



# Phylogeny of Ephemeroptera (mayflies) based on molecular evidence

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## Abstract

This study represents the first molecular phylogeny for the Order Ephemeroptera. The analyses included 31 of the 37 families, representing ~24% of the genera. Fifteen families were supported as being monophyletic, five families were supported as nonmonophyletic, and 11 families were only represented by one species, and monophyly was not testable. The suborders Furcatergalia and Carapacea were supported as monophyletic while Setisura and Pisciforma were not supported as monophyletic. The superfamilies Ephemerelloidea and Caenoidea were supported as monophyletic while Baetoidea, Siphonuroidea, Ephemeroidea, and Heptagenioidea were not. Baetidae was recovered as sister to the remaining clades. The mayfly gill to wing origin hypothesis was not supported nor refuted by these data. Mandibular tusks were supported as having at least one loss in Behningiidae and, together with the burrowing lifestyle, possibly two origins. The fishlike body form was supported as plesiomorphic for mayflies with multiple secondary losses. Topological sensitivity analysis was used as a tool to examine patterns concerning the stability of relationships across a parameter landscape, providing additional information that may not have been acquired otherwise.

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## 1. Introduction

Ephemeroptera (mayflies) is a monophyletic group of semi-aquatic pterygote insects, comprising 3083 species, 376 genera, and 37 described families (Brittain and Sartori, 2003). They are present on all continents, excluding Antarctica, and are associated with freshwater and brackish water habitats. Nymphs have much longer antennae, functioning mandibulate mouthparts, and are aquatic, in contrast to the adults which lack mouthparts and do not feed, relying on the nutritional buildup from the immature stages. Mayfly diversity is greatest in lotic habitats in the temperate and tropic regions, where they are an important part of the food chain, consuming primary producers such as algae and plants, and as a food source for vertebrate predators like fish. Additionally, these insects are used as bioindicators of

pollution and water quality. The adults are soft-bodied insects possessing short antennae, vestigial mouthparts, two long cerci, and typically possess a medial caudal filament originating from the last abdominal segment. Adult mayflies typically have two pairs of wings, however, the second pair is considerably smaller than the first and in some species is absent altogether. Ephemeroptera is unique among pterygote insects in possessing functional wings at the penultimate molt (subimago stage), prior to the full development of genitalia; in all other insects the presence of functional wings occurs only after the final molt (Brittain, 1982; Brittain and Sartori, 2003; Edmunds, 1996).

Ephemeroptera has been considered by many to be sister to Odonata + Neoptera (Fürst von Lieven, 2000; Kristensen, 1991; Staniczek, 2000; Wheeler et al., 2001; Whiting et al., 1997) although alternate hypotheses have been suggested (Boudreaux, 1979; Brodsky, 1994; Hennig, 1981; Hovmöller et al., 2002; Kukalova-Peck, 1991, 1997; Martynov, 1924; Matsuda, 1970; Riek and Kukalova-Peck, 1984). Recently, it was shown that, while data from three

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molecular loci ambiguously resolved basal pterygote relationships, total evidence analysis (combined molecular and morphological data) strongly supports the position of mayflies as sister to all other extant pterygotes (Ogden and Whiting, 2003).

Since, the current suite of evidence supports mayflies as sister group to all other winged insect orders, a robust phylogeny for Ephemeroptera should shed light on proposed hypotheses concerning the evolution of wings in insects. Specifically, the proposed hypothesis of pleural origins for wings from gills could be examined (Brodsky, 1994, 1978; 1983, 1991). One of the underlying assumptions of this hypothesis is that articulated pleural extensions, initially used as gills for respiration, served as a morphological transition to wings from the immature to the mature life stages. This particular assumption could be examined in a phylogenetic framework. For example, if the basal lineages of mayflies do not have highly innervated, movable, paddle-like gills, with well-developed associated musculature, then the proposed “mayfly gills to wing origin” hypothesis loses strength. Additionally, the pattern of loss of the imago of certain mayfly lineages could elucidate hypotheses concerning the homologies between mayfly life stages (subimago, imago) and life stages of other winged insects (imago). Moreover, these patterns could support or reject the notion that flight evolved out of the water as opposed to a terrestrial origin. There are also some interesting evolutionary trends within the mayflies that could be examined given a robust topology, such as the burrowing lifestyle and associated morphological features (i.e., mandibular tusks, gills, etc.), the fishlike body form and swimming behavior, and the presence of a carapace (fused pronotum, mesonotum, and wing buds), among others. Therefore, a robust phylogeny for Ephemeroptera should assist further investigation of important evolutionary trends, not only within the mayflies, but in the winged insect groups as well.

## 2. Review of mayfly classification and phylogeny

After the earliest taxonomic treatments (Leach, 1815; Linnaeus, 1758; Pictet, 1843–1845), more comprehensive works began to subdivide mayflies into more taxa based on diagnosed adult characters, with some larval structures depicted in the descriptions (Eaton, 1871, 1883–1888, 1968). Later classifications began to use more larval characters, due to their apparent usefulness in determining natural groupings (Ulmer, 1920) and this practice for proposing classifications continued up to the early 1970s (Demoulin, 1958; Edmunds, 1972; Edmunds and Traver, 1954; Tshernova, 1970). One occasion during this time period is worthy of mentioning. It was a symposium dealing with the phylogeny and higher classification of the Ephemeroptera that was held in Tallahassee, Florida in 1970. A number of phylogenetic schemes were presented at this meeting based on differing data and ideas (Edmunds, 1973; Koss, 1973; Landa, 1973; Riek, 1973). A discussion took place follow-

ing George Edmunds’ talk, which is summarized and discussed by McCafferty (1991b). The basic argument was whether classification systems should reflect phylogenetic branching sequences or not. It was recognized by many that, while conserving communicable nomenclatorial groupings is desirable in systematics, monophyly derived from synapomorphy should be the driving force behind any taxonomic classification (Farris, 1979; Hennig, 1966, 1979).

The most widely followed classification system to come out of the early attempts was that of McCafferty and Edmunds (1979), which was, in part, based on their earlier work (McCafferty and Edmunds, 1976). In this system, two suborders were delimited (Schistonota and Pannota) and a phylogeny was proposed (Fig. 1A). The major character that was used to distinguish between the two suborders was the extent of the fusion of the forewing pads to the thorax (fused in Pannota and divided in Schistonota). Other characters were suggested to support the monophyly of Pannota, such as: (1) reduced and protected gills in larvae; (2) relatively slow moving, inactive crawling or clinging behavior in larvae; (3) improved tracheal system in larvae; and (4) in the adults, highly tapered mesoscutellum extending posteriorly. Some taxa were recognized as exceptions to these character distributions, and no formal analysis was performed.

The 1979 classification was broadly used until McCafferty (1991b) proposed three different suborders (Pisciforma, Setisura, and Retracheata) and depicted the putative phylogenetic relationships within and among the suborders (Fig. 1B). Retracheata was defined by: (1) ventral tracheal anastomes present in abdominal segments 4–7 in addition to 8 and 9; and (2) abdominal visceral tracheae in segments 3–8 or 4–8. Setisura was proposed based on the following characters (Landa, 1973; Landa and Soldan, 1985): (1) highly developed maxillary and labial vestiture; (2) fusion of 2nd and 3rd segments of maxillary palps; (3) fusion of 2nd and 3rd segments of labial palps; (4) labial palp width broadened; (5) filamentous tufts on lamella and basal abdominal gills; (6) main anterior branch of tracheal trunk leads to labium; (7) gonads in dorsolateral or lateral position; and (8) forewing cubital intercaleries subparallel to CuA (McCafferty, 1991a). Pisciforma (nominally, an allusion to the minnow-like bodies and actions of the larvae) was not designated with any specific characters but was grouped based on similarities in leg segment proportions, claw development, ambulatory and swimming behavior, and cubital venation. McCafferty (1991b) choose not to propose familial phylogenetic relationships within the two larger assemblages of Pisciforma mayflies. As with the 1979 intuitive topology, taxonomic exceptions for the presence and absence of characters existed and these relationships were not based on any formal phylogenetic analysis, except within the suborder Setisura, where a cladistic analysis was performed (McCafferty, 1991b).

Concurrent to McCafferty’s work, Nikita Kluge (1988) independently proposed two suborders for Ephemeroptera.

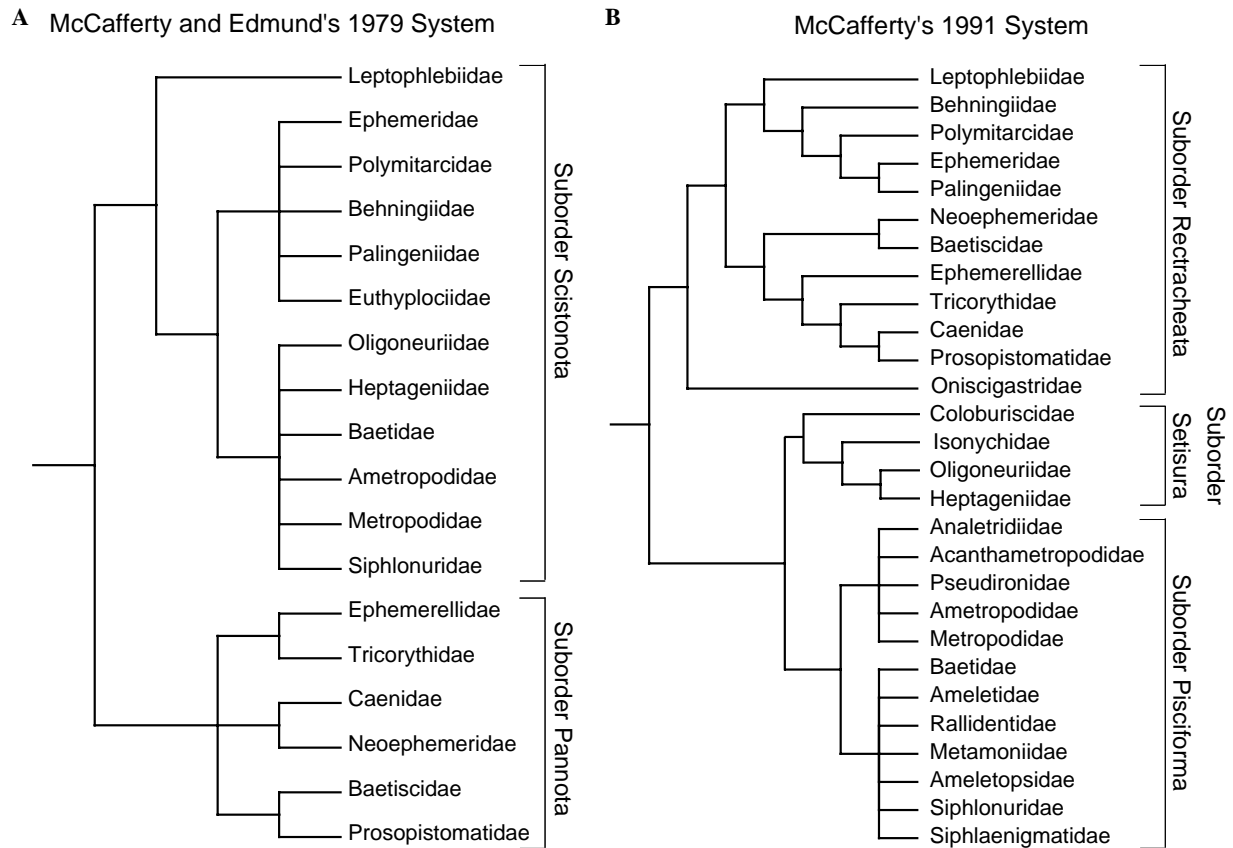


Fig. 1. (A) Topology representing the higher classification system of McCafferty and Edmunds (1979). (B) Topology summarizing the phylogenetic and classification system of McCafferty (1991b).

His suborder Furcatergalia is equivalent to McCafferty's Retracheata, except that Oniscigastridae is excluded from Furcatergalia. The other suborder proposed (Kluge, 1988) was Costatergalia, which is equal to McCafferty's (1991b) Pisciforma + Setisura + Oniscigastridae. While Kluge's work was based on extensive examination of morphological characters, no formal analysis was performed.

Since the McCafferty 1991 proposal, morphological cladistic analyses have been performed on some of the subgroups within Ephemeroptera, but not across Ephemeroptera as a whole. Table 1 summarizes studies focused on phylogenetic relationships for the major lineages of mayflies (i.e., family level and above) since the early 90s. Kluge's, 2004 topology (Fig. 2A) is a summary taken from his recently published book (Kluge, 2004) and was not based on any formal analysis of character data. The adjacent tree diagrammed in Fig. 2B (McCafferty's, 2004 hypothesis) is a compilation based partially on cladistic analysis for the Pannota (McCafferty and Wang, 2000) and Setisura (McCafferty, 1991a; Wang and McCafferty, 1995), from published trees (McCafferty, 1991b, 1997), and from personal communication with McCafferty.

The most recent systems for McCafferty and Kluge (2004) are mostly congruent (Fig. 2). We will follow, for the most part, McCafferty's nomenclatorial system comprised of 37 families placed in four suborders. Within mayflies,

both systems suggest that there are two major clades. Carapacea is currently considered the sister to the remaining taxa (Furcatergalia, Setisura, and Pisciforma). The characters that support this clade are: (1) notal shield or carapace; (2) tornus of forewing behind apex of CuP; (3) CuA, CuP, and AA are non-branched and nearly parallel to MP2; (4) synganglion in basisternum of mesothorax; and (5) imaginal and subimaginal furcasternal protuberances are contiguous medially (Kluge, 2004). The suborder Furcatergalia is placed as sister to Setisura + Pisciforma. The characters that support this grouping are similar to Retracheata above and Kluge (2004) describes two additional characters: (1) modified pleura of prothorax; and (2) 1st tarsal segment is strongly shortened in imago and subimago, although, some exceptions to these characters were discounted post hoc as secondary changes. Within Furcatergalia, Pannota, containing the sister groups Caenoidea (two families) and Ephemerelloidea (eight families), is sister to the clade Leptophlebiidae + (Behningiidae + Ephemeroidea). Note that McCafferty considers the Behningiidae, a group of burrowing mayflies that lack mandibular tusks, as a separate lineage not nested within the other five families of burrowing mayflies. The suborder Setisura (superfamily Heptagenioidea) is comprised of six families, three of which are monogeneric (Isonychiidae, Pseudironidae, and Arthropleidae). The characters listed above for

Table 1  
Review of Ephemeroptera studies since McCafferty's 1991 system that have investigated relationships above the family level

Families included in study	Analysis	# of characters	Author(s)
Order Ephemeroptera			
Most major lineages	Cladistic	45	(Tomka and Elpers, 1991)
Most major lineages	Intuitive	N/A	(McCafferty, 1997)
Most major lineages	Intuitive	N/A	McCafferty, 2003 (Website)
Most major lineages	Intuitive	N/A	(Kluge, 2004)
Suborder Pisciforma			
Acanthametropodidae, Siphuriscidae, Siphonuridae	Cladistic	11	(McCafferty and Wang, 1994)
Nesameletidae	Cladistic	16	(Hitchings and Staniczek, 2003)
Siphonuridae, Dipteromimidae, Ameletidae, Metropodidae, Acanthametropodidae, Ametropodidae, Oniscigastridae, Nesameletidae, Rallidentidae, Ameletopsidae	Intuitive	N/A	(Kluge et al., 1995)
Metropodidae, Ametropodidae, Siphonuridae	Cladistic	6	(Studemann and Tomka, 1991)
Siphuriscidae, Siphonuridae, Nesameletidae	Intuitive	N/A	(Zhou and Peters, 2003)
Suborder Setisura			
Heptageniidae, Oligoneuriidae, Isonychiidae, Coloburiscidae	Cladistic	36	(McCafferty, 1991a)
Heptageniidae, Pseudironidae, Arthropleidae	Cladistic	10	(Wang and McCafferty, 1995)
Suborder Furcatergalia			
Neophemeridae, Caenidae	Intuitive	N/A	(Wang et al., 1997)
Teloganodidae, Vietnamellidae	Cladistic	30	(McCafferty and Wang, 1997)
Neophemeridae, Caenidae, Teloganodidae, Vietnamellidae, Ephemerellidae, Teloganellidae, Ephemerithidae, Machadorythidae, Tricorythidae, Leptohiphidae	Cladistic	49	(McCafferty and Wang, 2000)
Potamanthidae	Cladistic	45	(Bae and McCafferty, 1991)
Potamanthidae, Euthyplociidae, Ichthybotidae, Ephemeridae, Polymitarciidae, Behningiidae	Intuitive	N/A	(Kluge, 2003)

In the Analysis column, the term Cladistic refers to studies that used coded characters and formally tested them; while the term Intuitive indicates that no coding or formal analysis was performed.

Setisura mostly apply for this grouping, and Kluge (2004) suggests a couple others: (1) strongly shortened prealar bridge of mesothorax; and (2) eggs have knob terminated coiled threads. The suborder Pisciforma is comprised of two superfamilies (Baetoidea and Siphonuroidea) containing the remaining 12 families. McCafferty again gives no specific characters for this group except the idea of a fish-like body form and swimming movement. Kluge recognizes that most included taxa present three dentisetae with exceptions in *Ameletus*, *Metreletus*, and *Acanthametropus*, yet he also concluded that his Tridentiseta is most likely not a monophyletic assemblage (Kluge, 2004). Both McCafferty and Kluge refrain from making any hypotheses concerning the relationships within the Pisciforma except for the division of the two superfamilies.

The purpose of this article is to present the first quantitative analysis of phylogenetic relationships within the order Ephemeroptera, with emphasis on reconstructing higher-level relationships. Specifically, we address: (1) Are the proposed suborders, superfamilies, and families monophyletic? (2) What are the relationships among these major lineages? (3) What nodal stability and support do these data provide for addressing these questions? (4) What evolutionary trends do these data support, specifically concerning the mayfly gill to wing origin hypothesis, fishlike body form, mandibular tusks and burrowing lifestyle, and presence of notal shield or carapace in larvae.

### 3. Materials and methods

#### 3.1. Taxon sampling

Taxonomic sampling consisted of exemplars representing 94 spp. of Ephemeroptera, 9 spp. of Odonata, and 5 spp. of non-apterygote insects for a total of 108 taxa (Table 2). All direct optimization analyses were rooted to the Collembola (Hypogastruridae). Within Ephemeroptera, 89 genera, from all four suborders, and from 31 families, representing ~24% of the genera and 84% of families were included. Numerous genera from large, diverse families were included to better represent the major lineages within these families. For example, 12 species of Heptageniidae and 8 species of Baetidae, two of the largest of the mayfly families, were sampled. Only the families Vietnamellidae, Ephemerithidae, Machadorythidae, Teloganodidae, Tricorythidae, and Teloganellidae were not represented. These are, in most cases, monogeneric families from the Old World, and material has not yet been acquired. The representatives from the families Ephemerellidae and Leptohiphidae should be sufficient to address the position of Ephemerelloidea. No morphological matrix exists across all mayflies, thus morphological data were not included in this analysis. Collaboration is currently under way to code morphological characters across all major lineages of mayflies and outgroups. Nevertheless, the evolutionary morphological trends that we discuss (i.e., movable gills, fishlike body form, mandibular tusks, burrowing lifestyle,

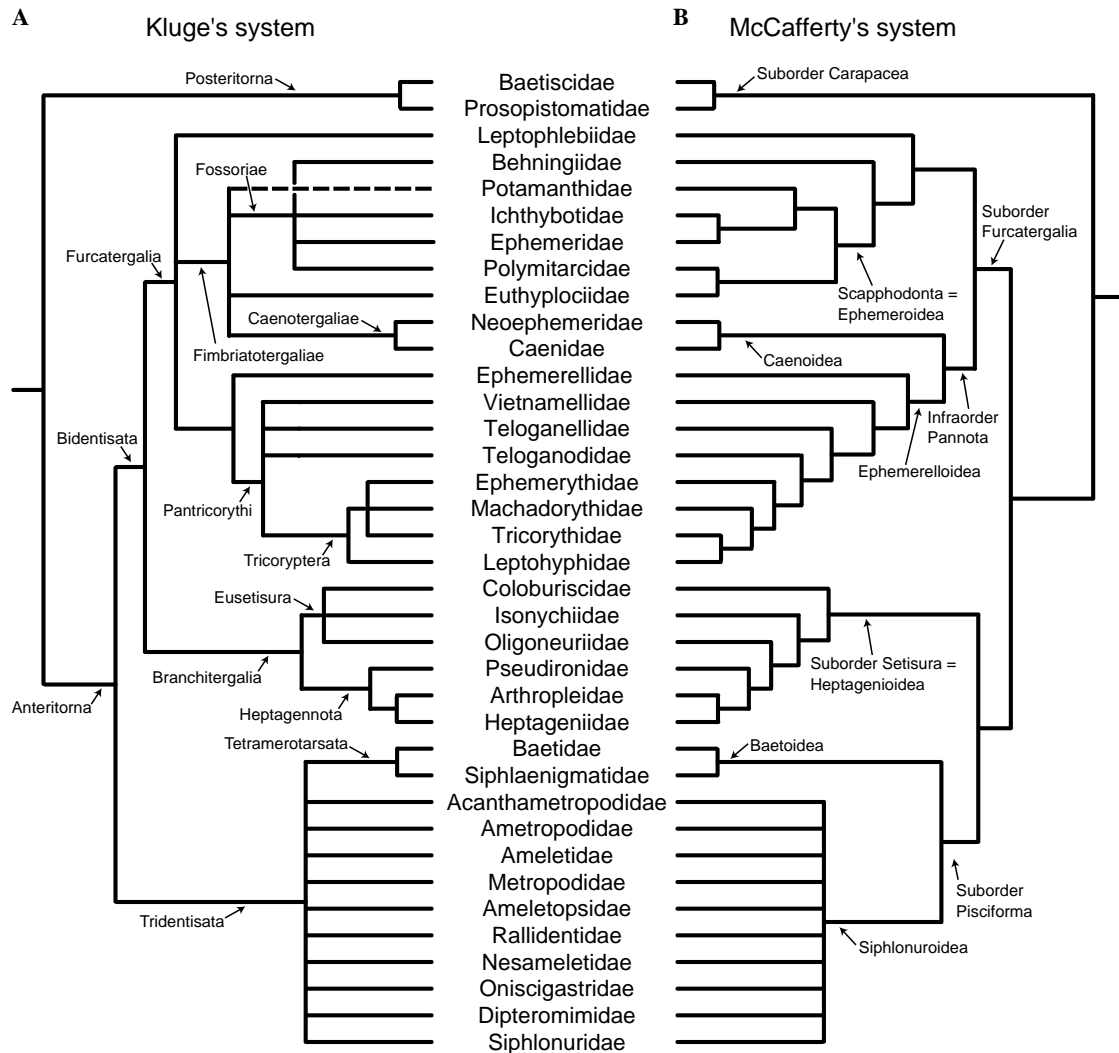


Fig. 2. Topological comparison of the two most recent systems of mayfly classification. (A) Topology summarizing the phylogenetic relationships of the non-Linnaean nomenclatorial system hypothesized by Kluge (2004). (B) Topology synthesizing the previous studies and personal communications of McCafferty.

and carapace), were coded and parsimony character optimization was performed on the most parsimonious topology and the likelihood topology in MacClade (Maddison and Maddison, 2000).

Muscle tissue was dissected, incubated, and DNA was extracted following the Qiagen DNeasy protocol for animal tissue (Valencia, CA). Genomic DNA vouchers and specimen vouchers were deposited at the Insect Genomics Collection (IGC), M.L. Bean Museum, Brigham Young University. Templates and controls were amplified in a Perkin-Elmer 9700 thermocycler using primers modified for insects. Five genes were targeted for amplification and sequencing: 18S rDNA (18S), 28S rDNA (28S), 16S rDNA (16S), 12S rDNA, and Histone three protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are given in Whiting (2002). Mayfly specific primers for certain regions of 28S are presented in this study (Table 3). Primer sequences for the gene H3 are given in Ogden and Whiting (2003). Primers for 12S rDNA are: 12Sai: 5' AACTACGATTAGATACCCTATTAT 3';

12Sbi: 5' AAGAGCGACGGGCGATGTGT 3'. Primers for 16S rDNA are: 16Sa: 5'GCCTGTTTATCAAAA CAT 3'; 16Sb: 5' CTCCGGTTTGAACCTCAGATCA 3'. Product yield, specificity, and potential contamination were monitored via agarose gel electrophoresis. The successful amplicons were purified and cycle-sequenced using ABI Prism Big Dye Terminator version 3.0 chemistry. The sequencing reactions were column purified and analyzed with the ABI 3100 automated sequencer. In all cases, DNA was sequenced from complementary strands, with sufficient overlap for the larger genes to ensure accuracy of the results. Manual correction of chromatography data was facilitated by the program Sequencher 4.0 (GeneCodes, 1999). GenBank Accession Numbers are given in Table 1.

### 3.2. Phylogenetic analyses

Sequences were initially assembled in Sequencher 4.0 (GeneCodes, 1999). The protein coding H3 gene was manually

Table 2  
Taxon list and GenBank Accession Numbers

Order	Family	Genus	Species	12S	16S	18S	28S	H3
Collembola	Hypogastruridae			<u>AY191995</u>	<u>AY191995</u>	<u>AY338691</u>	<u>AY338648</u>	<u>AY338616</u>
Diplura	Campodeidae			<u>AF252400</u>	<u>AF370868</u>	<u>AY338692</u>	<u>AY338649</u>	—
Archaeognatha	Machilidae			<u>AF252403</u>	<u>AF370871</u>	<u>AY338689</u>	<u>AY338646</u>	<u>AY338614</u>
Zygentoma	Lepismatidae	<i>Thermobia</i>	sp.	—	<u>AF370872</u>	<u>AY338726</u>	<u>AY338683</u>	<u>AY338644</u>
	Lepidotrichidae	<i>Tricholepidion</i>	sp.	<u>AY191994</u>	<u>AY191994</u>	<u>AY338727</u>	<u>AY338684</u>	<u>AY338645</u>
Ephemeroptera	Acanthametropodidae	<i>Analetris</i>	<i>eximia</i>	<u>AY749669</u>	<u>AY749797</u>	<u>AY338697</u>	<u>AY338654</u>	<u>AY338620</u>
	Ameletidae	<i>Ameletus</i>	sp.	<u>AY749644</u>	<u>AY749767</u>	<u>AY338712</u>	<u>AY338669</u>	<u>AY338632</u>
	Ameletopsidae	<i>Ameletopsis</i>	<i>perscitus</i>	<u>AY749685</u>	<u>AY749820</u>	<u>AY749895</u>	<u>AY750020</u>	—
	Ameletopsidae	<i>Chaquihua</i>	sp.	<u>AY749659</u>	<u>AY749784</u>	<u>AY338715</u>	<u>AY338672</u>	<u>AY338635</u>
	Ameletopsidae	<i>Chiloptorer</i>	sp.	<u>AY749651</u>	<u>AY749776</u>	<u>AY749852</u>	<u>AY749939</u> ,	—
							<u>AY749940</u> ,	
							<u>AY749941</u>	
	Ameletopsidae	<i>Mirawara</i>	sp.	<u>AY749661</u>	<u>AY749788</u>	<u>AY749867</u>	<u>AY749963</u> ,	<u>AY749720</u>
							<u>AY749964</u>	
	Ametropodidae	<i>Ametropus</i>	<i>neavei</i>	<u>AY749671</u>	<u>AY749800</u>	<u>AY338700</u>	<u>AY338657</u>	<u>AY338622</u>
	Arthropleidae	<i>Arthroplea</i>	<i>bipunctata</i>	<u>AY749683</u>	<u>AY749817</u>	—	<u>AY750015</u>	—
	Baetidae	<i>Baetis</i>	sp.	—	—	<u>AY338695</u>	<u>AY338652</u>	<u>AY338619</u>
	Baetidae	<i>Baetis</i>	<i>tricaudatus</i>	—	<u>AY749771</u>	<u>AY749848</u>	<u>AY749932</u> ,	<u>AY749708</u>
							<u>AY749933</u>	
	Baetidae	<i>Callibaetis</i>	sp.	—	<u>AY749764</u>	<u>AY749842</u>	<u>AY749925</u>	<u>AY749703</u>
	Baetidae	<i>Centroptilum</i>	<i>luteolum</i>	<u>AY749649</u>	<u>AY749774</u>	<u>AY749850</u>	<u>AY749935</u>	<u>AY749710</u>
	Baetidae	<i>Dipheter</i>	sp.	—	<u>AY749808</u>	<u>AY749881</u>	<u>AY749989</u> ,	<u>AY749733</u>
							<u>AY749990</u>	
	Baetidae	<i>Jugabaetis</i>	sp.	—	<u>AY749813</u>	<u>AY749887</u>	<u>AY750002</u> ,	<u>AY749738</u>
							<u>AY750003</u>	
	Baetidae	<i>Platybaetis</i>	<i>probus</i>	—	<u>AY749812</u>	<u>AY749886</u>	<u>AY750000</u> ,	<u>AY749737</u>
							<u>AY750001</u>	
	Baetidae 2	<i>Mystaxiops</i>	<i>venatoris</i>	<u>AY749647</u>	<u>AY749772</u>	<u>AY749849</u>	<u>AY749934</u>	<u>AY749708</u>
	Baetiscidae	<i>Baetisca</i>	<i>lacustris</i>	—	—	<u>AY749865</u>	<u>AY749960</u> ,	—
							<u>AY749961</u>	
	Baetiscidae	<i>Baetisca</i>	sp.	—	—	<u>AY338707</u>	<u>AY338664</u>	<u>AY338627</u>
	Behningiidae	<i>Behningia</i>	sp.	<u>AY749678</u>	<u>AY749809</u>	<u>AY338703</u>	<u>AY338660</u>	—
	Behningiidae	<i>Dolania</i>	<i>americana</i>	<u>AY749673</u>	<u>AY749802</u>	<u>AY749875</u>	<u>AY749978</u>	—
	Caenidae	<i>Brachycercus</i>	<i>harrisella</i>	<u>AY749687</u>	<u>AY749822</u>	<u>AY749896</u>	<u>AY750021</u>	—
	Caenidae	<i>Caenis</i>	sp.	<u>AY749641</u>	<u>AY749763</u>	<u>AY338710</u>	<u>AY338667</u>	<u>AY338630</u>
	Caenidae	<i>Genus Y</i>	sp.	<u>AY749679</u>	—	<u>AY749883</u>	<u>AY749993</u> ,	—
							<u>AY749994</u> ,	
							<u>AY749995</u>	
	Caenidae	<i>Madecocercus</i>	sp.	—	—	<u>AY749906</u>	<u>AY750035</u>	<u>AY749748</u>
	Caenidae	<i>Tasmanocaenis</i>	sp.	<u>AY749689</u>	<u>AY749824</u>	<u>AY749898</u>	<u>AY750023</u> ,	—
							<u>AY750024</u> ,	
							<u>AY750025</u>	
	Coloburiscidae	<i>Coloburiscoides</i>	sp.	—	<u>AY749789</u>	<u>AY749868</u>	<u>AY749965</u>	<u>AY749721</u>
	Coloburiscidae	<i>Coloburiscus</i>	<i>humeralis</i>	<u>AY749686</u>	<u>AY749821</u>	<u>AY338706</u>	<u>AY338663</u>	<u>AY338626</u>
	Coloburiscidae	<i>Murphyella</i>	sp.	—	—	<u>AY749861</u>	<u>AY749956</u>	<u>AY749716</u>
	Dipteromimidae	<i>Dipteromimus</i>	sp.	<u>AY749684</u>	<u>AY749819</u>	<u>AY749894</u>	<u>AY750018</u> ,	<u>AY749744</u>
							<u>AY750019</u>	
	Ephemerelliidae	<i>Attenella</i>	<i>margarita</i>	<u>AY749676</u>	<u>AY749805</u>	<u>AY749879</u>	<u>AY749985</u> ,	<u>AY749731</u>
							<u>AY749986</u>	
	Ephemerelliidae	<i>Caudatella</i>	<i>hystrix</i>	—	<u>AY749787</u>	<u>AY749866</u>	<u>AY749962</u>	<u>AY749719</u>
	Ephemerelliidae	<i>Drumella</i>	<i>doddsi</i>	—	<u>AY749756</u>	<u>AY749836</u>	<u>AY749915</u> ,	<u>AY749698</u>
							<u>AY749916</u>	
	Ephemerelliidae	<i>Ephemerella</i>	sp.	—	—	<u>AY749838</u>	<u>AY749918</u> ,	<u>AY749700</u>
							<u>AY749919</u>	
	Ephemerelliidae	<i>Hyrtanella</i>	<i>pascalae</i>	—	<u>AY749815</u>	<u>AY749891</u>	<u>AY750012</u>	<u>AY749742</u>
	Ephemeridae	<i>Hexagenia</i>	sp.	—	—	<u>AY121136</u>	<u>AY125276</u>	<u>AY125223</u>
	Ephemeridae	<i>Plethogenesia</i>	sp.	<u>AY749674</u>	<u>AY749803</u>	<u>AY749876</u>	<u>AY749979</u> ,	<u>AY749728</u>
							<u>AY749980</u>	
	Euthyplociidae	<i>Euthyplocia</i>	<i>hecuba</i>	<u>AY749653</u>	<u>AY749779</u>	<u>AY749855</u>	<u>AY749946</u>	<u>AY749712</u>
	Euthyplociidae	<i>Polyplacia</i>	sp.	—	—	<u>AY338705</u>	<u>AY338662</u>	<u>AY338625</u>
	Heptageniidae	<i>Afronurus</i>	<i>peringueyi</i>	<u>AY749662</u>	<u>AY749790</u>	<u>AY749869</u>	<u>AY749966</u> ,	—
							<u>AY749967</u>	
	Heptageniidae	<i>Atopopus</i>	sp.	<u>AY749681</u>	—	<u>AY749885</u>	<u>AY749998</u> ,	<u>AY749736</u>
							<u>AY749999</u>	

Table 2 (continued)

Order	Family	Genus	Species	12S	16S	18S	28S	H3
	Heptageniidae	<i>Cinygma</i>	sp.	<u>AY749675</u>	<u>AY749804</u>	<u>AY749878</u>	<u>AY749983</u> , <u>AY749984</u>	<u>AY749730</u>
	Heptageniidae	<i>Cinygmina</i>	sp.	<u>AY749680</u>	<u>AY749811</u>	<u>AY749884</u>	<u>AY749996</u> , <u>AY749997</u>	<u>AY749735</u>
	Heptageniidae	<i>Cinygmula</i>	sp.	<u>AY749640</u>	<u>AY749760</u>	<u>AY338704</u>	<u>AY338661</u>	<u>AY338624</u>
	Heptageniidae	<i>Ecdyonus</i>	<i>dispau</i>	<u>AY749650</u>	<u>AY749775</u>	<u>AY749851</u>	<u>AY749936</u> , <u>AY749937</u> , <u>AY749938</u>	<u>AY749711</u>
	Heptageniidae	<i>Epeorus</i>	<i>longimanus</i>	<u>AY749638</u>	<u>AY749757</u>	<u>AY749837</u>	<u>AY749917</u>	<u>AY749699</u>
	Heptageniidae	<i>Heptagenia</i>	sp.	—	<u>AY749762</u>	<u>AY338709</u>	<u>AY338666</u>	<u>AY338629</u>
	Heptageniidae	<i>Leucrocuta</i>	<i>maculipennis</i>	—	—	<u>AY749864</u>	<u>AY749959</u>	—
	Heptageniidae	<i>Macdunnoa</i>	<i>hipawinia</i>	<u>AY749668</u>	<u>AY749796</u>	<u>AY749874</u>	<u>AY749977</u>	<u>AY749727</u>
	Heptageniidae	<i>Rithrogena</i>	sp.	<u>AY749642</u>	<u>AY749765</u>	<u>AY749843</u>	<u>AY749926</u>	<u>AY749704</u>
	Heptageniidae	<i>Stenonema</i>	<i>vicarium</i>	<u>AY749645</u>	<u>AY749769</u>	<u>AY749845</u>	<u>AY749928</u>	—
	Ichthybotidae	<i>Ichthybotus</i>	<i>hudsoni</i>	<u>AY749682</u>	<u>AY749816</u>	<u>AY749892</u>	<u>AY750013</u> , <u>AY750014</u>	<u>AY749743</u>
	Isonychiidae	<i>Isonychia</i>	sp.	—	<u>AY749761</u>	<u>AY338708</u>	<u>AY338665</u>	<u>AY338628</u>
	Isonychiidae	<i>Isonychia</i>	sp.	—	—	<u>AY749889</u>	<u>AY750006</u> , <u>AY750007</u> , <u>AY750008</u>	<u>AY749740</u>
	Leptohyphidae	<i>Allenhyphes</i>	<i>flinti</i>	<u>AY749677</u>	<u>AY749807</u>	<u>AY749880</u>	<u>AY749987</u> , <u>AY749988</u>	<u>AY749732</u>
	Leptohyphidae	<i>Callistina</i>	<i>panda</i>	<u>AY749693</u>	—	<u>AY749907</u>	<u>AY750036</u> , <u>AY750037</u>	—
	Leptohyphidae	<i>Leptohyphes</i>	<i>zalope</i>	<u>AY749648</u>	<u>AY749773</u>	<u>AY338714</u>	<u>AY338671</u>	<u>AY338634</u>
	Leptohyphidae	<i>Travehyphes</i>	<i>indicator</i>	<u>AY749652</u>	<u>AY749778</u>	<u>AY749854</u>	<u>AY749943</u> , <u>AY749944</u> , <u>AY749945</u>	—
	Leptohyphidae	<i>Tricorythodes</i>	sp.	<u>AY749639</u>	<u>AY749758</u>	<u>AY749839</u>	<u>AY749920</u> , <u>AY749921</u>	—
	Leptohyphidae	<i>Yaurina</i>	<i>mota</i>	—	<u>AY749777</u>	<u>AY749853</u>	<u>AY749942</u>	—
	Leptophlebiidae	<i>Adenophlebia</i>	<i>peringueyella</i>	<u>AY749663</u>	<u>AY749791</u>	<u>AY749870</u>	<u>AY749968</u> , <u>AY749969</u> , <u>AY749970</u>	<u>AY749722</u>
	Leptophlebiidae	<i>Austrophleboides</i>	sp.	—	—	<u>AY749846</u>	<u>AY749929</u>	<u>AY749706</u>
	Leptophlebiidae	<i>Choroerpes</i>	sp.	—	—	<u>AY749844</u>	<u>AY749927</u>	<u>AY749705</u>
	Leptophlebiidae	<i>Meridialaris</i>	<i>diguillina</i>	<u>AY749657</u>	—	<u>AY749859</u>	<u>AY749952</u> , <u>AY749953</u>	<u>AY749714</u>
	Leptophlebiidae	<i>Paraleptophlebia</i>	<i>vaciva</i>	—	<u>AY749759</u>	<u>AY749840</u>	<u>AY749922</u> , <u>AY749923</u>	<u>AY749701</u>
	Leptophlebiidae	<i>Penaphlebia</i>	sp.	<u>AY749656</u>	<u>AY749782</u>	<u>AY749858</u>	<u>AY749950</u> , <u>AY749951</u>	<u>AY749713</u>
	Leptophlebiidae	<i>Thraulodes</i>	sp.	—	—	<u>AY749841</u>	<u>AY749924</u>	<u>AY749702</u>
	Leptophlebiidae			<u>AY749646</u>	<u>AY749770</u>	<u>AY749847</u>	<u>AY749930</u> , <u>AY749931</u>	<u>AY749707</u>
	Metropodidae	<i>Metretopus</i>	<i>borealis</i>	—	<u>AY749798</u>	<u>AY338698</u>	<u>AY338655</u>	<u>AY338621</u>
	Metropodidae	<i>Siphloplecton</i>	<i>interlineatum</i>	<u>AY749667</u>	<u>AY749795</u>	<u>AY749873</u>	<u>AY749976</u>	<u>AY749726</u>
	Neophemeridae	<i>Neophemera</i>	<i>youngi</i>	—	<u>AY749806</u>	<u>AY338702</u>	<u>AY338659</u>	—
	Nesameletidae	<i>Metamonius</i>	sp.	<u>AY749658</u>	<u>AY749783</u>	<u>AY749860</u>	<u>AY749954</u> , <u>AY749955</u>	<u>AY749715</u>
	Nesameletidae	<i>Nesameletus</i>	<i>ornatus</i>	<u>AY749665</u>	<u>AY749793</u>	<u>AY749872</u>	<u>AY749974</u> , <u>AY749975</u> , <u>AY749973</u>	<u>AY749724</u>
	Oligoneuriidae	<i>Elassoneuria</i>	sp.	—	—	<u>AY749905</u>	<u>AY750034</u>	<u>AY749747</u>
	Oligoneuriidae	<i>Homoeoneuria</i>	<i>alleni</i>	—	—	<u>AY749902</u> , <u>AY749903</u>	<u>AY750031</u>	—
	Oligoneuriidae	<i>Lachlania</i>	<i>dominguez</i>	<u>AY749654</u>	<u>AY749780</u>	<u>AY749856</u>	<u>AY749947</u>	—
	Oligoneuriidae	<i>Lachlania</i>	<i>saskatchewanensis</i>	<u>AY749672</u>	<u>AY749801</u>	<u>AY338701</u>	<u>AY338658</u>	<u>AY338623</u>
	Oligoneuriidae	<i>Oligoneuriella</i>	<i>rhenana</i>	<u>AY749688</u>	<u>AY749823</u>	<u>AY749897</u>	<u>AY750022</u>	<u>AY749745</u>
	Oniscigastridae	<i>Oniscigaster</i>	<i>distans</i>	—	<u>AY749818</u>	<u>AY749893</u>	<u>AY750016</u> , <u>AY750017</u>	—
	Oniscigastridae	<i>Siphlonella</i>	sp.	—	<u>AY749785</u>	<u>AY749862</u>	<u>AY749957</u>	<u>AY749717</u>
	Oniscigastridae	<i>Tasmanophlebia</i>	sp.	<u>AY749690</u>	<u>AY749825</u>	<u>AY749899</u>	<u>AY750026</u> , <u>AY750027</u>	—

(continued on next page)

Table 2 (continued)

Order	Family	Genus	Species	12S	16S	18S	28S	H3
	Polymitarciidae	<i>Proboscidoplocia</i>	sp.	<u>AY749692</u>	—	<u>AY749901</u>	<u>AY750030</u>	<u>AY749746</u>
	Polymitarciidae	<i>Tortopus</i>	sp.	<u>AY749655</u>	<u>AY749781</u>	<u>AY749857</u>	<u>AY749948</u> , <u>AY749949</u>	—
	Potamanthidae	<i>Anthopotamus</i>	sp.	<u>AY749643</u>	<u>AY749766</u>	<u>AY338711</u>	<u>AY338668</u>	<u>AY338631</u>
	Potamanthidae	<i>Rhoenanthus</i>	sp.	—	—	<u>AY749890</u>	<u>AY750009</u> , <u>AY750010</u> , <u>AY750011</u>	<u>AY749741</u>
	Potamanthidae	<i>Stygifloris</i>	sp.	—	<u>AY749814</u>	<u>AY749888</u>	<u>AY750004</u> , <u>AY750005</u>	<u>AY749739</u>
	Prosopistomatidae	<i>Prosopistoma</i>	sp.	—	<u>AY749827</u>	<u>AY749904</u>	<u>AY750032</u> , <u>AY750033</u>	—
	Prosopistomatidae	<i>Prosopistoma</i>	<i>wouerae</i>	—	<u>AY749810</u>	<u>AY749882</u>	<u>AY749991</u> , <u>AY749992</u>	<u>AY749734</u>
	Pseudironidae	<i>Pseudiron</i>	<i>centralis</i>	<u>AY749670</u>	<u>AY749799</u>	<u>AY338699</u>	<u>AY338656</u>	—
	Rallidentidae	<i>Rallidens</i>	<i>mcfarlanei</i>	<u>AY749666</u>	<u>AY749794</u>	<u>AY338696</u>	<u>AY338653</u>	<u>AY749725</u>
	Siphlaenigmatidae	<i>Siphlaenigma</i>	<i>janae</i>	<u>AY749664</u>	<u>AY749792</u>	<u>AY749871</u>	<u>AY749971</u> , <u>AY749972</u>	<u>AY749723</u>
	Siphonuridae	<i>Ameletoides</i>	sp.	<u>AY749691</u>	<u>AY749826</u>	<u>AY749900</u>	<u>AY750028</u> , <u>AY750029</u>	—
	Siphonuridae	<i>Edmundsius</i>	<i>agilis</i>	—	—	<u>AY749877</u>	<u>AY749981</u> , <u>AY749982</u>	<u>AY749729</u>
	Siphonuridae	<i>Paramaetus</i>	<i>columbiae</i>	—	<u>AY749768</u>	<u>AY338713</u>	<u>AY338670</u>	<u>AY338633</u>
	Siphonuridae	<i>Siphonurus</i>	sp.	<u>AY749660</u>	<u>AY749786</u>	<u>AY749863</u>	<u>AY749958</u>	<u>AY749718</u>
Odonata	Aeshnidae	<i>Anax</i>	<i>junius</i>	<u>AY749694</u>	<u>AY749829</u>	<u>AY749908</u>	<u>AY750038</u> , <u>AY750039</u>	<u>AY749749</u>
	Coenagrionidae	<i>Hesperagrion</i>	sp.	<u>AY749697</u>	<u>AY749832</u>	<u>AY749911</u>	<u>AY750043</u> , <u>AY750044</u>	<u>AY749752</u>
	Diphlebiidae	<i>Diphlebia</i>	<i>coerulescens</i>	—	<u>AY749833</u>	<u>AY749912</u>	<u>AY750045</u> , <u>AY750046</u>	<u>AY749753</u>
	Epiophlebiidae	<i>Epiophlebia</i>	<i>superstes</i>	<u>AF26094</u>	<u>AF26094</u>	<u>AF461247</u>	<u>AF461208</u>	—
	Gomphidae	<i>Progomphus</i>	<i>obscurus</i>	<u>AY749695</u>	<u>AY749830</u>	<u>AY749909</u>	<u>AY750040</u> , <u>AY750041</u>	<u>AY749750</u>
	Isostictidae	<i>Labidiosticta</i>	<i>vallisi</i>	—	<u>AY749834</u>	<u>AY749913</u>	<u>AY750047</u> , <u>AY750048</u> , <u>AY750049</u>	<u>AY749754</u>
	Lestidae	<i>Lestes</i>	sp.	—	<u>AY749828</u>	<u>AY338721</u>	<u>AY338677</u>	—
	Libellulidae	<i>Erythemis</i>	sp.	<u>AY749696</u>	<u>AY749831</u>	<u>AY749910</u>	<u>AY750042</u>	<u>AY749751</u>
	Megapodagrionidae	<i>Griseargiolestes</i>	<i>olbesens</i>	—	<u>AY749835</u>	<u>AY749914</u>	<u>AY750050</u> , <u>AY750051</u>	<u>AY749755</u>

Table 3  
Ephemeroptera specific primers for 28S rDNA

Primer Name	Primer Sequence (5' to 3')	Approximate bp position
28S EP2a	GAGTCGGGTTGCTTGAGAGTG	170
28S EP3a	AGTACCGTGAGGGAAAGTTG	250
28S EP4a	CGTCTTGAACACGGACCAA	780
28S EP5a	GGTTGCTTAAGACAGCAGGA	1400
28S EP2b	CACTCTCAAGCAACCCGACTC (Reverse complement of 28S EP2a)	170
28S EP3b	CAACTTTCCTCACGGTACT (Reverse complement of 28S EP3a)	250
28S EP4b	TTGGTCCGTGTTTCAAGACG (Reverse complement of 28S EP4a)	780
28S EP5b	TCCTGCTGTCTTAAGCAACC (Reverse complement of 28S EP5a)	1400

These primers were used in conjunction with the Whiting (2002) 28S rDNA primers.

aligned with reference to the amino acid sequence. For the ribosomal genes, a gross alignment was performed by manually aligning the conserved domains across the taxa. The 18S

and 28S sequences were then sectioned into fragments at the conserved domains, since this results in finding more optimal solutions more efficiently (Giribet, 2001). This resulted in seven fragments for 18S and 10 fragments for 28S. For 18S, fragments 1, 2, 3, 4, 6, and 7 correspond to the named regions V2, V3, V4, V5, V7, and (V8 + V9) from RNA secondary structure studies (De Rijk et al., 1992). For 28S, fragments (3 + 4), 5, 6, 8, 9, and 10 correspond to the regions D2, D3, (D4 + D5), D6, D7a, D7b, respectively. Fragment 9 (region D7a) contained a highly length-variable insertion region and was excluded because the sequence fragments were judged non-homologous. The DNA fragments of this excluded region ranged from 77 bp, in one taxa (*Baetidae* sp.1), to 758 in another (*Siphonella*). Some taxa had missing data in one or more of the DNA fragments given to POY to align, as indicated in Table 2. These data were analyzed via direct optimization in the program POY version 3.0 (Gladstein and Wheeler, 1999). POY was implemented on an IBM SP 2 supercomputer [316 Power3 processors @ 375 Mhz; 31 Winterhawk nodes (4 processors each); 12 Nighthawk II nodes (16 processors each); 348 GB total memory]. POY command



files were as follow: -outgroup CB002-fitchtrees-numslave-processes 8-onan-onannum 1-parallel-noleading-norandomizeoutgroup-sprmaxtrees 1-IMPLIEDALIGNMENT-tbrmaxtrees 1-maxtrees 5-holdmaxtrees 50-slop 5-checkslop 10-buildspr-buildmaxtrees 2-replicates 8 -stopat 25-nomultirandom-tree-fuse-fuselimit 10-fusemingroup 5-fusemaxtrees 100-ratchetspr 2-ratchettbr 2-checkslop 10-repintermediate-seed-1.

A variety of cost parameter values were investigated to explore data sensitivity (Fig. 3). We selected 36 values to explore sensitivity to gap/nucleotide change ratios (ranging from 1 to 100) and transition/transversion ratios (ranging from 1 to 100). Although one could essentially have an infinite number of ratio combinations for these three parameters, we believe that these representative ratios are sufficient to address the goals of this research (Giribet, 2001; Wheeler, 1995). Bootstrap values (Felsenstein, 1985) (1000 replicates with 20 random additions per replicate) were computed in PAUP\* 4.0b10 (Swofford, 2002) based on the implied alignment from the 1:1:1 parameter set. Partitioned Bremer values (Bremer, 1988), based on the implied alignment, were calculated using a PAUP block generated by TreeRot (Sorenson, 1999). A majority rule consensus tree was computed in PAUP\* of the most parsimonious topologies across all parameter sets.

Three replicates of a direct optimization likelihood analysis in POY were executed with the following commands: -numslaveprocesses 2-onan-onannum 1-parallel-likelihood-jobspernode 2-dpm-norandomizeoutgroup-maxtrees 5-hold-maxtrees 25-seed-1-slop 5-checkslop 5-multirandom-replicates 1-treefuse-fuselimit 5-fusemingroup 5-fusemaxtrees 25-noestimateparamsfirst-noestimatep-noestimateq-freq-model f5-invariantsitesadjust-gammaclases 4-trullytotallikelihood-seed-1. The optimal topology, and implied alignment that was constructed based on this topology, were used to construct the likelihood phylogram in PAUP\*. MODELTEST (Posada and Crandall, 1998) was used to identify the most “justified” model for likelihood settings, and branch lengths were calculated in PAUP\* for the phylogram. The model selected was GTR+G+I using and the following parameters were implemented in the PAUP block: Base=(0.2213 0.2244 0.2808), Nst=6, Rmat=(0.9031 3.1000 2.4666 0.7836 4.1811), Rates = gamma, Shape=0.5717, and Pinvar = 0.3057.

#### 4. Results

All of the amplified H3 sequences exhibited a conserved reading frame. A total of 365 bases from this gene were used in phylogenetic reconstruction and were treated as pre-aligned data. The longest complete mayfly sequences and average lengths (respectively) for the remaining genes were: 385 (*Epeorus* sp.) and ~380 bp for 12S; 593 (*Tricorythodes* sp. and *Neoephemera youngi*) and ~570 bp for 16S; 1932 (*Yarina mota*) and ~1850 bp for 18S; and 3223 (*Hexagenia* sp.) and ~3100 bp for 28S.

Direct optimization of the 1:1:1 (gap:tv:ts) ratio parameter set resulted in two most parsimonious topologies. A strict consensus of these two trees is depicted in Fig. 3, with

a length of 22491. This topology will be referred to in the discussion section (below).

Partitioned Bremer, and bootstrap values are reported in Table 4 for corresponding numbered nodes on the tree (Fig. 3). The relative Bremer support (total Bremer for the partition/total Bremer for all partitions) for each partition shows that 49.6% of the signal comes from the 28S data set, 18S contributes 23.7%, and 16S contributes 15.3%. The other two partitions, 12S and H3, contribute considerably less with the remaining 8.7 and 2.7% of the relative Bremer support (Table 4). Table 5 depicts relative Bremer supports for two subsets of the nodes that we will refer to as “Higher” and “Lower” level relationships. The “Higher” level consists of all the nodes above the family level, while the “Lower” level refers to the nodes at the familial level and below. Interestingly, the proportions are relatively the same. Therefore, it appears that all five markers are contributing information at both levels (Higher and Lower), and their respective contribution percentages is similar at both levels as well.

The likelihood direct optimization analyses resulted in one optimal topology. This optimal likelihood topology and its corresponding implied alignment were used to construct the phylogram (Fig. 5) with relative branch lengths based on the number of substitutions per site. The branches between the orders are relatively long compared to the backbone within ephemeropteran lineages (i.e., the branches that support the higher level groupings of mayflies). A few mayfly taxa, for example the genera *Neoephemera* (Neophemeridae), *Hytanella* (Ephemerellidae), *Paraleptophlebia* (Leptophlebiidae), *Dipheter* (Baetidae), *Homoneuria* (Oligoneuriidae), and *Cinygmula* (Heptageniidae), present relatively long branches.

Generally, when we talk of nodal support we will refer to the Bremer and bootstrap values, and when we talk of stability, we are referring to the results of the sensitivity analysis. All nodes were assigned to two different groups; (1) higher taxonomic level, above the familial status; and (2) lower taxonomic level, family status and below. The nodal support for this division is summarized (Table 5). The results from the sensitivity analyses are summarized in the landscape plots below each corresponding node (Fig. 3). The POY implied alignment resulted in a total base pair length of 7551. The implied alignment(s), data sets and trees may be downloaded at the following website: <http://whitinglab.byu.edu/Ephemeroptera/datasets.htm> or acquired through the corresponding author.

#### 5. Discussion

##### 5.1. Higher level

The direct optimization analyses of the data strongly support a monophyletic Ephemeroptera with a Bremer support value (Bs) of 84, and bootstrap value (bt) of 100. Nodal support for the placement of Baetidae as sister to all remaining clades is strong (Bs=40 and bt=100), however,

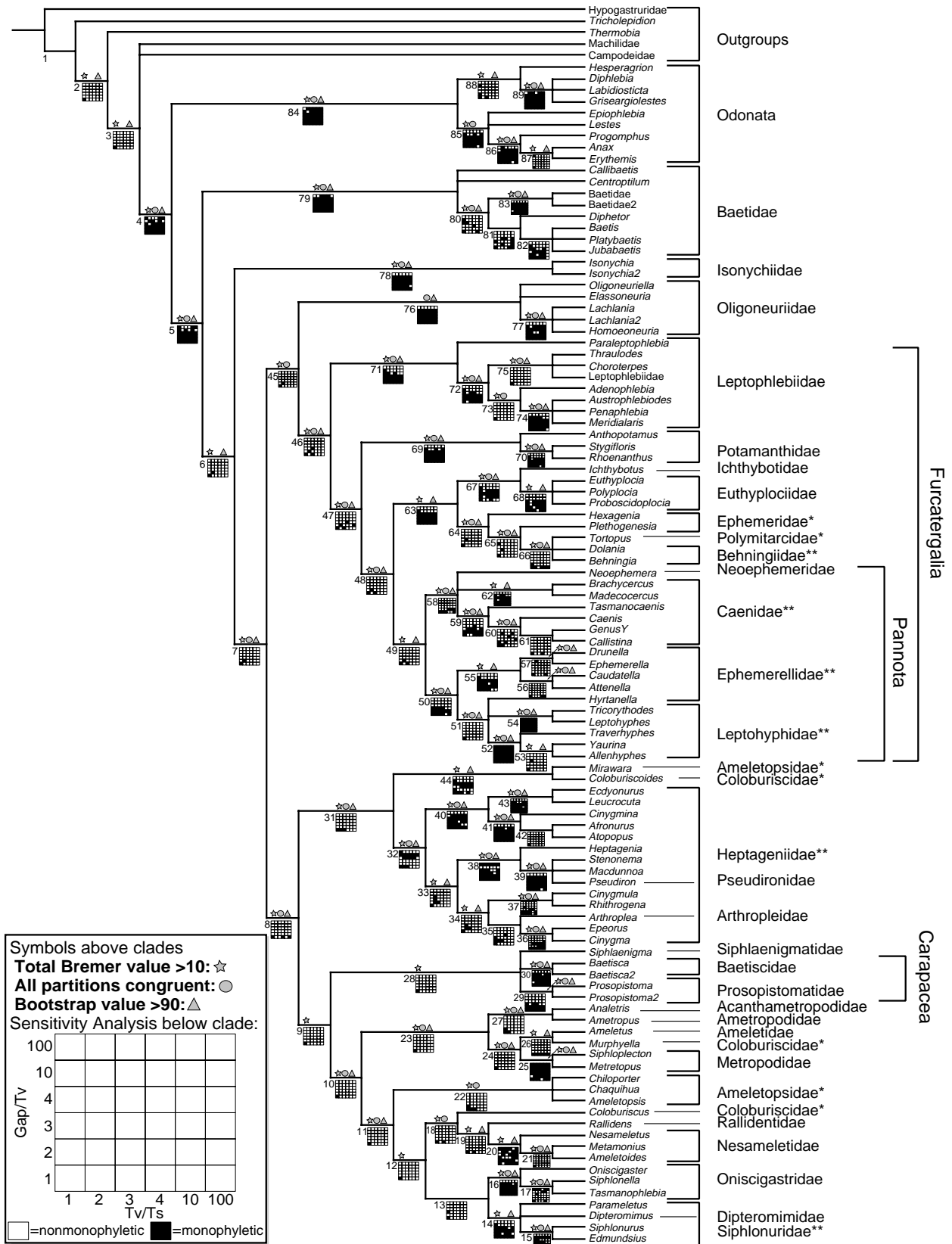


Fig. 3. Strict consensus of two most parsimonious trees analyzed in POY (direct optimization) under a 1:1:1 (gap:tv:ts) ratio parameter set. Each node has been numbered and corresponding nodal support values are found in Table 4. The parameter landscape has been plotted under each node. The symbols (☆, ●, ▲) plotted above each node indicate a total Bremer value >10, all partitions (12S, 16S, 18S, 28S, and H3) are congruent (i.e., no partition presented a negative Bremer value), and a bootstrap value >90, respectively. (\*not supported as monophyletic; \*\*not supported as monophyletic in 1:1:1 topology, but see text for discussion).

Table 4

Bremer support (Bs) values, bootstrap values, sensitivity analysis percent (SA%) score, and taxonomic level division of “Higher” and “Lower” (see text) for each node on the 1:1:1 topology (Fig. 3)

Node	Total Bremer	Bremer 12S	Bremer 16S	Bremer 18S	Bremer 28S	Bremer H3	bootstrap	SA (%)	Taxon level
1	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	113	0	24	27	71	-9	100	2.78	Higher
3	72	0	20	13	42	-3	100	2.78	Higher
4	73	12	10	2	44	5	100	80.56	Higher
5	127	0	0	85	42	0	100	83.33	Higher
6	40	0	10.5	18	12.5	-1	100	5.56	Higher
7	24	0	4	0.5	17.5	2	100	5.56	Higher
8	20	0	0	10	4	6	93	8.33	Higher
9	11	7	5	-1	-1	1	72	2.78	Higher
10	13	5	2	0	6	0	91	2.78	Higher
11	11	2	1	0	8	0	91	2.78	Higher
12	14	5.7	0.7	-0.3	5.3	2.6	82	2.78	Higher
13	4	3	3	0	-2	0	66	5.56	Higher
14	13	0	1	7	6	-1	94	58.33	Lower
15	40	0	0	8	25	7	100	66.67	Lower
16	17	0	1	2	14	0	100	80.56	Lower
17	19	0	3	1	15	0	100	27.78	Lower
18	13	1	5	0	6	1	80	5.56	Higher
19	17	5	2	1	10	-1	94	5.56	Higher
20	58	4	0	10	47	-3	100	72.22	Lower
21	16	5	3	3	5	0	97	2.78	Lower
22	11	3	8	0	0	0	89	11.11	Lower
23	19	4	5	3	7	0	98	2.78	Higher
24	16	9	0	1	6	0	96	2.78	Higher
25	60	0	0	28	34	-2	100	94.44	Lower
26	19	0	0	12	8	-1	98	16.67	Higher
27	35	12	1	4	13	5	100	8.33	Higher
28	20	0	12	0	11	-3	88	2.78	Higher
29	56	0	0	44.3	11.7	0	100	41.67	Lower
30	11	0	0	6	5	0	100	77.78	Lower
31	20	0	0	10	4	6	98	8.33	Higher
32	97	8	3	29	56	1	100	38.89	Lower
33	16	1	2	10	4	-1	94	13.89	Lower
34	13	6	5	-1	5	-2	94	11.11	Lower
35	3	0	3	1	-1	0	50	16.67	Lower
36	30	15	4	2	9	0	100	41.67	Lower
37	16	-1	2	4	10	1	98	36.11	Lower
38	26	0	8.5	1.5	15	1	100	75.00	Lower
39	11	0	4	0	7	0	94	80.56	Lower
40	40	1	7	13	18	1	100	72.22	Lower
41	21	5	0	2	14	0	95	80.56	Lower
42	4	2	0	-2	4	0	59	2.78	Lower
43	11	2	0	0	9	0	92	75.00	Lower
44	78	0	7	-6	76	1	100	44.44	Higher
45	16	2	12	0	2	0	86	5.56	Higher
46	29	2.5	1.5	11.5	11	2.5	100	11.11	Higher
47	41	6	2	14	19	0	100	13.89	Higher
48	28	9	7	1.5	9.5	1	100	8.33	Higher
49	44	15	6	-4	19	8	100	8.33	Higher
50	98	20	14	17	48	-1	100	41.67	Higher
51	49	0	2	8	34	5	100	2.78	Lower
52	47	0	28	16	3	0	100	100.00	Lower
53	11	0	12	1	-2	0	97	5.56	Lower
54	75	25	27	0	23	0	100	100.00	Lower
55	54	0	9	10.5	36	-1.5	100	61.11	Lower
56	23	0	6	9	7	1	96	8.33	Lower
57	35	0	0	8.5	21	5.5	100	8.33	Lower
58	31	0	8	3	20	0	100	19.44	Higher
59	23	15	0	2	6	0	100	41.67	Lower
60	11	5	0	0	6	0	97	22.22	Lower
61	5	3	0	-2	4	0	83	5.56	Lower
62	13	0	0	-1	14	0	96	72.22	Lower

(continued on next page)

Table 4 (continued)

Node	Total Bremer	Bremer 12S	Bremer 16S	Bremer 18S	Bremer 28S	Bremer H3	bootstrap	SA (%)	Taxon level
63	117	1	5	56	59	-4	100	69.44	Higher
64	18	0	0	6	9	3	99	5.56	Higher
65	25	0	0	8	17	0	100	8.33	Higher
66	42	9	10	11	12	0	100	16.67	Higher
67	44	6	3	11	20	4	99	52.78	Higher
68	51	9	0	38	8	-4	100	55.56	Higher
69	27	0	0	10	15	2	100	75.00	Lower
70	2	0	0	4	0	-2	57	63.89	Lower
71	33	0	11	2	19	1	100	61.11	Lower
72	26	0	6	7.5	12	0.5	100	58.33	Lower
73	14	4	2	2	4	2	83	2.78	Lower
74	24	0	0	2	18	4	100	75.00	Lower
75	11	0	0	0	11	0	96	2.78	Lower
76	20	0	0	10	4	6	100	83.33	Lower
77	3	0	0	3	0	0	96	75.00	Lower
78	40	0	0	13	22	5	100	80.56	Lower
79	72	0	35	14	20	3	100	91.67	Lower
80	25	0	6	11	7	1	98	16.67	Lower
81	6	0	0	-4	7	3	78	27.78	Lower
82	6	0	0	-2	6.7	1.3	79	47.22	Lower
83	34	0	0	8	17.5	8.5	100	80.56	Lower
84	198	0	22	45	131	0	100	94.44	Higher
85	15	0	4	2	9	0	67	72.22	Higher
86	18	10	6	2	0	0	97	80.56	Higher
87	38	1	36	-2	4	-1	100	5.56	Higher
88	33	0	6	2	26	-1	100	11.11	Higher
89	46	0	0	1	32	13	99	94.44	Higher
Total	2969	259.2	453.2	703.5	1473.7	79.4			
Average	33.7%	2.9%	5.2%	8.0%	16.7%	0.9%	0.0%	37.8%	
Bs contribution		8.7%	15.3%	23.7%	49.6%	2.7%			
Total # PIC	2967	386	365	611	1467	138			
Total Bs/PIC		0.67	1.24	1.15	1.00	0.58			

SA%, number of parameter sets monophyletic divided by 36 (total number of parameter sets). PIC, the number of parsimony informative characters.

Table 5

Summary of Bremer support (Bs) values, bootstrap values, and sensitivity analysis percent (SA%) score for the taxonomic level division of "Higher" and "Lower" (see text)

	Total Bremer	Bremer 12S	Bremer 16S	Bremer 18S	Bremer 28S	Bremer H3	bootstrap	SA%
"Lower" total sum	1278	103	198.5	301.3	627.9	47.3		
"Lower" average (%)		8.1	15.5	23.6	49.1	3.7	94.00	48.90
"Higher" total sum	1691	156.2	254.7	402.2	845.8	32.1		
"Higher" average (%)		9.2	15.1	23.8	50.0	1.9	94.85	24.44

SA%, number of parameter sets monophyletic divided by 36 (total number of parameter sets).

the node is not stable in the parameter landscape, being present in only one other parameter set (2:2:1). The character state reconstruction in MacClade for gill movability is equivocal when mapped on the parsimony tree (Fig. 6D) or the likelihood tree (Figs. 5 and 6G), which did not support Baetidae as sister to all other mayflies. Thus, a better taxon sampling and perhaps more specific gill structure characters are needed to address the flight hypothesis further. Still, the placement of Baetidae in the parsimony analysis may lend some support for the origin of wing hypothesis which assumes that highly movable and muscled gills evolved into wings, because many baetids present this type of gill. For example, *Callibaetis*, which possesses highly movable gills that are used to create water currents to facilitate respiration in slow moving or static water habitats was recovered as the basal taxon within Baetidae in 53% of the

sensitivity analyses (Fig. 4A). *Centroptilum*, which was recovered as the next lineage in Baetidae (Fig. 3), also presents highly movable gills. On the other hand, the position of the next clade, Isonychiidae, which was well supported (Bs = 24 and bt = 100) but not vary stable, may contradict the gill to wing theory. The gills of all isonychiids and some baetids, such as the *baetis* group, have little or no mobility, suggesting the gill to wing hypothesis may be inaccurate. Furthermore, highly movable gills are present in other clades, such as Siphonuridae, Leptophlebiidae (in part, ex. *Paraleptophlebia* and *Choroerpes*), Metropodidae, Heptageniidae (in part, ex. *Heptagenia*, *Stenonema*, *Macdunnnoa*), suggesting that movable gills may be a plastic character. Therefore, these data neither unambiguously support nor refute the mayfly gill to wing hypothesis. However, given the aquatic environment in which mayflies live,

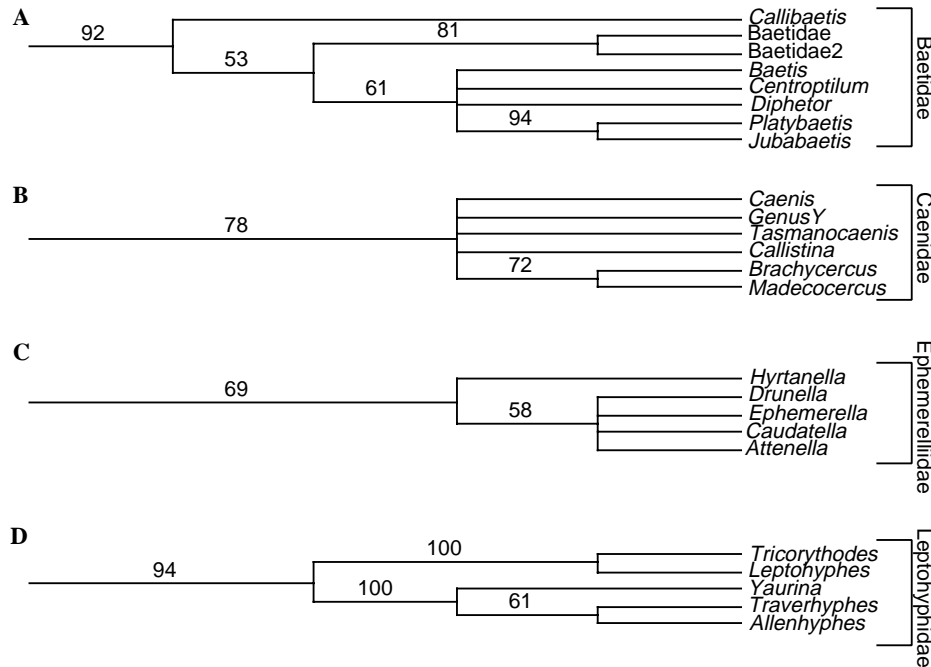


Fig. 4. These four clades are derived from a 50% consensus tree of the most parsimonious trees found for each parameter set (36 total). The four clades, which depict important differences from Fig. 3, are: (A) clade containing Baetidae, (B) clade containing Caenidae, (C) clade containing Ephemerellidae, and (D) clade containing Leptohyphidae. The numbers above each node are percent consensus values.

selection for movable gills could have allowed for multiple independent gains to adapt to lentic aquatic systems with low levels of dissolved oxygen. Clearly, more work needs to be done concerning this issue.

### 5.2. Pisciforma

The suborder Pisciforma was not supported as monophyletic because of the position of Baetidae as sister to all remaining clades and *Mirawara* (Ameletopsidae) nesting within a clade sister to Heptageniidae, while *Siphlaenigma* (Siphlaenigmatidae) nested within a clade including Prosoptomatidae and Baetiscidae. McCafferty's Pisciforma (from Fig. 2) was never recovered in any of the optimal topologies from the other parameter sets. The two proposed superfamilies of this putative suborder, Baetoidea and Siphonuroidea, were not supported as monophyletic due to the same reasons as explained above. Moreover *Siphlaenigma* was never recovered as sister to Baetidae in any of the parameter sets. These data support a plesiomorphic condition for the fishlike body form, as most parsimonious, with multiple losses in Carapacea, Heptageniidae, and Oligoneuriidae + Furcatergalia (Fig. 6E). Alternatively, the optimization on the likelihood tree supports a single evolution of the fishlike body form, on an internal node within Ephemeroptera, with a loss in the Heptageniidae lineage (Fig. 6H).

### 5.3. Setisura

Setisura (Heptagenioidea) was also recovered as nonmonophyletic because of the placement of *Mirawa*, but more importantly, the family Oligoneuriidae was supported

(Bs = 16 and bt = 86) as sister to Furcatergalia, although this placement was not particularly stable. Interestingly, the relationship of Setisura + Pisciforma – (Baetidae and Isonychiidae) is fairly stable and well supported (Bs = 20 and bt = 93).

### 5.4. Carapacea

Carapacea (Prosopistomatidae + Baetiscidae) was not supported as monophyletic (Bs = 20 and bt = 88) due to the inclusion of *Siphlaenigma* in the unresolved node. However, Carapacea was recovered as monophyletic in four parameter sets (8:4:1; 10:10:1; 20:10:1; and 100:100:1). In contrast to the hypotheses of other investigators (Kluge, 1998; Landa and Soldan, 1985; McCafferty, 1991b), Carapacea was never recovered as sister to the remaining mayflies. While neither the 1:1:1 parsimony or likelihood trees supported the monophyly of Carapacea, the sensitivity analysis lends some support for this grouping, which may substantiate the notion that there was a single origin for the notal shield or carapace, with subsequent morphological specialization.

### 5.5. Furcatergalia

The Suborder Furcatergalia was strongly supported as monophyletic (Bs = 29 and bt = 100), and was present in four parameter sets. The positions of many of the families were not supported as previously proposed (compare Figs. 2 and 3). However, Leptophlebiidae was supported as the basal lineage of Furcatergalia, corroborating McCafferty's hypothesis. Nevertheless, his Ephemeroidea was supported as nonmonophyletic due to the nesting of the family

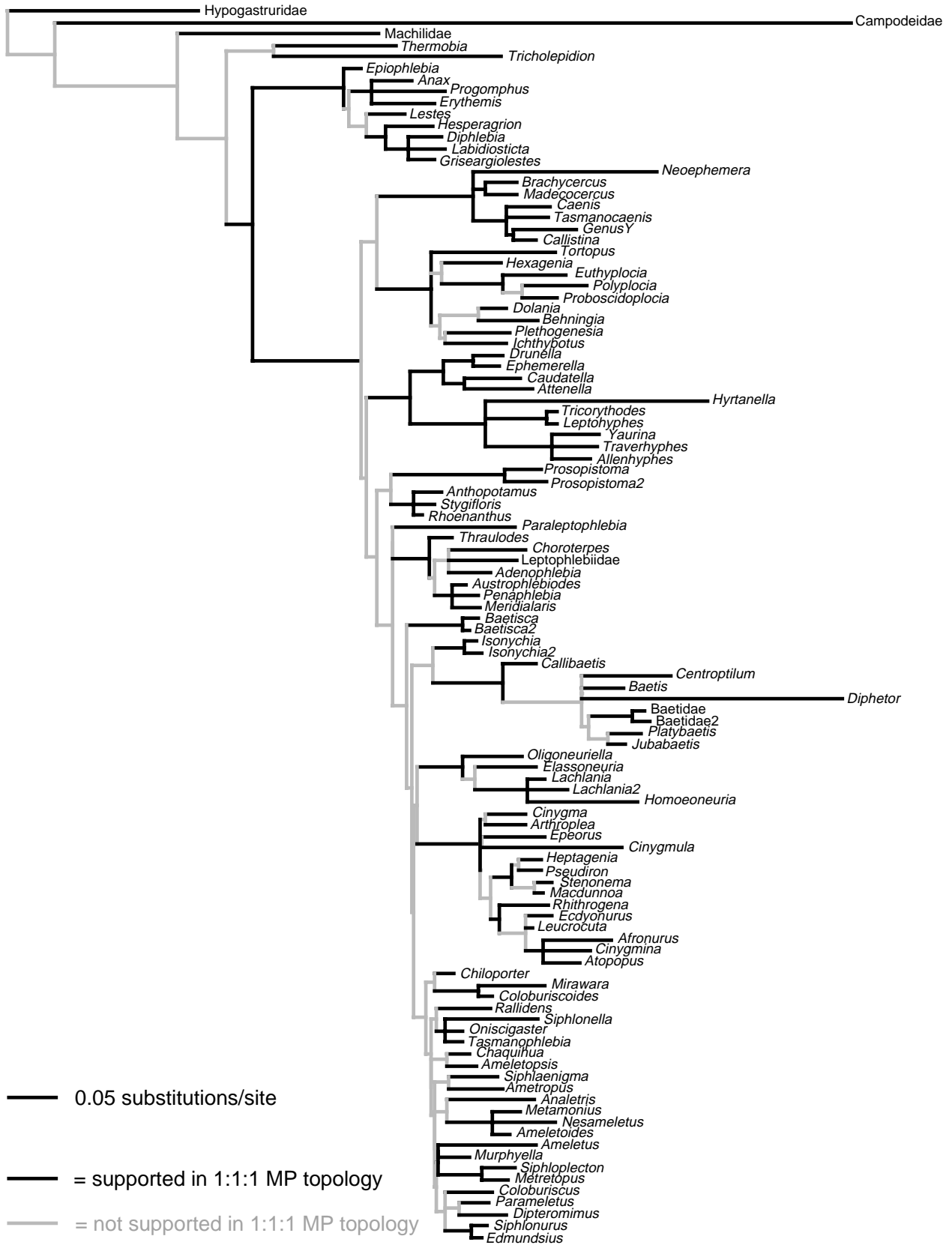


Fig. 5. Likelihood phylogram with relative branch lengths. Branches in grey were not congruent with relationships supported in the 1:1:1 parsimony reconstruction (Fig. 3). Branches in black are congruent with relationships supported in the 1:1:1 parsimony reconstruction.

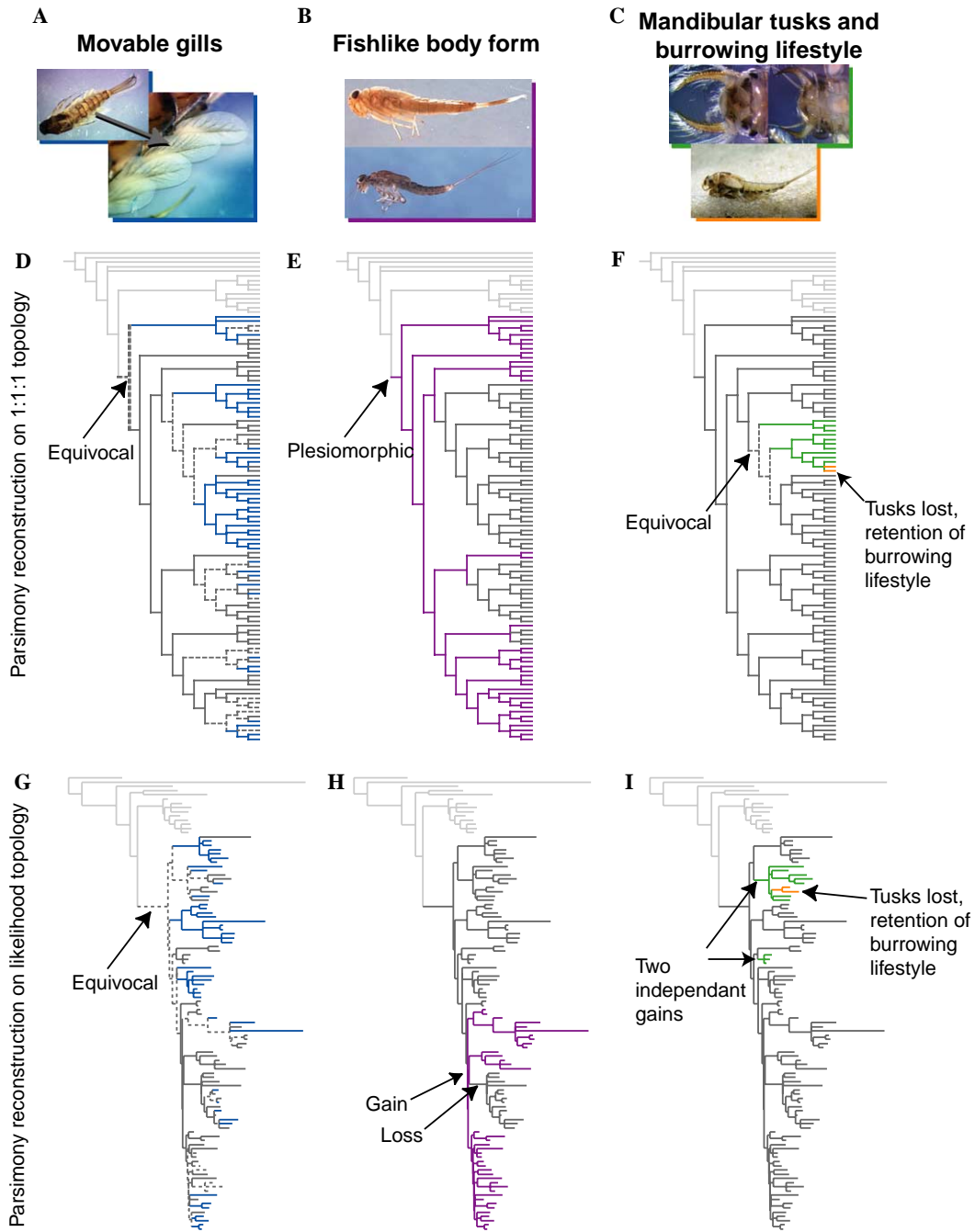


Fig. 6. Parsimony character optimizations mapped on the 1:1:1 parsimony topology and likelihood topology. (A) Dorsal view and close-up of movable abdominal gill of *Edmundsius*; (B) Lateral view two mayfly larvae with a fishlike body form (pisciform); (C) head with mandibular tusks of *Rhoenanthus* (top left) and *Polyplocia* (top right), and lateral view of tuskless *Dolania* (Behningiidae); (D and G) presence of highly movable gills (in blue) on parsimony and likelihood topologies; (E and H) presence of fishlike body form and swimming behavior (in purple) on parsimony and likelihood topologies; (C and I) burrowing lifestyle (in green), and burrowing lifestyle without mandibular tusks (in orange) on parsimony and likelihood topologies. The dotted line indicates that the character state is equivocal. Outgroups are in light grey.

Potamanthidae outside the rest of Ephemeroidea and because Behningiidae nested within this clade. The support values and parameter landscape for node 48 ( $B_s = 28$  and  $bt = 100$ ) indicate evidence for the nonmonophyly of burrowing mayflies. Additionally, the likelihood phylogram does not support their monophyly (Fig. 5). Therefore, there was either a single origin for mandibular tusks and burrowing lifestyle on the node subtending these taxa with a loss

on the branch leading to Pannota or there were two independent gains for Potamanthidae and for the remaining burrowers (Fig. 6F). Furthermore, these data strongly support nesting Behningiidae within the tusked burrowing mayflies as sister to the genus *Tortopus* (Polymitarcidae). Thus, a secondary loss of the mandibular tusks occurred in the behningiids, while the burrowing lifestyle was retained (Fig. 5C). The reconstruction on the likelihood tree would

suggest two origins with a loss in Behningiidae as well (Fig. 6I). Finally, Pannota was well supported as monophyletic (Bs=44 and bt=100), and was relatively stable (present in three parameter sets), although not supported under likelihood. Therefore, the extent of fusion of the forewing pads to the thorax appears to be a synapomorphic character, except in the case of the Carapacea. These data strongly supports the monophyly of the superfamilies Caenoidea (Bs=31 and bt=100) and Ephemerelloidea (Bs=98 and bt=100), and these groups were recovered with relatively high stability.

The likelihood phylogram (Fig. 5) differs substantially from the topology in Fig. 3, especially among the higher level relationships. This is not surprising though, because these nodes are the nodes that are also sensitive to parameter perturbation. Still, when comparing the likelihood topology to the traditional classifications of Kluge and McCafferty, except for the superfamilies Caenoidea and Ephemerelloidea, none of the proposed higher level groupings (above family level) were recovered. Nevertheless many of the familial and lower level relationships were recovered in both the likelihood and parsimony frameworks (indicated by the black branches in Fig. 5), and being supported throughout a majority of the sensitivity landscape.

### 5.6. Familial monophyly

Baetidae is strongly supported as monophyletic (Bs=72 and bt=100) and was very stable (92% of the landscape parameter sets). A majority rule consensus of Baetidae shows that *Callibaetis* was recovered as sister to the remaining baetids in 53% of the parameter sets (Fig. 4A). The sister group relationship of *Platybaetis* and *Jubabaetis* was also recovered in a large proportion of the parameter sets (94%) while not supported in the 1:1:1 set (Fig. 4A).

Isonychiidae, Baetiscidae, and Prosopistomatidae are monogeneric families, and were represented by two species and were recovered as monophyletic lineages. Ametropodidae (*Ametropus*), Arthropleidae (*Arthroplea*), Pseudironidae (*Pseudiron*), Dipteromimidae (*Dipteromimus*), Ichthybotidae (*Ichthybotus*), Rallidentidae (*Rallidens*), Polymitarciidae (*Tortopus*), and Siphlaenigmatidae (*Siphlaenigma*) are monogeneric families as well. However, only one species of each genus (represented in parentheses) was included in this analysis. Hence, monophyly was not specifically tested. Nevertheless the results suggest that some of these “families” are just apomorphic genera of other families or that the other families are paraphyletic.

For example, the genera *Pseudiron* and *Arthroplea* nest within the Heptageniidae, with high nodal support (Br=97 and bt=100) and stability. Some have suggested that “*Pseudiron*, *Arthroplea*, and all other genera of the Heptageniidae complex from three monophyletic lineages” (Jensen and Edmunds, 1973; Wang and McCafferty, 1995), however, only one character has been formally described and tested to support this claim (Wang and McCafferty,

1995). The data support that *Arthroplea* and *Pseudiron* be included in the Heptageniidae as proposed by earlier investigators (Edmunds and Traver, 1954). The family Oligoneuriidae was well supported as being monophyletic (Bs=3 and bt=100) but not as sister to the Heptageniidae–*Pseudiron*–*Arthroplea* clade, as proposed by Wang and McCafferty (1995).

Among the remaining “Pisciform” families, Metropodiidae (Bs=60 and bt=100), Nesameletidae (Bs=58 and bt=100) and Oniscigastridae (Bs=17 and bt=100) were recovered with high nodal support and stability. Node 14 containing Siphonuridae + *Dipteromimus* was also very stable and relatively well supported (Bs=13 and bt=94). These data suggest either a nonmonophyletic Siphonuridae or that Dipteromimidae should not have familial status, but rather be considered as a lineage within Siphonuridae.

Within Furcatergalia, Leptophlebiidae was recovered as monophyletic (Bs=33 and bt=100) and the genus *Paraleptophlebia* was well supported (Bs=26 and bt=100) as sister to the remaining leptophlebiid genera sampled. This was to be expected as *Paraleptophlebia* belongs to the subfamily Leptophlebiinae, while all the other genera represented belong to the Atalophlebiinae. The family Potamanthidae was strongly supported as monophyletic (Bs=27 and bt=100). Euthyplociidae was recovered as a robust monophyletic group. The positions of the borrowing mayflies representing the family Ephemeridae did not support its monophyly. Clearly, a better sampling is needed to more thoroughly examine the relationships within the burrowing mayflies. Behningiidae, while not supported as monophyletic in the 1:1:1 topology, was recovered in 89% of the parameter sets and the likelihood topology (Fig. 5).

Within Pannota, The monophyly of Caenidae was fairly stable being present in 78% of the parameter sets (Fig. 4B). Ephemerellidae was not supported as monophyletic in the 1:1:1 parameter set with *Hyrtanella* nesting outside. However, *Hyrtanella* did group with the other ephemerellids in 69% of the parameter landscape, as seen in the majority rule consensus topology (Fig. 4C). The relatively long branch length of *Hyrtanella* may be playing a role in its instability to nest within the ephemerellids, however, the likelihood reconstruction also supported placement of *Hyrtanella* with the leptohyphids (Fig. 5). Because of the placement of *Hyrtanella* in the 1:1:1 topology (Fig. 3), Leptohyphidae was recovered as nonmonophyletic, but across all parameter sets it was recovered as monophyletic in 94% of the analyses (Fig. 4D). While there is evidence that this family is monophyletic, the subfamilies Leptohyphinae and Tricorythodinae were strongly supported as nonmonophyletic, contrary to previous hypotheses (Wiersema and McCafferty, 2000). A more thorough representation of all genera is necessary to robustly explain subfamilial relationships within the Leptohyphidae.

There are patterns that are seen across the 1:1:1 topology and the majority rule consensus tree that can be examined through sensitivity analysis. First, the shallower nodes



(family level and below) and the very deep nodes (Ordinal level) are much more stable than the intermediate nodes (interfamilial level). In fact, using the designation of “Higher” and “Lower” for the nodes as before, the average percent of the parameter sets that were supported for the “Higher” level was 24.4 and 48.9% for the “Lower” level (Table 5). Another important pattern that can be observed is that the nodes are more sensitive to change in gap:nucleotide cost than the tv:ts ratio cost. This is visualized by identifying that there is more congruence horizontally across the landscapes than vertically. For example, the landscapes below the clades Leptophlebiidae, Potamanthidae, and the remaining burrowing mayflies, present high congruence for any tv:ts ratio horizontally, but once the vertical threshold (gap:nucleotide) of 10 is reached, congruence is minimal. Therefore, in a large data set like this one, it appears that the gap cost is a parameter that can influence more (i.e., change topological relationships) in the outcome than the tv:ts ratio parameter.

While other authors suggest that there can be no objective, frequency-based probability relating to the necessarily unique events of the past (Grant and Kluge, 2003; Siddall and Kluge, 1997), and that there are no known means of determining a priori which alignment parameters are appropriate for recovering evolutionary relationships (Phillips et al., 2000; Wheeler, 1995), it has also been proposed that inferences of indels and base transformations performed during the primary homology process (alignment) are unavoidable assumptions, and simple homogenous weighting during this process does not avoid the issue of arbitrary, yet crucial, assumptions (Phillips et al., 2000; Wheeler, 1995). These two juxtaposed ideas illustrate the usefulness of topological sensitivity analysis in a phylogenetic framework. We are interested in classifying nodes on the topology that are robust (stable to parameter value perturbations) and well supported (Bremer's, bootstraps, jackknives, etc.). We suggest that through examination of multiple parameters one can distinguish non-robust nodes, which may be more easily falsified in future studies. Again, we are not trying to accurately model the means by which the sequences evolved, because this is unknowable, or minimally, inapplicable in most cases. We are only using sensitivity analysis as a means of acquiring additional information that we might not have acquired otherwise. For example, while many nodes on the mayfly topology were apparently well supported (for example nodes 10, 11, 23, 21, 51, and 75 among others), the landscape indicated that they were sensitive to parameter perturbation. Interestingly, the nodes that were present in the likelihood tree (Fig. 5) were in all cases nodes that would be identified as robust under the sensitivity analysis. However, many robust nodes, based on sensitivity analysis, were not recovered from the likelihood topology. For example nodes 14, 36, 37, 39, 43, 67, 70, 82, and 85 were highly supported and very robust under parsimony and not supported under likelihood. In summary, the additional information supplied by a sensi-

tivity analysis could be used to direct future analyses, taxon sampling, and gene targeting for sequencing. Therefore, topological sensitivity analysis in phylogenetics is a useful tool to explore DNA sequences, of varying lengths, when inferring phylogenetic relationships.

## 6. Conclusion

This analysis represents the first formal analysis across almost every major lineage of mayflies and is the first molecular phylogeny for the Order Ephemeroptera. The analyses included 31 of the 37 families, representing ~24% of the genera. 11 families were supported as being monophyletic, although four others (Behningiidae, Caenidae, Ephemerellidae, and Leptohyphidae) were recovered in a large portion of the parameter landscapes supporting their monophyly as well; five families were supported as nonmonophyletic, although two of these (Heptageniidae and Siphonuridae), were considered as such because of the inclusion of a monogeneric lineages from other families; and 11 families were only represented by one species, and monophyly was not testable. The suborders Furcatergalia and Carapacea were supported as monophyletic under parsimony while Setisura and Pisciforma were not monophyletic. The superfamilies Ephemerelloidea and Caenoidea were supported as monophyletic under both parsimony and likelihood while Baetoidea, Siphonuroidea, Ephemeroidea, and Heptagenioidea were not. Baetidea was supported as sister to the remaining clades. The mayfly gill to wing origin hypothesis was not supported nor refuted by these data. This scenario will be scrutinized further in future analyses. Mandibular tusks were supported as having either two unique origins in the burrowing mayflies or an initial gain and a secondary loss on the branch subtending Pannota. The placement of Behningiidae indicates a secondary loss of tusks in this group with a retained lifestyle of burrowing. The extent of the fusion of the forewing pads to the thorax appears to be a synapomorphic character for Pannota. The monophyly of Carapacea supports the homologous nature of the notal shield character, as well as the other characters described for this group. These data strongly support a single origin for the fishlike body form with multiple losses under both parsimony and likelihood, although under parsimony the character was supported as plesiomorphic for mayflies. Topological sensitivity analysis was shown to be a tool to examine patterns concerning the stability of relationships across a parameter landscape, providing additional information that may not have been acquired otherwise.

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