Odonatologica 36(4): 365-372 *December 1, 2007*

A REVISED MOLECULAR PHYLOGENY OF THE CALOPTERYGINAE (ZYGOPTERA: CALOPTERYGIDAE)

H.J. DUMONT, A. VIERSTRAETE and J.R. VANFLETEREN
Department of Biology, Ghent University, Ledeganckstraat 35, B-9000 Gent, Belgium
Henri.Dumont@UGent.be

Received June 16, 2007 / Reviewed and Accepted August 2, 2007

An updated version of an ITS-based phylogeny of the Calopteryginae, using sequences of 31 ingroup taxa, is given. The subfamily consists of 3 main clades, each with 2 subclades. Only clade 1 (*Calopteryx* s. s.) is not exclusively Asian but extends to Europe and North America. In the East-Asian clade 2, the genus *Matrona* is found to be descended from an *Atrocalopteryx*-like ancestor. Several so-called South-East Asian *Calopteryx* probably either belong to *Atrocalopteryx* or to as yet unnamed genera near *Atrocalopteryx*. *Archineura* consists of 2 spp., limited to China and Indo-China, and is rather basal to clade 3. The subclade *Neurobasis-Matronoides* is worthy of further analysis.

INTRODUCTION

The dragonfly cohort Caloptera, composed of seven families, has recently been subjected to a phylogenetic analysis using the nuclear ribosomal genes 18S, 5.8S, and the internal transcribed spacers ITS1 and IRS2 (DUMONT et al., 2005). From this analysis, it appeared that the 5.8 and 18S genes in this group evolve at such a slow rate that almost all phylogenetically resolving power lies with the ITSses.

The Calopterygidae and the Haeterinidae constitute the two most speciose families of the cohort, and within the Calopterygidae, the largest subunit is the Calopteryginae, with currently about nine genera recognized.

In this subfamily, DUMONT et al. (2005) created a new genus, *Atrocalopteryx*, based on the position of a single species examined, *A. atrata*, distributed from the coastal fringe of Eastern Siberia to north-eastern China and Korea. However, this does not mean that the genus is monotypic: several species orginally described

in *Calopteryx* and occurring further south in China and Vietnam-Laos, are suspected to belong here, but none was so far available for molecular analysis.

A Chinese endemic, *Archineura incarnata*, was found to cluster with *Matrona* and *Atrocalopteryx*, a surprising result, since by habitus and morphology, this taxon looked closely related to yet another calopterygine, "*Leucopteryx*" *haeterinoides*, from Laos and Vietnam. The quotes suggest the uncertainty that surrounded (and still surrounds) the generic name *Leucopteryx*, since classical morphology led to the expectation that *haeterinoides* was a true *Archineura*. Shortly after the publication of DUMONT et al. (2005), the suspicion about the placement of *Archineura incarnata* was confirmed, when it was found that this taxon had been identified and sequenced using a female that in fact belonged to

Table I
Alphabetical list of taxa and specimens examined

	Geographical origin	Collector
Archineura hetaerinoides	Laos	M. Hämäläinen
Archineura incarnata	Nankunshan, Guangdong, China	Boping Han
Atrocalopteryx atrata	Dokigawe River, Japan	K. Inoue
Atrocalopteryx atrocyana	Nanling, Guangdong, China	K.D.P. Wilson
Atrocalopteryx atrocyana	Tian Men Gao, Guangdong, China	H.J. Dumont
"Calopteryx" coomani	Vietnam	M. Hämäläinen
Calopteryx aequabilis	Wisconsin, USA	S.W. Dunkle
Calopteryx amata	New Brunswick, Canada	S.W. Dunkle
Calopteryx cornelia	Yuragawe River, Japan	K. Inoue
Calopteryx exul	Ifrane, Atlas, Marocco	H.J. Dumont
Calopteryx haemorrhoidalis	Ifrane, Atlas, Morocco	H.J. Dumont
Calopteryx maculata	Oklahoma, USA	S.W. Dunkle
Calopteryx virgo virgo	Laon, France	H.J. Dumont
Calopteryx xanthostoma	Argens, Chateauvert, France	M. Papazian
Echo modesta	Kanchanaburi, Thailand	M. Hämäläinen
Matrona basilaris	Omei Shan, Sichuan, China	Su Rong
Matrona basilaris	Nankunshan, S. Guangdong, China	H.J. Dumont
Matrona basilaris	Tian Men Gao, C. Guangdong, China	H.J. Dumont
Matrona "basilaris"	Hainan Island, China (2005)	K.D.P. Wilson
Matrona "basilaris"	Hainan Island, China (2006)	H.J. Dumont
Matrona cyanoptera	Wasli, Taipei, Taiwan	W.C. Yeh
Matrona nigripecta	Chiang Mai, Thailand	M. Hämäläinen
Matronoides cyaneipennis	Mt. Kinabalu, Borneo	M. Hämäläinen
Mnais andersoni	Chiang Mai, Thailand	M. Hämäläinen
Mnais mneme	Hainan, China	K.D.P. Wilson
Neurobasis chinensis	Kanchanaburi, Thailand	M. Hämäläinen
Neurobasis chinensis	Asan lake, N. India	H.J. Dumont
Neurobasis chinensis	Hainan Island, China	K.D.P. Wilson
Psolodesmus dorothea	South Taiwan	W.C. Yeh
Psolodesmus mandarinus	North Taiwan	W.C. Yeh
Psolodesmus mandarinus kuroiwae	Mt. Omoto, Ishikagi, Japan	K. Watanabe

Matrona or *Atrocalopteryx*. Thus, new material was collected from Guangdong, South China, to rectify this error. In the same general area (South China) and in Vietnam, more fresh specimens belonging to *Matrona* suspected to belong to *Atrocalopteryx* were collected as well, in order to substantiate better these subdivisions of the calopterygine clade.

Here, we sequence and analyse the internal transcribed spacers and intervening 5.8 S rDNA of these additional (seven in all) insects, with an aim at obtaining an improved phylogeny of the subfamily. Beside the *Archineura*, three were suspected to belong to *Atrocalopteryx* (two to the taxon *atrocyana*, and one to *coomani*), and three to *Matrona*. They were inserted in a tree of Calopteryginae composed of 31 ingroup taxa, plus three outgroup taxa of the zygopteran families Chlorocyphidae, Diphlebiidae, and Megapodagrionidae. The origin and collectors of the specimens analysed is given in Table I.

MATERIAL AND METHODS

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING – The origins of the samples used in this study are listed in Table I. All were males that had been fixed and preserved in 70-80% ethanol immeditaly upon collection ion the field. In the laboratory, muscular tissue was isolated from the synthorax and genomic DNA was extracted, using the protocol of the Puregene™ DNA isolation kit type D-5000A (Gentra Systems Inc., BIOzym, Netherlands),or using a modified CTAB protocol (KOCHER et al., 1989). Briefly, muscle tissue was crushed using a beadbeater and subsequently incubated for a minimum of 3 h at 60°C in 500 µl CTAB buffer to which 6 µl proteinase K (10 mg/ml) was added. Next, 250 µl 7.5 M ammonium acetate was added and the mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was saved and DNA was precipitated by adding an equal volume of isopropanol. The sediment was washed with 70% ethanol and redissolved in 25 µl water. Small aliquots (usually 1 µl) were used as template for PCR. The complete region separating the SSU and LSU genes and comprising the ribosomal spacers ITS1 and ITS2, and the conserved 5.8S gene, was amplified using the polymerase chain reaction. The total length of the fragment sequenced amounted to 712 bp. Finding primer pairs that yielded sequenceable amplicons was a tedious task. In all, we used the following primers:

Vrain 2f : 5'-CTT TGT ACA CAC CGC CCG TCG CT- 3'
Ferris 2f : 5'-RGY AAA AGT CGT AAC AAG GT- 3'
Vrain 2r : 5'-TTT CAC TCG CCG TTA CTA AGG GAA TC- 3'
28R1 : 5'- TGA TAT GCT TAA NTT CAG CGG GT - 3'
5.8 f1 : 5'- TCG AAT TGT GAA CTG CAG GAC ACA T - 3'
5.8 r3 : 5'- TCC GTG GGC TGC AAT GTG CGT TCG AA - 3'

The PCR conditions were 30 s at 94°C, 30 s at 54°C and 2 min at 72°C for 40 cycles. After treatment with shrimp alkaline phosphatase and exonuclease I the PCR products could be used as template for cycle sequencing without further purification.

PHYLOGENETIC ANALYSIS – The DNA sequences were aligned with MUSCLE (EDGAR, 2004) using default settings. Analyses were performed under unweighted parsimony, maximum likelihood and Bayesian inference. Trees were displayed with TREEVIEW 1.6.6 (PAGE, 1996). Parsimony analysis was performed using PAUP 4.0b10 (SWOFFORD, 2003) with the following heuristic search settings: 100 random taxon addition replicates, followed by tree-bisection-reconnection branch (TBR) swapping (10^7 rearrangements). Gaps were treated as missing data. Nodal support was assessed by calculating bootstrap values from 100 bootstrap replicates obtained by heuristic search with 10 ran-

dom sequence additions each (FELSENSTEIN, 1985).

Maximum likelihood analysis was performed using the general time-reversible substitution model with a gamma correction for among-site variation and corrected for invariable sites (GTR + I + G). This model was identified as the best-fit model of DNA evolution for maximum likelihood analysis of the data-set by the likelihood-ratio test (LRT) and Akaike Information Criteria (AIC), implemented in ModelTest 3.7 (POSSADA & CRANDALL, 1998). Maximum likelihood was performed using the heuristic search option with stepwise taxon addition, TBR branch swapping, MulTrees option in effect, no steepest descent, and rearrangements limited to 100.000. Bootstrap values were determined from 100 bootstrap replicates by heuristic search with 10 random sequence addition replicates each (FELSENSTEIN, 1985).

Bayesian analysis was performed using MrBayes, version 3.1.2 (HUELSENBECK & RONQUIST, 2001). MrModeltest 2.2 (NYLANDER, 2004) also identified GTR + I + G as the best-fit model for Bayesian inference. The parameters for base frequencies, substitution rate matrix, gamma rate distribution and shape and proportion of invariant sites were allowed to vary throughout the analysis.

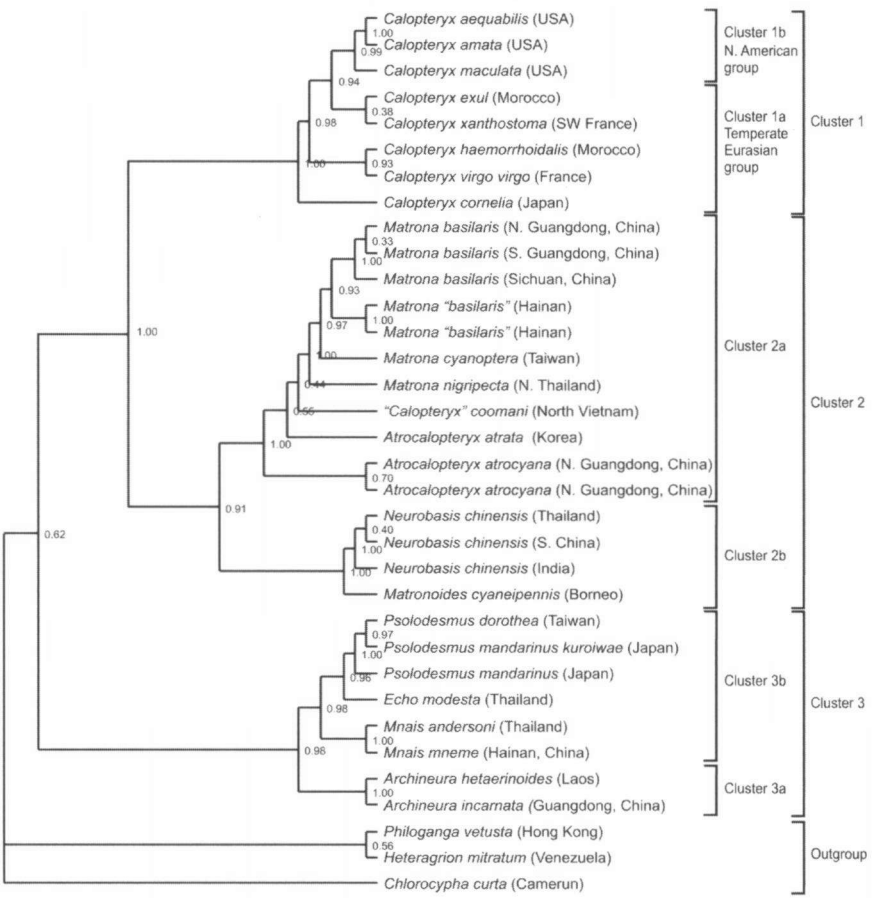

Fig. 1. Bayesian probability estimate of the phylogeny of the Calopteryginae based on internal transcribed spacer (ITS 1 and 2) data.

The Markov chain Monte Carlo process was run over four parallel chains (one cold and three heated) for 1,000,000 generations with trees being sampled every 100 generations. The burn-in value was set to 1000 trees (i.e. 100,000 generations) equating the number of generations needed to reach a stable value of all variable parameters in a preliminary run. Majority rule consensus trees were reconstructed after discarding the burn-in.

RESULTS

We obtained the unambiguous sequences of the ITSes of all taxa listed in Table I, from which we computed four phylogenetic trees. The length of ITS varied from163 to 203 bp, that of ITS2 from 206 to 229 bp, and that of 5.8 S from 163 to 166 bp. The Bayesian and maximum-likelihood-based trees (heuristic tree only) are shown in Figs 1 and 2. Four most parsimonious trees were found, differing only

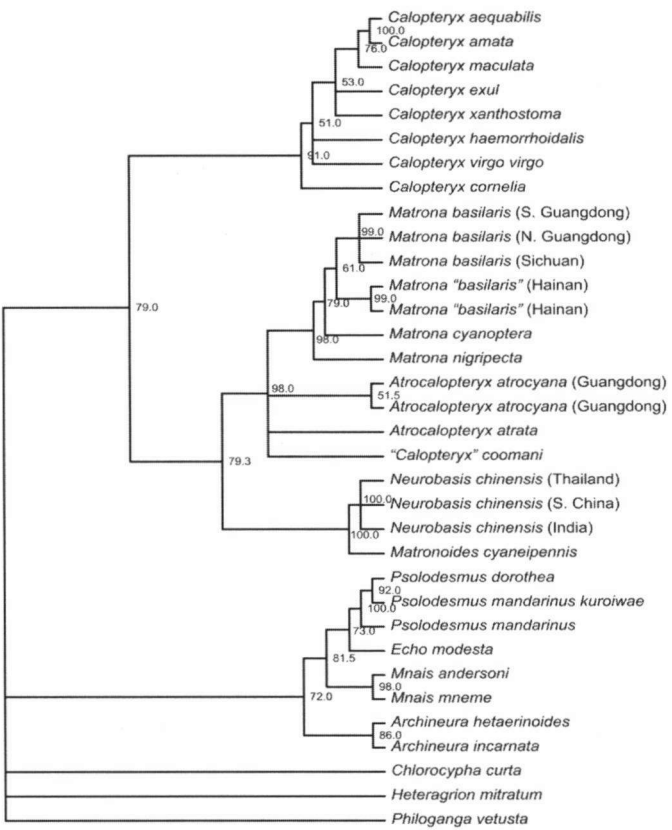

Fig. 2. Maximum likelihood estimate of the phylogeny of the Calopteryginae based on internal transcribed spacer (ITS1 and 2) data. Bootstrap support based on 100 replicates and expressed as a percentage.

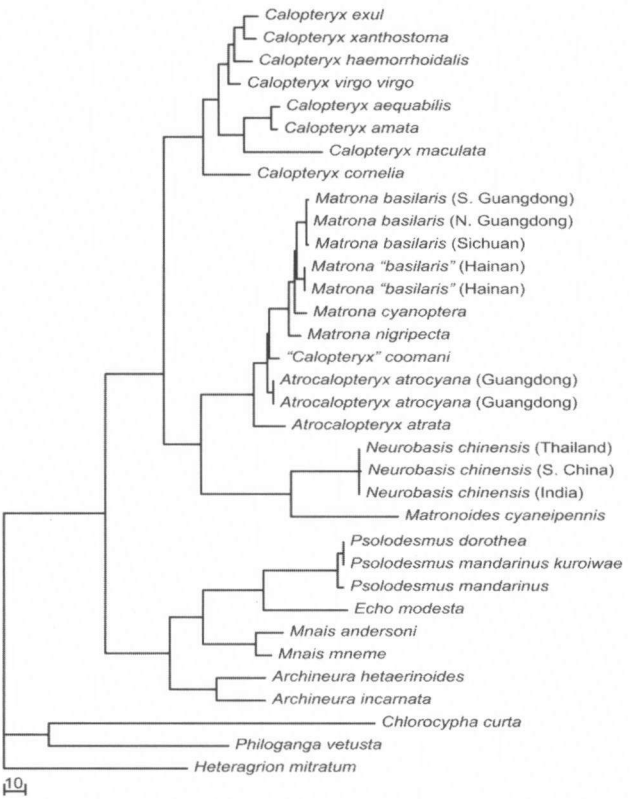

Fig. 3. Heuristic Maximum Parsimony tree with branch lengths of the phylogeny of the Calopteryginae based on internal transcribed spacer (ITS1 and 2) data. The MP analysis generated 4 most parsimonious trees of 1118 steps each. – [The scale bar represents 10 steps]

in minor details (the branching order of species within the genus *Calopteryx*). Only the consensus tree is shown (Fig 3). We also derived a neighbor-joining tree, but since this fully confirmed the previous ones, it is not reproduced here.

The Calopteryginae form three well-defined clusters, each of which can be subdivided into two subclades. The vast majority occur in East and South-East Asia; only cluster 1 (*Calopteryx* s. s.) extends west across the southern half of Siberia and central Asia to reach Europe and the Mediterranean basin. Cluster 1 also has a subcluster that occurs in Canada and the USA.

DISCUSSION

Most branches of our trees are well supported, allowing some rather robust conclusions to be drawn. Weaker support is only found in the relationship between two Asian genera, *Matrona* and *Atrocalopteryx*, and some uncertainty also remains as to the descendence order within the genus *Atrocalopteryx*.

All tree topologies reveal an undisputed monophyletic origin for the Calopteryginae (Bayesian probability 1.00; Maximum Parsimony, Maximum likelihood, and Neighbor joining all supported by 100% of the bootstraps executed), as established earlier by DUMONT et al. (2005), and the three main clusters found in

that paper are also confirmed (Figs 1-3). However, some important internal shifts fall to be recorded. *Archineura incarnata* is now closely clustering with *"Leucopteryx" haeterinoides*, forming a subclade that is well supported (Bayesian probability 0.99), as well as showing a close affinity between its two members (BP 1.00). The generic status of *Archineura* is thus accepted, and the need for a separate genus
("Leucopteryx") for taxon *hetaerinoides* lapses. New findings also include the fact that the position of *Archineura* is now fully embedded withinin cluster 3, and shows a sister-group relationship with the three other genera that consitute that cluster. Its relationship with such genera as *Calopteryx, Matrona*, and *Atrocalopteryx* is comparatively remote, not close as claimed in DUMONT et al. (2005).

Cluster 2 is now better structured, with the clade *Neurobasis-Matronoides* in sister position to a clade composed of *Matrona* and *Atrocalopteryx. Matrona* comes out of the analysis as a coherent, monophyletic genus, that is clearly descended from an *Atrocalopteryx*-like ancestor. The relationships within *Atrocalopteryx* are less straightforward, although clearly, *"Calopteryx" coomani* is seen to belong in *Atrocalopteryx* or a closely related taxon, and could well be the closest ancestor to *Matrona*. It certainly is not a true *Calopteryx*, and this position is supported by all three analyses independently.

Cluster 2 is also characterized by only South-East Asiatic taxa, and suggests local evolution in the absence of major environmental disturbances such as glaciations. This involves quite a bit of cryptic speciation, well illustrated by *Matrona*, where the *basilaris*-group is clearly composed of several species that have so far not been well characterized morphologically (e.g. a taxon, provisionally called *"basilaris"*, on the island of Hainan). In cluster 2b, the endemic *Matronoides* of Borneo is behaving as a genus distinct from *Neurobasis*, with the Indian specimen of *Neurobasis chinensis* suggesting at least evolution at the subspecies level within this widespread oriental taxon. It will be interesting to add to the analysis representatives of *Neurobasis* from Borneo, Sulawesi and The Phillipines, where this genus is rather speciose, to further refine the relationships within this cluster.

Cluster 2 furthermore raises the problem whether true *Calopteryx*, a genus of temperate and continental Eurasia and North America, really occurs south of the latitude of Japan. The little-known taxa *C. melli, C. oberthueri* and *C. laosica* from South China, Laos and Vietnam may well, like *coomani*, turn out to belong to different, still undescribed, genera.

Cluster 1 (*Calopteryx*) and its two subclusters (an Eurasian and a North American one) remain unaltered as compared to the analysis of DUMONT et al. (2005), as well as the genera composing cluster 3 (except for the addition of *Archineura*).

CONCLUSION

The subfamily Calopteryginae is composed of predominantly Asian representatives. A basal cluster of four genera (*Psolodesmus, Echo, Mnais*, and *Archineura*) is currently restricted to subtropical-tropical East Asia. A second cluster (four genera, but probably one or two remaining to be defined) extends from the Asian tropics to temperate East Asia. *Calopteryx*, finally, is a monogeneric cluster that is relatively species-poor and the only component of the Calopteryginae that extends through North-Central Asia to Europe, reaching North Africa in the west, invading North America in the East.

ACKNOWLEDGEMENTS

We thank all colleagues who provided the material necessary for this study. MATTI HÄMÄLÄINEN provided material and gave invaluable advice about a number of taxa. PETER WEEKERS' technical help with DNA extraction, amplification, and sequencing is appreciated. BOPING HAN made it possible for the senior author to visit Guangdong Province and Hainan Island (China), and collect a number of critical taxa there.

REFERENCES

DUMONT, H.J., J.R. VANFLETEREN, J.F. DE JONCKHEERE & P.H.H. WEEKERS, 2005. Phylogenetic relationships, divergence time estimation, and global biogeographic patterns of Calopterygoid damselflies (Odonata, Zygoptera) inferred from ribosomal DNA sequences. *Syst. Biol.* 54: 347-362.

EDGAR, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32: 1792-1797.

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

HUELSENBECK, J.P. & F.R. RONQUIST, 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

KOCHER, T.D., W.K. THOMAS, A. MEYER, S.V. EDWARDS, S. PÄÄBO, F.X. VILLABLANCA & A.C. WILSON, 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. natn Acad. Sci. USA* 86: 6196-6200.

NYLANDER, J.A.A., 2004. *MrModeltest v2*. Program distributed by the author. Evolutionary Biol Centre, Uppsala Univ.

PAGE, R.D.M., 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applics Biosci.* 12: 357-358.

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

SWOFFORD, D.L., 2003. *PAUP^{RM}: Phylogenetic analysis using parsimony (^{RM} and other methods)*. [Version 4]. Sinauer Associates, Sunderland, Massachusetts.

THOMPSON, J.D., D.G. HIGGINS & T.J. GIBSON, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.