A Phylogeny of the Damselfly Genus *Calopteryx* (Odonata) Using Mitochondrial 16S rDNA Markers

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We seek to reconstruct the phylogenetic relationships of the damselfly genus *Calopteryx,* for which extensive behavioral and morphological knowledge already exists. To date, analyses of the evolutionary pathways of different life history traits have been hampered by the absence of a robust phylogeny based on morphological data. In this study, we concentrate on establishing phylogenetic information from parts of the 16S rDNA gene, which we sequenced for nine *Calopteryx* species and five outgroup species. The mt 16S rDNA data set did not show signs of saturated variation for ingroup taxa, and phylogenetic reconstructions were insensitive to variation of outgroup taxa. Parsimony, neighbor-joining, and maximum-likelihood reconstructions agreed on parts of the tree. A consensus tree summarizes the significant results and indicates problematic nodes. The 16S rDNA sequences support monophyly of the genera *Mnais, Matrona,* and *Calopteryx.* However, the genus *Calopteryx* may not be monophyletic, since *Matrona basilaris* and *Calopteryx atrata* are sister taxa under every parameter setting. The North American and European taxa each appear as monophyletic clades, while the Asian *Calopteryx atrata* and *Calopteryx cornelia* are not monophyletic. Our data implies a different paleobiogeographic history of the Eurasian and North American species, with extant Eurasian species complexes shaped by glacial periods, in contrast to extant North American species groups.   © 2000 Academic Press

INTRODUCTION

It has become part of the biological orthodoxy that comparative analysis of character evolution is incomplete or even misleading without the context provided by a robust phylogeny (Patterson, 1987; Harvey and Pagel, 1991; Harvey and Purvis, 1991; Brooks and McLennan, 1991; Balter, 1997). In this work, we present a phylogenetic hypothesis for the evolutionary relationships of the damselfly genus *Calopteryx.*

Within this genus there is a remarkable variety of

morphological and behavioral traits which have been extensively studied in the context of sexual selection theory. As a consequence, the genus provides a powerful system for exploring and testing different hypotheses about character evolution, i.e., the evolution of genitalia via sexual selection, the significance of different character sets for reproductive isolation, and the adaptive value and the evolutionary pathways of behavioral traits (Gibbons and Pain, 1992; Heymer, 1973; DeMarchi, 1990; Meek and Herman, 1990; Siva-Jothy and Tsubaki, 1989; Siva-Jothy and Hooper, 1995; Thornhill and Alcock, 1983; Waage, 1973, 1979a,b; Zahner, 1960).

Despite this potential, a comparative (evolutionary) analyses of such life history traits has been hampered by the lack of a robust phylogeny. Not only are the phylogenetic relationships within the genus *Calopteryx* and its phylogenetic position within the Calopterygidae still under debate but the monophyly of both clades has also not yet been demonstrated.

Calopteryx damselflies are a holarctic, species-poor assemblage of perhaps 20 species ("perhaps", because the taxonomic status of some members of this group is still controversial). The genus contains 5 North American species, *Calopteryx amata, C. angustipennis, C. dimidiata, C. aequabilis,* and *C. maculata,* 2 exclusively Asian species, *C. cornelia* and *C. atrata* (it is unclear whether the described *C. atrocyana* and *C. grandaeva* represent distinct species of *C. atrata*), and 3 Eurasian species complexes, *C. haemorrhoidalis, C. splendens,* and *C. virgo.* The *C. splendens* group, including the species *C. (splendens) xanthostoma,* and the *C. virgo* group contain numerous morphologically distinct subgroups of unclear taxonomic status (Buchholtz, 1955; Maibach, 1986; DeMarchi, 1990; Anders and Rüppell, 1997). In particular, *C. (splendens) xanthostoma* has been the subject of debate regarding its status as a full-fledged reproductively isolated species. *C. virgo* is the most widely distributed Eurasian *Calopteryx* species, with subspecies *C. virgo japonica* in Japan as the easternmost representative of this taxon (but the status of *C. virgo japonica* as a subspecies or a

full-fledged reproductively isolated species is still under discussion). The Asian species *C. atrata* and the 2 closely related species *C. atrocyana* and *C. grandaeva* are known from Japan and the Asian continent, and *C. cornelia* is endemic in Japan. The North American species are widely distributed over the continent, with *C. dimidiata* as the southernmost species of this clade. South America and Africa both lack damselflies of the genus *Calopteryx.*

Miyakawa's (1982) analysis of behavioral traits suggests certain phylogenetic affinities; in particular, he notes similarities between *C. atrata* and *C. aequabilis,* between *C. virgo japonica* and *C. virgo,* and between *C. cornelia* and *C. maculata.* These evolutionary relationships remain to be tested by molecular methods. If Miyakawa's hypothesis, for example, holds, this would imply either that the genus *Calopteryx* radiated before the Asian and North American continents split (i.e., the current distribution pattern would be the result of vicariance phenomena) or that *Calopteryx* species invaded the North American continent several times, most probably during interglacial periods.

In this study, we explicitly address both the paleogeographic and the life history implications of the evolutionary history of the genus *Calopteryx.*

MATERIAL AND METHODS

Source Materials

In addition to the majority of North American (*C. dimidiata, C. aequabilis,* and *C. maculata*), European (*C. virgo, C. splendens, C. haemorrhoidalis,* and *C. xanthostoma*), and Asian (*C. atrata* and *C. cornelia*) *Calopteryx* species, we also included *Hetaerina americana* (Hetaerininae, Calopterygidae), a very distinct Calopterygid, and *Mnais costalis* and *Matrona basilaris* (both Calopteryginae and presumed close relatives to the *Calopteryx* species) in our analyses. *Neoneura esthera* (Protoneuridae) and *Palaemnema* sp. (Platistictidae) were included as outgroups.

Template Preparation and PCR Procedures

DNA extractions were performed according to protocols of Hadrys *et al.* (1992). For amplification of a 570-bp fragment of the mitochondrial 16S rDNA gene fragment, we used the primers LR-J-12887 (5′ CCG-GTCTGAACTCAGATCACGT 3′) and LR-N-13398 (5′ CGCCTGTTTAACAAAAACAT 3′) (Simon *et al.,* 1994). Numbering of the primers refers to positions in the *Drosophila yakuba* mt 16S rDNA sequence.

All reactions were carried out in 50-μl volumes, containing 4 units of *Taq* Polymerase (MBI, Fermentas), 10 mM Tris–HCL, 2 mM MgCl2, 50 mM KCL, pH 8.3. Amplification was performed under standard PCR conditions on a Perkin–Elmer 9600, with an initial 2-min denaturation step at 92°C, followed by 30 cycles of 92°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension step of 2 min at 72°C. Unincorporated

nucleotides and primers were removed by passing the PCR products over Sephadex columns (Qiagen) following the manufacturer's protocol. Purified PCR products were sequenced directly using the ABI Prism Dye Terminator Cycle sequencing kit (Perkin–Elmer) and separated on an Applied Biosystems 373 automated sequencer. Both strands were sequenced.

Sequence Analysis and Phylogenetic Reconstruction

Sequences were initially aligned with the CLUSTAL IV program package (Higgins and Sharp, 1989) and subsequently corrected by eye. To determine alignment-ambiguous positions, we varied alignment parameter specifications and compared resulting alignments (gap opening costs: 5.00–20.00; gap extention costs: 2.50–10.00; DNA transition weight: 0.50; DNA user matrix: IUB; delay divergent sequences: 40%). We analyzed saturation of variation by plotting number of transitions or transversions against p-distances as implemented in the MUST program package (Philippe, 1998). Tree reconstructions were carried out using maximum-parsimony, neighbor-joining with ML distances, and maximum-likelihood methods. Bootstrapping of maximum-parsimony, distance-based reconstructions entailed 1000 replications, with random additions of taxa, on informative sites only in maximum-parsimony and TBR branch swapping. The PAUP*4.2a program package (Swofford, 1999) was used for parsimony and distance reconstructions and the PUZZLE package (Strimmer, 1998) for maximum-likelihood reconstructions. Parsimony reconstructions used informative characters only, with gaps treated as a fifth character state, assuming a transition/transversion ratio (TS/TV) of either 1 or 2 implemented by a stepmatrix, and with all character weights of 1. Alternatively, we used scaled character weights and unordered character types on informative characters only. All parsimony reconstructions were performed with the heuristic search option, random addition of taxa, and TBR branch swapping. For neighbor-joining reconstructions we used HKY + %I + Γ distances calculated with the maximum-likelihood method.

For maximum-likelihood calculations, we chose the HKY + %I + Γ model because this model clearly fit the data better. A likelihood-ratio test (Goldman, 1993; Yang *et al.,* 1995) was used to assess fit of models with different degrees of complexity (Table 1). There is some controversy about the appropriateness of using the χ^2 distribution for assessing significance values of the test (see Goldman, 1993), but we think that using this test still provides a rough guidance for proper model selection. We used the most-parsimonious tree of the maximum-parsimony reconstructions for the model fit calculations. Values of support for single nodes in the maximum-likelihood tree are quartet puzzle support values. In all tree reconstructions, *N. esthera* and *Palaemnema* sp. were used as outgroup taxa.

TABLE 1

−ln Likelihoods of the Most-Parsimonious Tree under 12 Different Models, Relative to the HKY+%I+Γ Model

	Model		
Rate variation	JC	F81	HKY85
Equal rates	+250.20	+201.26	+153.7
%I	+145.67	+78.96	+13.62
Γ	+138.03	+72.06	+1.69
%I+Γ	+136.57	+70.04	0

Note. The −ln L of the most complex model is 2660.01. The values shown are the increase in −ln L relative to the HKY + %I + Γ model. The Γ distribution was approximated with 16 categories for all models. The differences in −ln L are all significant employing the likelihood-ratio test and a X^2-distribution, except for the 2 most complex models. For all maximum-likelihood reconstructions we used the HKY + %I + Γ model.

RESULTS

Sequences have been deposited in GenBank under Accession Nos. AF170948–AF170961. For all alignment parameter specifications, alignments were nearly identical, except for three positions. Therefore, we decided to keep all nucleotide positions for subsequent phylogenetic analyses. The aligned 16S rDNA sequences constituted a data matrix of 546 characters. Of those, 146 characters were parsimony informative. The mt 16S sequences had a A–T content of 67%. This is relatively low compared to that of other insect groups (see Simon *et al.,* 1994). This base compositional bias was incorporated in maximum-likelihood reconstructions.

Analysis of saturation of nucleotide variation showed that the frequency of transitions reaches a plateau at roughly 15% p-distances (Fig. 1a), whereas the frequency of transversions increases proportionally to the p-distance values (Fig. 1b). This can be taken as a sign of saturated transitional substitutions. We further checked whether saturation of transitions is restricted to comparisons of outgroup and ingroup taxa (Figs. 1c and 1d), which is indeed the case. Saturation plots for the ingroup taxa show no sign of saturation for transitions (Fig. 1c) or transversions (Fig. 1d).

In our maximum-parsimony analysis (informative characters only, equal character weights, transitions downweighted by a factor of 2, implemented through a stepmatrix, gaps as fifth character state) we found one most-parsimonious tree (TL = 563, CI = 0.567, HI = 0.433, RI = 0.535) (Fig. 2). In this reconstruction, *Mnais* emerged as the sister group to *Matrona* and *Calopteryx. H. americana* was at the stem of the sampled Calopterygidae. The European and the North American *Calopteryx* taxa are monophyletic. Bootstrap analysis recovered an identical tree (see Fig. 4). We explored the sensitivity of the analysis to different parameter settings by reanalyzing the data without downweighting transitions and obtained a single most-parsimonious and identical tree (TL = 410, CI = 0.624, RI = 0.557, HI = 0.376). Different character weighting schemes can also influence tree reconstructions. Reconstructions with scaled character weights and unordered character types also resulted in one most-parsimonious tree reiterating the topology already found. This demonstrates that the data set is insensitive to the various parameter settings in the maximum-parsimony approach.

We performed neighbor-joining reconstructions with distances corrected with the HKY + %I +Γ model used in the maximum-likelihood reconstructions. All characters were included in this analysis. The neighbor-joining method reproduced the parsimony-based topology, except for the position of *C. cornelia,* which here was placed at the stem of the North American clade (Fig. 3). Bootstrap values for the neighbor-joining reconstructions are given in Fig. 4.

We performed the maximum-likelihood reconstructions with the HKY + %I + Γ model fitted to our data. Character change was considered independent among sites. The resultant puzzling tree was not completely resolved; nodes with puzzling support below 50% are collapsed. Nodes which had a low bootstrap support in the maximum-parsimony reconstructions are collapsed in the puzzling tree. However, the monophyly of the North American group and the European group is still supported (Fig. 4).

Finally we checked for the influence of outgroup taxa on tree reconstructions. Since the genus *Mnais* invariably appears as a sister taxon to the *Matrona/Calopteryx* lineage, we used *Mnais* as an outgroup to the genera *Matrona* and *Calopteryx* and removed the more distant *Neoneura, Palaemnema,* and *Hetaerina* from the analysis. This did not significantly alter the TS/TV ratio; the resulting most-parsimonious tree was identical to the appropriate branch of Fig. 2, except that the positions of *C. dimidiata* and *C. aequabilis* flipped.

In summary, analyses incorporating a variety of assumptions converged on a tree in which *Matrona* and *Calopteryx* are linked together as a sister taxon to *Mnais* (Fig. 4). This conclusion is robust for all reconstruction methods. The European and North American *Calopteryx* species are each monophyletic clades, supported under a variety of assumptions. Interestingly, the Asian *M. basilaris* and *C. atrata* form a monophyletic clade with strong bootstrap and puzzle support for all reconstruction methods. The position of *C. cornelia* is at the base of the European species with bootstrap support of 71% in parsimony reconstructions but is not resolved in the maximum-likelihood puzzling tree. The position of *C. cornelia* is different in the distance reconstructions. Therefore, we leave the position of this species unresolved. *H. americana* emerges at the base
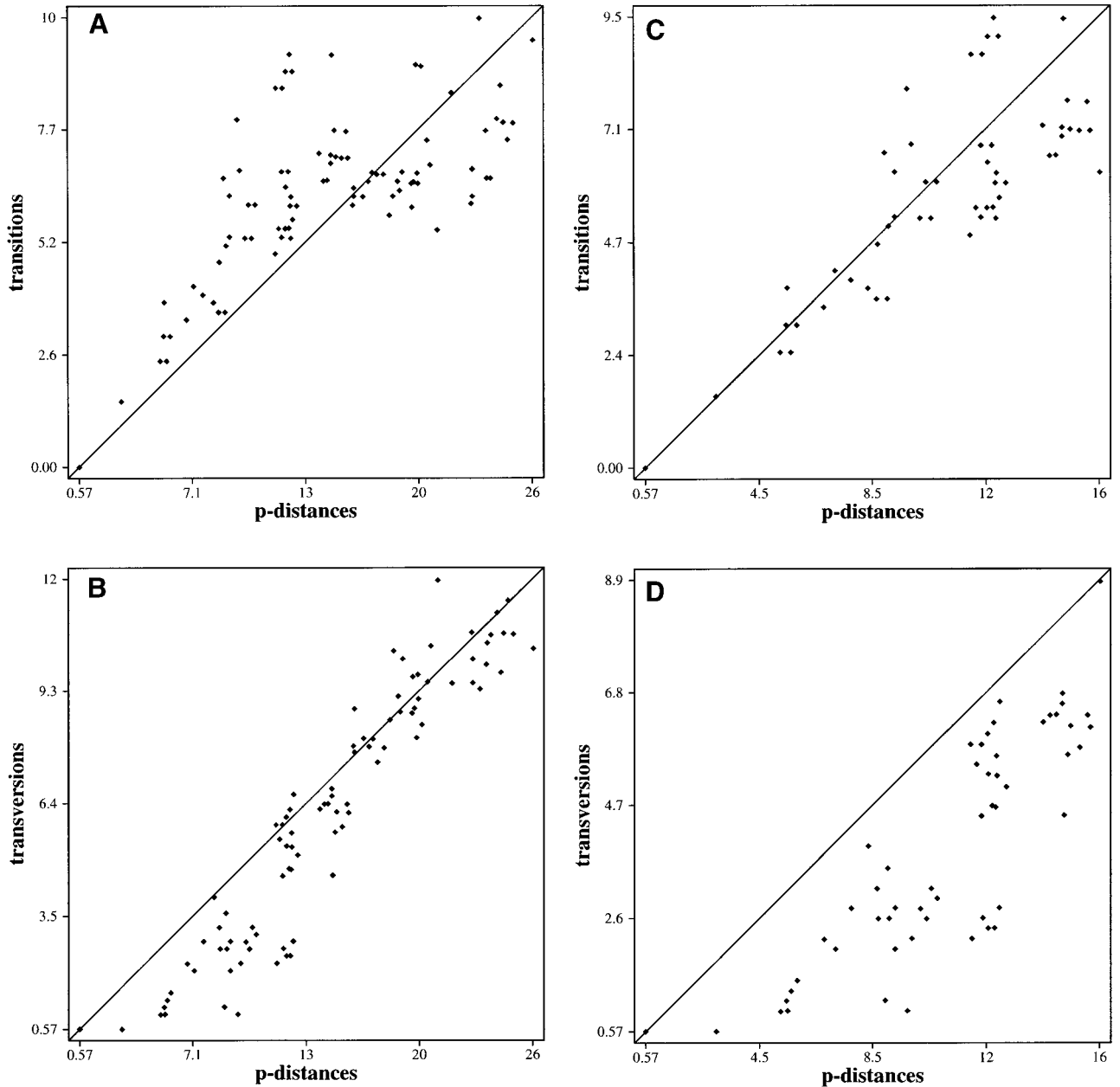
FIG. 1. *Analysis of substitutional variation.* In (a) the total number of transitions for each pairwise comparison in the data set is plotted against the appropriate p-distances. The p-distances are calculated as the total number of differences in percentage between two taxa. A plateau of transitional frequency is obvious at roughly 15% p-distance values. In (b) the total number of transversions for each pairwise comparison is plotted against the p-distances. The frequency of transversions increases almost linearly with the p-distances. In (c) the total number of transitions and in (d) the total number of transversions are plotted against p-distances for just the ingroup taxa. In both plots the number of substitutions increases with p-distances.

of the monophyletic Calopteryginae (*Mnais, Matrona,* and *Calopteryx*).

DISCUSSION

In this work, we present a molecular phylogeny adducing some of the evolutionary relationships within the damselfly genus *Calopteryx.* Traditional morphol-

ogy-based phylogenetic analyses of secondary sexual characters within Calopterygidae (Lindeboom, 1996) have not addressed phylogenetic relationships for this genus.

In our search for a plausible and robust phylogenetic hypothesis, we compared analyses of different tree-reconstruction algorithms to obtain information on the robustness of the conclusions (see Kim, 1993). A consen-
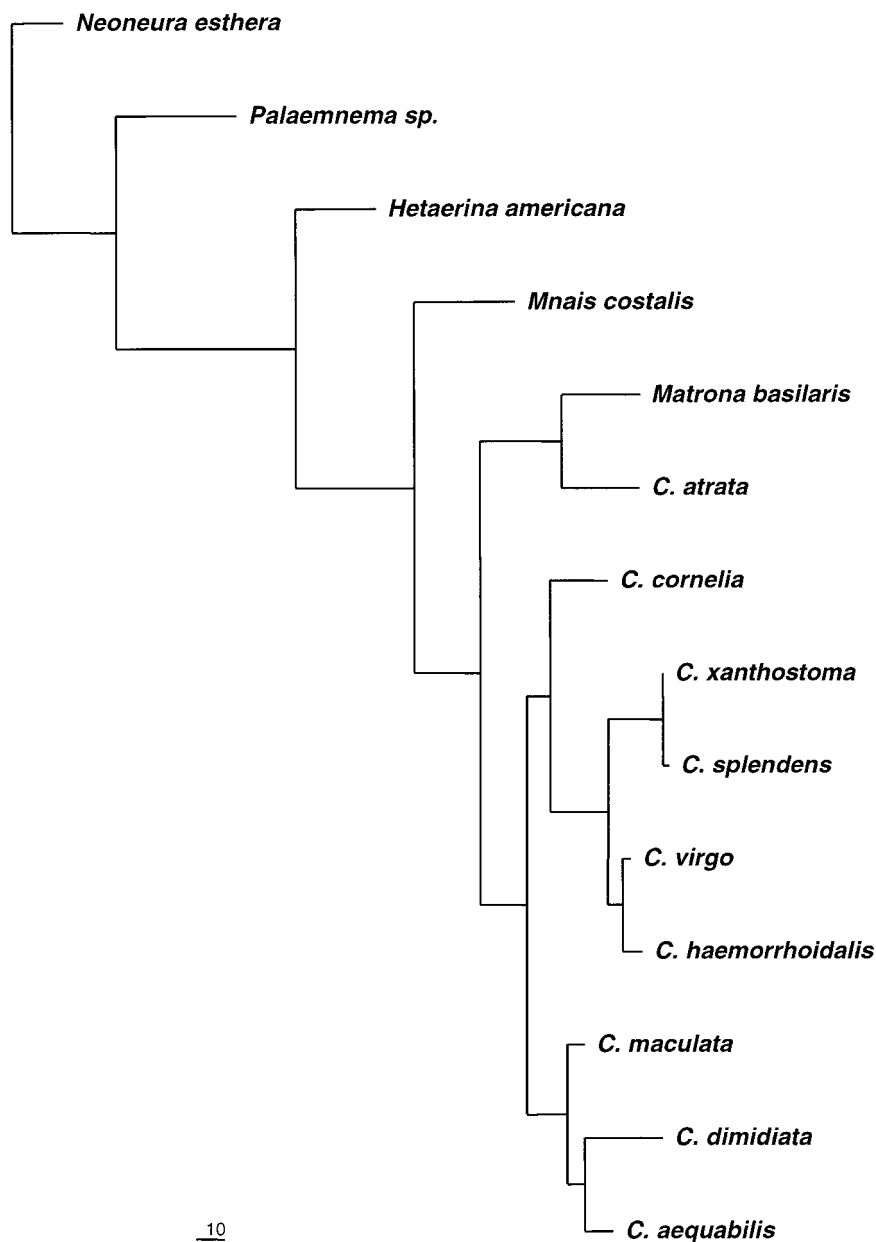
FIG. 2. *Maximum-parsimony Tree.* The single most-parsimonious tree was generated with the branch-and-bound algorithm, uninformative characters excluded, gaps treated as fifth character states, equal character weight, a transition/transversion ratio of 2 implemented by a step matrix, and *N. esthera* and *Palaemnema* sp. as outgroups (TL = 563, CI = 0.567, HI = 0.433, RI = 0.535). Bootstrap support values are given in Fig. 4. The parsimony reconstruction agrees with the neighbor-joining tree in most parts, except for the position of *C. cornelia,* and is compatible with the ML tree.

sus diagram summarizes what we believe are the significant, well-supported results of our analyses (Fig. 4). All three sets of reconstructions agree that *Hetaerina* is best placed at the stem of the clade composed of the Calopteryginae, *Mnais, Matrona,* and *Calopteryx* (Fig. 4). This result supports the morphology-based (comparison of wing venation) arguments developed by Fraser (1957).

Mnais, Matrona, and *Calopteryx* form a monophyletic group (Fig. 5) with *Mnais* most likely as the sister taxon to *Matrona* and *Calopteryx.* Interestingly, in every reconstruction, the Asian *M. basilaris* and *C. atrata* form with high bootstrap support a monophyletic clade at the base of the *Calopteryx* radiation. This makes the genus *Calopteryx* almost certainly paraphyletic or possibly polyphyletic. Moreover, morphology also tends to support this clustering (Fraser, 1957); e.g., *Matrona* and *Calopteryx* species share structures of the female pseudostigma. However, this could as well be a plesiomorphic condition for this clade. Nel and Brisac
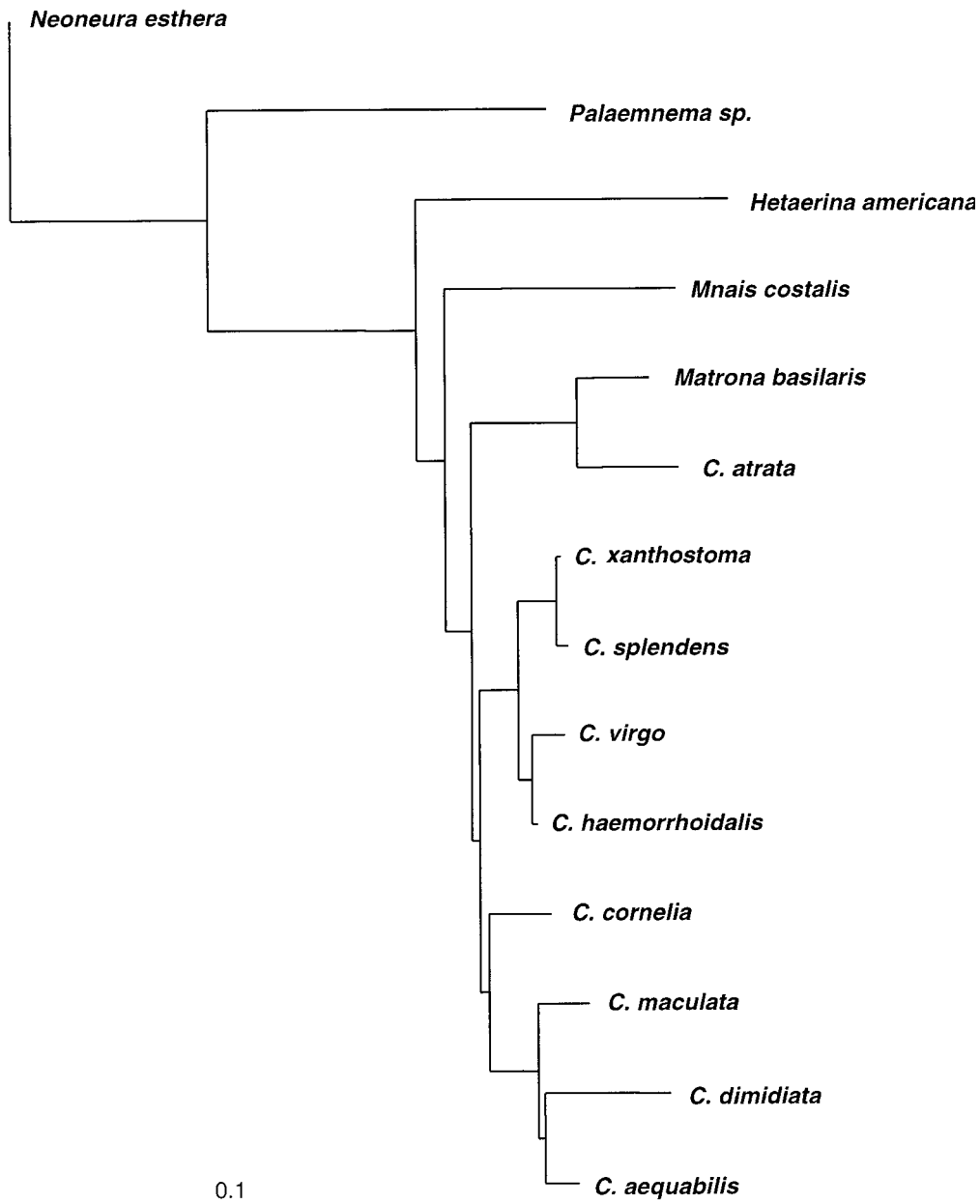
FIG. 3. *Neighbor-joining tree constructed with ML distances.* The neighbor-joining tree was constructed with the HKY + %I + Γ maximum-likelihood distances in PAUP*4.2a. The maximum-likelihood distances were used since the model fit procedure provided optimized distance corrections (model parameters: nucleotide frequencies, pi(A) = 38.7%, pi(C) = 19.1%, pi(G) = 13.5%, pi(T) = 28.7%; estimated transition/transversion parameter = 2.36 (SE = 0.33); expected transition/transversion ratio = 2.03; expected pyrimidine transition/purine transition ratio = 1.05; fraction of invariable sites = 50.4%; Γ parameter α = 0.26 (SE = 0.03); number of rate categories = 16). The neighbor-joining tree resembles the most-parsimonious tree, except for the position of *C. cornelia,* which is at the stem of the North American species. Bootstrap support values for the neighbor-joining tree are presented on the consensus cladogram in Fig. 4.

(1994) identified a Miocene calopterygid fossil from European deposits as a form closely related to *C. atrata.* Our molecular analysis and the fossil record imply that *M. basilaris* and *C. atrata* could be representatives of an early Eurasian radiation of the genus *Calopteryx.* This, in turn, implies that the current distribution of Eurasian *Calopteryx* species is likely the result of extinction, recolonization, and recent specia-

tion resulting from periods of glaciation. The Japanese endemic *C. cornelia* might also be a member of this early radiation. Our parsimony analyses place *C. cornelia* at the base of the European clade. This result is supported only in parsimony reconstructions and is in need of further investigation but all reconstructions clearly do not support the monophyly of the two Asian species *C. atrata* and *C. cornelia.* Furthermore, se-
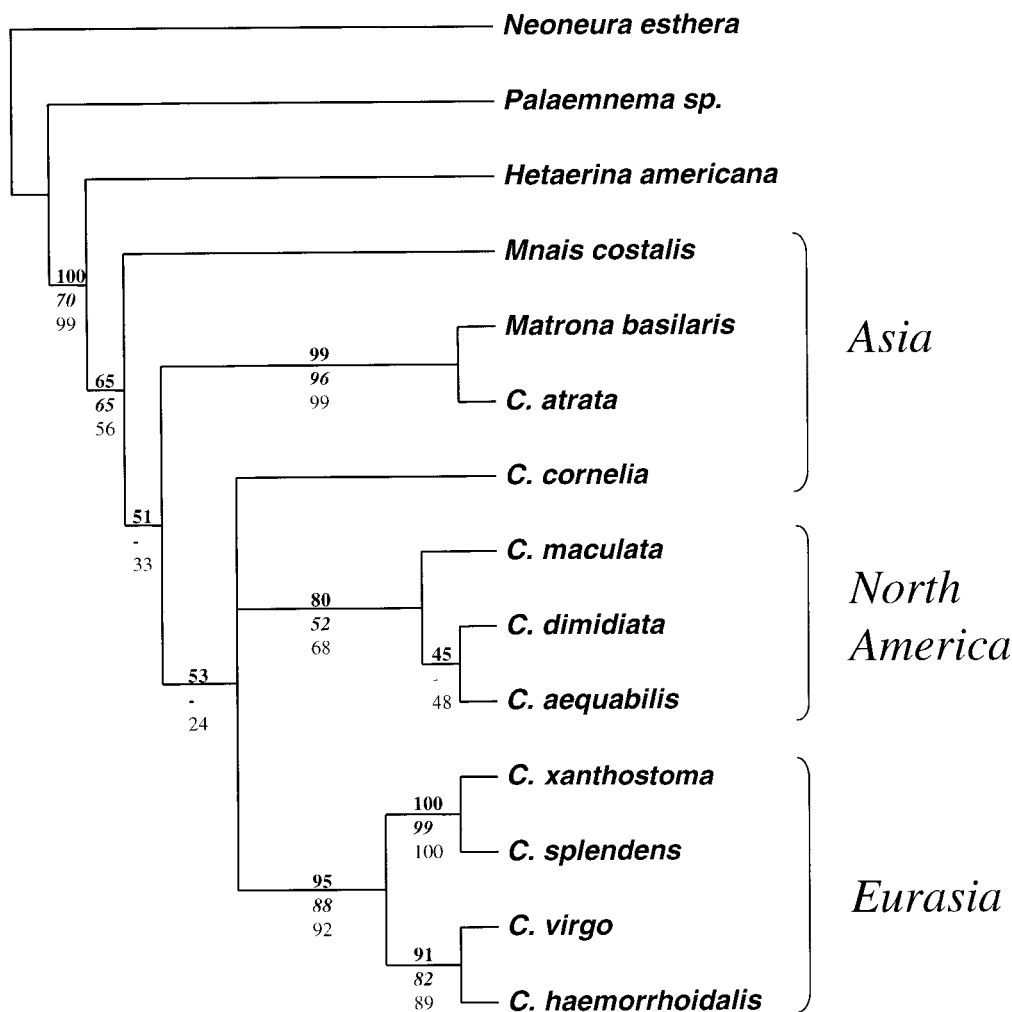
FIG. 4. *Consensus diagram of proposed phylogenetic relationships.* The consensus diagram summarizes the results of the three different reconstruction methods and presents bootstrap and puzzle support for single nodes on the tree. Bootstrap support is based on 1000 replicates for both maximum-parsimony and neighbor-joining methods. For parsimony, bootstrapping was performed on parsimony-informative characters, with random addition of taxa, a transition/transversion ratio of 2 implemented by a step matrix, and heuristic search algorithm with TBR branch swapping. Puzzle support was calculated with the PUZZLE program under the HKY + %I + Γ maximum-likelihood model. Parsimony support is given in boldface numerals, neighbor-joining support in lightface numerals, and puzzle support in italic numerals.

quence variation is highest among the Asian *Mnais, Matrona,* and *Calopteryx* species, supporting the evidence for the East Asian origin of the genus *Calopteryx* (for comparison of sequence data see *www.evolution.uni-bonn.de/calopteryx*).

All methods support the monophyly of European and North American *Calopteryx* lineages. This result was stable under all parameter settings. Although the relationships of the North American species are not well resolved, it is clear that the North American lineage diverged relatively early, considerably before the cladogenetic events that gave rise to the modern European (Eurasian) *Calopteryx* species. Since all North American *Calopteryx* species included in this investigation derive from a single lineage, we conclude that there have probably been no successful episodes of

colonization by other congenerics during interglacial periods.

A comparison of the molecular distances between the North American and the Eurasian clades (Table 2) implies that paleobiogeography of Eurasian and North American *Calopteryx* species is somewhat different. The existing species composition in the palearctic region is a consequence of extinction, subsequent recolonization, and probably relatively recent speciation phenomena driven by climatic changes (see above). The molecular differentiation between North American *Calopteryx* species does not indicate recent speciation phenomena, based on the assumption that rates of molecular change are roughly comparable between the North American and the Eurasian clades. The inclusion of *C. amata* and *C. angustipennis* in future investiga-
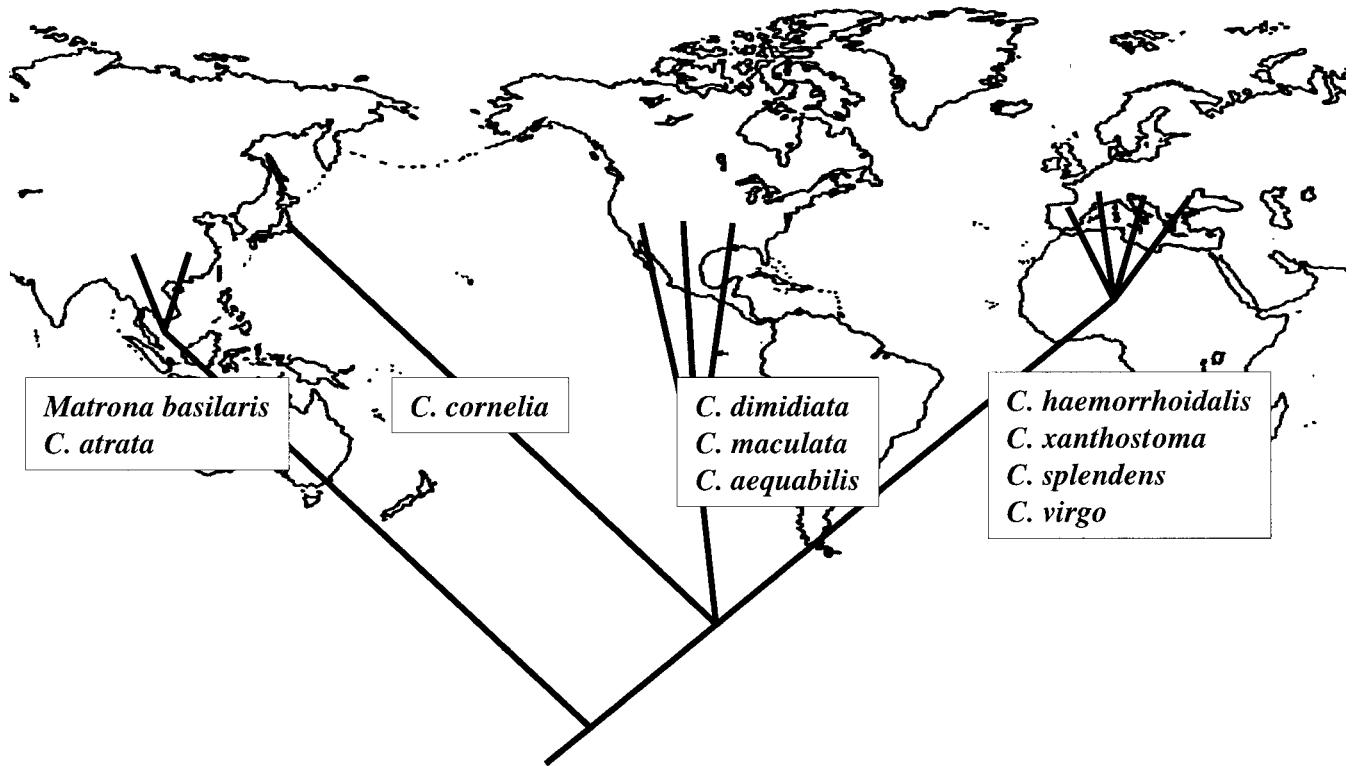
FIG. 5. *Consensus diagram associated with geographical distribution.* The figure maps the proposed phylogenetic relationships of the investigated *Calopteryx* species onto their geographical distributions. The proposed relationship is quite congruent with the distributional pattern and clearly at odds with the interpretation of the behavioral results. The inclusion of *C. amata* and *C. angustipennis* in future studies will be a good test of the current interpretation of the molecular results.

tions will show whether this conclusion can be confirmed.

The European clade is unambiguously monophyletic, with *C. virgo* and *C. haemorrhoidalis* as sister taxa. The previously postulated close association of *C. virgo* and *C. haemorrhoidalis* and the association of *C. splendens* and *C. xanthostoma,* based, for example, on analyses of different flight parameters, is consistent with our results (e.g., Anders and Rüppell, 1997). Further molecular analyses that compared *C. xanthostoma* with different populations and subspecies within the *C. virgo* and the *C. splendens* groups confirms, however, Dumont's conclusion of the "good species" status for *C. xanthostoma* (Hadrys *et al.,* in prep.; Dumont, 1972). In his analyses Dumont postulated that specific color characteristics of the wing and body,

TABLE 2

Pairwise Maximum-Likelihood Distances

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14
N. esthera	*													
Palaemnema sp.	31.6	*												
M. basilaris	31.9	46.5	*											
H. americana	43.9	49.2	35.9	*										
Mn. costalis	39.4	46.3	23.2	35.0	*									
C. atrata	39.2	50.3	10.3	35.1	28.06	*								
C. xanthostoma	30.8	41.3	15.7	25.8	20.69	16.6	*							
C. splendens	32.3	42.4	16.6	27.1	21.77	16.6	00.6	*						
C. virgo	30.3	40.4	17.0	27.7	23.50	17.9	04.7	05.3	*					
C. haemorrhoidalis	28.1	37.9	16.5	25.2	21.10	16.3	03.8	04.3	02.2	*				
C. cornelia	33.0	36.6	17.3	26.5	21.68	16.9	08.7	09.3	08.6	06.9	*			
C. dimidiata	43.8	50.5	21.4	33.6	21.05	21.6	15.6	16.4	16.3	14.6	15.4	*		
C. maculata	36.4	43.3	17.6	25.3	22.25	19.6	11.3	12.0	11.7	10.6	07.6	10.9	*	
C. aequabilis	35.3	41.4	16.4	26.4	21.13	18.4	10.6	11.3	10.6	09.4	09.9	09.4	05.4	*

differences in sexual behavior, and particulars of habitat selection are (in addition to structural parameters) valid character sets for achieving reproductive isolation between *C. xanthostoma* and *C. splendens.*

Miyakawa's behavioral analysis of *Calopteryx* species of all three continents is clearly at odds with the molecular reconstruction of relationships. Resemblances between *C. atrata* and *C. aequabilis* or among *C. virgo, C. cornelia,* and *C. maculata* in reproductive behavioral traits are most likely convergent or symplesiomorphic character states for these taxa and probably do not indicate close relationships.

Interesting, however, is the behavioral-based relationship between *C. virgo* and *C. maculata,* which is in accordance with male and female genitalic similarities that separate *C. virgo* from *C. xanthostoma* and *C. haemorrhoidalis* (Cordoba-Aguilar, 1999). Testing current models of sexual selection, Cordoba-Aguilar found size differences in penis structure related to different mechanisms of sperm removal. Whereas *C. virgo* and *C. maculata* are able to mechanically remove rival sperm from female sperm storage organs (paired spermathecae and bursa copulatrix), *C. xanthostoma* and *C. haemorrhoidalis* are not able to physically remove spermathecal sperm. Males of these species stimulate the female sensory system to induce ejection of rival sperm.

In summary, analysis of mt 16S rDNA gene fragments partially resolved relationships within the genus *Calopteryx* and between Calopterygid forms and seems to bode well for future, more exhaustive, analyses of this family of damselflies. The small number of informative character changes warrants additional sequence information of more rapidly evolving genes. Improved resolution will help to clarify puzzles of geographic distribution of *Calopteryx* ssp. Moreover, this phylogeny should prove very useful as an evolutionary framework in which to (i) compare and interpret the existing data on *Calopteryx* behavior and morphology and (ii) select "key species" for further studies.

ACKNOWLEDGMENTS

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