

Molecular Phylogenetic Analysis of the Dragonfly Genera *Libellula*, *Ladona*, and *Plathemis* (Odonata: Libellulidae) Based on Mitochondrial Cytochrome Oxidase I and 16S rRNA Sequence Data

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Molecular phylogenetic relationships among members of the odonate genus *Libellula* (Odonata: Anisoptera: Libellulidae) were examined using 735 bp of mitochondrial COI and 416 bp of 16S ribosomal RNA gene sequences. Considerable debate exists over several relationships within *Libellula*, as well over the status of two putative genera often placed as subgenera within *Libellula*: *Ladona* and *Plathemis*. Parsimony and maximum-likelihood analyses of the separate and combined data sets indicate that *Plathemis* is basal and monophyletic and that *Ladona* is the sister clade to the remainder of *Libellula sensu stricto* (*s.s.*) (all species within the genus *Libellula*, excluding *Plathemis* and *Ladona*). Moreover, two European taxa, *Libellula fulva* and *L. depressa*, were found to occupy a sister group relationship within the *Ladona* clade. Relationships within *Libellula s.s.* are less well resolved. However, monophyletic lineages within the genus are largely consistent with morphologically based subgeneric classifications. Although tree topologies from each analysis differed in some details, the differences were in no case statistically significant. The analysis of the combined COI and 16S data yielded trees with overall stronger support than analyses of either gene alone. Several analyses failed to support the monophyly of *Libellula sensu lato* due to the inclusion of one or more outgroup species. However, statistical comparisons of topologies produced by unconstrained analyses and analyses in which the monophyly of *Libellula* was constrained indicate that any differences are nonsignificant. Based on morphological data, we therefore reject the paraphyly of *Libellula* and accept the outgroup status of *Orthemis ferruginea* and *Pachydiplax longipennis*. © 2001 Academic Press

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INTRODUCTION

The genus *Libellula* Linnaeus 1758 (Odonata: Libellulidae) is a common, widespread, and diverse genus of principally Holarctic dragonflies and is the type genus for the odonate family Libellulidae. Twenty-one species are known from North America north of Mexico, and approximately 32 species are described worldwide (Table 1). The oldest fossil records for members of this genus date from the Oligocene (25–36 MY; Carpenter, 1992).

Members of this genus are large, abundant, and conspicuous predators in many freshwater ecosystems and play an important role in structuring aquatic communities (Needham and Westfall, 1955; Wissinger, 1989). In addition, this genus displays remarkable diversity in behavior and morphology and consequently has been the focus of studies on genital morphology and sperm competition (Kennedy, 1922a,b; Restifo, 1972; Miller, 1991), sexual selection (Moore, 1990), comparative population ecology (Wissinger, 1987, 1988, 1989), phylogeography (Garrison, 1976; Kiauta and Kiauta, 1991), and the evolution of mating behaviors (Campanella, 1975; Hilton, 1983; Koenig, 1987; Moore, 1987, 1989; Alcock, 1989; Convey, 1989; Utzeri and Dell'Anna, 1989; DeBano, 1993, 1996). However, these studies were conducted without knowledge of the phylogenetic relationships of the taxa. A complete understanding of the diverse morphology, ecology, behavior, and distribution of *Libellula* species must include evolutionary historical factors, and future studies on the evolution of traits in this genus should be conducted with reference to a phylogenetic framework.

Despite several attempts to resolve the taxonomy of the genus (Ris, 1910; Kennedy, 1922a,b; Bennefield, 1965; Dunkle, 1992; May, 1992) the systematics of the group remains poorly understood. Previous systematic studies did not survey the entire genus, were based on few characters, and/or did not employ rigorous phylogenetic methods. Consequently, there remains a gen-

TABLE 1

Taxonomic Summary of the Genus *Libellula* Showing the Geographic Location of Each of the Species and the Area Where Specimens Used in This Study Were Collected^a

Species	Geographic distribution	Collection location
<i>Pachydiplax longipennis</i> Burmeister, 1839 ^b	North America	Gainseville, FL, USA
<i>Erythemis simplicicollis</i> Say, 1839 ^b	North America	Chaffey's Locks, ON, CA
<i>Orthemis ferruginea</i> Fabricius, 1775 ^b	North America	Gainseville, FL, USA
<i>Libellula angelina</i> Selys, 1883	North China, Japan	Matsumoto, Kobe, JP
<i>L. auripennis</i> Burmeister, 1839	North & Central America	Gainseville, FL, USA
<i>L. axilena</i> Westwood, 1837	North America	Gainseville, FL, USA
<i>L. comanche</i> Calvert, 1907	North America	Glen Rose, TX, USA
<i>L. composita</i> Hagen, 1873	North America	Santa Rosa Co., NM, USA
<i>L. croceipennis</i> Selys, 1869	North & Central America	Cedarville, CA, USA
<i>L. cyanea</i> Fabricius, 1775	North America	Morris Co., TX, USA
<i>Libellula (Ladona) deplanata</i> Rambur, 1842 ^c	North America	New London Co., CT, USA
<i>Libellula (Plathemis) depressa</i> Linnaeus, 1758 ^c	Europe, West Asia	Limburg, NL
<i>Libellula (Ladona) exusta</i> Say, 1839 ^c	North America	New London Co., CT, USA
<i>L. flavida</i> Rambur, 1842	North America	Montgomery Co., AR, USA
<i>L. foliata</i> Kirby, 1889	Mexico	
<i>L. forensis</i> Hagen, 1861	North America	Lina Co., OR, USA
<i>Libellula (Ladona) fulva</i> Müller, 1764 ^c	Europe, Middle East	Noord-Holland, NL
<i>L. gaigei</i> Gloyd, 1838	Central America	
<i>L. herculea</i> Karsch, 1889	Central & South America	
<i>L. incesta</i> Hagen, 1861	North America	Gainseville, FL, USA
<i>L. jesseana</i> Williamson, 1922	North America	Gainseville, FL, USA
<i>Libellula (Ladona) julia</i> Uhler, 1857 ^c	North America	Chaffey's Locks, ON, CA
<i>L. luctuosa</i> Burmeister, 1839	North America	Chaffey's Locks, ON, CA
<i>Libellula (Plathemis) lydia</i> Drury, 1770 ^c	North America	Chaffey's Locks, ON, CA
<i>L. mariae</i> Garrison, 1992	Central America	
<i>L. melli</i> Schmidt, 1948	China	
<i>L. needhami</i> Westfall, 1943	North America	Gainseville, FL, USA
<i>L. nodistica</i> Hagen, 1861	North America to Venezuela	Modoc Co., CA, USA
<i>L. pulchella</i> Drury, 1770	North America	Chaffey's Locks, ON, CA
<i>L. quadrimaculata</i> Linnaeus, 1758	North America (holarctic)	Chaffey's Locks, ON, CA
<i>L. saturata</i> Uhler, 1857	North America	Pima Co., AZ, USA
<i>L. semifasciata</i> Burmeister, 1839	North America	Green Co., OH, USA
<i>Libellula (Plathemis) subornata</i> Hagen, 1861 ^c	North America & Mexico	Cochise Co., NM, USA
<i>L. vibrans</i> Fabricius, 1793	North America	Gainseville, FL, USA

^a See also Kambhampati and Charlton (1999).

^b Outgroup taxa.

^c Putative genera indicated in parentheses.

eral lack of consensus regarding relationships within the genus and the position of two putative genera, *Ladona* and *Plathemis*.

One recent study has significantly enhanced our understanding of the systematics of this genus. Using mitochondrial 16S rRNA sequence data, Kambhampati and Charlton (1999) reconstructed the phylogeny of the genus and concluded that there is evidence to suggest that *Ladona* and *Plathemis* are distinct monophyletic lineages within *Libellula sensu stricto* (*s.s.*). However, Kambhampati and Charlton (1999) were unable to adequately resolve relationships within *Libellula sensu lato* (*s.l.*). Their study may have been limited by the small number of parsimony-informative characters. Moreover, their study did not include many of the species in the genus. In particular, *Libellula depressa* and *L. fulva*, whose status within *Libellula s.l.* are contested, were not included.

A well-supported phylogeny of this group would have at least two important benefits: (1) it would provide the systematic framework upon which subsequent studies of the evolution of characters could be based and (2) it would resolve taxonomic questions of relationships among taxa within the genus and of the monophyly of *Libellula s.s.* The principle objectives of this study were to use mitochondrial cytochrome oxidase I (COI) and ribosomal rRNA 16S data to assess the major lines of phylogenetic descent within the genus and to assess the taxonomic status of nominal genera (i.e., *Ladona*, *Plathemis*). The utility of mitochondrial genes for resolving genus-level phylogenetic relationships is well known (Simon *et al.*, 1994). We used a region of COI that has been sequenced in several insect taxa (Crozier *et al.*, 1989; Brower, 1994; Sperling and Hickey, 1994; Sperling *et al.*, 1997), including Odonata (Lunt *et al.*, 1996). We also incorporated data from another mito-

chondrial gene, 16S rRNA (see Kambhampati and Charlton, 1999), and evaluated congruence among phylogenetic hypotheses based on separate and combined data sets. Our study represents a critical step in determining the historical relationships among members of the genus and establishes a framework for subsequent comparative studies of behavior and evolution within the group.

MATERIALS AND METHODS

Acquisition of Specimens

Adult dragonflies representing 26 *Libellula* species and 3 outgroup species were collected and preserved in 100% nondenatured EtOH when possible. In some cases, DNA was extracted from dried specimens, which typically had been collected within the previous 5 years, thereby minimizing DNA degradation. Of the three outgroup taxa, *O. furriginea* was chosen because it is a purported sister genus, and *P. longipennis* and *E. simplicicollis* were chosen because they are members of the same family.

Sequence data (419 bp) for 13 *Libellula* taxa and 5 outgroup taxa were obtained from GenBank for the mitochondrial ribosomal rRNA gene, 16S (Accession Nos. AF037171–AF037193). GenBank sequence data for 16S were not available for 9 additional taxa used in this study: *Libellula angelina*, *composita*, *fulva*, *depressa*, *forensis*, *needhami*, and *flavida*, and *Orthemis ferruginea*. To obtain data for these taxa, primers described in Kambhampati and Charlton (1999) were used. DNA isolation, PCR, and sequencing techniques were similar to those described below.

DNA Isolation

DNA extractions were performed using a modified CTAB/DTAB protocol (Phillips and Simon, 1995; Chipindale *et al.*, 1998). Thoracic muscle was dissected from fresh adult specimens, and the entire thorax containing flight muscle was used from dried specimens. Muscle tissue was dried, macerated in 300 μ l of TE Buffer and 600 μ l of DTAB, and incubated for ca. 2 h at 68°C. The solution was chloroform-extracted by adding 600 μ l of chloroform and centrifuging at 10,000g for 2 min. The aqueous solution was transferred and the chloroform extraction repeated. The aqueous layer from the second extraction was added to a solution of 900 μ l ddH₂O and 100 μ l CTAB and centrifuged at 10,000g for 2 min. The supernatant was discarded, and the pellet was resuspended in 300 μ l of 1.2 M NaCl. Following centrifugation at 10,000g for 2 min, the pellet was washed in 750 μ l 100% EtOH and centrifuged at 10,000g for 10 min. The pellet was resuspended in 300 μ l 70% EtOH and centrifuged at 10,000g for 5 min. The pellet was dried in a SpeedVac and resuspended in ca. 50 μ l of ddH₂O.

TABLE 2

Sequences and Locations of Primers^a Used to Amplify Regions of the mtDNA Gene Cytochrome Oxidase I Gene in Dragonflies

Primer name	Primer sequence	Position spanned
Cl-13 ^b	5'-ATAATTTTTTTTATAGTTATACC-3'	1662–1687
Cl-14 ^b	5'-GTTTCTTTTTTTCCTCTTTC-3'	2347–2366
Cl2161 (Jerry)	5'-CAACATTTATTTTGATTTTTGG-3'	2161–2183
Cl2570 (Ben)	5'-GTACTATGKATAATRCACAWCAG-3'	2570–2592
Cl1731 (Stimpy)	5'-AGCACCTGATATGGCTTTCCC-3'	1731–1751
Cl2523 (Ren)	5'-CCAAATTGTCCTCATMAAGATCG-3'	2523–2545

^a The position of each primer refers to the COI gene of *Drosophila yakuba*.

^b Primer sequences provided by Eisuke Hasegawa.

DNA Amplification and Sequencing

Amplifications were conducted in a 50- μ l total volume, containing 5 μ l of 10 \times Pääbo buffer (70 mM Tris-HCl, pH 8.8, 2.5 mM MgCl₂, BSA 200 ng/ μ l), 5 μ l dNTPs, 2.5 μ l of each primer (1.0 μ M each), 2.5 μ l of template (ca. 10 ng), 2 units of *Taq* polymerase, and 32.5 μ l of sterile water. Approximately 850 bp of the COI gene were amplified as a single product using two flanking primers or as two products using two pairs of primers that yielded overlapping regions (Table 2). The standard PCR profile consisted of 30 cycles of 94°C/60 s denaturation, 50°C/60 s annealing and 72°C/90 s extension. A negative control consisting of PCR solution and no template and a positive control were used. Amplified DNA was resolved by electrophoresis on 1.5% agarose gel stained with ethidium bromide. PCR product was purified with spin columns (QIAGEN Inc.) and cycle-sequenced using the big dye-primer *Taq* cycle sequencing reaction (ABI) and an automated DNA sequencer (Applied Biosystems 377A). Sequences were determined for both strands. Sequences were aligned using the multiple sequence alignment program Clustal W (Thompson *et al.*, 1994) and confirmed by visual inspection of both the nucleotide and the amino acid sequences. Sequence data for COI and 16S generated from this study are available from GenBank (Accession Nos. AF195726–AF195763).

Phylogenetic Analysis

The data consist of 735 bp of mitochondrial COI and 416 bp of mitochondrial 16S ribosomal RNA gene sequences for 27 *Libellula* and 4 outgroup species. No COI sequence was obtained for *Sympetrum*. Maximum parsimony (MP) and maximum-likelihood (ML) analyses were carried out separately on the COI data set (29

spp.) and the 16S data set (30 spp.), as well as on the data set formed by the combination of the two (30 spp.).

Parsimony analyses. Parsimony analyses were implemented in the computer program PAUP 4.0b2 (Swofford, 1999) using the heuristic search option with TBR branch-swapping and with parsimony-uninformative characters excluded. To insure that multiple "islands" of most-parsimonious trees were identified (Maddison, 1991), 100 random-addition replicate analyses were carried out for both the unweighted and the successive approximations-weighted analyses. For the successive approximations-weighted analyses, character weights were based on the maximum value of the rescaled consistency index and iterative rounds were continued until character weights stabilized (Farris, 1969; Carpenter, 1988). Bootstrapping (Felsenstein, 1985) under parsimony utilized 1000 pseudoreplicates, with 10 random-addition replicates per pseudoreplicate; parsimony-uninformative characters were excluded. Decay index ("Bremer support") values (Bremer, 1988, 1994; Donoghue *et al.*, 1992) for each branch were obtained by analyzing the data under reverse constraints using the search parameter values outlined above; constraint-tree parenthetical notation was automatically generated using the program AutoDecay 4.01 (Eriksson, 1998).

Maximum-likelihood (ML) analyses. To obtain an appropriate substitution model and model parameter values, as well as an optimal starting tree for branch-swapping under ML, the set of most-parsimonious trees and (where different) the set of successive approximations-weighted trees were evaluated under 40 "models" of evolution, with 10 basic substitution models, including JC (Jukes and Cantor, 1969), K2P (Kimura, 1980), TrNef (Tamura and Nei, 1993; but with equal base frequencies), K3P (Kimura, 1981), SYM (Zarkikh, 1994), F81 (Felsenstein, 1981), HKY85 (Hasegawa *et al.*, 1985), TrN (Tamura and Nei, 1993), K3Puf (Kimura, 1980; but with unequal base frequencies), and GTR (Rodriguez *et al.*, 1990). All models were evaluated with and without rate heterogeneity. Rate heterogeneity was accommodated in three ways: using a gamma model, using an invariant sites model, and using a gamma plus invariant sites model (as in Frati *et al.*, 1997). In one case (the 16S data set) in which the number of optimal parsimony trees was prohibitively large, a subset of those trees was evaluated. Using a standard likelihood ratio test, the likelihood scores of each of the parsimony trees were compared across nested models using the computer program Modeltest 2.1 (Posada and Crandall, 1998). In pairwise comparisons in which the improvement in likelihood imparted by a more complex model was not found to be significant, the simpler model was chosen.

Employing the adopted model and using the parsimony tree found to be most likely under that model as the

starting tree for branch-swapping, five iterative rounds of maximum-likelihood analyses were carried out, proceeding from those using less intensive to those using more intensive branch-swapping regimens. The most likely tree identified during each of the first four ML search rounds was used in the next search round for the calculation of parameter values and for initiation of branch-swapping. Branch-swapping regimens in the five rounds were, respectively: (1) nearest-neighbor interchange (NNI), (2) subtree pruning-regrafting (SPR), (3) SPR, (4) tree bisection-reconnection (TBR), and (5) TBR. In all rounds except round 4, the Rogers-Swofford approximation limit was set to 0.05 ("approxlim=5") and all optimal trees were saved during swapping. In round 4, the Rogers-Swofford approximation limit was set to 0.02 ("approxlim=2") and only one optimal tree was saved during swapping ("mulpars=no"). Based on analyses of diverse data sets, the foregoing method of optimizing topology and model parameter values under the ML criterion has been found to be vastly more computationally efficient than initially proceeding with TBR branch-swapping with a Rogers-Swofford approximation limit of 0.05 and with fixed parameter values derived from a parsimony tree (T. R. Schultz, unpublished; e.g., Mueller *et al.*, 1998). Bootstrapping (Felsenstein, 1985) under the ML criterion utilized 100 pseudoreplicates, with a single random-addition starting tree per pseudoreplicate and TBR branch-swapping. The Rogers-Swofford approximation limit was set to 0.05. To render bootstrapping under ML computationally tractable, ML model parameter values were set to the optimal (i.e., final) values estimated during the likelihood search procedure described above.

To conduct tests of the strength of support for ingroup (*Libellula*) monophyly, two parsimony- and likelihood-based tree-comparison tests were carried out in PAUP 4.0b2 (Swofford, 1999), the Kishino-Hasegawa (K-H) parametric test (parsimony and likelihood) (Kishino and Hasegawa, 1989) and the Wilcoxon's signed-rank (WSR) test (parsimony) (Templeton, 1983; Felsenstein, 1985). We used these tests to contrast the optimal trees found in the unconstrained analyses described above with the most optimal trees that are topologically consistent with alternative, conflicting hypotheses of ingroup monophyly/nonmonophyly. The latter "constraint" trees were generated with additional parsimony and likelihood analyses that imposed the minimum topological constraint of the presence (or, in the case of the COI parsimony analysis, the absence) of the single branch separating the ingroup (*Libellula*) from the four outgroup (non-*Libellula*) species. Except for this imposed constraint, all such searches followed exactly the methods described above.

Minimum-evolution (ME) analyses. Minimum-evolution analyses were conducted on the combined (COI + 16S) data set using the neighbor-joining algorithm of Saitou and Nei (1987) as the starting tree.

TABLE 3

Table of Nucleotide Composition (%) of All Constant and Nonconstant Sites (COI and 16S) and by Codon Position (COI only)

Gene	No. of sites	Nucleotide			
		A	C	G	T
16S—all	416	37.6–39.7 (38.4)	15.4–17.4 (16.7)	11.1–13.1 (12.1)	32.2–34.7 (32.8)
16S—nonconstant	116	40.5–50.4 (45.7)	8.7–15.9 (13.1)	3.5–10.6 (7.1)	31.9–40.9 (34.1)
COI—all	735	28.9–31.6 (30.5)	16.6–21.8 (17.8)	16.3–18.1 (17.2)	31.2–36.2 (34.4)
COI—nonconstant	272	37.9–43.4 (41.3)	9.6–22.5 (14.7)	4.0–8.1 (5.7)	29.0–42.6 (38.2)
Pos. 1	245	26.3–28.6 (26.9)	14.8–20.5 (16.5)	30.7–32.9 (31.9)	20.8–26.9 (24.6)
Pos. 2	245	14.7–16.7 (15.6)	26.2–28.8 (27.0)	14.0–16.0 (15.5)	40.1–43.0 (41.9)
Pos. 3	245	44.8–51.0 (48.9)	5.5–14.3 (10.1)	1.6–6.5 (4.3)	28.2–41.2 (36.7)
Pos. 1 nonconstant	44	11.6–18.4 (15.2)	20.9–47.4 (30.6)	6.5–14.6 (11.3)	26.3–54.5 (42.8)
Pos. 2 nonconstant	7	14.3–42.9 (28.9)	16.7–50.0 (27.8)	0.0–16.7 (14.4)	14.3–42.9 (28.9)
Pos. 3 nonconstant	221	42.9–50.5 (46.9)	6.5–19.4 (11.2)	1.4–6.8 (4.4)	28.1–41.2 (37.5)

Note. Ranges are shown with mean nucleotide composition in parentheses.

The distance measure employed was corrected for multiple hits using maximum-likelihood estimation under four substitution models, with and without rate heterogeneity: Jukes–Cantor (Jukes and Cantor, 1969), Kimura two-parameter (Kimura, 1980), Tamura–Nei (Tamura and Nei, 1993), and general time-reversible (GTR) (Yang *et al.*, 1994). All four analyses yielded the same topology. This topology was evaluated for 40 models of evolution using the Modeltest program (Posada and Crandall, 1998) and, not surprisingly, the GTR substitution model with rate heterogeneity accommodated using invariant sites plus gamma-distributed rates (GTR + I + G) was found to be significantly better fitting than the next-best-fitting model. Using the likelihood parameter values estimated for this model and this topology, 1000 neighbor-joining bootstrap replicates were carried out with maximum-likelihood distances under GTR + I + G and the minimum-evolution criterion. A separate minimum-evolution analysis, starting with the construction of a neighbor-joining tree and including 1000 neighbor-joining bootstrap replicates, was carried out using the GTR + I + G model and an alternative set of parameter values, those estimated in the maximum-likelihood phylogenetic analysis (see below). The results of this alternative minimum-evolution analysis were identical to those described above.

RESULTS

Sequence Characteristics

The alignment of 16S rRNA sequences produced 419 bp including gaps. Of these, 117 sites were variable (28.13%) and 78 were parsimony informative (18.75%). The 735 bp of COI sequences contained 272 variable sites (37.01%) of which 227 were parsimony informative (30.88%). Considering both genes together, uncorrected sequence divergences ranged from 1% between

L. axilena, *vibrans*, *auripennis*, and *jesseana* to 15% between *L. semifasciata*, *exusta*, and *deplanata*. Both genes showed considerable A + T nucleotide bias (Table 3), which is consistent with other insect mitochondrial genes (Crozier and Crozier, 1993; Simon *et al.*, 1994; Frati *et al.*, 1997; Chippindale *et al.*, 1999). Using χ^2 tests implemented in PAUP 4.0b2 (Swofford, 1999), we were unable to reject homogeneity of base frequencies among sequences for either gene for all sites (COI: $\chi^2_{(87)} = 33.428$, $P = 1.00$; 16S: $\chi^2_{(87)} = 7.299$, $P = 1.00$) or for only those sites observed to be variable (COI: $\chi^2_{(84)} = 92.373$, $P = 0.25$; 16S: $\chi^2_{(84)} = 35.352365$, $P = 1.00$). Thus, within the limitations of the test, we have little reason to suspect nonstationarity of base substitution processes across the phylogeny relating these sequences.

The circumstances under which separate data sets should or should not be combined are a subject of debate (Bull *et al.*, 1993; Eernisse and Kluge, 1993; deQueiroz *et al.*, 1995; Carpenter and Nixon, 1997), but none of the disparate viewpoints on this topic objects to combining data sets that are not demonstrably incongruent. To test the congruence of the separate 16S and COI data sets, we employed the Incongruence Length Difference test of Farris *et al.* (1995), implemented as the “Partition Homogeneity Test” in PAUP 4.0b2 (Swofford, 1999) with 1000 replicates, 10 random-addition tree searches per replicate, and invariant sites excluded (Cunningham, 1997). The results indicate that the null hypothesis of congruence cannot be rejected ($P = 0.106$) and that it is thus logical to combine and analyze the COI and 16S data sets as a single unit.

Phylogenetic Analysis of COI

Parsimony analysis of the COI data set identified six most-parsimonious trees (MPTs) (Fig. 1A) of length 964 steps, C.I. = 0.359, R.I. = 0.529, differing only in their placement of *L. incesta*, *L. jesseana*, *L. auripen-*

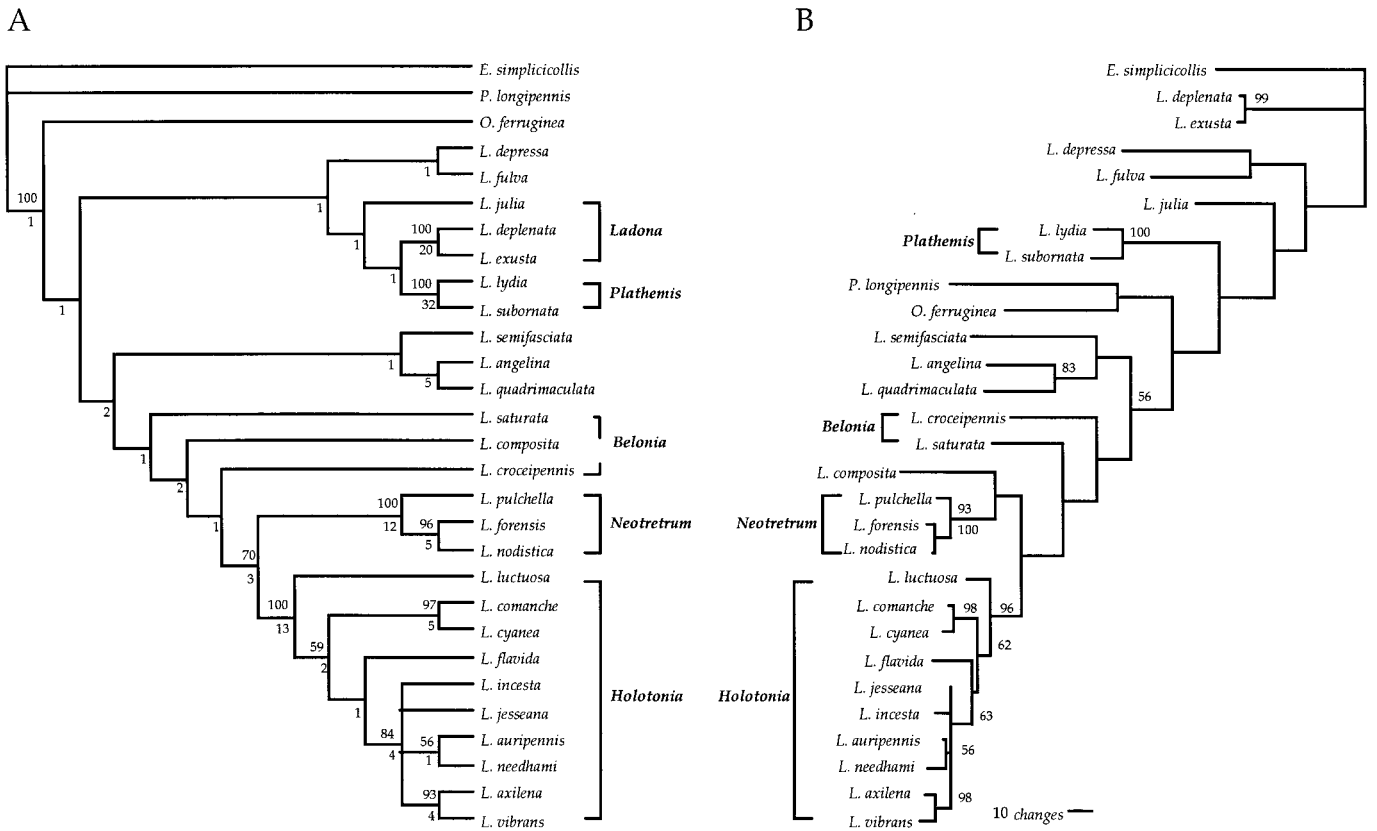


FIG. 1. (A) Strict consensus tree of the six MPTs based on COI data only. Bootstrap values (based on 1000 replicates) $>50\%$ are shown above branches, and decay index values (Bremner support) are shown below branches. (B) Phylogeny inferred from a maximum-likelihood analysis (GTR + I + G) based on COI data only. Bootstrap values (based on 100 replicates) $>50\%$ are shown above branches. Putative subgenera assigned by Kennedy (1922a,b) are indicated by brackets.

nis + *L. needhami*, and *L. axilena* + *L. vibrans*. Analyses using successive approximations weighting identified three additional trees (SWTs) with an equally weighted length 1 step longer (965) than the MPTs and with C.I. and R.I. identical to the equally weighted result.

A strict consensus tree of the six MPTs suggests the monophyly of *Libellula s.s.* (Fig. 1A); however, *Plathemis*, *Ladona*, and *Libellula fulva* and *depressa* do not form separate monophyletic groups, but together form a single basal clade that is the sister clade to *Libellula s.s.* This result is not strongly supported by bootstrap ($<50\%$) or decay analyses, indicating that the COI data have little to say about these basal relationships.

When the six MPTs and three SWTs were evaluated under four models of evolution (all nested variants of the general time reversible model), the most parameter-rich model, GTR + I + G, was found to be significantly better fitting than the next best model (GTR + G) ($P < 0.001$) and one of the three SWTs was found to be the most likely of the nine trees.

Using the most likely SWT as the starting tree for branch-swapping, ML analysis ultimately identified a single most likely tree, differing in topology from the

parsimony trees, with a log likelihood of -5140.21048 (Fig. 1B). The ML analysis does not support the monophyly of *Libellula s.l.* (Fig. 1B). Excluding *L. fulva* and *depressa*, all 19 species of *Libellula s.s.* formed a clade. Interestingly, whereas in the most-parsimonious and in the successive approximations-weighted trees *Libellula* is monophyletic, in the most likely tree it is paraphyletic because, when the tree is rooted using *E. simplicicollis* as the outgroup, a clade consisting of 2 of the outgroup species (*P. longipennis* + *O. ferruginea*) arises at least four nodes within *Libellula*. However, ML bootstrap support for all relationships in this region of the tree are below 50%, and any conclusions based on the COI data for basal relationships within *Libellula* are thus unreliable by this criterion. The basal taxa comprising *Ladona* spp. and *Libellula fulva* and *depressa* do not form a monophyletic group.

Phylogenetic Analysis of 16S

Parsimony analysis of the 16S data set identified 98 most-parsimonious trees (Fig. 2A) of length 207 steps, C.I. = 0.589, R.I. = 0.747. Analysis using successive approximations weighting identified 36 additional trees (SWTs), all with equally weighted lengths 2 steps

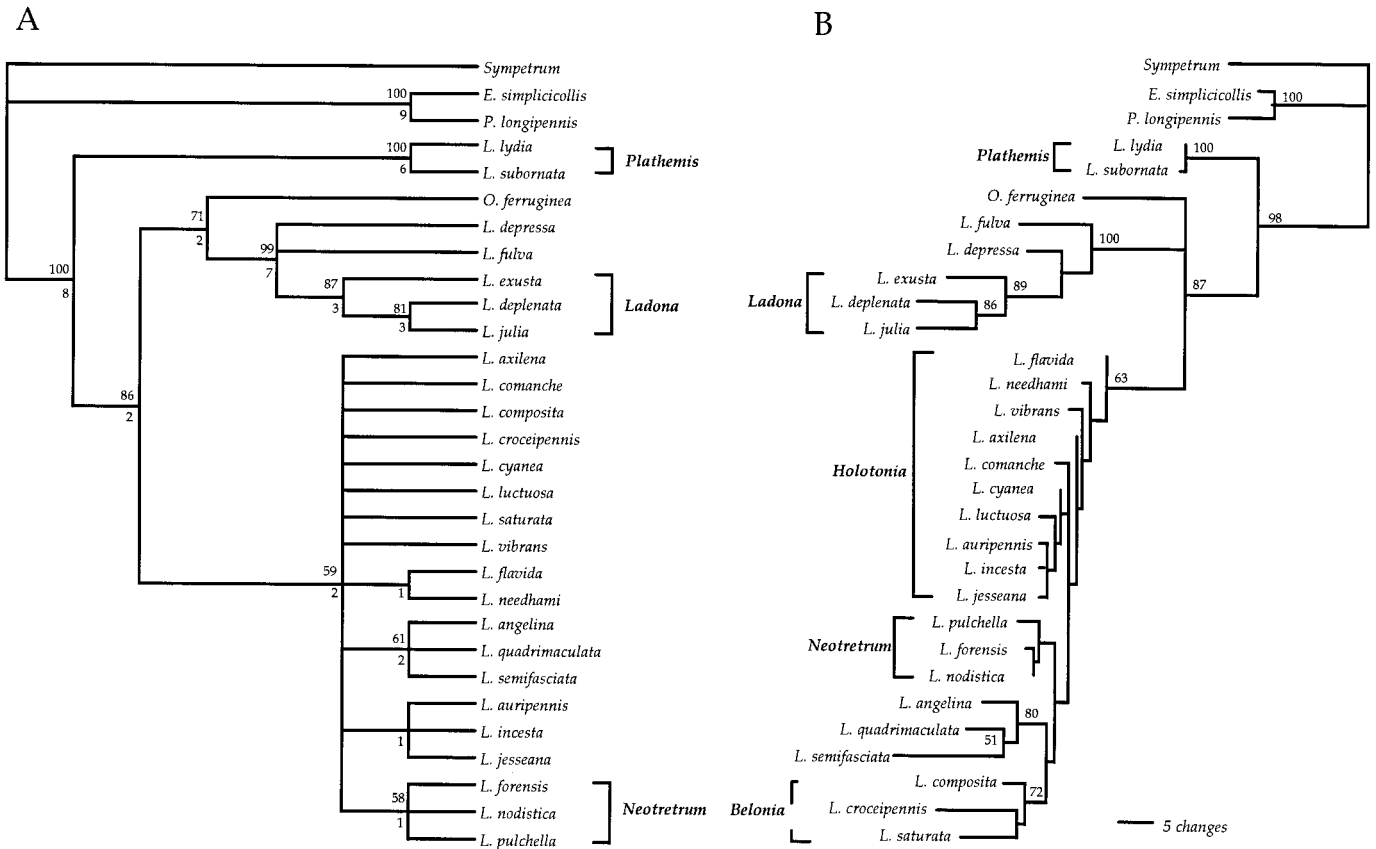


FIG. 2. (A) Strict consensus tree of the 98 MPTs based on 16S data only. Bootstrap values (based on 1000 replicates) $>50\%$ are shown above branches, and decay index values (Bremer support) are shown below branches. (B) Strict consensus tree of two trees inferred from a maximum-likelihood analysis (GTR + I + G) based on 16S data only. Bootstrap values (based on 100 replicates) $>50\%$ are shown above branches. Putative subgenera assigned by Kennedy (1922a,b) are indicated by brackets.

longer (length = 209) than the MPTs, and all with equally weighted C.I. = 0.584 and R.I. = 0.741. A strict consensus of the MPTs does not support the monophyly of *Libellula s.l.* (Fig. 2A). In the strict consensus tree, 19 *Libellula s.s.* species form a clade that excludes only *Libellula fulva* and *depressa*, the sister group of which includes all 3 *Ladona* species plus *Libellula fulva* and *depressa*. However, one of the outgroups, *O. ferruginea*, is the sister species to this clade. A basal clade consists of the 2 *Plathemis* species.

A subset of the parsimony trees, consisting of 6 of the MPTs and 6 of the SWTs, was chosen and evaluated under 40 models of evolution (all variants of the 10 basic substitution models as discussed above). Under the assumption that adjacent trees in the MPT treefile generated by the parsimony analysis are topologically more similar than those that are widely separated, the 6 trees from each file were chosen to maximize the distance between trees and thus, hopefully, to produce the widest variety of topologies. For the 12 trees evaluated, the most parameter-rich model (GTR + I + G) is clearly not significantly better fitting than the next best model (GTR + G) ($P > 0.995$). However, it is more

difficult to judge the significance of the difference in fit to the data of the GTR + G model versus the next-best-fitting model (K3Puf + G), a comparison of which produces a likelihood-ratio significance range of $0.069888 > P > 0.027206$ across the 12 trees evaluated. For the most likely tree under either model, MPT20, the significance level of the increase in goodness-of-fit of GTR + G versus K3Puf + G has $P = 0.034520$. Although this value would seem to recommend the use of the more complex model at the 0.05 significance level, two ML searches were nonetheless conducted, one under each of the two models, to determine whether the analyses would produce differing results. In both cases, MPT20 was used as the starting tree for branch-swapping.

Under GTR + G, ML analysis ultimately identified two most likely trees, each with a log likelihood of -1844.61544 (Fig. 2B). Under K3Puf + G, ML analysis also identified two trees, each with a log likelihood of -1847.85387 and with topologies identical to those found under GTR + G. Excluding *Libellula fulva* and *depressa*, all 19 *Libellula s.s.* species form a clade in these trees. This clade is related by a trichotomy with

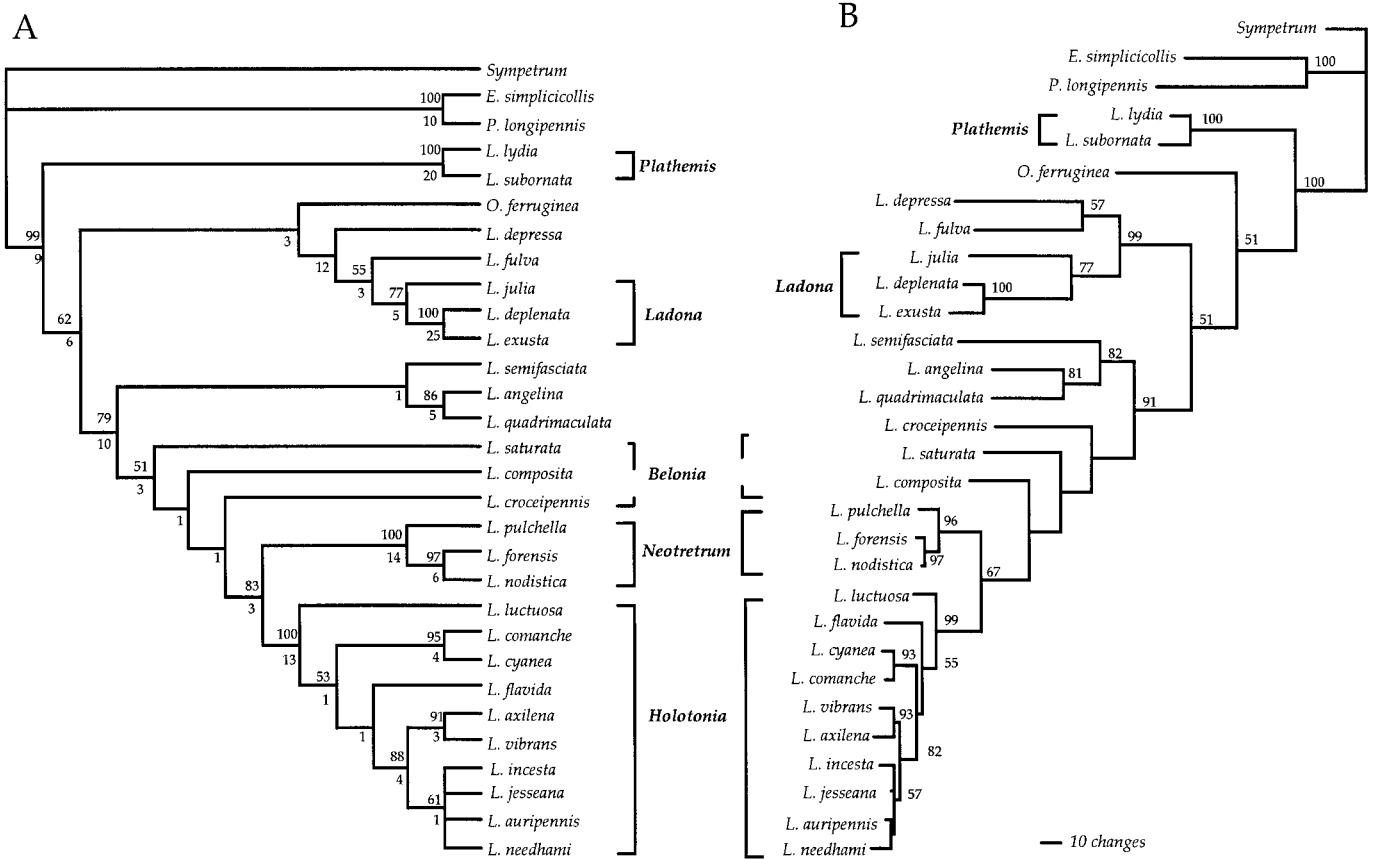


FIG. 3. (A) Strict consensus tree of the six MPTs based on COI and 16S data. Bootstrap values (based on 1000 replicates) >50% are shown above branches, and decay index values (Bremer support) are shown below branches. Putative subgenera assigned by Kennedy (1922a,b) are indicated by brackets. Dashed line around *Belonia* indicates that *Libellula composita* was not assigned to this subgenus by Kennedy. (B) Strict consensus tree of two trees inferred from a maximum-likelihood analysis (GTR + I + G) based on COI and 16S data. Bootstrap values (based on 100 replicates) >50% are shown above branches. Putative subgenera assigned by Kennedy (1922a,b) are indicated by brackets. Dashed line around *Belonia* indicates that *Libellula composita* was not assigned to this subgenus by Kennedy.

O. ferruginea on the one hand and on the other hand with a clade comprised of *Ladona* spp. and *Libellula fulva* and *depressa*. *Plathemis* forms a basal clade. The 16S data are generally uninformative for resolving many of the relationships within *Libellula s.s.* (Fig. 2B).

Phylogenetic Analysis of 16S and COI

The results of an ILD (partition homogeneity) test indicated that the two data sets are not significantly incongruent at the 95% level ($P = 0.07$), and combined data set analyses were conducted. Parsimony analysis of the combined (COI + 16S) data set identified six most-parsimonious trees (MPTs) (Fig. 3A) of length 1187 steps, C.I. = 0.394, R.I. = 0.564. Analysis using successive approximations weighting identified three additional trees (SWTs) with an equally weighted length 1 step longer (1188) than the MPTs, and with C.I. = 0.394, R.I. = 0.563.

Analysis of the combined data set produced results that are most consistent with the existing taxonomy of

Libellula s.l. Parsimony analysis yielded six equally parsimonious trees with a length of 1187 steps. A strict consensus of these trees supports the monophyly of *Libellula s.s.* (Fig. 3A). Within *Libellula s.s.*, groupings are strongly consistent with the subgenera proposed by Kennedy (1922a,b). Excluding *L. composita*, all species listed as belonging to the subgenus *Holotonia* form a monophyletic group, as do the three species belonging to *Neotretum*. The two species belonging to *Belonia* do not form a clade, but are adjacent on the tree. Although Kennedy proposed separate subgeneric status for each of the three species *Libellula angelina*, *quadrimaculata*, and *semifasciata*, our results provide evidence that these three species instead form a single monophyletic group. In the COI + 16S parsimony tree, the sister group to *Libellula s.s.* is a clade consisting of *Ladona*, *Libellula fulva* and *depressa*, and *O. ferruginea*. The genus *Plathemis* is basal, monophyletic, and the sister group to the remainder of *Libellula*. Many of the branches in the parsimony tree are strongly sup-

ported by the bootstrap criterion, including those of the clades defining the genera and subgenera (Fig. 3A).

When the six MPTs and three SWTs were evaluated under 40 models of evolution, the most parameter-rich model, GTR + I + G, was found to be significantly better fitting than the next best model (GTR + G) ($P < 0.001$) and one of the three SWTs was found to be the most likely of the nine trees.

Using the most likely SWT as a starting tree, ML analysis ultimately identified a single most likely tree (differing in topology from the parsimony trees) with a log likelihood of -7198.05338 (Fig. 3B).

The results of the maximum-likelihood analysis of the combined data set are very similar to those of the parsimony analysis (Fig. 3B), with all genera and subgenera similar to those described for the parsimony analysis. In the ML analysis, however, there is weak support for *O. ferruginea* as a sister species to all of *Libellula* except *Plathemis*, rather than as part of *Ladona s.l.* Again, bootstrap analyses under ML provide strong support for the generic and subgeneric nodes (Fig. 3B).

The minimum-evolution tree does not differ significantly from the trees obtained from parsimony and ML analyses of the combined data sets (Fig. 3). The positions of *Plathemis* and *Ladona* are the same, and the subgeneric classifications of Kennedy (1922a,b) are similarly supported. The ME tree differs only in the arrangement of some of the taxa in the subgenus *Holotonia*.

The Phylogenetic Position of O. ferruginea

In the MP analyses of both the 16S data set and the combined data set, and in the ML analyses of all three data sets, *Libellula* is found to be paraphyletic with respect to *O. ferruginea* (most analyses) or to (*O. ferruginea* + *P. longipennis*) (COI ML analysis only). Only in the parsimony analysis of the COI data set is *Libellula* found to be monophyletic. Under both MP and ML, in the 16S and combined COI + 16S data analyses *O. ferruginea* is nested within *Libellula*. In the ML analysis of the COI data, under the assumption that *E. simplicicollis* is the outgroup, *P. longipennis* and *O. ferruginea* form a clade that is nested well within *Libellula*. Based on both MP and ML bootstrap values, this placement of *O. ferruginea* within *Libellula* appears to be driven mainly by characters in the 16S data set (Fig. 2). For the 16S data set analyzed under MP, this placement is supported by two branches having bootstrap values of 86 and 71%; under ML it is supported by a single branch with a bootstrap value of 87%. This bias of the 16S data is somewhat eroded in the tree resulting from an analysis of the combined data set; under MP the single branch supporting an internal position for *O. ferruginea* has a bootstrap value of 62% and under ML the corresponding branch has a value of 51%. Bootstrap support of

greater than 50% for a paraphyletic *Libellula* is entirely lacking in the COI data.

To determine whether any of the data sets provide significant support for distinguishing between the monophyly vs paraphyly of *Libellula*, a series of "constraint" analyses was carried out to determine whether the most-parsimonious and most likely phylogenetic trees obtained for the three data sets differed significantly from the most-parsimonious/most likely trees obtained when *Libellula* is constrained to be monophyletic (or, in the case of the COI MP analysis, nonmonophyletic).

COI. Parsimony analysis of the COI data set under the constraint of *Libellula* nonmonophyly identified 15 MPTs of length 965 steps (only 1 step longer than the most-parsimonious trees in which *Libellula* is consistently monophyletic) with C.I. = 0.359 and R.I. = 0.529. These trees fall into two categories: those in which *Libellula* is paraphyletic with respect only to *O. ferruginea* (8 trees) and those in which it is paraphyletic with respect to (*O. ferruginea* + *P. longipennis*) (7 trees). Not surprisingly, when these 15 trees were compared with the 6 MPTs obtained from the unconstrained parsimony analysis, the differences were found to be nonsignificant in both tests: K-H: $0.9096 \leq P \leq 0.9272$; WSR: $0.8467 \leq P \leq 0.9514$. ML analysis of the COI data set under the constraint of *Libellula* monophyly identified a single tree with log likelihood = -5145.42282 . The difference between this value and that of the tree identified by the unconstrained search (log likelihood = -5140.20866), in which *Libellula* was found to be nonmonophyletic, was found to be nonsignificant by the K-H test ($P = 0.5459$). Thus, we conclude that the COI data set is unable to distinguish between the monophyly vs nonmonophyly of *Libellula*.

16S. Parsimony analysis of the 16S data set under the constraint of *Libellula* monophyly identified 96 MPTs with length 210 steps (3 steps longer than the unconstrained trees), C.I. = 0.581, and R.I. = 0.738. In all possible pairwise comparisons of these 96 constraint MPTs with the 98 MPTs identified in the unconstrained analyses, all three tests found the differences to be nonsignificant: K-H: $0.3173 \leq P \leq 0.5518$; WSR: $0.3173 \leq P \leq 0.5485$. ML analysis of the 16S data set under the constraint of *Libellula* monophyly identified a single most likely tree with log likelihood of -1851.25882 ; the difference between this value and those of the two equally likely trees identified by the unconstrained ML analysis (-1844.61544), in which *Libellula* was found to be paraphyletic, was found to be nonsignificant by the K-H test ($P = 0.2788$). Thus, we conclude that, like the COI data set, the 16S data set provides nonsignificant support for favoring the monophyly vs nonmonophyly of *Libellula*.

TABLE 4

Bootstrap Support Values for the Subgenera of Kennedy (1922a,b) Resulting from Separate Analyses of the COI, 16S, and Combined Data Sets

Clade	COI		16S		Combined	
	MP	ML	MP	ML	MP	ML
<i>Ladona</i> (excl. <i>La. fulva</i> + <i>La. depressa</i>)	20*	0*	87	89	80	77
<i>Plathemis</i>	100	100	100	100	100	100
<i>Neotetrum</i>	100	93	58	70	100	96
<i>Belonia</i>	0*	8*	35*	36*	0*	20*
<i>Holotania</i>	100	96	5*	0*	100	99
Average	64.0	59.4	57.0	59.0	76.0	78.4

Note. An asterisk (*) indicates that a node did not appear in the optimal tree for that analysis.

COI + 16S. Parsimony analysis of the combined COI + 16S data set under the constraint of *Libellula* monophyly identified 18 MPTs with length 1193 steps (6 steps longer than the unconstrained trees), C.I. = 0.392, and R.I. = 0.560. In all possible pairwise comparisons of these 18 MPTs with the 6 MPTs identified in the unconstrained analyses, both tests found the differences to be nonsignificant: K-H: $0.3772 \leq P \leq 0.4063$; WSR: $0.3763 \leq P \leq 0.4284$. ML analysis of the combined COI + 16S data set under the constraint of *Libellula* monophyly identified a single most likely tree with log likelihood of -7198.77888 ; not surprisingly, the difference between this value and that of the tree identified by the unconstrained ML analysis (-7198.05338), in which *Libellula* was found to be paraphyletic, was found to be nonsignificant by the K-H test ($P = 0.8366$). Again, we conclude that the combined COI + 16S data set cannot distinguish between the monophyly vs nonmonophyly of *Libellula*.

DISCUSSION

Molecular Systematics

There is considerable disagreement over whether data sets should be combined or considered separately in phylogenetic analyses (Hillis, 1987; Kluge, 1989; Chippindale and Weins, 1994; Olmstead and Sweere, 1994; Miyamoto and Fitch, 1995). Hillis (1987) suggests that the best phylogenetic hypotheses are obtained by including all relevant data and that congruence among separate data sets enhances confidence in the overall phylogenetic hypothesis. A combined-evidence approach has heuristic appeal, as it increases the number of parsimony-informative characters and maximizes the explanatory power of all the data (Kluge, 1989). Several studies have concluded that the most reliable phylogenetic hypotheses are obtained from combined-data set analyses (Dietrich *et al.*, 1997; Volger and Welsh, 1997; Chippindale *et al.*, 1999; Flook *et al.*, 1999). The results of this study indicate that,

first, the two data sets are not significantly incongruent at the 95% confidence level and, second, that the combined data set analyses (Fig. 3) provide greater resolution than do separate data set analyses (Fig. 1). In general, trees obtained from combined-data set analyses have fewer unresolved polytomies and higher bootstrap and decay index values than do trees obtained from the single-gene analyses (see Table 4). Where differences in topology between separate and combined analyses exist, there is generally weak bootstrap support favoring relationships in a single-gene analysis versus a better-supported alternative arrangement in the combined analyses. Therefore, except where noted, we limit our discussion of phylogenetic relationships to the results from combined-data set analyses.

Monophyly of Libellula

Several analyses failed to support the monophyly of *Libellula s.l.* because one or two outgroup species were found to be positioned within *Libellula s.l.* (Figs. 1–3). The significance of these results was tested using constraint analyses. Specifically, the most-parsimonious and most likely trees supporting the paraphyly of *Libellula* were compared with the most-parsimonious and most likely trees, respectively, supporting *Libellula* monophyly. In none of the comparisons was the difference between trees found to be statistically significant.

Although morphologically and behaviorally similar to *Libellula*, the genus *Orthemis* differs from *Libellula* in characters of wing venation arrangement (Kirby, 1887; Borror, 1945; Needham and Westfall, 1955; Dunkle, 1989). Given the indecisiveness of the molecular data about an ingroup vs an outgroup position for *O. ferruginea* with respect to *Libellula*, we suggest that the weight of the evidence (supplied by the morphological data) favors the current null hypothesis of *Libellula* monophyly and a corresponding outgroup position for *O. ferruginea* (e.g., Fig. 1A).

It is possible, however, that wing venation in Anisoptera may not be an infallible character for delineating natural groups. Although it has long been used by odonate taxonomists, it is conceivable that wing venation may be subject to variation, parallelism, or reversal, just like any other morphological character. In Hawaiian *Megalagrion* damselflies, wing venation is generally unreliable, since each wing on an individual specimen may exhibit a different pattern of venation (Perkins, 1913; Zimmerman, 1948). Thus, because the molecular data presented here weakly suggest that *Orthemis* may occupy a basal position within *Libellula*, we suggest that generic definitions across the Libellulidae should be the subject of an ongoing reevaluation based on a broader selection of both molecular genes and morphological characters. Such a study should employ robust phylogenetic methodologies in an effort to identify potentially homoplastic characters.

The distributions of branch support values on the trees in Figs. 1 and 2 strongly indicate that the COI data set is most informative about more recent relationships in *Libellula*, i.e., branches near the tips have the highest support values (Fig. 1), whereas the opposite is true for the 16S data set, which strongly supports basal relationships and which provides virtually no information about recent relationships within the genus (Fig. 2). Coupled with the nonsignificant results of the ILD test for incongruence (discussed above), this complementarity of the two data sets strongly recommends a combined-data analysis. Indeed, in the trees produced by the combined parsimony analyses, branch support values are generally elevated (Fig. 3A) and basal relationships are entirely congruent with those found in the 16S-only tree (Fig. 2A). Likewise, the relationships in the more recent branches of the combined-data tree (Fig. 3A) are entirely congruent with those found in the COI-only tree (Fig. 1A). The same general pattern is found in the maximum-likelihood trees (Figs. 1B, 2B, and 3B). However, branch support values for a few nodes are decreased in the combined analysis, most notably the two basal branches that are critical for understanding the position of *O. fer-runginea* and the associated question of *Libellula* monophyly. In this case it is possible that "noise" in the COI data may be overwhelming the phylogenetic "signal" in the 16S data set. As indicated by the results of the various constraint analyses, however, noise (or, for that matter, conflicting signal) in the COI data set cannot entirely account for the indecisiveness of the combined data regarding *Libellula* monophyly, because even when the 16S data are analyzed alone the problem of monophyly remains unresolved. The question of *Libellula* monophyly will only be resolved by additional data, either from 16S or from another gene more appropriate to the problem.

Status of Libellula, Ladona, and Plathemis

Considerable disagreement exists as to whether *Ladona* Needham, 1897 and *Plathemis* Hagen, 1861 should be accorded full generic status, be considered subgenera of *Libellula*, or be synonymized under *Libellula*. Needham (1897) assigned these taxa full generic status, and this interpretation has been widely adopted since (Needham and Westfall, 1955; Carle, 1978; May, 1992; Schmidt, 1987; Westfall and Tennenssen, 1996; Steinmann, 1997). Several other authors, however, have suggested that these two groups be assigned subgeneric status (Kennedy 1922a,b; Borror, 1945; Bennefield, 1965; Walker and Corbet, 1975; Allen *et al.*, 1985; Tsuda, 1986). Still other authors have concluded that *Ladona* and *Plathemis* do not deserve even subgeneric status and have synonymized them under *Libellula s.l.* (Ris, 1910; Garman, 1927; Byers, 1930). The situation is further complicated by the uncertain assignment of two European *Libellula* species, *L. depressa* and *L. fulva*, that have been variously assigned to *Plathemis* and *Ladona* (Hagen, 1861; Needham, 1897; Needham and Westfall, 1955; Schmidt, 1987), included within *Libellula s.s.* (Kirby, 1887; Tsuda, 1986), or even given monotypic subgeneric rank (Kennedy, 1922a,b).

The results of our study indicate that *Plathemis* and *Ladona* are indeed distinct monophyletic lineages within *Libellula s.l.* The combined-evidence analysis in particular suggests that *Plathemis* forms the basal sister group to the remainder of *Libellula s.l.*, and that *Ladona* (defined to include *L. depressa* and *L. fulva*) is the next most basal clade within the *Libellula* lineage. The combined-evidence analyses utilizing parsimony, maximum-likelihood, and minimum-evolution criteria all support the monophyly of *Plathemis* and *Ladona*, with bootstrap values >90% and Bremer support values >5 steps. These results support the original classification proposed by Needham, with the exception that *L. depressa* and *L. fulva* must be included within *Ladona s.l.* were it accorded generic or subgeneric status. Based on our results, we propose that separate generic or subgeneric status be adopted for *Plathemis* and *Ladona* within Libellulidae. This conclusion is supported by a previous molecular phylogenetic study on these groups (Kambhampati and Charlton, 1999) based on the 16S gene alone and on fewer taxa (see below).

Phylogenetic Relationships within Libellula

Phylogenetic relationships within *Libellula* are also controversial. There is disagreement over whether some species are truly distinct or are merely races or subspecies. For example, *L. julia* and *L. deplanata* have been synonymized with *L. exusta* (Muttkowski, 1910; Byers, 1930; Dunkle, 1989), whereas other authors have treated them as distinct species (Bennefield, 1965; May, 1992). Several authors have assigned

specific rank to *L. jesseana* (Kennedy, 1922a,b; Williamson, 1922; Dunkle, 1989), whereas others (Westfall, 1943; Needham and Westfall, 1955) synonymized it with *L. auripennis*. Using several morphological characters, Dunkle (1992) included three species in the *L. vibrans* group (*L. vibrans*, *axilena*, and *incesta*), whereas Kennedy (1922a,b) included two other species (*L. comanche* and *cyanea*) in this complex based on the morphology of the genitalia. The earliest exhaustive effort to resolve the taxonomy of the genus (Kennedy, 1922a, b) was based on the morphology of the genitalia, recognizing 10 subgenera within *Libellula*. In addition to the generic classifications listed above, Kennedy erected 3 subgenera that included only one species (*Eolibellula semifasciata*, *Syntetrum angelina*, and *Libellula quadrimaculata*) and one subgenus with three species (*Neotetrum forensis*, *pulchella*, and *nodistica*). Kennedy also recognized Kirby's (1887) major subdivision of *Libellula* into 2 subgenera, *Belonia*, which included *foliata*, *saturata*, *croceipennis*, and *herculea*, and *Holotania*, which included *axilena*, *composita*, *jesseana*, *flavida*, *auripennis*, *luctuosa*, *cyanea*, *comanche*, *incesta*, and *vibrans*. Calvert (1907) suggested that *L. comanche*, *flavida*, and *cyanea* are closely related based on wing venation.

The results of this study strongly support many of the taxonomic groups erected by Kennedy (1922a,b). In particular, the subgenera *Holotania* and *Neotetrum* were found to be monophyletic lineages within *Libellula* s.s. The notable exception is *Libellula composita*, which was assigned to *Holotania*, but found to occupy a position as part of a (sometimes paraphyletic) *Belonia*, within *Neotetrum*. Although not always forming a clade, the two species from the subgenus *Belonia* (*L. saturata* and *croceipennis*) surveyed in this study were found to be topologically adjacent on most trees. Moreover, we found evidence that *Libellula quadrimaculata*, *angelina*, and *semifasciata* form a monophyletic group and should not retain separate subgeneric status (Kennedy, 1922a,b). This study was unable to resolve some taxonomic disputes, such as the number of species in the *Libellula vibrans* group or the taxonomic status of *Libellula jesseana*. Poor resolution and low bootstrap support for several nodes within *Libellula* s.s. indicate that species in the subgenus *Holotania* are recently derived. This subgenus appears to be undergoing adaptive radiation and the genetic distances for many of these species are low.

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