatment of qualitatively different data, Chavarria and Carpenter (1995) proposed the total evidence method (i.e. combined analysis) in which all the data derived from different sources are combined to increase the phylogenetic signals in the combined data set. This method can be effective when the partitioned components of the data set include phylogenetic signals consistent with each other, but there are two major conditions that violate this consistency. First, if different sources have not experienced a common lineage, the two data sets could have inconsistent phylogenetic signals, and the combined data set would have a low phylogenetic resolution ability. Several studies have shown the incongruent lineage of nuclear DNA with mitochondrial DNA, and these incongruencies are probably due to past hybridization events (Mukai *et al.* 1997; Sota & Vogler 2001). Hybridization events might have been restricted among closely related taxa such as species complexes, and the situation may differ in the Odonata. Despite these events having occurred in the past, there is a possibility that their effects remain as incongruence in deep branching. Although lineage sorting and accumulation of changes in sequences over millions of years may have minimized the effects of hybridization in the past, combined analysis should not be the first choice. Second, the combined data would not be effective when independent sources include many convergences that have been accumulated by virtue of the marked difference in their evolutionary rates. The mitochondrial genome is known to evolve considerably faster than the nuclear genome (Brown *et al.* 1979, 1982). In fact, Misof *et al.* (2000) reported the decay of phylogenetic signals in the mitochondrial DNA of the Odonata. Recently, Lecoindre and Deleporte (2005) criticized the total-evidence method for these same reasons. Thus there are two good reasons why combined analysis should not be the first choice. The different sources should initially be analyzed separately and then combined if there is no major incongruence in the results (e.g. Farris *et al.* 1994).

In the present study, therefore, we inferred the phylogenetic relationships independently from the two sources and tested the robustness of each result by examining the sequence characteristics of each source. Based on the inferred phylogenetic hypothesis, we discuss the effectiveness of 28S rDNA in phylogenetic analysis of the Odonata, and also the monophyly of the Zygoptera and the phylogenetic position of *Epiophlebia*.

## MATERIALS AND METHODS

### Study organisms

From 1995 to 1998, we collected 32 species of Odonata from various regions in Japan. The collected samples

covered four of the 19 families in Zygoptera, the sole family in Anisozygoptera, and four of the seven families in Anisoptera. The species used are summarized in Table 1. The collected individuals were preserved in pure acetone until DNA extraction (see Fukatsu 1999). A mayfly, *Centroptilum* sp. from the Ephemeroptera, was selected as the outgroup based on the ordinal phylogeny of the Insecta (Wheeler *et al.* 2001).

### DNA preparation and sequencing

For each individual, total DNA was extracted from the thoracic muscles using a DNA extraction kit (DNeasy tissue kit; Qiagen, Tokyo, Japan) and suspended in 200 µL of the buffer supplied in the kit. Two primer

**Table 1** Samples and collection sites used (all sites are in Japan)

| Order         | Suborder       | Family           | Species  | Collection site   |
|---------------|----------------|------------------|--|---|
| Ephemeroptera |                |                  | <i>Centroptilum</i> sp.<br><i>Ephemera</i> sp.   | Umi, Fukuoka<br>Ebino, Miyazaki   |
| Odonata       | Zygoptera      | Coenagrionidae   | <i>Coenagrion lanceolatum</i> (Selys, 1872)<br><i>Coenagrion terue</i> (Asahina, 1949)<br><i>Ceriagrion melanurum</i> Selys, 1876<br><i>Ceriagrion nipponicum</i> Asahina, 1967<br><i>Cercion plagiosum</i> (Needham, 1930)<br><i>Cercion hieroglyphicum</i> (Brauer, 1865)<br><i>Cercion calamorum calamorum</i> (Ris, 1916)<br><i>Ischnura asiatica</i> (Brauer, 1865)<br><i>Ischnura senegalensis</i> (Rambur, 1842)  | Kawaguchi, Niigata<br>Kawaguchi, Niigata<br>Kasuya, Fukuoka<br>Kasuya, Fukuoka<br>Niigata, Niigata<br>Kasuya, Fukuoka<br>Kasuya, Fukuoka<br>Koga, Fukuoka<br>Kasuya, Fukuoka                                      |
|               |                | Platycnemididae  | <i>Copera annulata</i> (Selys, 1863)   | Niigata, Niigata  |
|               |                | Lestidae         | <i>Lestes japonicus</i> Selys, 1883  | Usa, Ooita  |
|               |                | Calopterygidae   | <i>Calopteryx japonica</i> Selys, 1869<br><i>Calopteryx atrata</i> Selys, 1853<br><i>Calopteryx cornelia</i> Selys, 1853<br><i>Mnais pruinosa pruinosa</i> Selys, 1853<br><i>Mnais pruinosa costalis</i> Selys, 1869<br><i>Epiophlebia superstes</i> (Selys, 1889)   | Ueda, Nagano<br>Ueda, Nagano<br>Maki, Niigata<br>Usa, Fukuoka<br>Iwamuro, Niigata<br>Otaru, Hokkaido  |
|               | Anisozygoptera | Epiophlebiidae   | <i>Epiophlebia superstes</i> (Selys, 1889)   | Maki, Niigata   |
|               | Anisoptera     | Petaluridae      | <i>Tanypteryx pryeri</i> (Selys, 1889)   | Umi, Fukuoka  |
|               |                | Gomphidae        | <i>Davidius nanus</i> (Selys, 1869)  | Umi, Fukuoka  |
|               |                | Cordulegastridae | <i>Anotogaster sieboldii</i> (Selys, 1854)   | Kasuya, Fukuoka   |
|               |                | Libellulidae     | <i>Pantala flavescens</i> (Fabricius, 1798)<br><i>Rhyothemis fuliginosa</i> Selys, 1883<br><i>Nannophya pygmaea</i> Rambur, 1842<br><i>Pseudothemis zonata</i> (Burmeister, 1839)<br><i>Crocothemis servilia mariannae</i> Kiauta, 1983<br><i>Orthetrum japonicum japonicum</i> (Uhler, 1858)<br><i>Orthetrum albistylum speciosum</i> (Uhler, 1858)<br><i>Orthetrum triangulare melania</i> (Selys, 1883)<br><i>Sympetrum pedemontanum elatum</i> (Selys, 1872)<br><i>Sympetrum frequens</i> (Selys, 1883)<br><i>Sympetrum infuscatum</i> (Selys, 1883)<br><i>Sympetrum eroticum eroticum</i> (Selys, 1883) | Kasuya, Fukuoka<br>Kasuya, Fukuoka<br>Simoda, Niigata<br>Tamazu, Hyogo<br>Kasuya, Fukuoka<br>Miki Niigata<br>Kasuya, Fukuoka<br>Umi, Fukuoka<br>Ueda, Nagano<br>Yahiko, Niigata<br>Niigata, Niigata<br>Usa, Ohita |

pairs were used to amplify either a part of the 16S rRNA gene (16sf, 5'-CGGCCTGTTTAAACAAAAACAT-3'; 16sr, 5'-CCCGGTTTGAAGTCAAGATCATGT-3'; product length approximately 550 bp) coded on the mitochondrial genome or a part of the 28S rRNA gene (28sf, 5'-AAGGTAGCCAAATGCCTCATC-3'; 28sr, 5'-AGTAGGGTAAAATAACCT-3'; product length approximately 750 bp) coded on the nuclear genome. For the 28S rRNA gene, the target region was amplified by polymerase chain reaction (PCR) in a programmable thermal cycler (PTC-100; MJ Research, Watertown, MA, USA). Each 100  $\mu$ L reaction volume contained the following components: 10  $\mu$ L of DNA solution (~200 ng), 10  $\mu$ L of 10 $\times$  Ex *Taq* buffer (Mg<sup>2+</sup> added), 10  $\mu$ L of each 2.5 mmol/L 4dNTP, 20 pmol each of the primer pairs, 1.0 U of *Taq* DNA polymerase (TaKaRa Ex *Taq*; TaKaRa, Osaka, Japan) and miliQ water. The reactions were incubated for 35 cycles of 30 s at 94°C, 45 s at 52°C, and 3 min at 65°C. The amplification products were purified by using the QIAamp PCR purification kit (Qiagen), and suspended in 22  $\mu$ L of Te buffer (0.1 mmol/L ethylenediamine tetraacetic acid (EDTA), 10 mmol/L Tris-HCl (pH 8.0)). For details regarding the amplification of mitochondrial DNA, see Hasegawa *et al.* (2001).

An automated sequencer (CEQ2000XL; Beckman Coulter, Fullerton, CA, USA) was used to sequence the amplified fragments. The reaction procedure was carried out in accordance with the protocol of a direct sequencing kit (DTCS Quick Start Kit; Beckman Coulter). The optimal concentration of the template DNA was checked by comparing the brightness of the amplified fragment with that of a band from a size marker ( *$\lambda$ HindIII*; Toyobo, Osaka, Japan) in 1.0% agarose gel electrophoresis, stained with ethidium bromide.

### Alignments of sequences

The sequences obtained for each gene were subjected to multiple alignment with the CLUSTAL X program (Thompson *et al.* 1997) using the profile alignment mode, enforcing a secondary structure model of wasp 28S rRNA or insect 16S rRNA (Schmitz & Moritz 1998; Buckley *et al.* 2000) as the profiles. In the alignment procedure, gaps in stem structures were penalized more heavily than those in loop structures (see Yoshizawa & Johnson 2003). The result was manually adjusted for the variable regions with indels (see later sections in this paper) because in the results curious alignments were apparently found at that region (e.g. insertion of a gap at a different site in the same sequence part). During the manual adjustment process, each

sequence was randomly numbered, and the adjuster (E. Hasegawa) was blinded to the taxon from which the sequence came. This treatment prevents the occurrence of unconscious biases based on previously known taxonomic information. The final result of alignment will be deposited in TREEBASE.

### Phylogenetic inference

Phylogenetic relationships were inferred with PAUP\* (version 4.0b10; Swofford 2002) using the neighbor-joining (NJ) method (Saitou & Nei 1987), the maximum parsimony (MP) method and the maximum likelihood (ML) method. A computer program (MODELTEST version 3.06; Posada & Crandall 1998) was used to select the most adequate substitution model for ML analysis. The selected model, the estimated proportion of invariant sites and the shape parameter of gamma distribution were used in phylogenetic inference (for details see later sections).

### Character weighting

There are variable and stable regions within rRNA gene sequences (Schmitz & Moritz 1998), and the observed substitution rate differs considerably between these regions. A fast substitution rate results in multiple hits at a nucleotide position, which leads to convergence. In addition, the substitution rate for transitions (A-G or T-C) is considerably faster than that for transversions (Brown *et al.* 1982). These facts suggest that transitional changes in variable regions are saturated considerably faster than substitutions in other regions and that variable regions contain a larger amount of convergence. In fact, the decay of phylogenetic signals at rapid-evolving sites has been detected for the 16S rRNA gene of anisopteran species (Misof *et al.* 2001). Hence, the phylogenetic information in the variable region is unclear and should therefore be given less importance when a large degree of saturation is observed.

To determine an adequate weight ratio of the variable regions to the stable regions, we compared the rate of substitution between the two regions. First, we calculated the distances among all the pairs of sequences by using Kimura's two-parameter method (K2D; Kimura 1980). Each distance was used as an index of time between two sequences after branching off. Second, with respect to each region, the proportion of substituted sites between the two sequences was plotted against the K2D of that pair. Regression equations were calculated by enforcing the Y intercept at zero. By calculating the slope of the regression equation for each region, we obtained an estimate of the substitution rate

for each region. The difference between the slopes represented the difference in substitution rate. Based on the degree of difference, we assigned several weight sets to each region and tested the robustness of tree topology with respect to changes in weight ratio. Weightings were used only in MP analysis because it is a useful approach for dealing with rate differences within a parsimony framework. In NJ and ML frameworks, rate differences were considered as gamma or invariant-proportion parameters, and these parameters were estimated from the data using PAUP\* or MODELTEST. Conventional bootstrap tests were also used to evaluate the reliability of tree topologies.

## RESULTS AND DISCUSSION

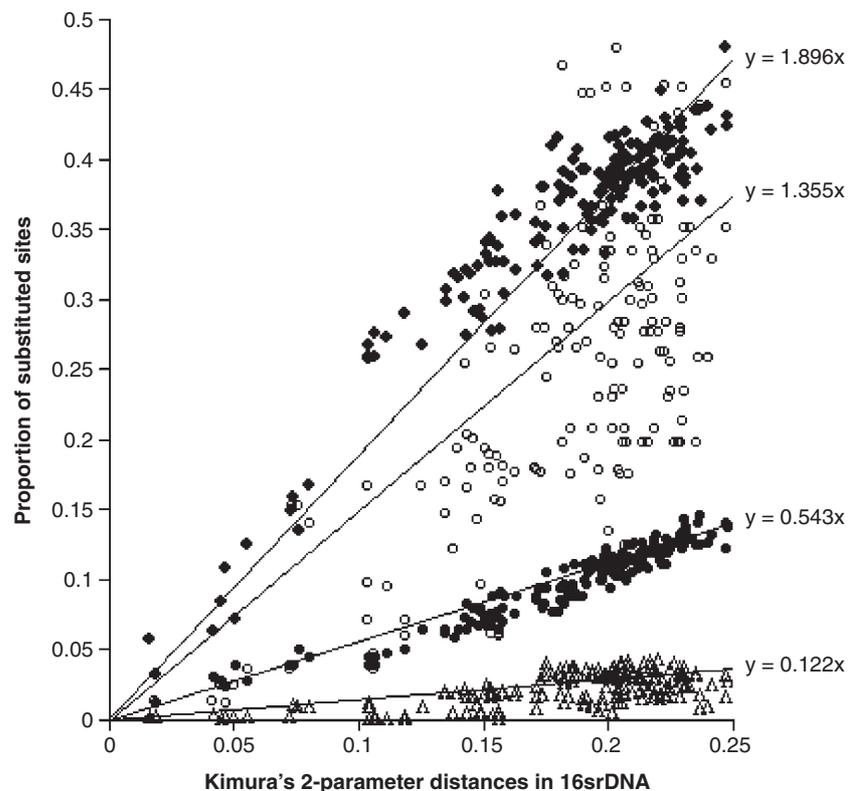
### Character evaluation and weighting

After alignment, we were able to obtain a 747 bp sequence for 28S rDNA and a 507 bp for 16S rDNA (the aligned data can be obtained from TREEBASE; for original sequences, GenBank accession numbers AB127406-AB127435, AB127056-AB127077). The number of variable regions was four in 16S rDNA and two in 28S rDNA. In the variable regions, taxonomi-

cally close species showed higher sequence similarity, while distant taxa showed much more divergence. This result suggests that the variable regions contain phylogenetic information, at least among closely related species, while the decay of phylogenetic signals among distant taxa is expected to be as shown by Misof *et al.* (2001). In addition, these findings indicate that the blind adjustment of the taxonomic information was effective because the adjuster did not know the taxonomic relationships of sequences during adjustment, but the results reflected presumable similarities among taxonomically close species.

For unknown reasons, the primer pairs used in the study failed to amplify the target region for two species of the genus *Ceriagrion* in the 28S rDNA region and for several species in the 16S rDNA region. Thus, these species could not be included in the analysis using each region.

To compare the relative evolutionary rates between the two genes, the proportion of substituted sites in both the genes was plotted against the K2D of the 16S rDNA using the species that were common to both the data sets (Fig. 1). The analysis showed that within the Odonata, the evolutionary rates were in the following order:



**Figure 1** Estimates of evolutionary rates at the variable and stable regions in the 16S rDNA and the 28S rDNA. (◆) Variable and (●) stable regions of the 16S rDNA sequence; (○) variable and (△) stable regions of 28S rDNA sequence. For each region, the value of the slope is given for the regression line with a zero intercept.

16S variable > 28S variable > 16S stable > 28S stable. By comparing the slopes of the regression lines, it was shown that the variable regions have evolved 3.5 times and 11.1 times as fast as the stable regions in the 16S and in the 28S rDNA, respectively.

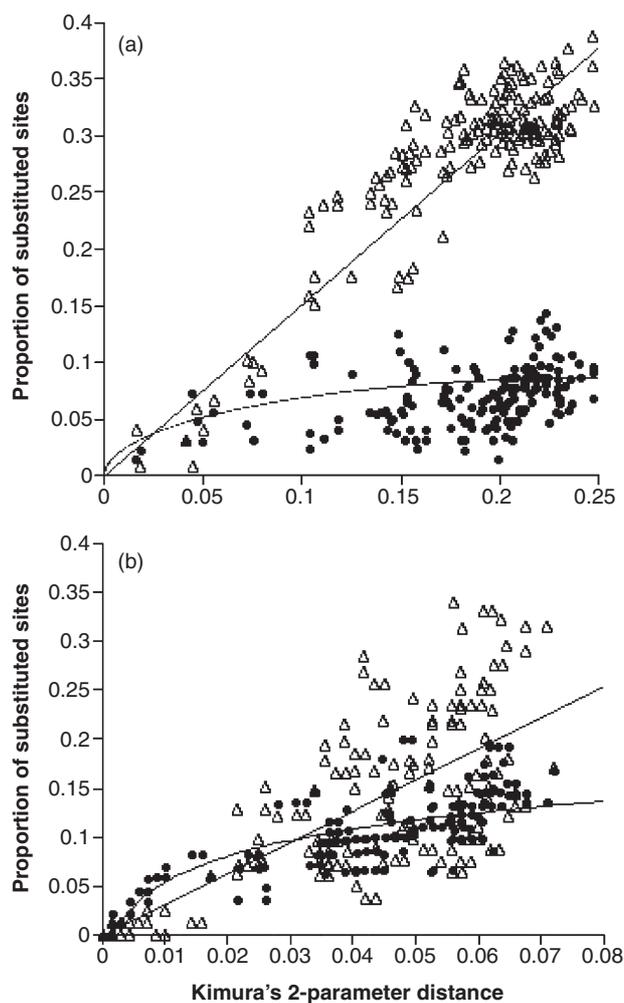
As shown in Fig. 1, the variable regions of the 16S rDNA showed a pattern of decreasing return to scale rather than of linear increase. Transitions and transversions were plotted separately against K2D for the variable regions of both genes, in order to check possible saturation (Fig. 2a,b). Transitional substitutions in the 16S rDNA showed a heavy degree of saturation (Fig. 2a), thereby suggesting a large amount of convergence in this region among distant taxa. This result is in accordance with that of Misof *et al.* (2001). The transitions were also saturated to a lesser extent in the 28S rDNA (Fig. 2b), probably due to a slow evolutionary rate for this gene. For the stable regions, saturation was not observed in either of the genes. These results imply that phylogenetic signals would be more decayed in the variable regions of the 16S rDNA than in 28S rDNA. In addition, in the stable regions, the 28S rDNA has evolved at a considerably slower rate than the 16S rDNA. All these results indicate that the 28S rDNA is a more suitable source to resolve deep branching among distant taxa. Based on the differences in substitution rates, in the following analyses, we used a weight ratio (stable : variable) of 1:1–5:1 to test the robustness of tree topology in MP analysis.

### Phylogenetic analyses based on 28S rDNA

Figure 3 shows the inferred phylogenetic relationships from the 28S rDNA sequences. A mayfly (*Centroptilum* sp.) was used as the outgroup. Gaps in the data set were treated as the fifth character in MP analysis. The branch and bound search with equal weight for all sites resulted in the 16 most parsimonious trees, thus we presented the strict consensus tree. For the ML analysis, we used the GTR + I + G model that was selected by MODELTEST based on the Akaike information criterion (AIC). The heuristic search option was used to find the optimal tree. In the NJ analysis, the ML distances calculated using the GTR + I + G model were used to make a distance matrix. All the methods resulted in similar branching patterns that suggested a basal, paraphyletic relationship in the Zygoptera and the position of *Epiophlebia* as the sister taxon of the monophyletic Anisoptera. In the following sections, we examine the robustness of the 28S tree.

Bootstrap probabilities from 1000 resamplings showed strong support (>80%) for most of the nodes

that resolved interfamily (or intersuborder) relationships in NJ and MP trees. However, support was considerably lower for the intra-family relationships, particularly within the family Libellulidae. Although the intrafamily relationships among *Cercion*, *Coenagrion* and *Ischnura* were incongruent, the result clearly showed a paraphyletic relationship within the suborder Zygoptera. The clade Anisoptera was supported, with bootstrap values of 60% in MP and 67% in NJ. The clade Anisoptera + Anisozygoptera was supported by a bootstrap value of 97% for both the MP and NJ methods. We tested the robustness of the paraphyly of Zygoptera and the position of *Epiophlebia* by excluding



**Figure 2** Evolutionary rates of (●) transitional and (Δ) transversional substitutions in the variable regions of (a) the 16S rDNA and (b) the 28S rDNA. Linear and logarithmic regression lines are fitted to each of the transversions and the transitions, respectively.

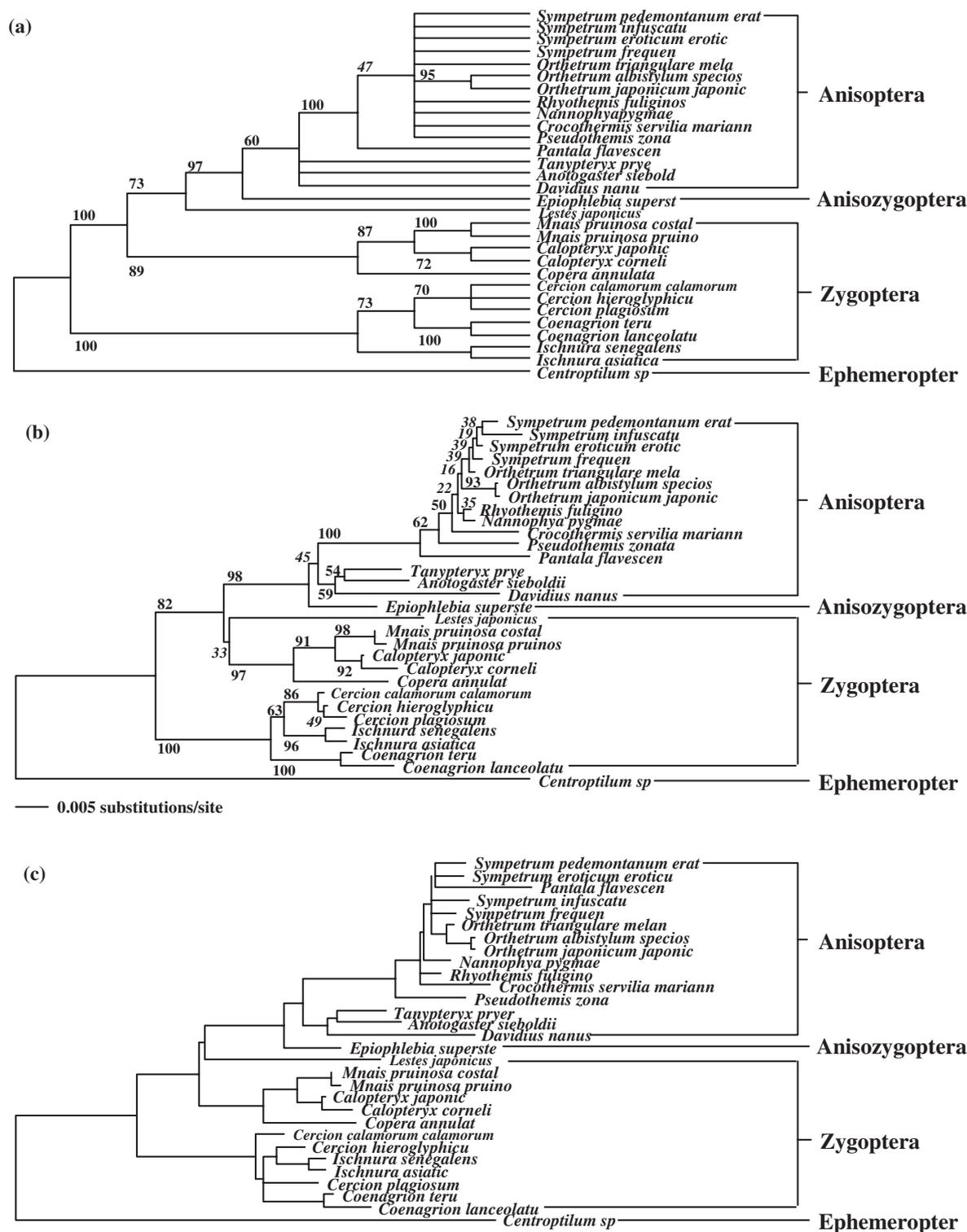


Figure 3 Inferred phylogenetic relationships obtained from the 28S rDNA; (a) strict consensus tree of the 16 maximum parsimony (MP) trees; (b) neighbor-joining (NJ) tree; and (c) maximum likelihood (ML) tree. The numbers on the nodes are bootstrap values from 1000 resamplings. Italics represent bootstrap values less than 50%.

all the gapped sites or all the variable regions from the data, but these relationships remained regardless of data exclusions. In addition, these relationships remained unchanged even when the gaps were treated as missing data.

The results mentioned here support the following hypothesis for Odonata phylogeny: basal and paraphyletic arrangement of the Zygoptera and the intermediate position of *Epiophlebia* between the Zygoptera and Anisoptera. The results also indicate that the 28S rDNA produces high resolution for deep branching (interfamily to intersuborder) rather than intrafamily relationships. These results differ from those based on nucleic 18S rDNA (Kjer 2004), in which the Anisozygoptera (*E. superstes*) were found to be the sister group to the other two subfamilies, and the Anisoptera had a paraphyletic arrangement between *Epiophlebia* and the monophyletic Zygoptera. However, Kjer (2004) noted that there are very few substitutions in 18S rDNA within the Odonata, and the result based on 18S is different from his unpublished results obtained using 28S rDNA and EF1- $\alpha$ . Thus, the result based on 18S rDNA might be doubtful. For the position of *E. superstes*, further analysis is provided later in this paper.

### Phylogenetic analysis based on 16S rDNA

The results obtained from 16S rDNA were more controversial than those obtained using 28S rDNA. For ML, MODELTEST selected the TVM + I + G model based on AIC. A gamma shape parameter (0.635) was estimated by MODELTEST and used in an NJ analysis with Kimura's two-parameter distance. An ML distance was not used for the 16S rDNA because of the appearance of a node having a negative distance. For MP and ML analyses, a heuristic search option was used to find the optimal tree. A nucleotide sequence of a mayfly (*Ephemera* sp.; GenBank accession no. AF266047) was obtained from the DNA Data Bank of Japan database and was used as an additional outgroup in the analysis.

Paraphyly of the Zygoptera was indicated by both NJ and MP methods; however, bootstrap values for these deep branchings were considerably lower compared with the 28S trees (see Fig. 4). This is similar to

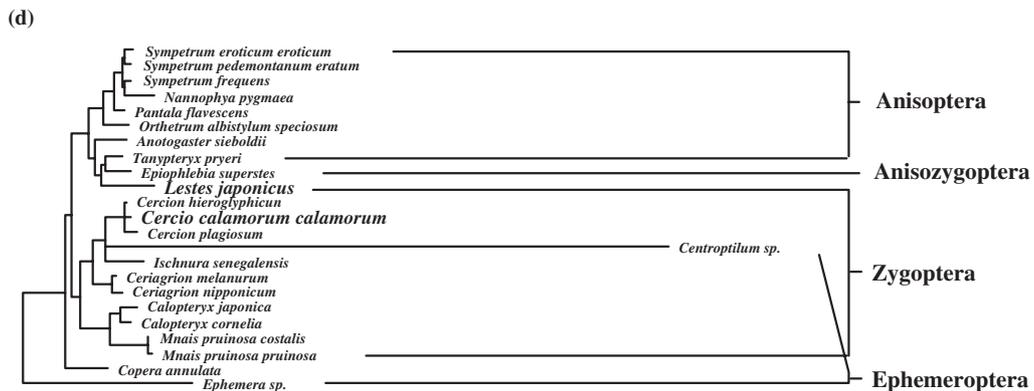
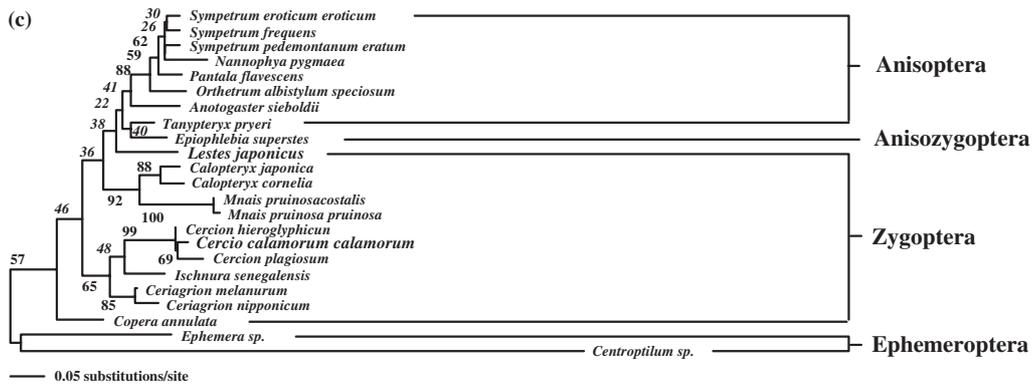
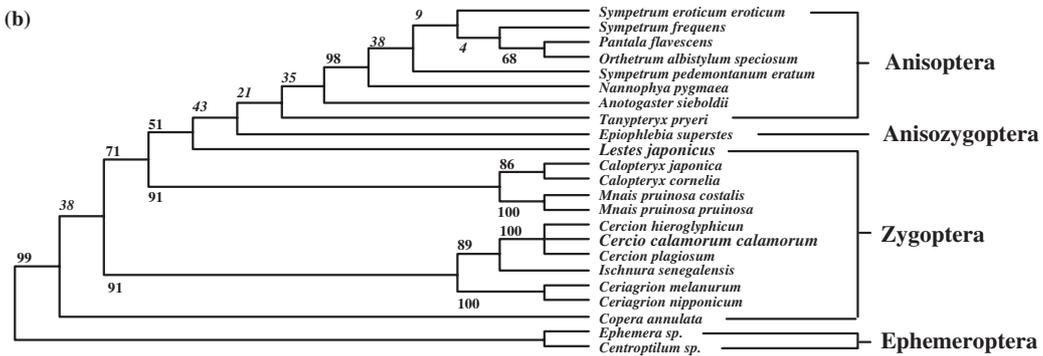
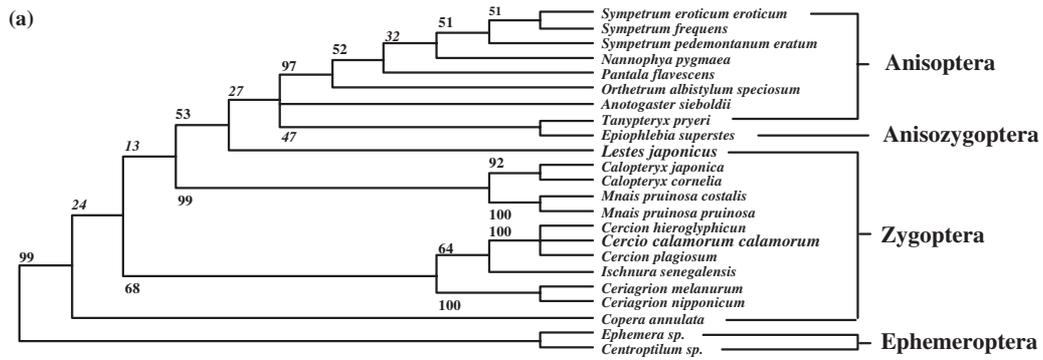
the result obtained using mitochondrial 12S rDNA (Saux *et al.* 2003). Bootstrap values were relatively high in shallow branchings (i.e. intra-family relationships). Three methods (unweighted MP, NJ and ML) placed *E. superstes* as the sister taxon of *Tanypteryx pryeri*, and the monophyly of the Anisoptera was not supported. This relationship (*E. superstes* + *T. pryeri*) was supported with a weak bootstrap value of 47% and 40% in unweighted MP and NJ, respectively (see Fig. 4), thus this clade is doubtful. Another reason that suggests the doubtfulness of this clade is the heavy saturation of transitions in the variable regions (Fig. 2a; see the following sections in this paper). In MP analysis, when a weight ratio of greater than 2:1 was assigned to stable : variable regions, the clade Anisoptera appeared and *E. superstes* was positioned as the sister taxon to all the anisopteran species as in the 28S trees (Fig. 4b). This result also suggests an incorrect positioning of *E. superstes* in the other three methods.

### Character reconstruction for incongruent topologies

A previous study indicated that the phylogenetic signals in the 16S rRNA gene were degenerate among distant taxa in the Anisoptera, particularly at rapid-evolving sites (Misof *et al.* 2001). In that study, when the sites with high substitution rates were removed from the data set, the resolution of the obtained tree improved for deep branching, suggesting considerable convergence in rapid-evolving sites. This result led us to the hypothesis that the observed monophyly of *E. superstes* with *T. pryeri* in the 16S trees was incorrect because of convergence.

To test this hypothesis, substitutions were reconstructed on the obtained MP trees that were incongruent with each other with respect to relationships among *E. superstes*, *T. pryeri*, *A. sieboldii* and the members of the Libellulidae, because reconstruction on a tree shows us synapomorphies that support the resultant cladogram. Thus, by comparing the robustness of these changes between incongruent trees, we can obtain information for the robustness of each tree. We used MacClade version 3.08 (Maddison & Maddison 1992) to

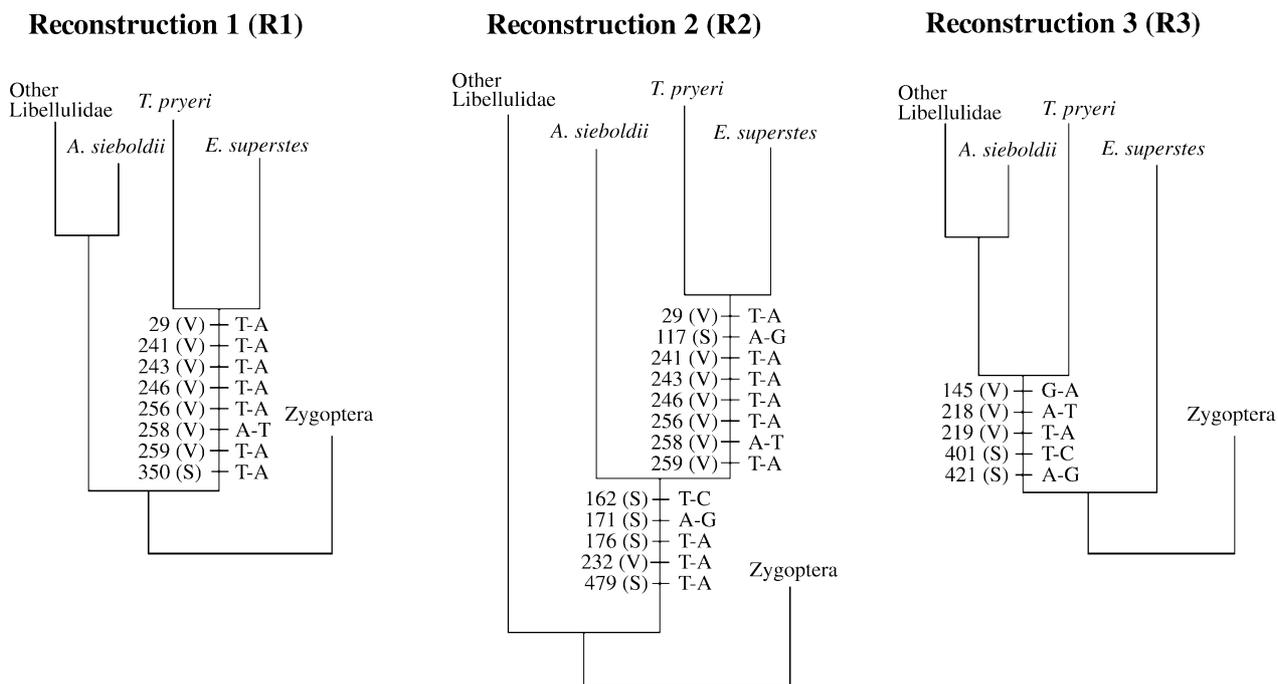
**Figure 4** Inferred phylogenetic relationships obtained from the 16S rDNA; (a) strict consensus tree of the 4 maximum parsimony (MP) trees obtained from unweighted analysis (stable : variable, 1:1); (b) strict consensus tree of the 2 MP trees inferred from a weighted analysis (stable : variable, 3:1); (c) neighbor-joining (NJ) tree; and (d) maximum likelihood (ML) tree. The numbers on the nodes are bootstrap values from 1000 resamplings. Italics represent bootstrap values less than 50%.



reconstruct character changes. There were four MP trees in the unweighted MP analysis (UW trees) and one MP tree in the 3:1 weighted analysis (W trees). The character reconstruction for these five trees resulted in the two most parsimonious reconstructions (R1 or R2) on the UW trees and a single reconstruction (R3) on the W trees (Fig. 5). In the R1 or R2, the basal node of the problematic clade (*E. superstes* + *T. pryeri*) was supported by eight changes, of which seven were within the variable regions (Fig. 5), whereas in the R3, two of the five changes were within the stable regions (Fig. 5), and three of the five changes were concerned with G or C. These results showed that the problematic clade was supported by more changes (eight changes) than the plausible clade (five changes), but many of the former are not reliable compared with the latter. Furthermore, weighting possibly recovered the plausible clade due to a heavy weight assigned to changes in the stable region.

Analyses for sequence characteristics showed that there would be multiple hits within the variable regions of the 16S rDNA (Fig. 2b). The reconstruction analyses

showed that most of the changes that support the problematic topology of the UW trees occurred within the variable regions, whereas a higher proportion of the changes occurred in stable regions in the topology of the W tree. (Fig. 5). These results suggest that the topologies of the UW trees were more doubtful than those of the W trees. This conclusion is also supported by another viewpoint. In the 16S rDNA of the Odonata, the base composition was considerably biased toward A and T (with respect to the data set in the present study, an average of 68.4% of bases were either A or T) as in other insect mitochondrial DNA (Clary & Wolstenholme 1985; Crozier & Crozier 1993; Flook *et al.* 1995). This fact leads to a simple prediction that substitutions concerning G or C are relatively rare compared with substitutions between A and T. Thus, the reconstruction analysis suggests that the W trees are more reliable than the UW trees because the events supporting the topology of the former are rare. Although the true phylogeny can not be determined (it can only be estimated), all the analyses presented here



**Figure 5** Reconstructions of character changes on nodes that were contradictory between the unweighted (UW) and the 3:1 weighting (W) methods in maximum parsimony (MP) analyses for the 16S rDNA. R1 and R2 are reconstructions on the 4 MP trees in the UW analysis, and R3 is that on the 4 MP trees in the W analysis. Numbers represent site positions in the aligned sequence at which the indicated change occurred (e.g. A-T means a change from A to T). Letters in parentheses indicate the type of sites (V, variable regions; S, stable regions).

suggest that *E. superstes* is the sister group to the Anisoptera, at least in the extant taxa.

### Combined analysis

A combined MP analysis was conducted on a data set that consisted of the species that were common to both the data sets. The result was controversial, as was the result for the 16S data set (results not shown). When all the positions were equally weighted, *E. superstes* formed a clade with *T. pryeri*, while when the 28S part was given more than twice the weight, the clade Anisoptera appeared. In addition, with the exception of a shallow clade, the bootstrap values of clades did not increase considerably. In general, the combined analysis did not improve the results of the separate analyses. In fact, it obscured the result of the separate analyses. Recently, Lecointre and Deleporte (2005) argued that when there are phylogenetically contradictory signals in partitioned components, the total evidence method (i.e. combined analysis) is not effective. As shown in the previous section, the 16S data indicates convergence between *E. superstes* and *T. pryeri* (see also Misof *et al.* 2000). These phylogenetically misleading characters would obscure clear phylogenetic signals from the 28S part. Therefore, the first choice for phylogenetic inference should be separate (not combined) analysis, and a mutual reference among the results is important to find the most plausible phylogenetic hypothesis from multiple data sets.

The present study indicated that sequence information from slowly evolving nuclear genes is useful to resolve deep branching within the Odonata. In addition, even by using rapidly evolving genes, character evaluation and adequate weighting can improve phylogenetic inferences. Information on the evolutionary characteristics of specific genes is accumulating rapidly (e.g. Kjer 2004). The phylogenetic history of many organisms will be clarified in future studies by selecting specific genes with adequate substitution rates for the focal taxa.

### ACKNOWLEDGMENTS

We thank Dr M. Ohara for providing information on the sampling sites of *Epiophlebia superstes*. We also acknowledge T. Aoki and K. Hayashi for assistance with sample collections. Dr A. Chittenden helped to improve the English of this manuscript. This work was partly supported by Grants-in-Aid (no. 08304049 to E. K. and no. 13440227 to E. H.) for Scientific Research from the Japan Society for the Promotion of Science.

### REFERENCES

- Artiss T, Schultz TR, Polhemus DA, Simon C (2001) Molecular phylogenetic analysis of dragonfly genera *Libellula*, *Ladona* and *Plathemis* (Odonata: Libellulidae) based on mitochondrial cytochrome oxidase I and 16S rRNA sequence data. *Molecular Phylogenetics and Evolution* **18**, 348–361.
- Bechly G (1996) Morphologische Untersuchungen am Flügelgäader der rezenten Libellen und deren Stammgruppenvertreter (Insecta; Pterygota; Odonata) unter besonderer Berücksichtigung der phylogenetischen Systematik und des Grundplanes der Odonata. *Petalura (Special Volume)* **2**, 1–402.
- Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA* **76**, 1967–1971.
- Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. *Journal of Molecular Evolution* **18**, 225–239.
- Buckley TR, Simon C, Flook PK, Misof B (2000) Secondary structure and conserved motifs of the frequently sequenced domain IV and V of the insect mitochondrial large subunit rRNA gene. *Insect Molecular Biology* **9**, 565–580.
- Chavarria G, Carpenter JM (1995) “Total evidence” and the evolution of highly social bees. *Cladistics* **10**, 229–258.
- Chippindale PT, Dave VK, Whitmore DH, Robinson JV (1999) Phylogenetic relationships of North American damselflies of the genus *Ischnura* (Odonata: Zygoptera: Coenagrionidae) based on sequences of three mitochondrial genes. *Molecular Phylogenetics and Evolution* **11**, 110–121.
- Clary DO, Wolstenholme DO (1985) The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *Journal of Molecular Evolution* **22**, 252–271.
- Crozier RH, Crozier YC (1993) The mitochondrial genome of the honey bee *Apis mellifera*: complete sequence and genome organization. *Genetics* **133**, 97–117.
- Farris JS, Källersjö M, Kluge AG, Bult C (1994) Testing significance of incongruence. *Cladistics* **10**, 315–319.
- Flook PK, Rowell CHF, Gellissen G (1995) The sequence, organization and evolution of the *Locusta migratoria* mitochondrial genome. *Journal of Molecular Evolution* **41**, 928–941.
- Fukatsu T (1999) Acetone preservation: a practical technique for molecular analysis. *Molecular Ecology* **8**, 1935–1945.
- Hasegawa E, Tinaut A, Ruano F (2001) Molecular phylogeny of two slave-making ants: *Rossomyrmex* and *Polyergus* (Hymenoptera: Formicidae). *Annals Zoologici Fennici* **39**, 267–271.
- Jordan S, Simon C, Polhemus D (2003) Molecular systematics and adaptive radiation of Hawaii's endemic damselfly

- genus *Megalagrion* (Odonata: Coenagrionidae). *Systematic Biology* 52, 89–109.
- Kambhampati S, Cherton RE (1999) Phylogenetic relationship among *Libellula*, *Ladona*, and *Plathemis* (Odonata Libellulidae) based on DNA sequence of mitochondrial 16S rRNA gene. *Systematic Entomology* 24, 37–49.
- Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Biology* 16, 111–120.
- Kjer KM (2004) Aligned 18S and insect phylogeny. *Systematic Biology* 53, 506–514.
- Lecointre G, Deleporte P (2005) Total evidence requires exclusion of phylogenetically misleading data. *Zoologica Scripta* 34, 101–117.
- Lohmann H (1996) Das Phylogenetische System der Anisoptera (Odonata). *Entomologische Zeitschrift* 106, 209–266.
- Maddison WP, Maddison DR (1992) *MacClade, Version 3: Analysis of Phylogeny and Character Evolution*. Sinauer Associates, Sunderland, MA, USA.
- May ML (2002) Phylogeny and taxonomy of the damselfly genus *Enallagma* and related taxa (Odonata: Zygoptera; Coenagrionidae). *Systematic Entomology* 27, 387–408.
- Misof B, Anderson CL, Hadrys H (2000) A phylogeny of the damselfly genus *Calopteryx* (Odonata) using mitochondrial 16S rDNA markers. *Molecular Phylogenetics and Evolution* 15, 5–14.
- Misof B, Fleck G (2003) Comparative analysis of mt LSU rRNA secondary structures of Odonates: structural variability and phylogenetic signal. *Insect Molecular Biology* 12, 535–547.
- Misof B, Rickert AM, Buckely TR, Freck G, Sauer KP (2001) Phylogenetic signal and its decay in mitochondrial SSU and LSU rRNA gene fragments of Anisoptera. *Molecular Biology and Evolution* 18, 27–37.
- Mukai T, Naruse K, Sato T, Shima A, Morisawa M (1997) Multiregional introgressions inferred from the mitochondrial DNA phylogeny of a hybridizing species complex of gobiid fishes, genus *Tridentiger*. *Molecular Biology and Evolution* 14, 1258–1265.
- Nel A, Martinez-Delclos X, Paicheler JC, Henrotay M (1993) Les “Anisoptera” fossiles: phylogenie et classification (Odonata). *Martinia* 3, 1–311.
- Pfau KH (1991) Contributions of functional morphology to the phylogenetic systematics of Odonata. *Advances in Odonatology* 5, 109–141.
- Polhemus DA (1997) Phylogenetic analysis of the Hawaiian damselfly genus *Megalagrion* (Odonata: Coenagrionidae): Implications for biogeography, ecology, and conservation biology. *Pacific Science* 51, 395–412.
- Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Rehn AC (2003) Phylogenetic analysis of higher-level relationships of Odonata. *Systematic Entomology* 28, 181–239.
- Rueppell G, Hilfert D (1993) The flight of the relict dragonfly *Epiophlebia superstes* (Selys) in comparison with that of the modern Odonata (Anisoptera: Epiophlebiidae). *Odonatologica* 22, 295–309.
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.
- Saux C, Simon C, Spicer GS (2003) Phylogeny of the dragonfly and damselfly order Odonata as inferred by mitochondrial 12s ribosomal RNA sequences. *Annals of the Entomological Society of America* 96, 693–699.
- Schmitz J, Moritz RFA (1998) Molecular phylogeny of Vespidae (Hymenoptera) and the evolution of sociality in Wasps. *Molecular Phylogenetics and Evolution* 9, 183–191.
- Sota T, Vogler AP (2001) Incongruence of mitochondrial and nuclear gene trees in the carabid beetles *Obomopterus*. *Systematic Biology* 50, 39–59.
- Swofford DL (2002) *paup\**. *Phylogenetic Analysis Using Parsimony (\*and Other Methods)*, version 4. Sinauer Associates, Sunderland, MA, USA.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.
- Trueman JWH (1996) A preliminary cladistic analysis of odonate wing venation. *Odonatologica* 25, 59–72.
- Weekers PHH, De Jonckheere JF, Dumont HJ (2001) Phylogenetic relationships inferred from ribosomal ITS sequences and biogeographic patterns in representatives of the genus *Calopteryx* (Insecta: Odonata) of the West Mediterranean and adjacent West European zone. *Molecular Phylogenetics and Evolution* 20, 89–99.
- Wheeler WC, Whiting M, Wheeler QD, Carpenter JM (2001) The phylogeny of the extant hexapod orders. *Cladistics* 17, 113–169.
- Yoshizawa K, Johnson KP (2003) Phylogenetic position of Phthiraptera (Insecta: Paraneoptera) and evaluated rate of evolution in mitochondrial 12S and 16S rDNA. *Molecular Phylogenetics and Evolution* 29, 102–114.