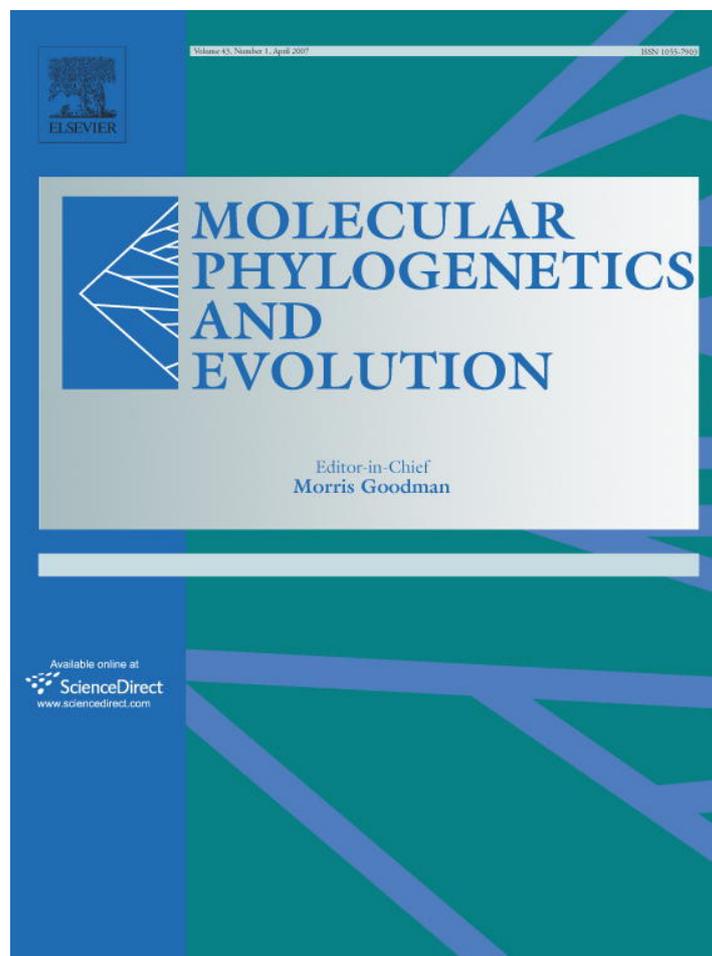


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Short communication

## Phylogenetic relationships among the Braconidae (Hymenoptera: Ichneumonoidea): A reassessment of Shi et al. (2005)

Kevin M. Pitz<sup>\*</sup>, Ashley P.G. Dowling, Barbara J. Sharanowski,  
C. Andrew Boring, Katja C. Seltmann<sup>1</sup>, Michael J. Sharkey

*Department of Entomology, University of Kentucky, Lexington, KY, USA*

Received 15 March 2006; revised 6 November 2006; accepted 8 November 2006  
Available online 21 November 2006

### 1. Introduction

Phylogenetic relationships among the Braconidae have been a source of contention, debate, and uncertainty for many years. This uncertainty has been evident in the conflicting relationships and lack of resolution obtained from various morphological (van Achterberg, 1984; Quicke and van Achterberg, 1990; Wharton et al., 1992) and molecular datasets (Dowton et al., 1998; Belshaw et al., 1998; Dowton et al., 2002). Recently, Shi et al. (2005) analyzed a large morphological and multi-gene dataset. The morphological components of their dataset were compiled from the previously published works of Quicke and van Achterberg (1990) and modified by van Achterberg and Quicke (1992) and Dowton et al. (2002). Their molecular dataset was compiled from published sequences from GenBank (<http://www.ncbi.nlm.nih.gov/>) and seven new sequences provided by the authors. The combined dataset was the largest employed in a systematic analysis of the Braconidae. However, the analysis performed by Shi et al. (2005) contained several methodological and conceptual errors. The purpose of the present paper is to address these errors, attempt to reproduce the original dataset, and offer a re-analysis of the molecular dataset in hopes to further the present knowledge of relationships among the Braconidae.

<sup>\*</sup> Corresponding author. Present address: Department of Zoology – Insects, Field Museum of Natural History, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA.

E-mail address: [braconid@gmail.com](mailto:braconid@gmail.com) (K.M. Pitz).

<sup>1</sup> Present address: School of Computational Sciences, Florida State University, 400 Dirac Science Library, Tallahassee, FL 32306, USA.

### 2. Critique of Shi et al. (2005)

Both the morphological and molecular data sets contained errors. Two taxa sequenced by the authors, *Aleiodes* (AY920277) and *Ascogaster* (AY920276), were found to be reverse compliments of the targeted 18S gene region. From GenBank, Shi et al. (2005) chose the wrong gene region for the 18S sequence of *Peristenus* (AF473538). This same *Peristenus* sequence was mistakenly combined with 28S and 16S data from a species of *Perilitus*.

Wharton et al. (1992) criticized the morphological data set of Quicke and van Achterberg (1990) for deficiencies in character state definition, *a priori* character weighting, and ground-plan coding for subfamilies. Based on current subfamily composition, Shi et al. (2005) scored all genera in each subfamily identically. Scoring genera based on putative subfamily relationships does not allow for objective tests of monophyly of these same subfamilies. Additionally, ground plan-coding at the subfamilial level is amplified when these data are combined with molecular data scored at the generic level.

Due to numerous coding errors, the 96-character morphological dataset could not be analyzed as published. Some taxa lacked the appropriate number of characters in each column. The published dataset was divided into columns of ten characters (Table 2, p. 108); in four places there are only nine characters, e.g. Alysinae (characters 71–80), Cheloninae (61–70), Exothecinae (91–100), and Pambolinae (91–100). Additionally, 11 characters were included in one section for the Miracinae (characters 91–100). These errors created 50 scores with uncertain alignment. The morphological dataset also contained ambiguity. For example, the polymorphic states of some taxa were not defined, represented only by a “P”.

The authors claimed they tested different alignment parameters, including gap cost and extension values. However, Shi et al. (2005) failed to mention the parameters or results of their test. Rather, they simply stated “we tested alignment using the Clustal X program with different gap opening and gap extension values, and resulted [sic] in different length of aligned sequences (p. 109)”. The authors chose to employ the default parameter values based on the mathematical origin of the value itself, not on the results of their test. Shi et al. (2005) also stated “downweighting transitions or treating gaps as fifth base did not markedly affect the results obtained (p. 109)”. Once again, the authors did not report the parameters used to test the effect of downweighting transitions.

Shi et al. (2005) stated “where more than one most parsimonious tree was found, a strict consensus tree was calculated (p. 109)”. Contrary to their claim, the authors did not present consensus trees for their parsimony analyses. After the initial branch swapping, they ran a second round of swapping where only a single tree was held, rather than holding multiple minimum length trees. Therefore, the authors chose a single, fully resolved tree for each analysis, and ignored all other minimum length trees. Shi et al. (2005) failed to provide justification for their choice of one tree over competing most parsimonious trees.

The Shi et al. (2005) interpretation of their results is not always supported by their data. The authors stated that the microgastroids, cyclostomes, and helconoids were resolved as monophyletic groups with high levels of support. However, the only monophyletic lineage found in all of their analyses was the microgastroid complex. Contradicting their earlier statement, the authors acknowledged that the cyclostomes were not monophyletic, as two cyclostome taxa (*Mesostoa* and *Aspilodemon*) fell within the helconoid complex in 3 of the 4 analyses. Additionally, recovery of the traditionally cyclostome group Aphidiinae (Sharkey, 1993) within the helconoid complex, contradicts the supposed monophyly of this lineage.

The authors stated that the relationship (Aphidiinae + (Euphorinae + Neoneurinae + Cenocoeliinae)) was found in all trees, supporting a relationship proposed by Čapek (1970). There are two problems with the Shi et al. (2005) interpretation. In Fig. 4 (Shi et al., 2005, p. 113), Cenocoeliinae was recovered as sister to ((Trachypetinae + (Euphorinae + Neoneurinae)) + Aphidiinae). Second, Čapek (1970), presented a significantly different topology than that recovered by Shi et al. (2005). Čapek (1970) treated Cenocoeliinae as a tribe within Helconinae and proposed the relationship (Helconinae + (Cheloninae + (Paxylomatinae + (Neoneurinae + (Aphidiinae + (Leiophroninae + Euphorinae)))))).

Shi et al. (2005) asserted that only Cenocoeliinae [*sensu* Belshaw and Quicke (2002)] and Neoneurinae were not monophyletic. However, both subfamilies were resolved as monophyletic in all four of their cladograms. The Euphorinae were rendered paraphyletic in all of the Shi et al. (2005) analyses by Cenocoeliinae or Neoneurinae. Shi et al. (2005) suggested that Cenocoeliinae might be treated as a tribe of

the Euphorinae, as three of their analyses placed Cenocoeliinae within the Euphorinae. They ignored the results of the Bayesian analyses, which placed the cenocoeliines outside the Euphorinae with very strong support (Fig. 4, p. 113), leaving the placement of the Cenocoeliinae in question. The authors suggested that the morphology of Cenocoeliinae supports its inclusion within Euphorinae, while historically cenocoeliines have been allied with the Helconinae based on morphological data (Muesebeck and Walkley, 1951; Čapek, 1970; van Achterberg, 1994).

Finally, the published methods for Shi et al.’s (2005) Bayesian analyses were lacking pertinent information necessary for replication. The authors reported the number of total generations and sample frequency used in calculating posterior probabilities, but did not mention how many generations were discarded as burnin after reaching stationarity. Furthermore, Shi et al. (2005) failed to state the model of evolution employed or how that model was chosen. Based on the lack of available information for accurate replication, we chose to replicate and re-analyze the parsimony-based analyses only (see Section 3).

### 3. Materials and methods

Our first analysis was a replication using the same methods and dataset as reported by Shi et al. (2005). The second phylogenetic test was a re-analysis of the molecular data, employing different methodologies and using sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>). In our replication experiment, we chose only to repeat the Shi et al. (2005) combined maximum parsimony analyses with the morphological characters equally weighted. The molecular dataset was obtained from published supplementary materials (<http://qpm.zju.edu.cn/alignment.htm>), but at the time of publication of this article, the link is no longer active. The data files, as we downloaded them, are available at <http://www.sharkeylab.org/sharkeylab/sharkeyMatrices.php>. As mentioned previously, the morphological dataset cannot be analyzed in the published form. We made some corrections to the morphological dataset by comparing it against the matrix of Dowton et al. (2002) to determine which characters were excluded or coded more than once in the Shi et al. (2005) matrix. An ‘A’ was inserted for character 66 under Alysiinae. A ‘0’ was inserted for character 51 under Cheloninae; a ‘0’ was inserted for character 90 under Exothecinae; a ‘?’ was deleted from character 89 under Miracinae, and a ‘?’ was inserted for character 89 under Pambolinae. Only nine characters were coded within characters 61–70 for Hydrangeocolinae, a taxon not coded in previous versions of this morphological data set. It is uncertain which character was omitted, thus a ‘?’ was arbitrarily inserted for character 70. All character states coded as ‘P’ were changed to a ‘?’, as PAUP\* (Swofford, 2001) does not recognize the character state ‘P’ as meaning polymorphic. Unknown and missing data were treated equally. We assumed this is how Shi et al. (2005) treated these character states and predicted that these changes would not significantly alter the results. However, we

cannot discount that these changes may have had an effect on the replication experiment. This version of the morphological matrix is available at <http://www.uky.edu/~mjshar0/HI/datamatrices/index.htm>.

Using the methodology reported by Shi et al. (2005), we reanalyzed the combined molecular and morphological dataset in PAUP\* 4.0b (Swofford, 2001) with 100 random addition replicates, TBR swapping, and gaps treated as missing data. Unlike Shi et al. (2005), we compiled a strict consensus tree of all minimum length trees. Support for nodes was calculated using 1000 bootstrap pseudoreplicates (Felsenstein, 1985), TBR branch swapping, and no more than 500 trees saved per replicate. We reported bootstrap values 50% or higher. We also analyzed their dataset using 10,000 random addition replicates with TBR swapping, to see if a more thorough analysis of the data would yield a shorter minimum length tree. Additionally, we obtained support values using 6000 bootstrap pseudoreplicates as these values have been reported to show sensitivity to low pseudoreplicate numbers (e.g. Hedges, 1992; Mort et al., 2000; Salamin et al., 2003; Freudenstein et al., 2004).

For the re-analysis, sequence files were obtained directly from GenBank based on the published accession numbers reported in Shi et al. (2005). In order to create an objective and repeatable alignment, knowledge of ribosomal secondary structure was incorporated. The rRNA unit has a specific secondary structure necessary for the formation and functioning of ribosomes (Gutell et al., 1994) and the basic structure is conserved across an array of divergent taxa (Hillis and Dixon, 1991). Nucleotide evolution of rRNA genes (rDNA) is constrained by secondary structure (Muse, 1995). Knowledge of secondary structure can be effectively used in multiple sequence alignment (for discussion, see Kjer, 1995; Hickson et al., 1996).

A preliminary alignment was obtained using Clustal X (Thompson et al., 1997), employing default settings. Using methodology developed by Kjer (1995) and modified by Yoder and Gillespie (2004), homologous stem and loop regions were reconstructed based upon complementary base-pairing and structural models of 18S and 28S of the Ichneumonoidea (Gillespie et al., 2005). Ambiguous regions, where alignment cannot be justified by complementary base-pairing, are delimited by brackets in the model and are typically excluded from the analysis. We chose to exclude the same ambiguous regions outlined in the model to maintain repeatability of the molecular alignment. Although alignment based on secondary structure information is labor intensive, it provides an objective and repeatable criterion for alignment (Kjer, 1995). The alignment can be found at <http://www.sharkeylab.org/sharkeylab/sharkeyMatrices.php>.

Maximum parsimony analyses were performed using PAUP\* 4.0b (Swofford, 2001). Heuristic searches involved 10,000 random sequence addition replicates and TBR branch swapping. All residues were weighted equally and gaps were treated as a fifth state. Support for nodes was calculated using 6000 bootstrap pseudoreplicates (Felsenstein, 1985), TBR branch swapping, and no more than 500 trees

saved per replicate. The morphological data set was not incorporated in the re-analysis due to incongruence between the ranks of the taxa coded for each dataset.

#### 4. Results and discussion

We were unable to recover the topology presented in Fig. 1 (Shi et al., 2005, p. 110, reproduced here in Fig. 1A). Our replication analysis, employing 100 random additions recovered four most parsimonious trees of length 7672. The strict consensus is depicted in Fig. 1B. Tree length could not be compared as it was never reported by Shi et al. (2005). Several clades, such as (*Zele*+*Centistes*), (*Callibracon* (*Bracon*+*Habrobracon*)) and (*Schizoprymnus*+*Eubazus*), were recovered in our strict consensus, but not recovered in the Shi et al. (2005) analysis, therefore eliminating the possibility that the topology of Shi et al. (2005) could be one of the four most parsimonious trees found. The small changes made to the morphological data matrix probably did not have a significant effect on the results, since the morphological dataset was coded at the subfamily level. For example, *Callibracon*, *Bracon*, and *Habrobracon* are all Braconinae, and thus, all coded identically for the morphological matrix. Therefore, generic relationships within the Braconinae would be obtained solely from information in the molecular dataset. Since we used the published alignment of Shi et al. (2005), we should have recovered the identical generic relationships. We also considered the possibility that the low number of replications (100) employed in the Shi et al. (2005) analysis may have accounted for differences between the topologies in Fig. 1A and B. However, our more in-depth analysis of 10,000 replications recovered the same four most parsimonious trees of length 7672, indicating that the lower number of replications was enough to converge on the most parsimonious trees.

A comparison of the clades recovered in both Fig. 1A and B revealed that bootstrap values were consistently inflated in the Shi et al. (2005) analysis. The support values for their nodes (Fig. 1A) were over 50% for all but 8 nodes, but in our analysis, over 40 nodes had less than 50% bootstrap support. In our replication, we did not recover monophyletic microgastroid, helconoid, or cyclostome lineages, as defined by Shi et al. (2005) in Table 1 (pp. 106–107). The only subfamilies recovered as monophyletic with bootstrap values over 50% were Cenocoeliinae and Betylobraconinae. These results differ sharply from those reported by Shi et al. (2005), where they claimed nearly all subfamilies were well supported and monophyletic.

Our re-analysis of the molecular data with alignment governed by secondary structure, recovered 8 most parsimonious trees of length 4747. The strict consensus (Fig. 2) demonstrated monophyly of Alysiinae, Betylobraconinae, Braconinae, Doryctinae, Opiinae, Rhyssalinae, Agathidiinae, Cenocoeliinae, Orgilinae, Cardiochilinae, Aphidiinae, and Meteorinae (included with Euphorinae by Shi et al., 2005). All of these groups had bootstrap values over 50%. We recovered the microgastroid complex as monophyletic, but not the helconoids or the cyclostomes. We recovered

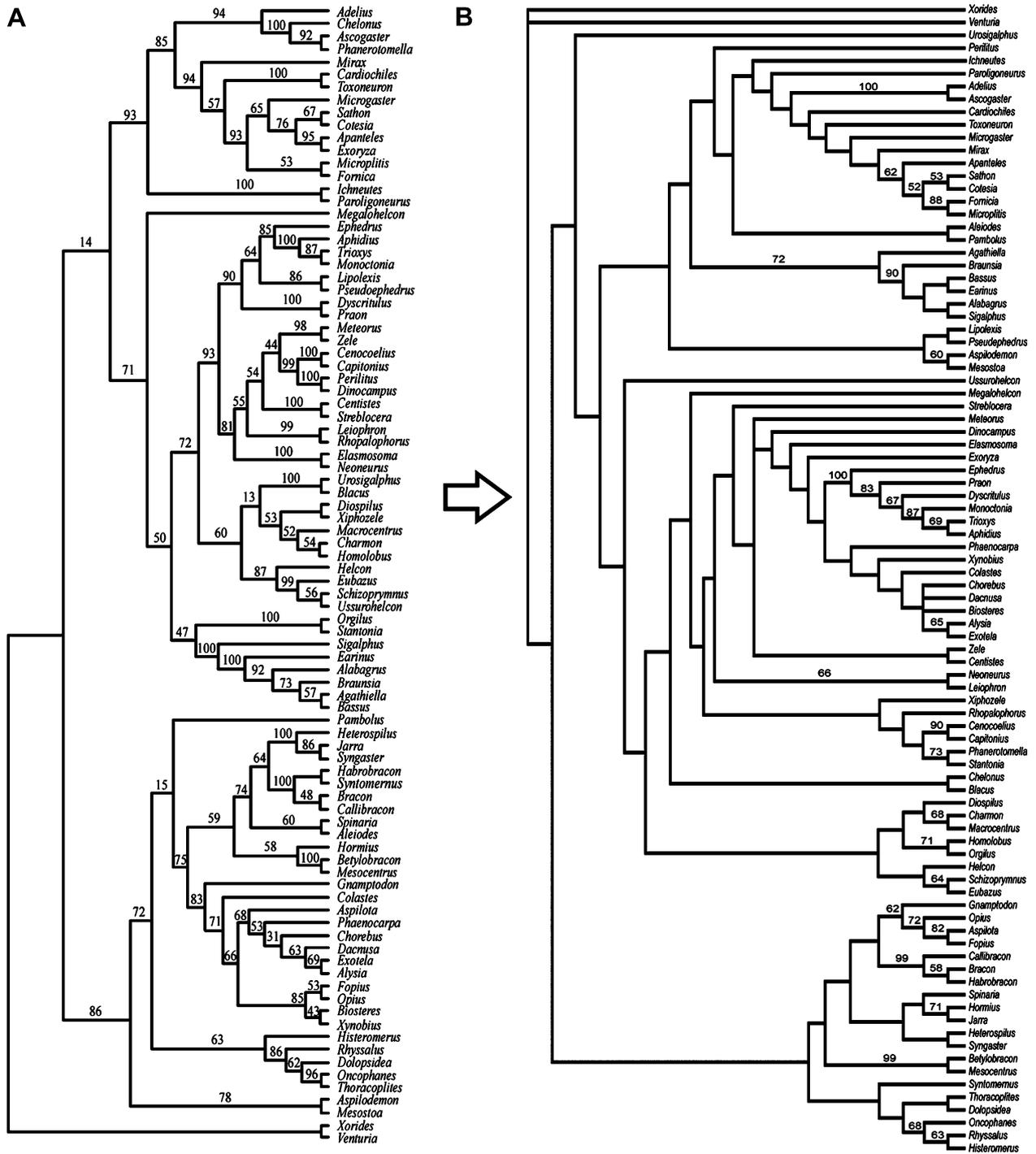


Fig. 1. (A) Phylogenetic hypothesis of braconid relationships presented by Shi et al. (2005) (Fig. 1, p. 110) based on a combination of three molecular markers (16S, 18S, and 28S) and morphology using maximum parsimony. Numbers at nodes represent bootstrap percentages based on 1000 pseudoreplicates. (B) Consensus of four minimum length trees ( $L = 7672$ ) from parsimony re-analysis of combined molecular and morphological data as presented by Shi et al. (2005). Numbers at nodes represent bootstrap percentages based on 1000 pseudoreplicates; nodes with less than 50% bootstrap support are not reported.

both Cardiochilinae and Cheloninae as monophyletic lineages within the microgastroids, but Cheloninae did not have bootstrap support over 50%.

The subfamilies comprising the helconoid complex were recovered in a variety of locations. The Euphorinae + Neoneurinae + Meteorinae (treated as part of Euphorinae by Shi et al., 2005) were recovered as sister to the microgast-

roid lineage (Fig. 2). Trachypetinae was recovered at the base of the phylogeny, sister to the clade containing all other taxa used in this analysis, and Blacinae + Acampsohelconinae were recovered as sister to a clade of cyclostome taxa. All other helconoid taxa were recovered as a monophyletic clade, sister to ((Euphorinae + Neoneurinae + Meteorinae) + microgastroid subfamilies). The two ichneu-

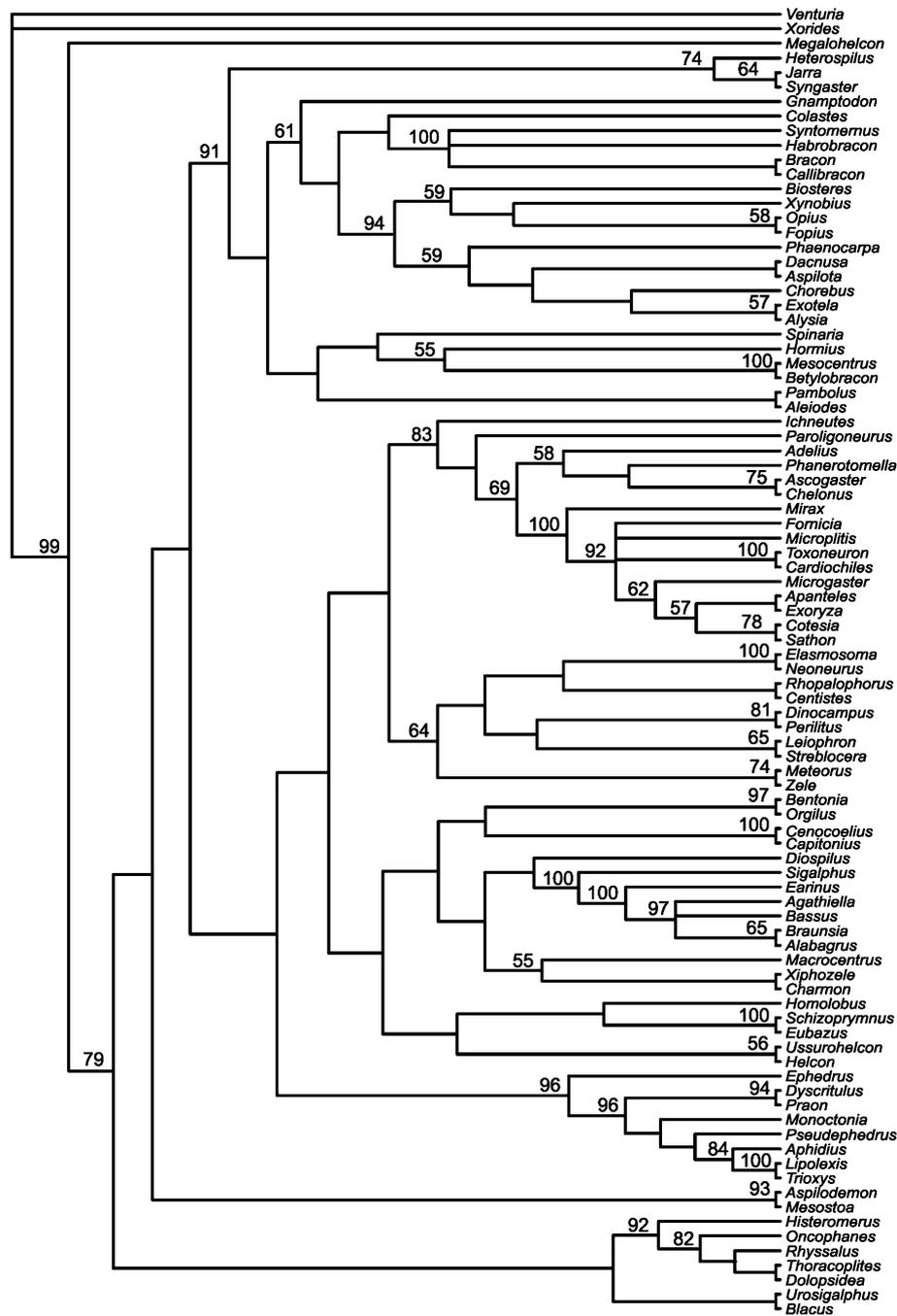


Fig. 2. Strict consensus of eight minimum length trees ( $L = 4747$ ) based on a secondary structure alignment of sequences retrieved from GenBank for three molecular markers (16S, 18S, and 28S) using maximum parsimony analysis. Numbers at nodes represent bootstrap percentages based on 6000 pseudoreplicates; nodes with less than 50% bootstrap support are not reported.

tine genera were recovered as a basal grade to the microgastroid subfamilies. Within the helconoids, Agathidinae, Cenocoeliinae, Neoneurinae, Meteorinae, and Orgilinae were all recovered as monophyletic lineages. Neoneurinae rendered Euphorinae polyphyletic, and Meteorinae was found as sister to (Euphorinae + Neoneurinae). This suggests that, contrary to the treatment by Shi et al. (2005), Meteorinae can be recognized as a distinct subfamily without making Euphorinae polyphyletic, but Neoneurinae may need to be synonymized with Euphorinae.

The cyclostome lineage formed a basal grade within the phylogeny recovered by analyses of our dataset (including the aforementioned helconoid taxa) (Fig. 2). Aphidiinae was recovered sister to the clade containing most helconoids + microgastroids. Within the cyclostomes, Opiinae, and Alysinae were recovered as monophyletic and as sister to each other. Braconinae was recovered monophyletic and sister to Exothecinae. Braconinae + Exothecinae were sister to the clade Opiinae + Alysinae. Interestingly, Rogadinae was not recovered as monophyletic, with *Aleo-*

ides being sister to *Pambolus* and *Spinaria* sister to (*Hormius* + (*Betylocracon* + *Mesocentrua*)). Rhysalinae was monophyletic, and sister to Histeromerinae. Hydrangeocolinae was sister to Mesostoinae.

Using our alignment, and neglecting morphological data, we hope to have established a clearer picture of the current understanding of braconid relationships at the generic and subfamilial levels. One possible explanation for the lack of resolution at the base of the phylogeny is that the dataset lacks a gene evolving at the appropriate rate to recover these relationships. Subfamilies such as Agathidinae, with previously established monophyly, were also recovered as monophyletic. However subfamilies with questionable monophyly, such as Helconinae, remain unresolved. It is this lack of resolution that is of interest, as it demonstrates how much we have yet to discover about braconid phylogeny.

## 5. Conclusion

Repeatability is an important component of scientific research. We were unable to reproduce the results of Shi et al. (2005) using their published methodologies, molecular alignment, and a slightly modified morphological dataset. Using a new alignment and excluding morphological data, we found the only demonstrably monophyletic subfamilies were Alysiniinae, Betylocraconinae, Braconinae, Doryctinae, Opiinae, Rhysalinae, Agathidinae, Cenocoeliinae, Orgilinae, Cardioc-hilinae, Aphidiinae, and Meteorinae. However, many of these subfamilies lacked sufficient numbers of exemplar taxa, potentially creating spurious results (e.g. Orgilinae and Doryctinae). Though we found a high level of resolution in the strict consensus tree of our parsimony analysis, there were many clades with less than 50% bootstrap support, suggesting that we have not sampled genes that are evolving at the proper rate to resolve many of the relationships within Braconidae, specifically those at the base of the tree.

## Acknowledgments

We thank three anonymous reviewers for comments on previous versions of this manuscript and Cheryl Lindsay for her help in production of the figures. Support for this research was provided by the Department of Entomology, University of Kentucky, and NSF Grants DEB-0205982 and EF-00337220 to M.J. Sharkey. This is paper 06-08-140 of the Agricultural Experiment Station, University of Kentucky.

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