

Phylogeny of the Dragonfly and Damselfly Order Odonata as Inferred by Mitochondrial 12S Ribosomal RNA Sequences

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ABSTRACT The phylogenetic relationships among members of the Odonates were inferred from mitochondrial DNA 12S ribosomal RNA sequence data. These data show support for a monophyletic Anisoptera suborder, which are consistent with previous phylogenetic work performed on the group. However, the Zygoptera are paraphyletic based on mitochondrial DNA evidence. In particular, the family Lestidae appears more closely related to the Anisoptera than the Zygoptera.

KEY WORDS Odonata, Anisoptera, Zygoptera, maximum likelihood, mtDNA

“A CLEAR RESOLUTION OF ODONATE higher relationships is needed to achieve a classification which reflects the phylogeny of the order, and to facilitate progress in evolutionary, ecological and biological studies, which rely on phylogenetic estimates for purposes such as modeling past relationships, making a distinction between the ecological correlation and co-inheritance of traits, and determining whether instances of apparent co-variation are statistically independent or historically linked” (Trueman 1996).

The reasons for pursuing this study parallel that of Trueman (1996) in that we realize a clear understanding of the relationships among the Odonata can have far reaching implications in Odonate biology. Over the past 45 yr, there have been many studies attempting to resolve the relationships within the Odonata (Fraser 1957; Hennig 1969, 1981; Carle 1982; Pfau 1991; Trueman 1996; Bechley 2002) based on morphological characters. Within the Odonates there are 11 families currently recognized in North America north of Mexico, which have been further divided into two clades or suborders: the Anisoptera, dragonflies, and the Zygoptera, damselflies. A third suborder, Anisozygoptera, is recognized, but is found only in Japan and the Himalayas and is not included in this study (Hennig 1969, 1981; Bridges 1993; Needham et al. 2000) (Table 1). Although the many morphological studies have attempted to use different characters to resolve the relationships of the Odonates based on wing venation (Fraser 1957, Carle 1982, Trueman 1996) and morphology of the flight apparatus and copulatory structures (Pfau 1991), none have been able to come to robust conclusions. Recently, a molecular phylogeny was employed for the Anisoptera (Misof et al.

2001). This was the first study to use molecular markers to investigate higher relationships within the Odonata.

In this work, we present a molecular phylogeny from mitochondrial DNA (mtDNA) sequence data encompassing the partial 3' region of the 12S ribosomal RNA (rRNA) gene (Hickson et al. 1996). This study is the first attempt to use mitochondrial sequence to investigate the relationships of the Odonata across both the Anisoptera and the Zygoptera.

Materials and Methods

Collection of Specimens. The species names and current classification of the specimens used in this study are listed in Table 1, which includes 24 species from 16 genera in seven families (Bridges 1993, Needham et al. 2000). The exact collection data for each specimen can be obtained from the authors. Voucher specimens have been deposited in the personal collection of Rosser W. Garrison (Los Angeles, CA), to be deposited in the United States National Museum in Washington, D.C. Specimens were collected and placed on wet ice in the field and then transferred to a -80°C freezer.

DNA Isolation. Total genomic DNA was isolated by grinding thoracic wing muscle with a Teflon grinding implement. This was performed in a 1.5-ml tube containing 500 μl of grinding buffer (0.1 M EDTA, 100 mM Tris, pH 8.0, 1% SDS, 0.2 M NaCl). The homogenate was incubated overnight at 65°C , and then extracted with equilibrated phenol several times until the supernatant was not cloudy or discolored. The supernatant was then extracted twice with chloroform, then with cold 100% ethanol, and finally several times with 70% ethanol at room temperature. The DNA was dried and resuspended in 200 μl of double distilled water.

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Table 1. Taxonomic samples used in this study

Taxonomic grouping	Species	Collecting locality
ZYGOPTERA		
COENAGRIONIDAE		
Ischnurinae	<i>Ischnura posita</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
	<i>Ischnura cervula</i>	USA: California, San Mateo Co. (G. S. Spicer)
	<i>Ischnura perparva</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Ischnura gemina</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Ischnura rumburii</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
	<i>Enallagina civile</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
	<i>Enallagina basidens</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
Pseudagrioninae		
	<i>Telebasis salva</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
Argiinae		
	<i>Argia scdula</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
CALOPTERYGIDAE		
Hetaerinae	<i>Hetaerina americana</i>	USA: California, Stanislaus Co. (G. S. Spicer)
LESTIDAE		
	<i>Lestes disjunctus</i>	USA: California, San Mateo Co. (G. S. Spicer)
ANISOPTERA		
AESHNIDAE		
	<i>Aeshna multicolor</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Aeshna californica</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Anax junius</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
GOMPHIDAE		
	<i>Octogomphus specularis</i>	USA: California, San Mateo Co. (G. S. Spicer)
CORDULEGASTRIDAE		
	<i>Cordulegaster dorsalis</i>	USA: California, San Mateo Co. (G. S. Spicer)
LIBELLULIDAE		
Sympettrinae		
	<i>Sympetrum illotum</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Erythemis simplicicollis</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
	<i>Pachydiplax longipennis</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
Palpopleurinae		
	<i>Perithemis tenera</i>	USA: Texas, Tarrant Co. (J. W. Robbinson)
Trameinae		
	<i>Tramea lacerata</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Tramea onusta</i>	USA: California, Santa Clara Co. (G. S. Spicer)
Libellulinae		
	<i>Libellula saturata</i>	USA: California, Santa Clara Co. (G. S. Spicer)
	<i>Libellula luctuosa</i>	USA: California, Santa Clara Co. (G. S. Spicer)
OUTGROUP		
	<i>Locusta migratoria</i>	

Polymerase Chain Reaction (PCR) Amplification. The conditions of the PCR (Mullis et al. 1987, Saiki et al. 1988) were varied depending on the specimen being amplified. Amplification primers for this region were designed to correspond to that of Shaw (1996). Primers correspond to *Drosophila yakuba* mtDNA (Clary and Wolstenholme 1985) sites 14588–14612 (12Sai, 5'-AAA CTA GGA TTA GAT ACC CTA TTA T) and site 14214–14233 (12Sbi, 5'-AAG AGC GAC GGG CGA TCT GT). The double-stranded amplification reaction volumes were usually 50- μ l solutions. Generally, the 5 \times buffer (300 mM Tris-HCl, 75 mM (NH₄)₂SO₄, pH 8.5) was used, along with 10 mM of dNTPs and 10 μ M of the primers. Both the Mg²⁺ concentration and pH were adjusted depending on the template. These were varied from a concentration of 7.5–17.5 mM of MgCl₂ and a pH 8.5–9.5 for the buffer. Between 30 and 35 cycles were used for the amplifications. The denaturing step was set at 94°C for 40 s, and the extension step was at 72°C for 1 min. The annealing step varied according to the specimen that was being amplified. Usually, this ranged from 48 to 54°C for 1 min. *Locusta migratoria* sequence is from Flook et al. (1995).

Template Purification. The double-stranded templates were purified using the Pharmacia Biotech (Uppsala, Sweden) MicroSpin S-300 HR columns, according to the protocol supplied by the manufacturer. The double-stranded DNA product was obtained by spinning the solution three times at 5,000 \times g in a Millipore 30,000 NMWL Ultrafree-MC microconcentrator.

Sequencing. All direct DNA sequencing of the double-stranded PCR products was performed using the USB Sequenase Kit (USB Corporation, Cleveland, OH), but not according to the manufacturer's guidelines. Instead, a modified protocol was followed (Casanova et al. 1990, Liu and Beckenbach 1992). The denaturing step consists of boiling the template, reaction buffer, and primer for 5 min, and then placing this into either a liquid nitrogen or dry ice/ethanol bath. The labeling reaction mixture is added while the sample is still frozen. The reaction mixture was microfuged for 30 s and then added to the extension mix. This was incubated at 37–42°C for 5 min, then terminated.

Sequence Alignment. All final sequences used were obtained by reconciling sequences from both the for-

ward and reverse sequencing runs. Conserved regions were first identified and aligned, and the gaps were assigned so that the fewest number of changes occurred. However, a secondary structure approach was used to construct the final alignment (Kjer 1995, Hickson et al. 1996). The *Ischnura cervula* (damselfly) sequence was used in the model building in Hickson et al. (1996) to determine the secondary structure of the third domain of animal 12S rRNA.

Preliminary Sequence Analysis. Sequences were then evaluated for overall base composition bias and among taxa base composition. The base composition bias statistic was calculated according to Irwin et al. (1991) and ranges in value from 0 to 1, 0 indicating no bias and 1 showing complete base composition bias. An extreme overabundance of 1 nucleotide state can increase the tendency for those sites to become saturated (Irwin et al. 1991). In addition, a strongly skewed mutation bias can violate the assumption in parsimony analysis that there is an equal probability of change at all sites (Perna and Koehler 1995). The heterogeneity chi-square test in PAUP*4.03b10 was used to test for bias among taxa.

Phylogenetic Analysis. A variety of model-based methods, in addition to maximum parsimony, was employed to infer phylogenetic relationships. Parsimony has been shown to be inconsistent under certain situations when dealing with molecular sequence data (Hasegawa and Fujiwara 1993, Kuhner and Felsenstein 1994, Huelsenbeck 1995), so model-based maximum likelihood approaches were also used. All parsimony and maximum likelihood analyses were performed using the computer program PAUP*4.03b10 (Swofford 2002).

Maximum parsimony searches were conducted using heuristic search methods with tree bisection-reconnection branch swapping, collapse of zero-length branches, and equal weighting of all characters for 300 iterations. To assess the confidence limits concerning the branching pattern, a bootstrap analysis was performed (Felsenstein 1985). A total of 100 replications was performed using the branch-and-bound algorithm. The result is presented as a majority rule consensus tree (Margush and McMorris 1981), which shows the most frequently occurring branching orders. In addition, to evaluate some alternative less parsimonious arrangements, tree manipulations were accomplished by using the program MacClade4.03 (Maddison and Maddison 2001).

In addition to searching for trees under the maximum parsimony criterion, we also searched for trees using maximum likelihood. To determine which model best fit the data, a series of nested (i.e., the null hypothesis [H_0] is a special case of the alternative hypothesis [H_1]) hypotheses were performed on various nucleotide substitution models. An initial neighbor-joining (NJ) tree based on the Jukes-Cantor distance (JC) was generated, and then a likelihood ratio test (LRT) was performed (Goldman 1993) to test the models. We calculated the test statistic as $2(\ln L_0 - \ln L_1) = -2\ln\Lambda$, where L_0 and L_1 are the likelihood values under the null and alternative hypotheses, re-

spectively. We calculated the associated probability using a χ^2 distribution with the degrees of freedom equal to the difference in number of free parameters between the two models. The models tested included the simplest substitution model, the Jukes-Cantor model (JC; Jukes and Cantor 1969), which assumes that all nucleotide substitutions are equally probable and that the nucleotides occur in equal frequencies. The more complicated Hasegawa, Kishino, and Yano model (HKY85; Hasegawa et al. 1985) allows the transition and transversion rate to differ and incorporates observed average nucleotide frequencies. Finally, the most parameter-rich model tested was the general time-reversible model (GTR; Lanave et al. 1984, Tavaré 1986, Rodríguez et al. 1990), which incorporates observed average base frequencies and allows for rate variation among six substitution types. In addition to the nucleotide models, other parameters were investigated. These included the extent of among site rate variation (α value of the Γ -distribution estimated with eight rate categories) along with the number of invariable sites (I). After the best-fit model was found, we performed a heuristic search using the same branch-swapping techniques as described when using maximum parsimony. The search was started using the initial parameter estimates from the NJ tree, but once a better tree was found we reestimated the parameters and searched again. This process was continued until it converged on the same maximum likelihood tree. Bootstrap tests were performed once again, using 100 replicates.

Maximum likelihood was also used for additional phylogenetic tests. To test the null hypothesis of a molecular clock for our data set, we used a procedure proposed by Felsenstein (1993). This test uses an LRT to determine whether there is a significant difference between the likelihood scores obtained from an analysis in which the branch lengths are unconstrained as compared with an analysis that constrains the branch lengths so that all the tips are contemporaneous. Once again, the likelihood test statistic is assumed to be approximately equal to a χ^2 distribution with $n-2$ degrees of freedom, where n equals the number of taxa sampled (Felsenstein 1981). In addition, competing tree topologies based on previous phylogenetic hypotheses were compared using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) to test for significant difference in tree lengths. This test was performed using RELL with 1,000 bootstrap replicates, and the results were evaluated as a one-tailed test.

Results

Simple Sequence Statistics. An aligned 346-bp fragment was sequenced for a region spanning the 3' part of the 12S rRNA gene, with 196 variable sites (148 positions were potentially parsimony informative). Sequences have been deposited in Genbank (accession numbers AY282544-AY282567). The *L. migratoria* sequence (Genbank accession number X80245) is from Flook et al. (1995). Examination of base composition

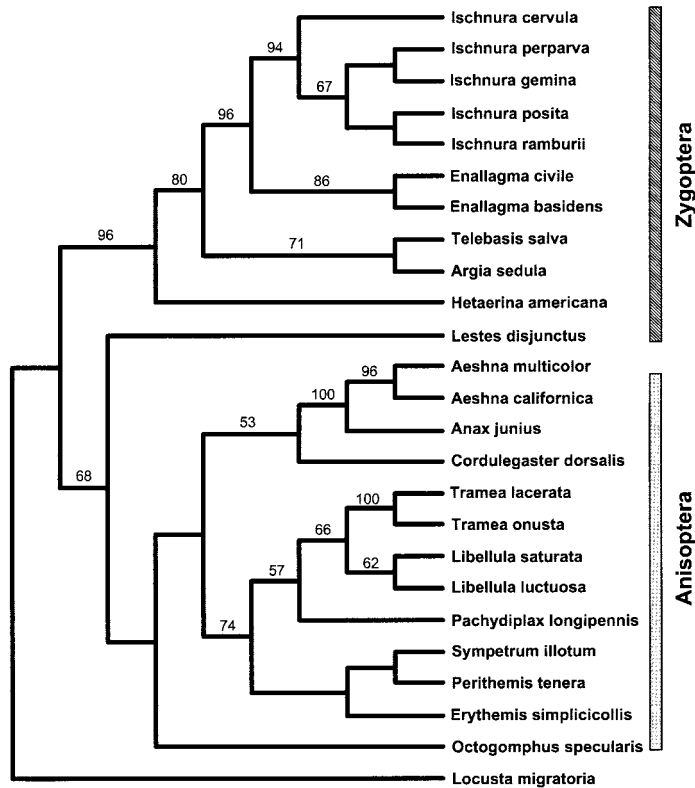


Fig. 1. Single most parsimonious tree topology for the mitochondrial 12S rRNA data set. Values above the branches represent bootstrap percentages >50%.

and base composition bias revealed that our data set has moderate bias (0.301) with the following empirical base frequencies: A = 0.323, C = 0.107, G = 0.166, T = 0.402. A χ^2 test for homogeneity of base frequency among taxa was nonsignificant when including all characters in the analysis ($P = 0.999$) and remained nonsignificant once uninformative sites were excluded ($P = 0.995$).

Phylogenetic Analyses of mtDNA. Parsimony analysis of the mtDNA data set resulted in a single minimum length tree of 646 steps, with a CI of 0.485 and an RI of 0.631 (Fig. 1).

The evaluation of the model for maximum likelihood determined using the LRT suggested that the best model for these data was the GTR + Γ with a score of $-\ln L = 3,139.48710$ (Fig. 2). The parameter values estimated from this tree were: A \leftrightarrow C, 1.739085; A \leftrightarrow G, 6.963124; A \leftrightarrow T, 1.869579; C \leftrightarrow G, 0.555109; C \leftrightarrow T, 9.911142; G \leftrightarrow T, 1.0 for the GTR model; estimated base composition was A = 0.33534, C = 0.06133, G = 0.11735, T = 0.48598, and $\alpha = 0.314775$ for the Γ distribution. Maximum likelihood was also used to test for a molecular clock. The molecular clock tree produced with the same parameter estimates above gave a likelihood score of $-\ln L = 8,430.99075$, which indicates that the molecular clock should be rejected ($\chi^2 = 97.4$, df = 23, $P < 0.0001$). In this instance, the difference among topologies was the nonresolution

among the interfamily relationships within the Anisoptera, although both lack bootstrap support for these relationships.

Phylogenetic Relationships Within the Odonata. All tree topologies show support for a monophyletic Zygoptera (minus Lestidae) lineage within Odonata. However, the monophyly of Anisoptera does not have any bootstrap support, unless the lestid is included as part of the Anisoptera (Figs. 1 and 2). Minimal support for the relationships among the Anisoptera taxa was recovered (Figs. 1 and 2). Relationships among the Zygoptera are more clearly resolved, with the exception of the Lestidae, the spread-winged damselflies, which remained outside of the Zygoptera.

One result consistent across all analyses was the finding of the Anisoptera as a monophyletic clade. The relationships within the Anisoptera correspond to those found by Misof et al. 2001. In their analysis, based on two mitochondrial gene fragments, they found the same relationships within the Anisoptera that our study uncovered (Misof et al. 2001).

Comparison of Competing Tree Topologies. Competing tree topologies based on previous phylogenetic hypotheses were compared using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) to test for significant difference in tree lengths (Table 2). In comparing tree topologies from previous phylogenetic hypotheses, our best tree was compared with the

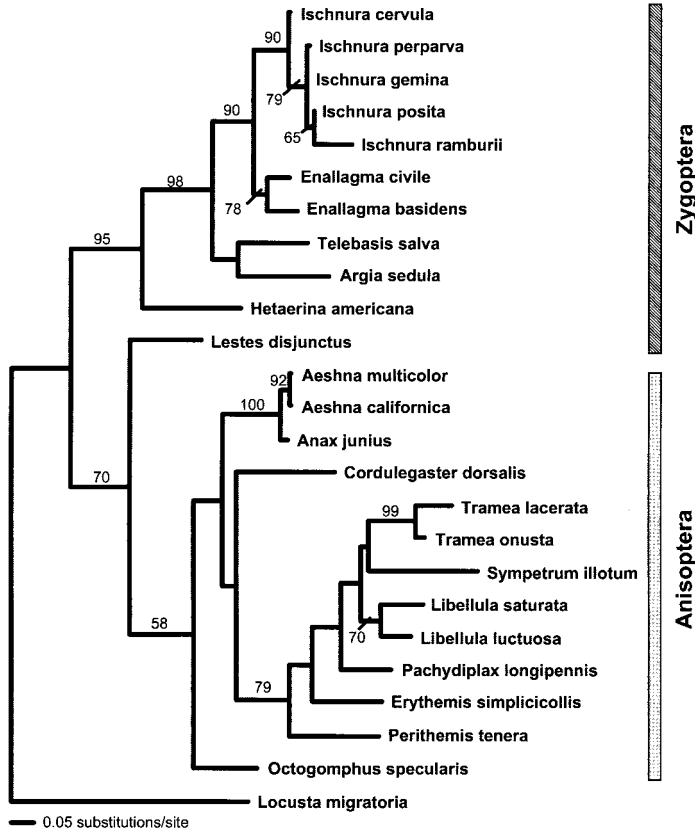


Fig. 2. Single tree inferred under maximum likelihood search with a GTR- Γ model of sequence evolution. All parameters used in the model were estimated using maximum likelihood. Values above the branches represent bootstrap percentages >50%.

trees hypothesized by Fraser (1957), Carle (1982), Pfau (1991), Trueman (1996), and Bechley (2002) (Fig. 3). The topology that was uncovered by this analysis was not statistically different at the ≥ 0.05 level than either Carle (1982) ($P = 0.444$) or Pfau (1991) ($P = 0.352$).

Discussion

This is the first molecular study to examine both the Zygoptera (damselflies) and Anisoptera (dragonflies). From our analysis, we are able to infer that Odonata contains a paraphyletic Zygoptera and a

monophyletic Anisoptera. The relationships among the Anisoptera were also investigated by Misof et al. (2001), and the relationships among the families are consistent with the relationships we found in our maximum likelihood tree.

Phylogenetic hypotheses inferred from the parsimony and maximum likelihood searches of this data set resulted in tree topologies consistent with those found based on other morphological data sets: Carle (1982) and Pfau (1991) (Table 2). Relationships resolved in this study cast doubt on the utility of solely using wing venation for understanding Odonate evolution. Several authors have attempted to use wing venation to resolve relationships among the Odonates (Carle 1982, Trueman 1996), with limited success. Pfau (1991) proposed that modes of sperm transfer to the female might shed some light into the relationships within the Odonata. Pfau (1991) believed that the sperm-transfer mode found in the Zygoptera is probably the primitive condition, which corresponds to the results found in this study.

Although our data set is limited in number of base pairs sequenced, bootstrap support for many of the clades is strong (>70%) (Fig. 3). One clade that has

Table 2. Shimodaira-Hasegawa test, evaluated by using RELL bootstrap (one-tailed test) with 1,000 replicates

	-ln L	Difference -ln L	P
Present study	3,139.48710	(best)	
Carle (1982)	3,143.27940	3.79230	0.444
Pfau (1991)	3,143.96026	4.47315	0.355
Bechley (2002)	3,152.58348	13.09637	0.023*
Fraser (1957)	3,152.75278	13.26568	0.020*
Trueman (1996)	3,153.70063	14.21353	0.019*

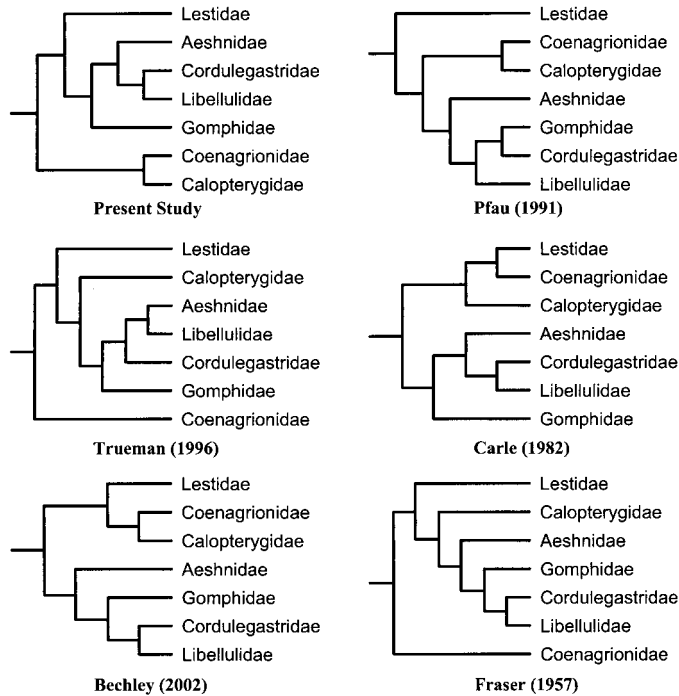


Fig. 3. Simplification of the phylogenetic relationships among the Odonata proposed by current and previous studies.

strong support is the clade containing the suborder Zygoptera minus Lestidae. Many have speculated that the spread-winged damselflies, Lestidae, may be more closely related to the Anisoptera or the Anisozygoptera than to the Zygoptera damselflies because of the belief that the ancestral odonate had narrow, zygopteran-like wings (Fraser 1957, Trueman 1996). Our analysis potentially suggests that the Lestidae in fact belong within the Anisoptera (70% bootstrap value; Fig. 3), unless the Zygoptera are accepted as a paraphyletic group. It is evident that more work is necessary to fully resolve the relationships within the Odonata. Future investigations should also include specimens from the Anisozygoptera suborder, which has morphological characters that place it as a sort of intermediate between the Anisoptera and Zygoptera (Fraser 1957, Carle 1982, Trueman 1996).

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