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A PHYLOGENY OF *CELITHEMIS* INFERRED FROM MITOCHONDRIAL AND NUCLEAR DNA SEQUENCE DATA AND MORPHOLOGY (ANISOPTERA: LIBELLULIDAE)

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The dragonfly genus *Celithemis* consists of 8 spp., some of them common and brightly colored, that are confined largely to eastern North America. Several spp. have been used in behavioral, ecological, and morphological studies, but their intrageneric phylogeny is unclear. In this paper is provided a phylogeny based on morphology and on data from mitochondrial and nuclear DNA sequences of multiple individuals of each species. The genus appears to be monophyletic, with one nested species pair (*C. amanda* + *C. martha*) receiving strong bootstrap support by both parsimony or maximum-likelihood criteria as well as high Bayesian posterior probability. A second group (*C. bertha, C. elisa, C. ornata* and *C. fasciata*) is well-supported in Bayesian analysis but only weakly by parsimony and maximum-likelihood bootstrap values. *C. verna* and *C. eponina* are probably basal to both these groups, but their relationship to each other is unclear. All individuals assigned to a species recognized on morphological grounds were recovered as monophyletic. The problematic taxa, *C. monomalaena* and *C. bertha leonora*, are shown definitively to be synonyms of *C. fasciata* and *C. bertha*, respectively.

INTRODUCTION

Aside from the inherent interest of their evolutionary history, Odonata have been important in research on freshwater systems as well as behavioral ecology, sexual selection, and insect physiology and functional morphology. The genus *Celithemis*, in particular, has been used in studies of functional morphology of

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the penis (MILLER, 1981), reproductive behavior (MILLER, 1982), competition (COWLEY & JOHNSON, 1992), energy balance (BENKE & BENKE, 1975, BENKE, 1976), and predation pressure (GRESENS et al., 1982). Understanding relationships among taxa is essential for comparative assessment of adaptive similarities and differences in behavioral ecology. Thus establishment of a reliable phylogeny is a prerequisite for any deep understanding of selection and character evolution in *Celithemis*, as in any group of organisms.

HAGEN (1861) established the genus *Celithemis* to include *Libellula eponina* Drury, *Celithemis superbum* sp. n. and the fossil species, *C. cellulosa* sp. n. *Celithemis superbum* was moved to the monotypic genus, *Pseudoleon* Kirby, 1881, and *C. cellulosa* is clearly distant from our current concept of *Celithemis* (see GARRISON et al., 2006, for a full diagnosis) by virtue of having the midrib of the anal loop strongly bent, and having many more cells in the forewing triangle and supratriangle and many more antenodal crossveins than in extant species. Thus these two species are not considered further.

Celithemis has not been revised since WILLIAMSON (1922), and no modern analysis of the species boundaries or relationships has been attempted. The named taxa, *C. monomalaena* Williamson, 1910, and *C. bertha leonora* Westfall, 1952, have recently been considered synonyms of *C. fasciata* Kirby, 1889, and *C. b. bertha* Williamson, 1922, respectively, but unequivocal data supporting synonymy have not been published. KORNELL (2003) investigated phylogeny and species distinctions using molecular and morphological data, but several gaps and inconsistencies in her data have encouraged this re-examination.

Our principal objective here is to derive a well supported phylogeny resolving the relationships among species of *Celithemis*. Since morphological features lead to little resolution, we combine this with sequence data from the mitochondrial protein coding gene cytochrome oxidase I (COI) and from the D2 region of the nuclear 28S rDNA gene. We also provide data supporting the synonymy of *C. monomalaena* and *C. bertha leonora*.

MATERIAL AND METHODS

MOLECULAR TECHNIQUES – Twenty-eight specimens for molecular analysis, including representatives of all putative species and subspecies of *Celithemis* and three outgroup species, *Cordulia shurtleffi*, *Libellula pulchella*, and *Leucorrhinia glacialis*, were field collected or taken from the collections of MLM and Rutgers University. Tissue was obtained by excising thoracic muscle or removing a leg, which was then placed in an Eppendorf tube. Extraction procedures followed manufacturer's recommendations (QIAGEN, DNeasy Tissue handbook).

After the DNA was extracted, polymerase chain reaction (PCR) was used for amplification of a COI fragment from the mtDNA and the D2 region of the large subunit (28S) nuclear ribosomal coding region. We used the C1731F and CI–14R primers of Artiss *et al.*(2001) for COI amplification and the D2ODUP and D2DnB primers (5'TGCTTGAGAGTGCAGCCCAA3' and 5'CCTTGGTCCGTGTTTCAAGAC3'). We amplified the DNA with 1 µl each of up and down primer, 1 to 5 µl of template DNA, 12.5 µl Master Mix solution, and 5.5 to 9.5 µl ddH,O.

Amplified DNA was separated on a 1.5% low melting agarose gel (FMC Bioproducts), then purified using QIAquick PCR Purification Kit Protocol (QIAGEN) according to the manufacturers instructions. The purified product was sequenced in both directions using BigDye Terminators Cycle Sequencing Kit (Applied Biosystems) and an ABI 3100 capillary sequencer. COI sequences were aligned using Clustal (THOMPSON, 1997) then, to assure homology, translated to the corresponding protein sequence in MacClade (MADDISON & MADDISON, 1992). D2 sequence alignments were made using Clustal, and the resulting files were then aligned manually in Microsoft Word using the structural methods described in KJER et al. (1994), KJER (1995), KJER et al., (2007) and secondary structure models based on GUTELL et al. (1993).

D2 sequences only were provided for *Leucorrhinia orientalis* (specimen obtained from Dr Elena Malikova) and a *Leucorrhinia* sp sequence obtained from GenBank (Acc. # AY859583)). COI sequences were scored as missing data for these two species.

Thirty-six morphological characters, including 3 larval characters, were scored for all putative taxa of *Celithemis* and for the 3 outgroup species. All adult characters were observed directly, mostly using a Wild™ stereomicroscope. Characters of the secondary genitalia were examined with an Hitachi S510 scanning electron microscope after sputter coating with gold-palladium. Data on larval characters were taken from NEEDHAM et al. (2000).

PHYLOGENETIC ANALYSIS – Each data partition was analyzed individually and in combination. The partitioned analyses were not seriously incongruent, although they differed in their level of resolution. Morphology and D2 gave poor resolution, while the COI tree was well resolved. The analyses of the combined data are presented hereafter. A maximum parsimony heuristic search of 1,000 random replicates was conducted using PAUP– 4.0b10 (SWOFFORD, 1999) with TBR branch swapping. To assess branch support, 400 bootstrap pseudoreplicates (FELSENSTEIN, 1985) were performed using 10 random addition searches per pseudoreplicate. Modeltest 3.06 (POSADA & CRANDALL, 1998) was used to obtain an evolutionary model for each molecular data set in the Bayesian and maximum likelihood analyses; in each case the GTR+I+ Γ was estimated to be the best model for molecular data, with mk model used for morphology. Using the program MrBayes (HUELSENBECK, 2000), Bayesian analysis (4 MCMC chains: 1 cold, 3 hot) was run on the combined data for 2,873,500 generations; the first 100,000 were discarded as burn-in.

MORPHOMETRIC ANALYSIS – WILLIAMSON (1910) distinguished *C. monomalaena* from *C. fasciata* based on size, wing coloration, and several venational characters. Numerous authors (e.g. NEEDHAM et al., 2000) have doubted the validity of *C. monomalaena*, and CARLE (1982) indicated that the supposed differences were either clinal or showed discordant variation. Nonetheless, no quantitative evaluation of the supposed distinctions has been published. We measured to the nearest 0.5 mm or counted the relevant characters (see Results) in 59-66 specimens from the National Museum of Natural History (NMNH), the Florida State Collection of Arthropods (FSCA), and the collection of MLM, using a Wild stereomicroscope when necessary. Data were plotted as a function of latitude and evaluated by linear regression to assess geographic variation of each character.

RESULTS

Figure 1 presents a strict consensus of the four most parsimonious trees (length 1065). *Celithemis* is recovered as monophyletic with good support. All named taxa are recovered except that the two *C. amanda* are in a polytomy with *C. martha*. Note also that *C. fasciata* and *C. monomelaena* are mutually polyphyletic and *C. b. bertha* is paraphyletic relative to *C. bertha leonora*. *C. verna* and *C. eponina* form a polytomy with an apparent clade that includes all other species (but this clade is supported with very low bootstrap support). The strongly supported *C.*

amanda and *C. martha* clade is apparently basal in the pectinate arrangement of the remaining species.

Figure 2 is the majority rule consensus tree obtained by Bayesian analysis. *Celithemis verna* is sister to the remaining species, followed successively by *C. eponina* and the *C. amanda* + *C. martha* clade and finally a polytomy comprising *C. bertha*, *C. ornata* + *C. elisa*, and *C. fasciata*. Posterior probabilities provide strong support for the monophyly of *Celithemis* and for that of most of the species ex-

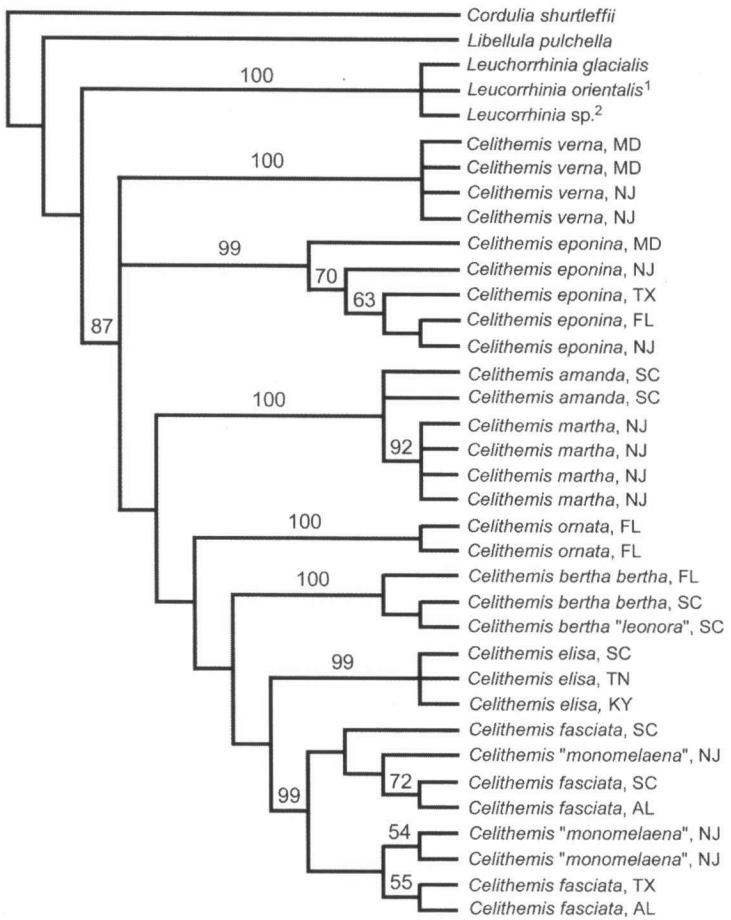

Fig. 1. Strict consensus of four most parsimonious trees of length 1065 based on combined data from COI, D2 and morphology. Numbers above the branches indicate bootstrap values > 50%. The names in quotations, "leonora" and "monomalaena", are regarded as synonyms of *C. bertha* and *C. fasciata*, respectively, but are retained in the tree to indicate specimens that exhibit characteristics of those supposed taxa. Superscripts indicate specimens for which sequence data were obtained from E. Malikova (1) or from GenBank (2).

cept *C. amanda*, which is, however, recovered as monophyletic. *C. fasciata* and *C. monomelaena* are again mutually polyphyletic, and *C. b. leonora* forms a clade with *C. b. bertha*. Species groupings are at least moderately well supported except for the terminal polytomy and within the latter, the possible clade including *C. ornata* and *C. elisa*. It is worth noting that Bayesian analysis of the COI data alone (which was the most informative data partition used) recovered *C. verna*, *C. eponina*, and *C. amanda* + *C. martha* in a basal polytomy but gave fairly strong support (93% posterior probability) to the *C. ornata* + *C. elisa* clade.

Morphometric analyses of specimens of the *C. fasciata*/*C. monomalaena* clade show gradual latitudinal clines in hindwing length, number of cells between veins A_2 and A_3 in the hindwing, number of hindwing marginal cells basal to the anal

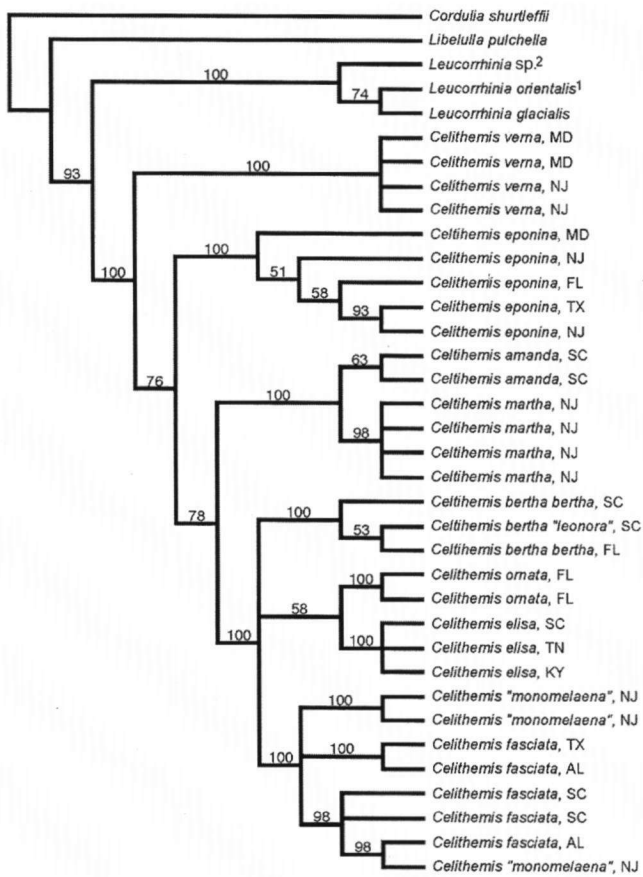

Fig. 2. Majority rule consensus of 11,096 trees obtained for combined COI, D2, and morphology data from Bayesian analysis. Posterior probabilities > 50% are shown above the corresponding branches. See Fig. 1 for explanation of quotation marks and superscripts.

loop (Fig. 3A), and extent of the forewing nodal spot; hindwing length also tends to increase clinally from east to west ($p = 0.09$, not shown). Characters claimed by WILLIAMSON (1910) to distinguish the taxa do not vary concordantly from north to south (Fig. 3B), although specimens from north of 38 N do tend on average to have fewer forewing triangle and posttrigonal cells and fewer basal paranal cells (see NEEDHAM et al., 2000). The proportion of specimens with yellow at the base of the hindwing, supposedly characteristic of nominate *C. fasciata*, was not quantified because of variation in its intensity and extent, but, although this color pattern was clearly more frequent in southern specimens, individuals with completely clear hindwings occurred as far south as 32.3° N (GA, Crawford Co.) and with distinct yellow basal areas as far a 42.2° N (NY, Chataqua Co.).

DISCUSSION

All 8 generally acknowledged species appear to be valid, monophyletic taxa, with the possible exception of *C. martha*, which could be a junior synonym of *C. amanda*; this pair is quite similar in general appearance and morphology except for the strong darkening of mature males of *C. martha*. We do not advocate a taxonomic change, however, pending a more thorough study of these two nominal taxa.

We suggest that the putative taxa *C. monomalaena* and *C. bertha leonora* be relegated to synonymy with *C. fasciata* and *C. bertha*, respectively. In the first case, individuals identified as *C. monomalaena* on morphological grounds are mingled haphazardly in a clade with typical *C. fasciata* (Figs 1-2). Furthermore, Figure 3 provides no morphological basis for distinguishing these taxa even as subspecies. In the case of *C. b. leonora* and *C. b. bertha*, recognition of "leonora" leaves the nominotypical form paraphyletic (Fig. 1). As already described by WESTFALL (1952), the subterminal dark wing spot distinguishing "leonora" varies from about 0.5 cm in diameter to vanishingly small, so that the supposed subspecies is doubtfully diagnosable. Moreover, the "leonora" form is not geographically distinct by virtue of being restricted to the Florida panhandle but also occurs at least in South Carolina, from which our sequenced specimen originated. Thus it is geographically broadly coextensive with *C. b. bertha* and hence does not fit the usual definition of a subspecies (e.g. MAYR, 1963).

No formal morphology-based phylogeny has been proposed nor, to our knowledge, has any other discussion of relationships within the genus appeared since WILLIAMSON's (1922) very brief treatment. One might assume a close relationship among *C. elisa, C. eponina*, and *C. fasciata* based on wing coloration, as Williamson did, or among *C. eponina, C. fasciata, C. ornata*, and *C. verna* based on larval features (NEEDHAM et al., 2000), especially the unusual eye morphology of *C. verna, C. fasciata,* and *C. ornata*, but neither of these groupings is supported here. Rather, elaborately patterned wings and conical larval eyes and long

abdominal spines are likely to have evolved independently multiple times. A strict consensus tree (not shown) based on morphology alone and analyzed using the maximum parsimony criterion showed little resolution within *Celithemis* except that it grouped *C. amanda* and *C. martha*, based on thoracic color and the shape of the cornu of the penis, and *C. elisa, C. eponina*, and *C. fasciata* based on wing coloration.

Our hypothesis is not without weakness. The parsimony tree (Fig. 1), although well-resolved, provides minimal support for interspecific relationships. The Baye-

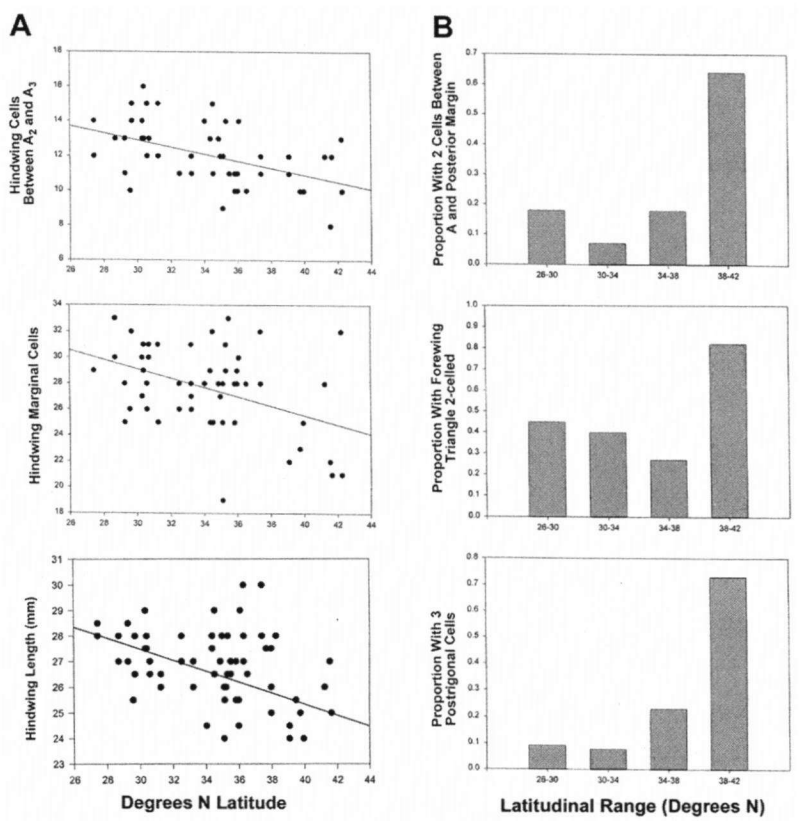

Fig. 3: (A) Relation of cell counts and hindwing length, suggested by WILLIAMSON (1910) to differentiate *C. fasciata* and *C. monomelaena*, as a function of latitude. Lines represent linear regression of the respective measurements on latitude. Each quantity declined significantly ($p < 0.001$) but smoothly with latitude, indicating a N-S cline; measurements of the transverse extent of the forewing nodal spot gave similar results (not shown; $p < 0.01$). – (B) Proportion of cell counts, suggested by Williamson to characterize the supposed northern species, *C. monomelaena*, as a function of latitudinal range. In each case the highest proportion occurs at the highest range but with secondary peaks at the lowest range and with minima discordant, at 30-34°N (marginal cells and postrigonal cells) or 34-38 °N (cells in triangle).

sian tree (Fig. 2), however, gives high posterior probabilities for most branches, and the two trees are concordant in most respects. The relative positions of *C. eponina* and the *C. amanda* + *C. martha* clade, and the relationships among *C. bertha, C. ornata, C. elisa* and *C. fasciata* are uncertain, but in other respects we think the phylogenies have numerous common features that can be accepted with some confidence.

We hope this study will encourage additional comparative investigations of this interesting genus. To mention only two intriguing possibilities: (1) since extensive wing maculation at or beyond the nodus appears to have evolved independently two or three times, studies of the effect of this character on intra- or interspecific signaling could be productive; (2) similarly, extremely conical larval eyes seem to have evolved independently two or three times, so the adaptive advantages and physiological consequences of this unusual morphology are open to investigation. Although the genus is a small one, it may well provide numerous other opportunities for comparisons of behavioral, morphological, and ecological properties.

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REFERENCES

ARTISS, T., T. SCHULTZ, D.A. POLHEMUS & C. SIMON, 2001. Molecular phylogenetic analysis of the odonate genus Libellula (Odonata: Libellulidae) based on mitochondrial cytochrome oxidase I and 16S rRNA sequence data. *Mol. Phylog. Evol.* 18: 348-361.

BENKE, A.C., 1976. Dragonfly production and prey turnover. *Ecology* 57: 915-927.

BENKE, A.C. & S.S. BENKE, 1975. Comparative dynamics and life histories of coexisting dragonfly populations. *Ecology* 56: 302-317.

CARLE, F., 1982. *A contribution to the knowledge of Odonata*, pt 2. PhD Diss., Virginia Polytech. Inst. St. Univ., Blacksburg, VA.

CROWLEY, P.H. & D.M. JOHNSON, 1992. Variability and stability of a dragonfly assemblage. *Oecologia* 90: 260-269.

DRURY, D., 1773. *Illustrations of natural history*, Vol. 2. White, London.

FELSENSTEIN, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

GARRISON, R.W., N. VON ELLENRIEDER & J.A. LOUTON, 2006. *Dragonfly genera of the New World. An illustrated and annotated key to the Anisoptera*. John Hopkins Univ. Press, Baltimore.

GENSENS, S.E., M.L. COTHRA & J.H. THORP, 1982. The influence of temperature on the functional response of the dragonfly Celithemis fasciata (Odonata: Libellulidae). *Oecologia* 53: 281-284.

GUTTELL, R.R., M.W. GRAY & M.N. SCHINARE, 1993. A compilation of large subunit (23S

and 23S-like) ribosomal RNA structures. *Nucleic Acids Res.* 21: 3055-3074.

HAGEN, H.A., 1861. Synopsis of the Neuroptera of North America, with a list of the South American species. *Smithson. misc. Collns* 4: 1-347.

HUELSENBECK, J.P., 2000. *MrBayes: Bayesian inference of phylogeny*. Distributed by the author. Dept Biol., Univ. Rochester.

KJER, K.M., 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions and example of alignment and data presentation from the frogs. *Mol. Phylog. Evol.* 5: 314-330.

KJER, K.M., J.J. GILLESPIE & K.A. OBER, 2007. Opinions on multiple sequence alignment, and an empirical comparison of repeatability and accuracy between POY and structural alignments. *Syst. Biol.* 56: 133-146.

KORNELL, D., 2003. *Phylogeny of Celithemis Hagen (Libellulidea: Odonata): a claudistic analysis*. M.S. Thesis, Rutgers Univ., New Brunswick, NJ.

MADDISON, W.P. & D.R. MADDISON, 1992. *MacClade version 3: Analysis of phylogeny and character evolution*. Sinauer Associates, Sunderland / MA.

MAYR, E., 1963. *Animal species and evolution*. Belknap Press, Cambridge, MA.

MILLER, P.L., 1981. Functional morphology of the penis of Celithemis eponina (Drury) (Odonata: Libellulidae). *Odonatologica* 10: 293-300.

MILLER, P.L., 1982. Observation on the reproductive behaviour of Celithemis eponina (Drury) (Odonata: Libellulidae) in Florida. *Ent. mon. Mag.* 117: 209-212.

NEEDHAM, J.G., M.J. WESTFALL & M.L. MAY, 2000. *Dragonflies of North America* [Revised edn]. Scient. Publishers, Gainesville, FL.

POSADA, D. & K.A. CRANDALL, 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

QIAGEN, 2002. QIAquick PCR Purification Kit Protocol. *QIAquick Spin Handbook*, p. 18.

QIAGEN, 2004. Protocol: purification of total DNA from animal tissues. *DNeasy Tissue Handbook*, pp. 18-20.

SWOFFOLD, D.L., 1999. *PAUP−. Phylogenetic Analysis Using Parsimony (and other methods)*. Version 4. Sinauer Associates, Sunderland, MA.

THOMPSON, J.D., T.J. GIBSON, F. PLEVNIAK, F. JEANMOUGIN & D.G. HIGGINS, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24: 4876-4882.

WESTFALL, M.J., 1952. Celithemis bertha in Florida, with a description of a new subspecies (Odonata). *Fla Ent.* 35: 109-116.

WILLIAMSON, E.B., 1910. A new species of Celithemis (order Odonata). *Ohio Naturalist* 10: 153-160.

WILLIAMSON, E.B., 1922. Notes on Celithemis with description of two new species (Odonata). *Occ. Pap. Mus. Zool., Univ. Mich.* 108: 1-22.