

Towards a new paradigm in mayfly phylogeny (Ephemeroptera): combined analysis of morphological and molecular data

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Abstract. This study represents the first formal morphological and combined (morphological and molecular) phylogenetic analyses of the order Ephemeroptera. Taxonomic sampling comprised 112 species in 107 genera, including 42 recognized families (all major lineages of Ephemeroptera). Morphological data consisted of 101 morphological characters. Molecular data were acquired from DNA sequences of the 12S, 16S, 18S, 28S and H3 genes. The Asian genus *Siphuriscus* (Siphuriscidae) was supported as sister to all other mayflies. The lineages Carapacea, Furcatergalia, Fossoriae, Pannota, Caenoidea and Ephemerelloidea were supported as monophyletic, as were many of the families. However, some recognized families (for example, Ameletopsidae and Coloburiscidae) and major lineages (such as Setisura, Pisciforma and Ephemeroidea among others) were not supported as monophyletic, mainly due to convergences within nymphal characters. Clade robustness was evaluated by multiple methods and approaches.

Introduction

Ephemeroptera (mayflies) constitute a well-supported monophyletic group of pterygote insects (Hovmöller *et al.*, 2002; Ogden & Whiting, 2003, 2005). Recent reconstructions and taxonomic works have greatly influenced the systematics of mayflies (e.g. Kluge, 2000, 2004; Molineri & Domínguez, 2003; Zhou & Peters, 2003; Ogden & Whiting, 2005; Ogden *et al.*, 2008), which nowadays encompass over 3000 species and over 400 genera, constituting at least 42 described families (Barber-James *et al.*, 2008). Mayflies occupy freshwater habitats across the world, with the exception of Antarctica. The immature aquatic stages or nymphs are characterized by a combination of the presence of abdominal tracheal gills, a medial terminal filament, a maxilla with medially fused galea and lacinia, and a three-jointed mandible. The presence of a subimago (characterized by possessing functional wings at the penultimate moult) is unique to pterygote insects and usually regarded as remnant of several imaginal moults as still present

in primarily wingless insects (Edmunds & McCafferty, 1988; Brittain & Sartori, 2003) (Archaeognatha and Zygentoma). The winged stages of mayflies lack functional mouthparts and, hence, are incapable of feeding as adults. Adult mayflies are more soft-bodied insects possessing short antennae, two long cerci, and may possess a medial caudal filament originating from the last abdominal segment. Adult mayflies have two pairs of wings, with the second pair being considerably smaller than the first and even absent in some species (Brittain, 1982; Edmunds, 1996; Brittain & Sartori, 2003). The mayfly wing present three axillary plates in the articulation and a hypertrophied costal brace (Willkommen, 2008). The winged stages of Ephemeroptera, as with Odonata, cannot fold their wings horizontally over the abdomen.

The Ephemeroptera (mayflies) are an ancient lineage of insects, dating back to the late Carboniferous or early Permian periods, some 290 Mya (Brittain & Sartori, 2003). The position of Ephemeroptera relative to other pterygote lineages (Odonata and Neoptera) has been controversial. Some have defended a sister relationship to Odonata + Neoptera (= Metapterygota) (Kristensen, 1991; Whiting *et al.*, 1997; Fürst von Lieven, 2000; Staniczek, 2000; Wheeler *et al.*,

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2001), whereas alternative hypotheses have been proposed, such as either Ephemeroptera + Odonata (Paleoptera) as the sister group to Neoptera or Odonata as the sister group to Ephemeroptera + Neoptera (Martynov, 1930; Matsuda, 1970; Boudreaux, 1979; Hennig, 1981; Riek & Kukulová-Peck, 1984; Kukulová-Peck, 1991, 1997, 2008; Brodsky, 1994; Hövmöller *et al.*, 2002; Kjer, 2004). Analyses of the combined available suite of morphological and molecular data for these pterygote lineages supported mayflies as sister to all other extant pterygotes (Ogden & Whiting, 2003), although a fairly recent combined analysis (with emphasis on Elliplura and including only two mayflies, two odonates and two neopterans) did not support this hypothesis (Giribet *et al.*, 2004). Nevertheless, morphological data alone support mayflies as sister to all remaining winged insects (Kristensen, 1991; Whiting *et al.*, 1997; Fürst von Lieven, 2000; Staniczek, 2000; Wheeler *et al.*, 2001; Ogden & Whiting, 2003). Furthermore, a recent mitochondrial genome analysis of insects (which included a mayfly) strongly supported Ephemeroptera as sister to Odonata + Neoptera (Zhang *et al.*, 2008).

Some higher-level relationships within Ephemeroptera have been as problematic as the position of the group within the other insect orders. General summaries of the history of mayfly systematics that highlight the suggested major relationships and classifications have been reviewed (Ogden & Whiting, 2005; Sun *et al.*, 2006). Here we offer a brief review of the most recent hypotheses of higher-level relationships, including the results from recent molecular data analyses.

The classification by McCafferty & Edmunds (1979) was used broadly until McCafferty (1991b) proposed a three suborder classification (Pisciforma, Setisura and Retracheata). This classification incorporated some changes already introduced by Kluge (1988), including the exclusion of the Carapacea (Prosopistomatidae + Baetiscidae) from the Pannota. Kluge's 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. Neither of these classification systems was based on formal analysis of characters in a coded matrix, and until Ogden & Whiting (2005) no formal analysis, cladistic-based or otherwise, had been performed across the entire order [see Ogden & Whiting (2005: table 1) for details on other lower-level studies performed]. To date, no morphological data matrix has been constructed across all mayflies, nonetheless, the most recent hypotheses of McCafferty and Kluge (Kluge, 2004) are mostly congruent with each other (Fig. 1). McCafferty's hypothesis is a compilation based partially on cladistic analyses for the Pannota (McCafferty & Wang, 2000; Jacobus & McCafferty, 2006) and Setisura (McCafferty, 1991a; Wang & McCafferty, 1995), from published trees (McCafferty, 1991b, 1997), and from personal communication with McCafferty. The molecular data study of Sun *et al.* (2006) is questionable, as the reanalysis (Ogden *et al.*, 2008) of the dataset demonstrated that most major conclusions were erroneous.

In contrast to previous hypotheses based on morphological observations, the relationships inferred from the molecular data (Ogden & Whiting, 2005) were congruent in some cases, but incongruent in others. For example, the groups Furcatergalia, Pannota, Carapacea, Ephemerelloidea and Caenoidea and 15 families were supported as monophyletic. On the other hand, Setisura, Pisciforma, Baetoidea, Siphonuroidea, Ephemeroidea, Heptagenoidea and five families (having more than one taxon represented) were not supported as monophyletic. The molecular data supported the inclusion of the genera *Arthroplea* and *Pseudiron* as lineages within Heptageniidae and not as separate families. Similarly, *Dipteromimus* nested within Siphonuridae, negating the validity of Dipteromimidae as a family. Although 94 species of Ephemeroptera were included in the study, representing ~24% of the genera and ~84% of the families, several families, namely Coryphoridae, Austremerellidae, Vietnamellidae, Ephemerythidae, Machado-rythidae, Teloganodidae, Tricorythidae, Teloganellidae and the recently described Siphuriscidae (Zhou & Peters, 2003) were not included. The monophyly of many other families was not tested, because the family is monotypic or because only one genus was available. As was recognized, certain portions of the phylogeny were judged non-robust and, thus, conclusions from these clades were difficult to assess (for example, the backbone was sensitive to parameter perturbation and contained low support measures). Likewise, some families (for example, Coloburiscidae and Ameletopsidae) that were not supported as monophyletic, as they possess putatively numerous morphological apomorphies, need to be examined further. The inclusion of morphological characters, in conjunction with an increased suite of molecular evidence, may elucidate some of the problematic relationships with Ephemeroptera.

We recognize that reconstructing ancient rapid radiations of insects, of which mayflies surely are a good example, is not straightforward and has many associated pitfalls (Whitfield & Kjer, 2008). Nevertheless, evidence supports the notion that combined data (morphology + molecular data) analysis provides a more robust estimate of phylogenetic relationships (Nixon & Carpenter, 1996; Gatesy *et al.*, 1999, 2003; Gadagkar *et al.*, 2005), particularly within the arthropods (Giribet *et al.*, 2005). Over the past few years, many phylogenetic analyses have been performed, using a combined approach of morphological and molecular data, to examine ordinal and interordinal relationships within the insects. For example, combined phylogenetic hypotheses have been presented for Odonata (Bybee *et al.*, 2008), Dermaptera (Jarvis *et al.*, 2005), Phasmida (Whiting *et al.*, 2003), Trichoptera (Kjer *et al.*, 2002), Lepidoptera (Wiegmann *et al.*, 2002), Paleoptera (Ogden & Whiting, 2003), Polyneoptera (Terry & Whiting, 2005), Insecta (Wheeler *et al.*, 2001; Whiting *et al.*, 1997) and Arthropoda (Giribet *et al.*, 2001, 2004, 2005) among others. Additionally, after reviewing nine combined insect dataset studies, Danforth *et al.* (2005) concluded that combining different molecular markers into one combined analysis may provide a more robust overall phylogeny. Furthermore, character coding and formal construction of morphological datasets (matrices) allows the investigator to examine the evolutionary trends of the characters in

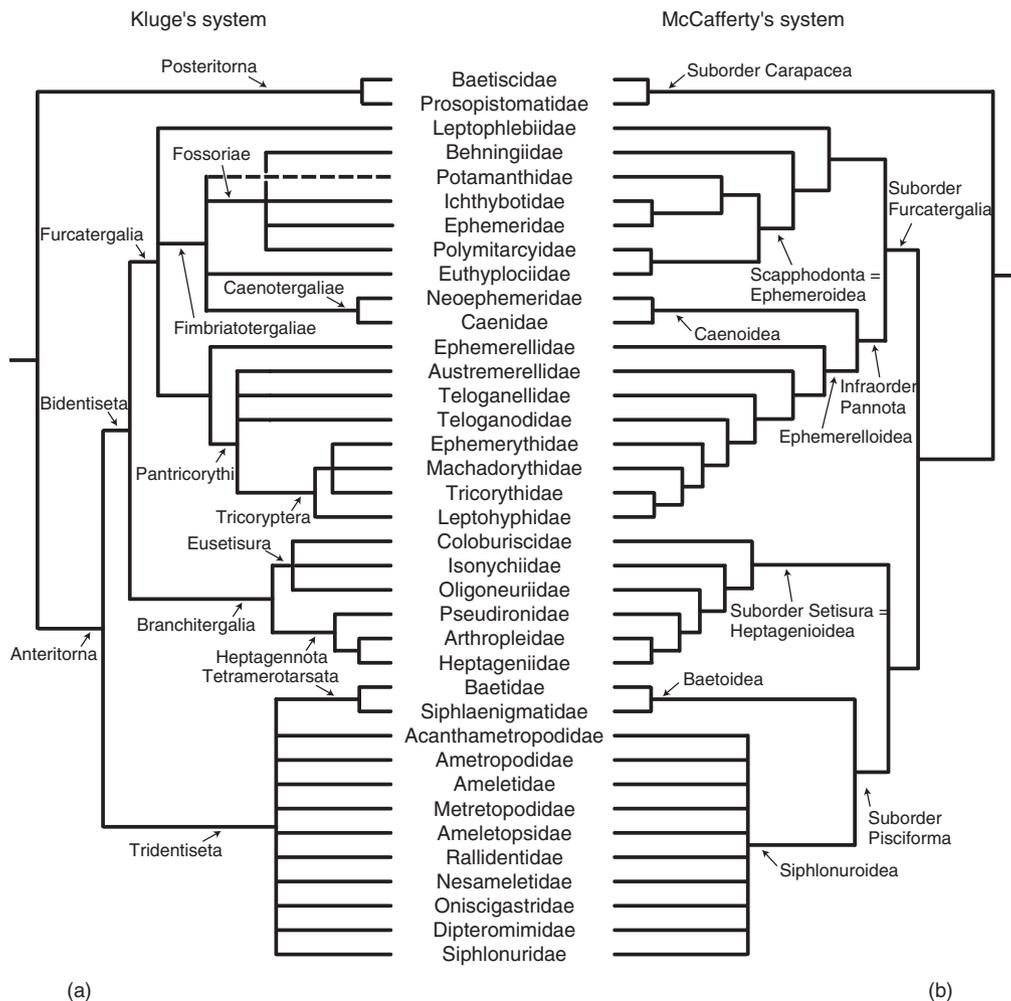


Fig. 1. Topological comparison of the two most recent systems of mayfly classification. (a) Topology summarizing the phylogenetic relationships of the non-Linnaean nomenclatorial system ('volumetric classification') hypothesized by Kluge (2004). (b) Topology synthesizing the previous studies and personal communications of McCafferty. Taken from Ogden & Whiting 2005: fig. 2.

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an objective and repeatable manner; under parsimony, likelihood, Bayesian or other approaches (Swofford & Maddison, 1987, 1992; Schultz *et al.*, 1996; Cunningham, 1999; Moers & Schluter, 1999; Pagel, 1999; Ogden *et al.*, 2005). Which method performs best on combined datasets (morphology and multiple genes) that require alignment has not been tested formally. However, recent simulation studies have shown that, on average, clustal alignments are more accurate than POY-implied alignments and that likelihood and Bayesian methods reconstruct the topologies more accurately than neighbour joining or parsimony (Fleissner *et al.*, 2005; Ogden & Rosenberg, 2006, 2007; but see Lehtonen, 2008). Still, any one dataset could be reconstructed more accurately in a direct optimization framework (such as POY), or by any other method (such as parsimony or Bayesian). Therefore, it may remain informative to examine different methods and frameworks when trying to identify nodal support, robustness and stability.

Here we present the first combined morphological and molecular analyses of phylogenetic relationships within the order Ephemeroptera. Specifically, we: (i) present the first character coded morphological matrix for Ephemeroptera; (ii) perform multiple analyses of the data in order to identify stable, robust and well-supported relationships; and (iii) examine evolutionary trends in light of the resulting phylogenetic topologies.

Materials and methods

Taxon sampling

Taxonomic sampling consisted of specimens representing 112 species of Ephemeroptera (including two extinct genera: *Kukalova* and *Protereisma*), five species of Odonata, three species of non-apterygote insects and one extinct basal pterygote

Table 1. List of taxa included in the different datasets. The total number of taxa for each dataset is given together with the methods used to analyse each dataset. Some of the species used to generate the molecular data for the genus were not the same species used to code the morphology for the genus (compare with Supporting Information Table S1).

Order	Included in morphological dataset	Included in reduced dataset	Included in total dataset	Family	Genus
Archeoagnatha		Y	Y	Machilidae	<i>Machilis</i>
Zygentoma	Y	Y	Y	Lepidotrichidae	<i>Tricholepidion</i>
	Y	Y	Y	Lepismatidae	<i>Thermobia</i>
Odonata		Y	Y	Aeshnidae	<i>Oplonaeschna</i>
	Y	Y	Y	Amphipterygidae	<i>Devadatta</i>
		Y	Y	Coenagrionidae	<i>Hesperagrion</i>
	Y	Y	Y	Epiophlebiidae	<i>Epiophlebia</i>
		Y	Y	Lestidae	<i>Lestes</i>
Meganisoptera	Y		Y	Namurotypidae	<i>Namurotypus</i> ^a
Ephemeroptera	Y		Y	Acanthametropodidae	<i>Acanthametropus</i>
	Y	Y	Y	Acanthametropodidae	<i>Analetris</i>
	Y	Y	Y	Ameletidae	<i>Ameletus</i>
	Y		Y	Ameletidae	<i>Metreletus</i>
	Y	Y	Y	Ameletopsidae	<i>Ameletopsis</i>
	Y	Y	Y	Ameletopsidae	<i>Chaquihua</i>
	Y	Y	Y	Ameletopsidae	<i>Chiloporter</i>
		Y	Y	Ameletopsidae	<i>Mirawara</i>
	Y	Y	Y	Ametropodidae	<i>Ametropus</i>
	Y	Y	Y	Arthropleidae	<i>Arthroplea</i>
	Y		Y	Austremerellidae	<i>Austremerella</i>
	Y	Y	Y	Baetidae	<i>Baetis</i>
	Y	Y	Y	Baetidae	<i>Callibaetis</i>
	Y	Y	Y	Baetidae	<i>Centroptilum</i>
	Y	Y	Y	Baetidae	<i>Cloeon</i>
	Y	Y	Y	Baetiscidae	<i>Baetisca</i>
	Y	Y	Y	Baetiscidae	<i>Baetisca2</i>
	Y	Y	Y	Behningiidae	<i>Behningia</i>
	Y	Y	Y	Behningiidae	<i>Dolania</i>
	Y		Y	Behningiidae	<i>Protobehningia</i>
	Y	Y	Y	Caenidae	<i>Brachycercus</i>
	Y	Y	Y	Caenidae	<i>Caenis</i>
	Y	Y	Y	Caenidae	<i>Clypeocaenis</i>
	Y	Y	Y	Caenidae	<i>Madecocercus</i>
		Y	Y	Caenidae	<i>Tasmanocoenis</i>
	Y	Y	Y	Coloburiscidae	<i>Coloburiscoides</i>
	Y	Y	Y	Coloburiscidae	<i>Coloburiscus</i>
	Y	Y	Y	Coloburiscidae	<i>Murphyella</i>
	Y		Y	Coryphoridae	<i>Coryphorus</i>
	Y	Y	Y	Dipteromimidae	<i>Dipteromimus</i>
	Y	Y	Y	Ephemerellidae	<i>Drunella</i>
	Y	Y	Y	Ephemerellidae	<i>Eurylophella</i>
	Y	Y	Y	Ephemerellidae	<i>Hyrtanella</i>
	Y	Y	Y	Ephemerellidae	<i>Serratella</i>
	Y	Y	Y	Ephemerellidae	<i>Timpanoga</i>
	Y	Y	Y	Ephemerellidae	<i>Torleya</i>
	Y		Y	Ephemeridae	<i>Eatonica</i>
	Y		Y	Ephemeridae	<i>Eatonigenia</i>
	Y	Y	Y	Ephemeridae	<i>Ephemera</i>
	Y	Y	Y	Ephemeridae	<i>Hexagenia</i>
	Y		Y	Ephemeridae	<i>Pentagenia</i>
	Y		Y	Ephemerythidae	<i>Ephemerythus</i>
	Y		Y	Euthyplociidae	<i>Campylocia</i>
	Y	Y	Y	Euthyplociidae	<i>Euthyplocia</i>
	Y		Y	Euthyplociidae	<i>Exeuthyplocia</i>

Table 1. Continued.

Order	Included in morphological dataset	Included in reduced dataset	Included in total dataset	Family	Genus
	Y	Y	Y	Euthyplociidae	<i>Polyplocia</i>
	Y	Y	Y	Euthyplociidae	<i>Proboscidoplocia</i>
	Y	Y	Y	Euthyplociidae	<i>Proboscidoplocia2</i>
	Y	Y	Y	Heptageniidae	<i>Afronurus</i>
	Y	Y	Y	Heptageniidae	<i>Cinygma</i>
	Y	Y	Y	Heptageniidae	<i>Ecdyonurus</i>
	Y	Y	Y	Heptageniidae	<i>Heptagenia</i>
	Y	Y	Y	Heptageniidae	<i>Rhithrogena</i>
	Y	Y	Y	Heptageniidae	<i>Stenonema</i>
	Y	Y	Y	Ichthybotidae	<i>Ichthybotus</i>
	Y	Y	Y	Isonychiidae	<i>Isonychia</i>
	Y	Y	Y	Isonychiidae	<i>Isonychia2</i>
	Y	Y	Y	Leptohyphidae	<i>Allenhyphes</i>
	Y	Y	Y	Leptohyphidae	<i>Leptohyphes</i>
	Y	Y	Y	Leptohyphidae	<i>Tricorythodes</i>
	Y		Y	Leptophlebiidae	<i>Castanophlebia</i>
	Y	Y	Y	Leptophlebiidae	<i>Choroterpes</i>
	Y	Y	Y	Leptophlebiidae	<i>Habroleptoides</i>
	Y	Y	Y	Leptophlebiidae	<i>Paraleptophlebia</i>
	Y	Y	Y	Leptophlebiidae	<i>Paraleptophlebia2</i>
	Y		Y	Machadorythidae	<i>Machadorythus</i>
	Y		Y	Melanemerellidae	<i>Melanemerella</i>
	Y		Y	Metretopodidae	<i>Metreplecton</i>
	Y	Y	Y	Metretopodidae	<i>Metretopus</i>
	Y	Y	Y	Metretopodidae	<i>Siphloplecton</i>
	Y	Y	Y	Neophemeridae	<i>Neophemera</i>
	Y	Y	Y	Neophemeridae	<i>Potamanthellus</i>
	Y	Y	Y	Nesameletidae	<i>Ameletoides</i>
	Y	Y	Y	Nesameletidae	<i>Metamonius</i>
	Y	Y	Y	Nesameletidae	<i>Nesameletus</i>
	Y		Y	Oligoneuriidae	<i>Chromarcys</i>
	Y	Y	Y	Oligoneuriidae	<i>Elassoneuria</i>
	Y	Y	Y	Oligoneuriidae	<i>Lachlania</i>
	Y	Y	Y	Oligoneuriidae	<i>Oligoneuriella</i>
	Y	Y	Y	Oniscigastridae	<i>Oniscigaster</i>
	Y	Y	Y	Oniscigastridae	<i>Siphlonella</i>
	Y	Y	Y	Oniscigastridae	<i>Tasmanophlebia</i>
	Y		Y	Palingeniidae	<i>Anagenesia</i>
	Y	Y	Y	Palingeniidae	<i>Cheirogenesis</i>
	Y	Y	Y	Palingeniidae	<i>Palingenia</i>
	Y	Y	Y	Palingeniidae	<i>Plethogenesis</i>
	Y	Y	Y	Polymitarciidae	<i>Ephoron</i>
	Y		Y	Polymitarciidae	<i>Povilla</i>
	Y	Y	Y	Polymitarciidae	<i>Tortopus</i>
	Y	Y	Y	Potamanthidae	<i>Anthopotamus</i>
	Y	Y	Y	Potamanthidae	<i>Potamanthus</i>
	Y	Y	Y	Potamanthidae	<i>Rhoenanthus</i>
		Y	Y	Potamanthidae	<i>Stygifloris</i>
	Y	Y	Y	Prosopistomatidae	<i>Prosopistoma</i>
	Y	Y	Y	Prosopistomatidae	<i>Prosopistoma2</i>
	Y		Y	Protereismatidae	<i>Kukalova^a</i>
	Y		Y	Protereismatidae	<i>Protereisma^a</i>
	Y	Y	Y	Pseudironidae	<i>Pseudiron</i>
	Y	Y	Y	Rallidentidae	<i>Rallidens</i>
	Y	Y	Y	Siphlaenigmatidae	<i>Siphlaenigma</i>
		Y	Y	Siphonuridae	<i>Edmundsius</i>

Table 1. Continued.

Order	Included in morphological dataset	Included in reduced dataset	Included in total dataset	Family	Genus
	Y	Y	Y	Siphonuridae	<i>Parameletus</i>
	Y		Y	Siphonuridae	<i>Siphonisca</i>
	Y	Y	Y	Siphonuridae	<i>Siphonurus</i>
	Y		Y	Siphonuridae	<i>Siphurella</i>
	Y	Y	Y	Siphuriscidae	<i>Siphuriscus</i>
	Y		Y	Teloganellidae	<i>Teloganella</i>
		Y	Y	Teloganodidae	<i>Derlethina</i>
	Y		Y	Teloganodidae	<i>Dudgeodes</i>
	Y	Y	Y	Teloganodidae	<i>Manohyphella</i>
	Y		Y	Tricorythidae	<i>Diceromyzon</i>
	Y	Y	Y	Tricorythidae	<i>Spinirythus</i>
	Y	Y	Y	Tricorythidae	<i>Tricorythus</i>
Total no. taxa included	113	96	122		
Methodological approaches performed	Parsimony	Parsimony Bayesian Likelihood poY	Parsimony Bayesian		^a Extinct

species (*Namurotypus*) for a total of 122 taxa (Table 1). Within Ephemeroptera, 107 genera, from 42 recognized families, representing ~30% of the genera and essentially all of the families (although familial ranks are in flux) were included. Numerous genera from large, diverse families were included in order to better represent the major lineages within these taxa. For some species it was impossible to collect fresh specimens to yield molecular characters, and therefore these species were only included in analyses using morphological characters. In addition to the morphological component, DNA data has been collected for several important families since the Ogden & Whiting (2005) analysis, such as Siphuriscidae, Teloganodidae and Tricorythidae.

Morphological dataset

The morphological dataset consisted of 101 characters coded for 113 taxa (Supporting Information Tables S2 and S3). All major lineages and families of mayflies were represented with morphological data. All pertinent characters used in previous analyses were included in the dataset (Supporting Information Table S3). Thirty-one characters were coded from the imaginal stage and 70 from the larval stage. Across the character matrix, nine characters were coded as ordered, 24 were coded as Dollo up, 57 as irreversible up, and 11 as unordered (as indicated in Supporting Information Table S1). The data matrix (Supporting Information Table S3) was analysed under parsimony criteria using PAUP* 4.0b10 (Swofford, 2002). Tree searches were conducted using the heuristic search option with tree bisection and reconnection (TBR) branch swapping. Both missing data and non-applicable characters were scored as ? and /, respectively. Branches collapsed (creating polytomies) if the maximum branch length was zero. A 50% majority-rule consensus tree was constructed

based on the most-parsimonious trees. Character states were mapped on one of the most-parsimonious trees using the 'list of changes' in the tree description option.

Molecular data

For the molecular data, taxonomic sampling consisted of exemplars representing 84 species of Ephemeroptera, five species of Odonata and three species of non-apterygote insects, for a total of 96 taxa (Table 1). Within Ephemeroptera, 79 genera, from all four suborders, and from 31 families (not counting *Arthroplea*, *Pseudiron* and *Dipteromimus* as families), representing ~25% of genera and 91% of families were included. Representatives of the families Coryphoridae, Austremereleididae, Vietnamellidae, Ephemerythidae, Machadorithidae and Teloganellidae (all of which are monotypic) were not available for sequencing or failed to obtain a result in polymerase chain reaction amplification. Most of the data came from Ogden & Whiting (2005), but new sequence data were acquired for several taxa. For all new data, muscle tissue was dissected and DNA extracted following the Qiagen DNeasy protocol for animal tissue (Valencia, CA, U.S.A.). Genomic DNA vouchers and specimen vouchers were deposited at the Insect Genomics Collection, 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, 28S rDNA, 16S rDNA, 12S rDNA and Histone 3 protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are given in Whiting 2001; for 12S rDNA, 16S rDNA and additional modified mayfly primers specific for 28S see Ogden & Whiting (2005). Product yield, specificity and potential contamination were monitored via agarose gel electrophoresis.

Successful amplifications were purified and cycle-sequenced using ABI Prism Big Dye[®] Terminator version 3.0 chemistry. Sequencing reactions were column purified and analysed 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 Supporting Information Table S1.

Phylogenetic analyses

The molecular data and the morphological characters were combined using several different strategies to examine the effects of missing data and the exclusion of problematic taxa on phylogenetic relationships. The different dataset combinations were: morphological dataset (113 taxa), consisting of the 101 morphological characters; reduced dataset (96 taxa), comprising taxa with the most molecular and morphological data; total dataset (122 taxa), consisting of the 16 taxa with only morphological data, nine taxa with only molecular data and 87 taxa with molecular and morphological data (Table 1).

Sequences were assembled initially in SEQUENCHER[®]4.0 (Genecodes, 1999). The protein coding H3 gene was aligned manually with reference to the amino acid sequence. For the ribosomal genes, a preliminary alignment was performed by aligning the conserved domains across the taxa. The 12S, 16S, 18S and 28S sequences were then sectioned into smaller fragments at the conserved domains in order to find optimal solutions more efficiently in the POY searches (Giribet, 2001). This resulted in four fragments for 12S, five fragments for 16S, 11 fragments for 18S and 12 fragments for 28S. Fragment 10 of 28S (corresponding to region D7a) contained a highly length-variable insertion region and was excluded because the sequence fragments were judged non-homologous. Multiple sequence alignment was performed on each of these same fragments from the ribosomal genes in CLUSTALW version 1.83 (Thompson *et al.*, 1994), using default settings. Although this approach has been shown to produce, on average, more accurate alignments and more accurate subsequent topologies compared with POY, when the trees are more pectinate, POY tends to recover more accurate topologies (Ogden & Rosenberg, 2007). Some taxa had missing data (see datasets, Supporting Information Table S1) in one or more of the DNA fragments that were submitted to CLUSTALW for alignment and to POY for direct optimization analysis.

The datasets resulting from CLUSTALW alignment were analysed in PAUP* 4.0b10 (Swofford, 2002) under maximum parsimony, with gaps treated as missing and as a fifth state character and under the maximum likelihood criterion (for reduced molecular dataset). Parsimony settings consisted of 100 random additions with TBR swapping. Strict and 50% consensus trees were examined. Bootstrap and Bremer values were calculated for the total and reduced datasets. The bootstrap analyses consisted of 1000 replicates with 20 random additions per replicate. Bremer values were computed in NONA (Goloboff, 1994). MODELTEST (Posada & 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 (GTR + G + I) 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. The same model was implemented across all molecular data under the combined analysis approach. Bayesian inference was performed on the total and reduced datasets in MRBAYES (v3.1) (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using mixed models, in order to include the morphological partition. In total, 1 500 000 generations, running on four chains, were sampled every 1000 generations. The first 500 000 generations were excluded (as the burn in).

Direct optimization, in the program POY version 3.0 (Gladstein & Wheeler, 1999), was implemented, for 50 random additions, under the following commands: -sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 25 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -stopat 25 -multirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 50 -ratchetspr 2 -ratchettr 2 -checkslop 10. All direct optimization analyses were rooted to *Machilis* (Machilidae: Archeognatha). This approach was used to identify areas sensitive to fixed multiple alignments, particularly if the topology is more pectinate (Ogden & Rosenberg, 2006).

Results

The clustal alignments for each gene consisted of 415, 591, 2010 and 2485 characters for 12S, 16S, 18S and 28S gene partitions, respectively. The aligned characters for these nuclear and mitochondrial ribosomal genes (5501 total), the H3 protein coding gene (379 nucleotides long) and the morphological data (101 characters) combined for a total of 5981 characters.

Parsimony analyses of the reduced dataset (96 taxa) using clustal alignment (Fig. 2)

When gaps were treated as missing characters, 2786 characters were constant and parsimony-uninformative, 744 were variable characters, but were parsimony-uninformative and 2451 characters were parsimony-informative. When gaps were treated as a fifth state character, 1651 characters were constant and parsimony-uninformative, 863 were variable characters and parsimony-uninformative and 3467 characters were parsimony-informative.

Ephemeroptera was strongly supported as monophyletic. The proposed suborders Furcatergalia and Carapacea were supported as monophyletic clades. However, Pisciforma and Setisura were not supported as being monophyletic. The taxon *Siphuriscus* (Siphuriscidae) was recovered as sister to all other mayflies, with high Bremer support and when gaps were treated as a fifth state character. The lineages Baetidae and

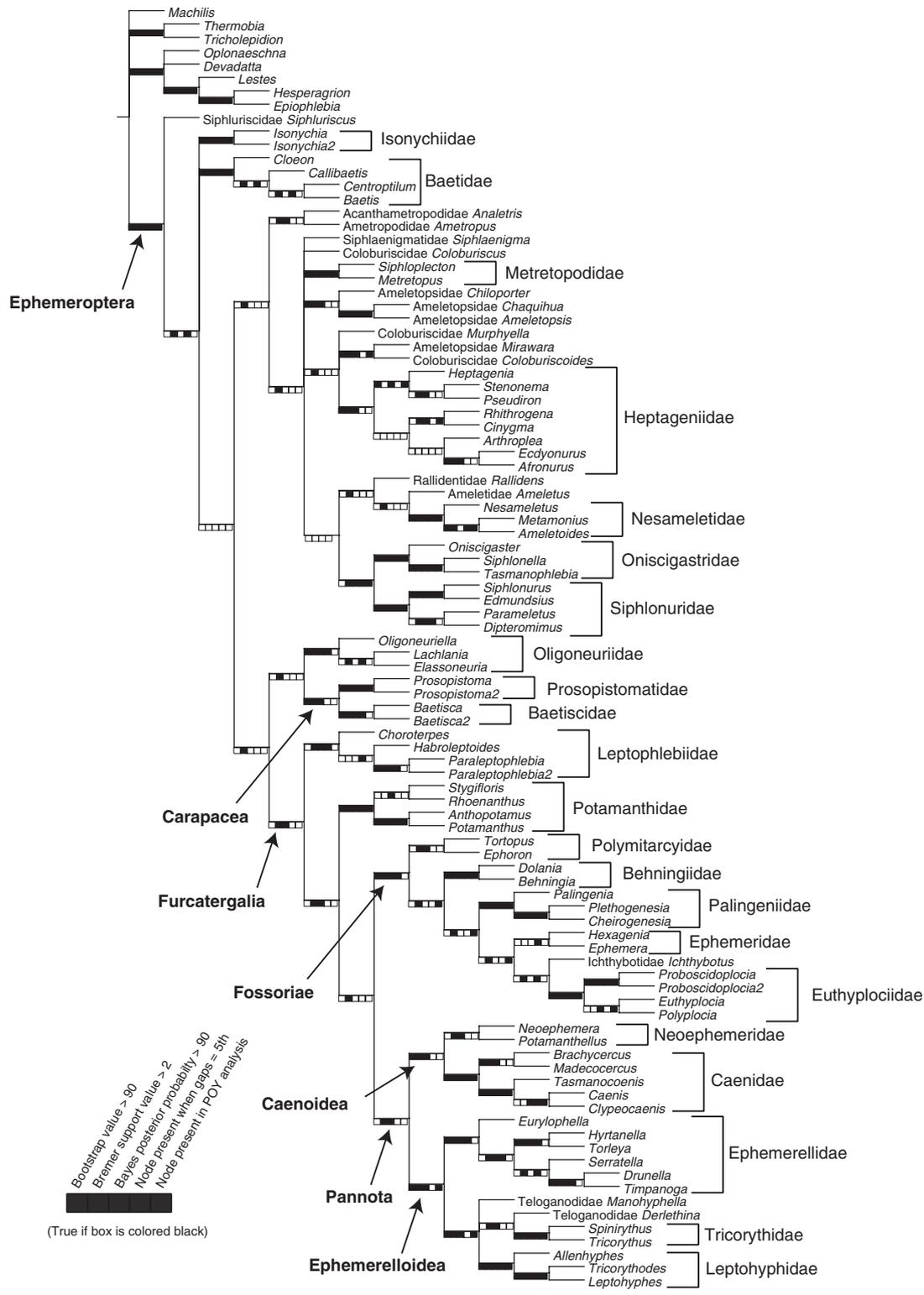


Fig. 2. Strict consensus topology of nine most-parsimonious trees of the reduced combined dataset parsimony analysis (96 taxa). The nodes that were also recovered with a bootstrap value >90, Bremer >2, Bayesian posterior probability >90, when gaps were treated as a fifth state character, and in the POY analysis are indicated by shaded boxes. Families that were recovered as monophyletic are represented by the name and reversed bracket. All other taxa were represented either by only one specimen or were not recovered as monophyletic in this analysis.

Isonychiidae were unresolved relative to the remaining clade that included all other mayfly taxa sampled.

The analysis recovered two large clades, one of which consisted of the majority of the pisciform mayflies (excluding Baetidae and Isonychiidae) and the Heptageniidae (including *Arthroplea* and *Pseudiron*). The families Heptageniidae (when including *Arthroplea* and *Pseudiron*), Siphonuridae (when including *Dipteromimus*), Acanthametropodidae, Nesameletidae and Oniscigastridae were supported as monophyletic. However, two currently proposed families, Ameletopsidae and Coloburiscidae, were not supported as monophyletic clades. There was strong support to recognize *Mirawara* (Ameletopsidae) as sister group of *Coloburiscoides* (Coloburiscidae). There was good support for the sister-group relationship of Oniscigastridae and Siphonuridae.

In the other large clade, Carapacea + Oligoneuriidae were recovered as sister group to Furcatergalia. The sister-group relationship of Prosopistomatidae and Baetiscidae was strongly supported. Within Furcatergalia, the monophyletic Leptophlebiidae was sister to all other clades. Potamanthidae was not supported as nesting within the clade containing the other burrowing mayflies (Fossoriae), rendering the proposed Ephemerioidea as non-monophyletic. Fossoriae and Pannota were recovered as sister groups. Fossoriae comprised the monophyletic families Polymitarciidae, Behningiidae, Palingeniidae, Ephemeridae (including *Pentagenia*) and Euthyplociidae. Within Pannota, the well-supported superfamily Caenoidea (Caenidae + Neophemeridae) was recovered as sister to Ephemerelloidea. Among this last superfamily, Ephemerellidae was recovered as sister to Tricorythidae, Leptohyphidae and Teloganodidae. The three families Ephemerellidae, Tricorythidae and Leptohyphidae were well supported as monophyletic.

Likelihood analyses of the reduced dataset using clustal alignment (Fig. 3)

The likelihood analyses of the clustal alignment also recovered a monophyletic Ephemeroptera with *Siphuriscus* as sister to all other mayflies (log likelihood = 102898.30715). The lineage Baetidae + Isonychiidae was recovered as sister to the remaining lineages of mayflies, which were basically composed of two main clades. However, the clades do not share the same sets of families as in the parsimony analyses. The major difference is that the carapace mayflies (Baetiscidae + Prosopistomatidae) were recovered in the opposing clade. Some differences in branching order within the pisciform + heptageniid assemblage can also be observed. The sister relationship of Oniscigastridae and Siphonuridae was supported as monophyletic. The relationships for the second main clade are very similar to the parsimony analysis with Oligoneuriidae as sister to Furcatergalia. Leptophlebiidae and Potamanthidae are in the same position as before nesting outside the pannota and Fossoriae clades. Pannota, however, was not supported as monophyletic because Caenoidea was recovered as sister to Fossoriae + Ephemerelloidea. As before, Ephemerellidae is sister to the other represented ephemerelloid

groups. Within the Fossoriae, the sister relationship of the families Ichthybotidae and Euthyplociidae was recovered. However, other familial relationships of the other burrowing groups conflict with the parsimony results, and Polymitarciidae was not supported as monophyletic.

Parsimony analyses of morphological data (Fig. 4)

The phylogenetic analyses recovered 512 247 most-parsimonious topologies and a 50% majority-rule consensus tree of 1020 steps was constructed (Fig. 4). The consistency index was 0.1490 and retention and homoplasy indexes were 0.8431 and 0.8510, respectively. Of the 101 characters used, only eight were autapomorphic and only four others had a consistency index superior or equal to 0.500. Therefore, for every ten evolutionary morphological transitions, eight or nine are probably due to homoplasy.

Unlike the previous results, Furcatergalia, Fossoriae, Pannota and Ephemerelloidea were not supported in the morphological topology. Nevertheless, the morphological data did support Carapacea and Caenoidea as monophyletic.

In contrast to the molecular analysis, the lineage Baetoidea (Baetidae + Siphlaenigmatidae) was supported by the morphological dataset. Baetoidea were recovered as the sister group of Leptophlebiidae and Ameletidae. These relationships were supported by the morphological analysis, and were surprising because they had not been previously hypothesized. The support comes from seven homoplastic characters, such as the dissimilar claws of the legs in the adult and the absence of posterolateral expansions on the abdomen in nymphs. Given that absence of a shared character may not be good evidence of common ancestry and given that the consistency indices were somewhat low (varying between 0.067 and 0.200), these relationships should be viewed with caution.

The majority of mayfly lineages constitute a monophyletic but poorly supported group. The Siphonuridae + Nesameletidae + Rallidentidae + Siphuriscidae clade was recovered with good support. Each of these families belongs to the putative suborder Pisciforma and superfamily Siphonuroidea.

The rest of the families are subdivided into two subgroups. The first subgroup contains the rest of the Siphonuroidea (Ametropodidae, Metretropodidae, Acanthametropodidae and Oniscigastridae) and the Pannota and the second subgroup comprises Setisura and Ephemerioidea. As already noted, Pannota was not supported as monophyletic, due to the inclusion of Carapacea, yet the relationships among this lineage were well supported. Ephemerellidae and Teloganodidae, together with two monotypic families (Austremerellidae and Melanemerellidae) were the sister group of the rest of the Pannota + Carapacea. The Carapacea (Prosopistomatidae + Baetiscidae) were supported as the sister group of Caenoidea (Caenidae + Neophemeridae).

In the second large subgroup, McCafferty's concept of Setisura was recovered except for the inclusion of the Behningiidae. Although the Coloburiscidae were supported

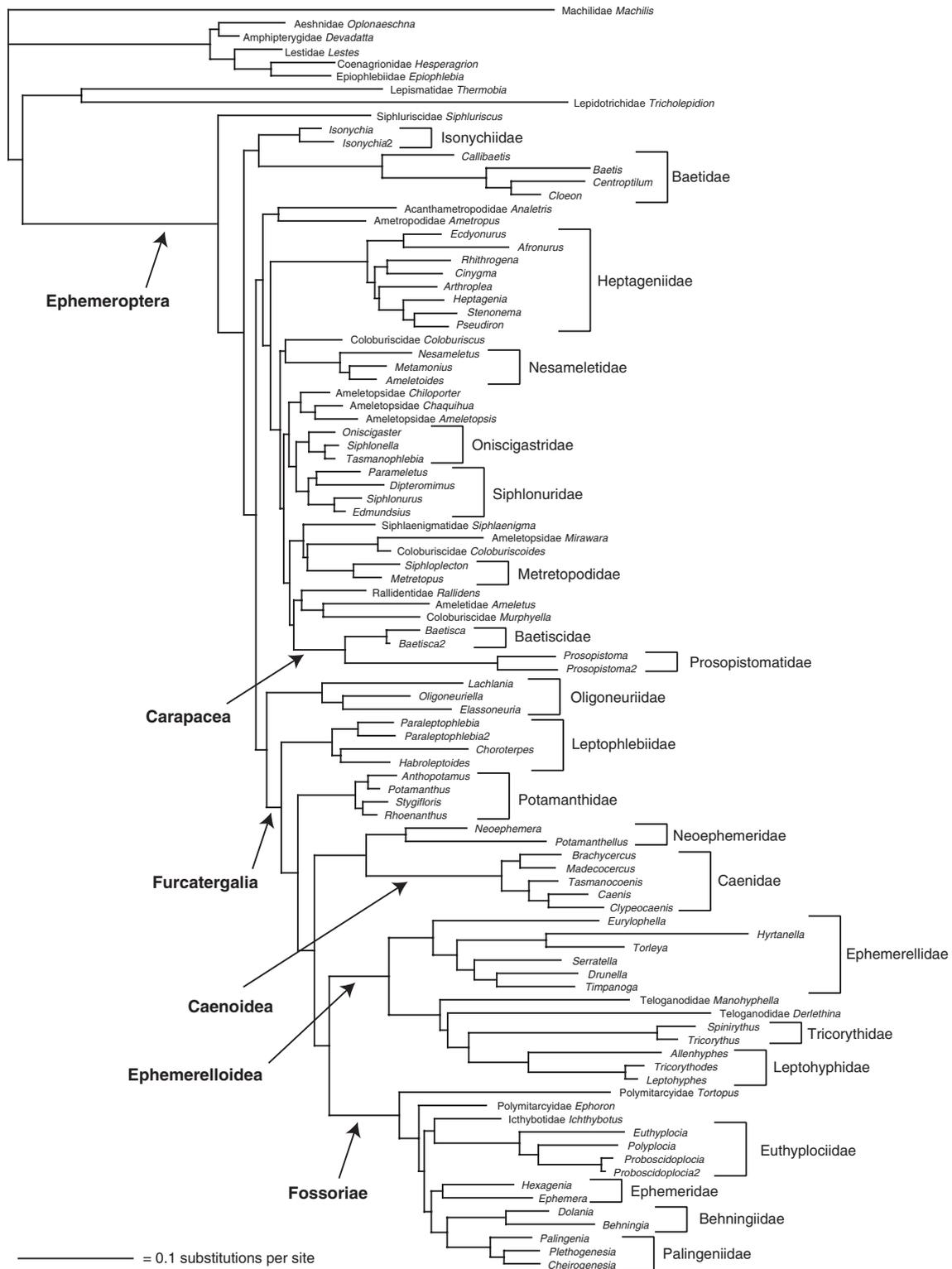


Fig. 3. Likelihood phylogram with relative branch lengths. Families that were recovered as monophyletic are represented by the name and reversed bracket. All other taxa were represented either by only one specimen or were not recovered as monophyletic in this analysis.

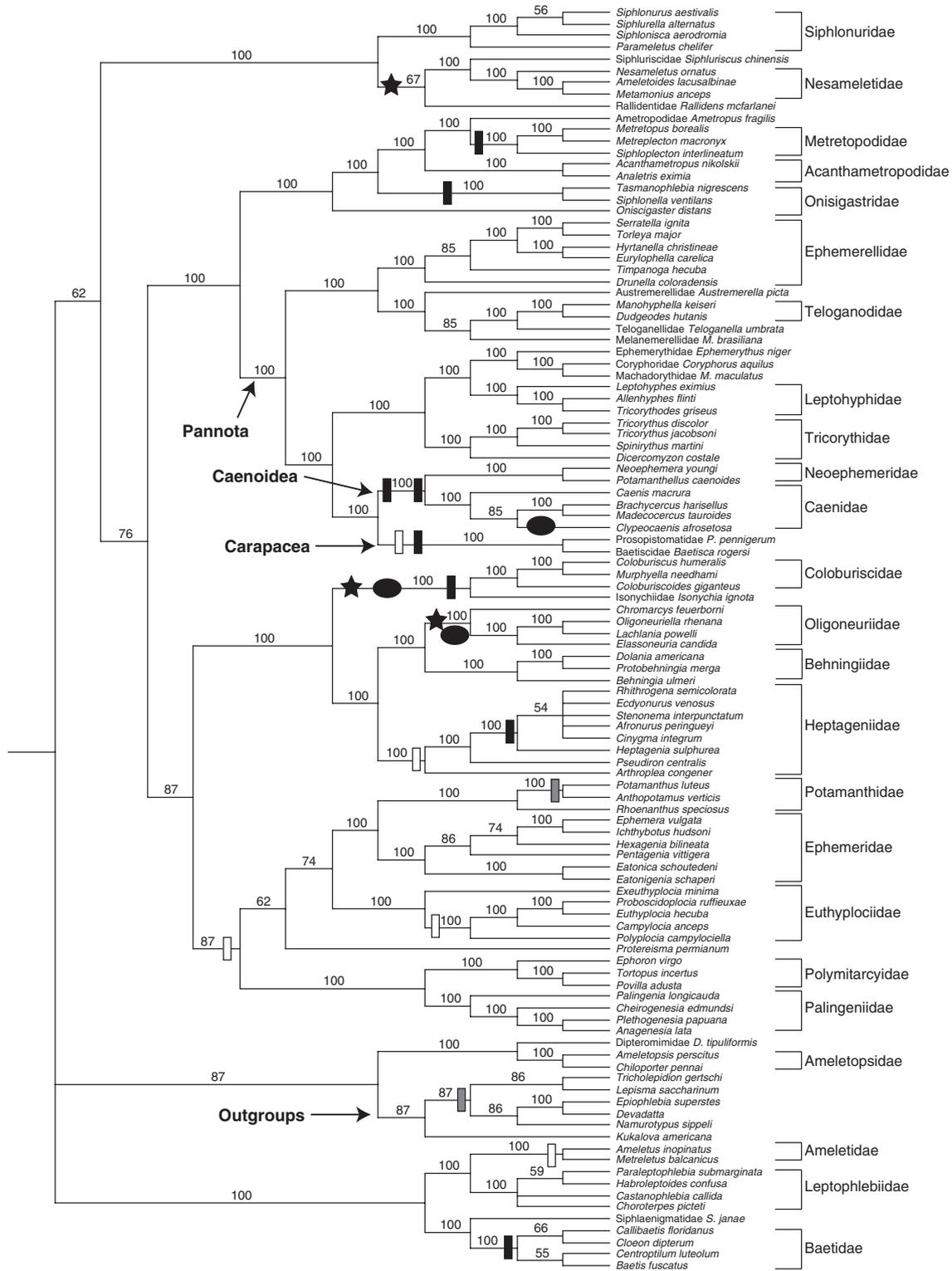


Fig. 4. Fifty per cent majority-rule consensus tree of the 512 247 most-parsimonious topologies of the morphological dataset (101 characters coded for 113 taxa). The numbers on the nodes are the consensus values. Only characters with consistency index ≥ 0.500 are mapped. Black boxes: autapomorphies; white boxes: homoplasies or transition states; grey boxes: reversion. Stars indicate lineages with maxillary gills; ellipses indicate lineages with filtering setae on forelegs.

to be monophyletic (polyphyletic in the molecular analysis), they were placed as the sister group to the Isonychiidae. Coloburiscidae + Isonychiidae were recovered as the sister group to a clade that comprised Heptagenioidea + (Behningiidae + Oligoneuriidae). The inclusion of the Behningiidae within the Setisura remained enigmatic, as previous workers have placed them commonly within the Ephemeroidea. *Pseudiron* and *Arthroplea* were sister groups of the rest of the Heptageniidae. Thus, the morphology data support a monophyletic Heptageniidae, which could even include *Pseudiron* and *Arthroplea*. Still, this topology does not corroborate *Pseudiron* and *Arthroplea* as lineages nesting within the other heptageniid taxa, as do the other analyses.

Ephemeroidea constituted a monophyletic group and some of the relationships between the different families were not always strongly supported. A monophyletic Palingeniidae was supported as the sister group of Polymitarciidae, rather than being more closely related to Ephemeridae. *Ichthybotus* was included in the Ephemeridae, challenging the concepts of these families. The fossil genus *Protereisma* was included by the present analysis in the Ephemeroidea, but relationships with the other families remained unclear.

Parsimony analyses of the total dataset (116 taxa) using clustal alignment (Fig. 5)

When gaps were treated as missing characters, 2772 characters were constant and parsimony-uninformative, 753 were variable characters, but were parsimony-uninformative and 2456 characters were parsimony-informative. When gaps were treated as a fifth state character, 1628 characters were constant and parsimony-uninformative, 884 were variable characters and parsimony-uninformative and 3468 characters were parsimony-informative.

Phylogenetic analyses recovered 170 most-parsimonious topologies and a strict consensus of these topologies was constructed (Fig. 5). The topology resembles the topology from the parsimony analyses of the reduced dataset (Fig. 2). *Siphuriscus* was again recovered as sister to all other mayflies, and Baetidae and Isonychiidae were in the same positions as before. Essentially, the same two large clades containing the remaining taxa were recovered as well. One clade similarly contained the pisciform mayflies (excluding Baetidae and Isonychiidae) and Heptageniidae. The second clade follows the same pattern as above for the Carapacea + Oligoneuriidae being sister to Furcatergalia. The relationships within Furcatergalia were also very much the same, although with a little less resolution in some areas (for example, Ephemerelloidea).

POY analyses of the reduced dataset (Fig. 6)

Implied alignment for each gene consisted of 771 characters for 12S, 1510 characters for 16S, 2718 characters for 18S and 3727 characters for 28S. The implied alignment characters for the ribosomal genes (8726 total), the H3 protein coding

gene (379 nucleotides long) and the morphological data (101 characters) combined for a total of 9206 characters.

The POY analyses recovered a monophyletic Ephemeroptera; however, there were major differences in the branching order as compared with the clustal alignment analyses (Fig. 5). Most notably, Ephemerelloidea was supported as sister to all other mayflies. Additionally, several families recovered as monophyletic in the clustal alignment analyses were not supported as monophyletic in the POY analysis (for example: Oligoneuriidae, Heptageniidae, Leptophlebiidae, Baetiscidae, Neoephemeridae, Ephemeridae, Polymitarciidae and Ephemerellidae). Only the family Teloganodidae was supported as monophyletic in POY but not in the other analyses.

Discussion

This study represents the first combined analysis of morphological and molecular data for Ephemeroptera as a whole. Although future data and analyses may imply different relationships within mayflies, a summary hypothesis (Fig. 7) of the major lineages, chiefly based on the total reconstruction, offers a view somewhat different from traditional hypotheses of Kluge and McCafferty.

All major proposed lineages were included in this study, even if only by morphological data for some taxa. Several new and important taxa have been sequenced and utilized in this study. One such taxon was *Siphuriscus chinensis*, the sole species attributed to the Siphuriscidae (Zhou & Peters, 2003), which was supported as sister to all other mayflies in the molecular and total dataset reconstructions. *Siphuriscus chinensis* was considered to be related most closely to Nesameletidae (Hitchings & Staniczek, 2003; Zhou & Peters, 2003). In describing the egg, larva and additional characters of the adult, Zhou & Peters (2003) suggested a relationship to the Middle Jurassic *Stackelbergisca* (originally associated with Acanthametropodidae) and they thus considered the Siphuriscidae to be a member of the perhaps oldest extant lineage. Although no dates have been estimated specifically for these analyses, the total dataset analysis included the fossil taxa. Lower Permian *Kukalova* (Protereismatidae) nested well within the pisciform main clade as sister to three of the ameletopsid taxa. This assumes that more ancestral nodes are at least as old as the Permian; however, the position of *Kukalova* was not well supported.

As proposed by Ogden & Whiting (2005), Baetidae were not supported as nesting within the other pisciform mayflies. Therefore, there was good support that the ancestor to mayflies presented a more torpedo- ('hydrodynamic'-) shaped body form adapted for swimming, instead of a robust body shape adapted for crawling and clinging. The phylogenetic positions of Baetidae and Isonychiidae are somewhat surprising and contradictory to previous hypotheses (non-molecular data) where Baetidae + Siphonematidae were proposed as the monophyletic group Baetoidea (Staniczek, 1997) and Isonychiidae was considered to be a member of the Setisura,

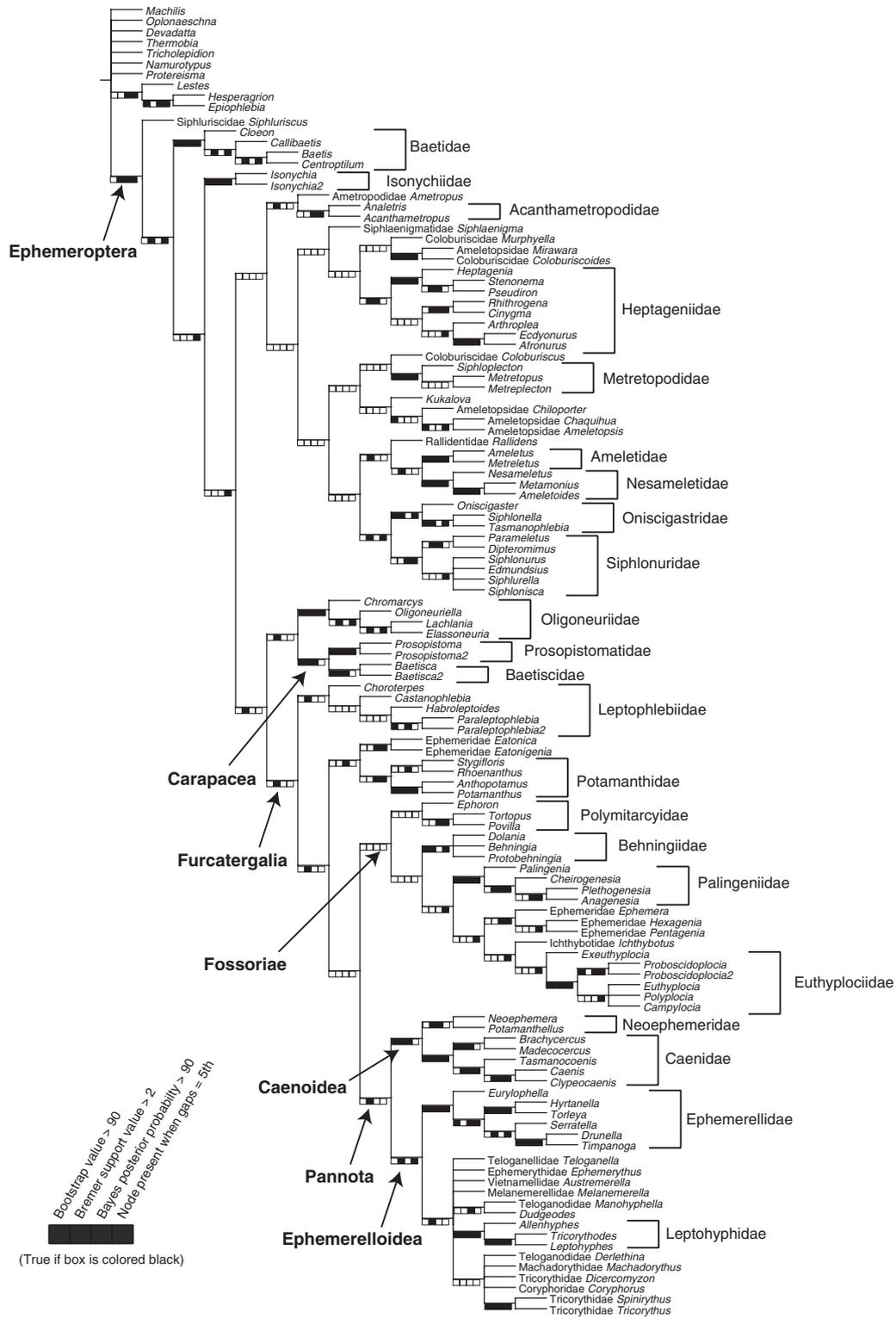


Fig. 5. Strict consensus topology of 140 most-parsimonious trees of the total combined dataset parsimony analysis (122 taxa). The nodes that were also recovered with a bootstrap value >90, Bremer >2, Bayesian posterior probability >90 and when gaps were treated as a fifth state character are indicated by shaded boxes. Families that were recovered as monophyletic are represented by the name and reversed bracket. All other taxa were represented either by only one specimen or were not recovered as monophyletic in this analysis.

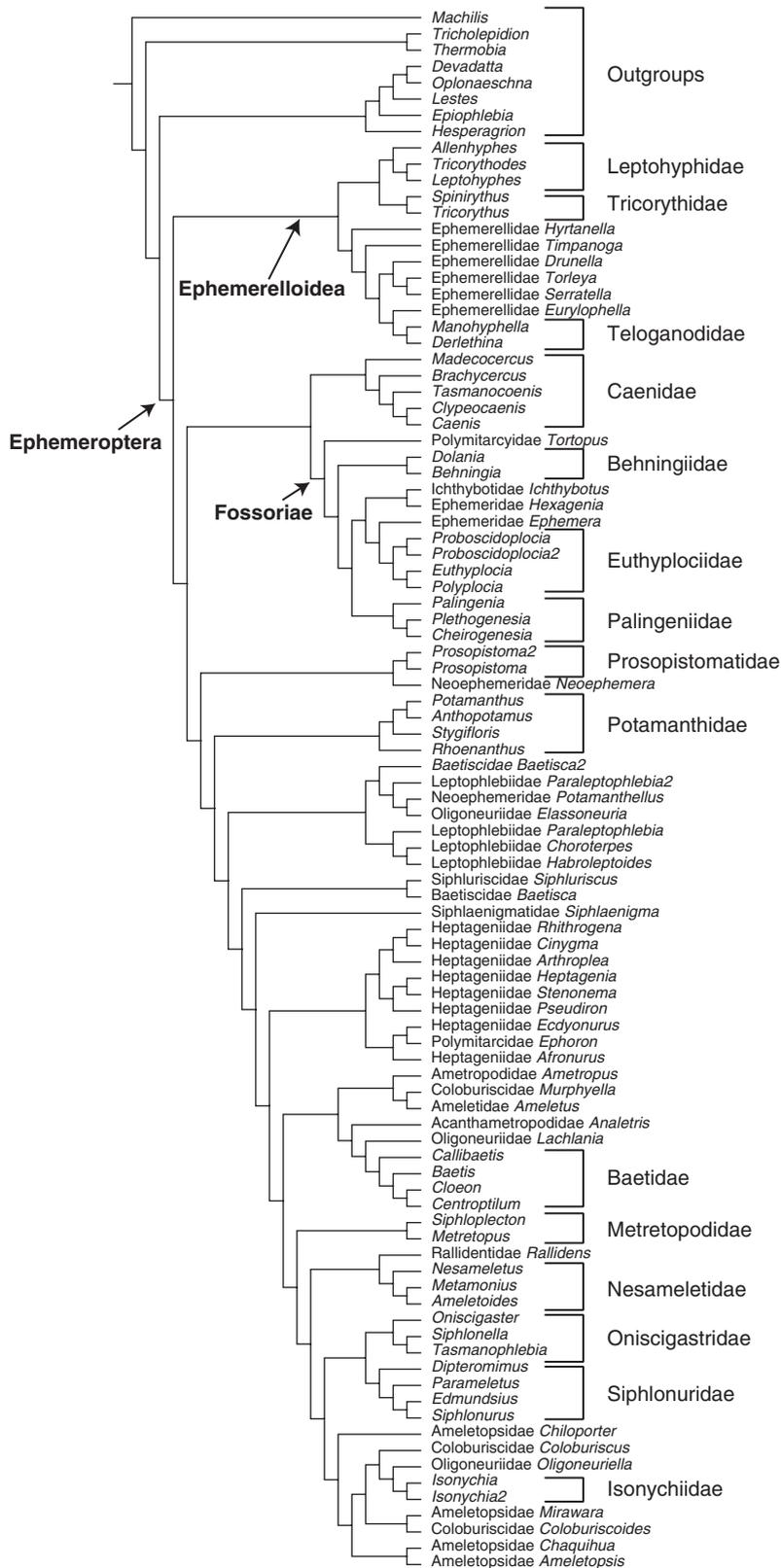


Fig. 6. POY (direct optimization) topology analysed under a 1:1:1 (gap : tv : ts) ratio parameter set. Families that were recovered as monophyletic are represented by the name and reversed bracket. All other taxa were either represented by only one specimen or were not recovered as monophyletic in this analysis.

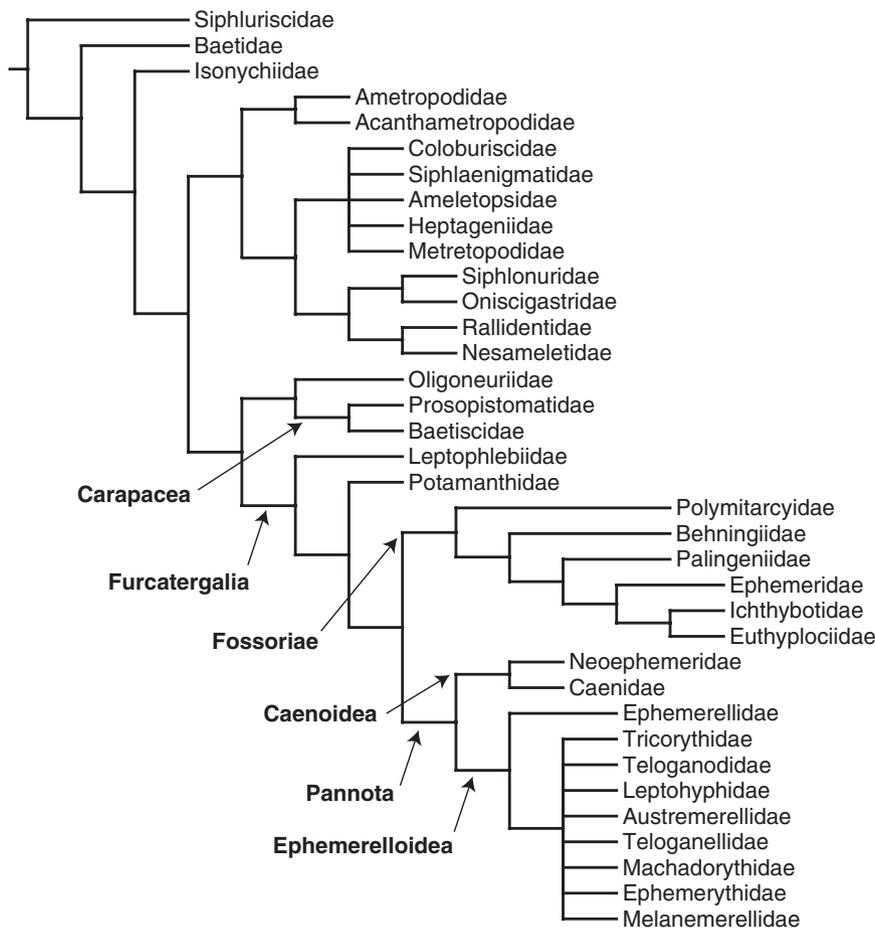


Fig. 7. Summary hypothesis of the major mayfly lineages based on the result of the total combined analysis. Polytomies represent relationships that were not well resolved given the current suite of data.

and closely related to Oligoneuriidae (Riek, 1973; McCafferty, 1991a). The morphological reconstruction alone recovered Baetoidea as monophyletic, but in an enigmatic position. On the basis of Staniczek (1997), Baetoidea were characterized by the following apomorphies: (1) larval antennae elongated, (2) middle and hind legs with four tarsomeres, (3) hindwing much reduced or missing, and (4) veins IMP and MP2 detached in forewing. Mapping of these characters in the trees indicated that three of them were also present in other clades and do not constitute autapomorphies of Baetoidea (Staniczek, 1997) (character 1 is also shared by some Leptophlebiidae or Ephemerelloidea, character 3 is common among Ephemerelloidea, character 4 also occurs in some Leptophlebiidae or Tricorythidae, for instance). Only character 2 seems autapomorphic for Baetoidea (mapped on the morphological tree), hence the name *Tetramerotarsata* used by Kluge (2004) (Fig. 1). Edmunds (1972) already pointed out that the siphlaenigmatid larval external morphology is baetid-like, but the ventral nerve cord and Malpighian tubes are closer to Nesameletidae. Internal anatomy and molecular characters suggest that the superficial similarities between Baetidae and Siphlaenigmatidae may be explained by convergent evolution rather than by homology.

Setisura (= Branchitergalia) were recovered as monophyletic in the morphological reconstruction, but included the Behningiidae, which have always been considered a member of Furcatergalia in all other analyses, as well as in previous reconstructions. All the different molecular reconstructions showed Setisura as highly polyphyletic as the different families belong to different clades. McCafferty (1991a) listed eight characters common to Setisura, of which only one is in the adult stage. Mapping of these characters in our reconstructions indicated that most may be subject to homoplasies, such as reduction in some mouthparts, or the abdominal gills with basal tufts, also present in Rallidentidae and Ameletopsidae. The supposed apomorphies in internal anatomy were misinterpreted by McCafferty (1991a) when compared with the original work by Landa & Soldán (1985). We may assume, as for Baetoidea, that Setisura is not monophyletic and the concept was based more on convergences and/or plesiomorphies than on unique apomorphies.

In previous hypotheses (Fig. 1), Isonychiidae, Coloburiscidae and Oligoneuriidae were considered as closely related (*Eusetisura*), based on some remarkable larval characters, such as maxillary gill tufts, as in Kluge 2004: figs 37C, 41D and Štys & Soldán 1980: fig. 13, or filtering setae on the forelegs in

Edmunds 1975: figs 25–28, but our combined and morphological analyses placed them in three different lineages. Therefore, we have to assume that such characters appear independently or reflect plesiomorphic conditions. The placement of Isonychiidae as the sister group of all extant families, excluding Siphuriscidae and Baetidae, is not so surprising, as *Isonychia* has been defined as remarkable a 'living fossil' as *Latimeria* (Edmunds, 1975). Therefore, the concept of Eusetisura is highly polyphyletic.

As proposed by others (Kluge *et al.*, 1995; Ogden & Whiting, 2005), these analyses of molecular and morphological data did not support 'Siphonuroidea' as a monophyletic group. The presence of accessory maxillary gill (Zhou & Peters, 2003: fig. 8; Štys & Soldán, 1980: fig. 18) mapped as synapomorphic for Rallidentidae + (Nesameletidae + Siphuriscidae) on the morphological topology, but as homoplasious on the other topologies that included molecular data. Interestingly, the Oniscigastridae was the only family not supported as monophyletic by the morphological reconstruction. The monophyly of Oniscigastridae has never been challenged, and was supported by the molecular data in this study. They are true vicariants in their amphinotic distribution, indicating a common ancestor: *Oniscigaster* is restricted to New Zealand, *Tasmanophlebia* being found only in Australia, whereas *Siphonella* is endemic in the Neotropic-Patagonian area. The larvae of the Oniscigastridae have a very specific general appearance; especially the gill morphology is unique within mayflies. However, otherwise the Oniscigastridae show significant plesiomorphies, e.g. the large hindwings and the forewings bearing many triads.

In all molecular reconstructions, Ameletopsidae and Coloburiscidae were polyphyletic, whereas in the morphological reconstruction both families were monophyletic with Coloburiscidae as the sister group to all other Setisura. Nodal support values strongly supported the two Australian endemic genera *Mirawara* (Ameletopsidae) and *Coloburiscoides* (Coloburiscidae) as sister groups. This is most surprising as the lineages differ by several well-established morphological characters. There was some nodal support for Siphonuridae (including *Dipteromimus*) as the sister group of Oniscigastridae, and Rallidentidae as the sister group of (Ameletidae + Nesameletidae). These relationships support the notion that these groups are of relatively ancient origin, with their ancestors going back to the Pangea, evolving in Laurasian (Siphonuridae, Ameletidae) and Gondwanian (Oniscigastridae, Rallidentidae, Nesameletidae) families after the break-off of the continental shield.

The monophyly of Carapacea (Baetiscidae + Prosopistomatidae) suggests that the evolution of the notal shield ('carapace'), which is fusion of the nymphal pronotum, mesonotum and forewing buds, together with the exceptional arrangement of the anal field of the adult forewing, and unique fusion of all thoracic and abdominal nerve ganglia to a single ganglionic mass (as in Landa & Soldán, 1985: figs 23, 24) are homologous for this group. In contrast to both McCafferty and Kluge's systems, no reconstruction recovered Carapacea as sister to all other mayflies; the concept of Kluge's Anteritorna was therefore not supported.

Our results confirm the monophyly of the Furcatergalia, as well as the position of Leptophlebiidae as its sister group. Compared with the previous molecular reconstruction (Ogden & Whiting, 2005), some new taxa were included within the burrowing mayflies (e.g. in the families Polymitarcyidae, Ephemeridae, Palingeniidae and Euthyplociidae). The increased taxon sampling supported the monophyly of all the families (Fig. 2). The different reconstructions also supported the classification of Palingeniidae as its own family, rather than being considered as a subfamily of the Ephemeridae (McCafferty, 1991b). Only morphological characters were available for *Eatonica* and *Eatonigenia*, and the morphological reconstruction (Fig. 4) clearly indicated that they belong to the Ephemeridae. The position of Potamanthidae outside Ephemeroidea supports Kluge's (2004) concept of Fossoriae, contrary to McCafferty's (2004) concept of the infraorder Scaphodonta. Regardless of the exact branching order of these clades in the different molecular and combined analyses, Behningiidae nested consistently within the Fossoriae burrowing mayflies. Thus, the hypothesis that the Behningiidae lost mandibular tusks, while retaining the burrowing life style (Ogden & Whiting, 2005), has been corroborated. Potamanthidae were recovered consistently as sister to the other burrowing mayflies + Pannota, supporting the proposal from Ogden & Whiting (2005) that the burrowing lifestyle either evolved twice (in Potamanthidae and Fossoriae) or once, prior to Potamanthidae, and lost subsequently in the Pannota.

In our reconstructions, Caenoidea is the sister group of Ephemerelloidea, corroborating the monophyletic clade of Pannota. The additional pannote taxa assisted in further resolving the relationships within this group. With the addition of *Potamanthellus*, Neoephemeridae was recovered as monophyletic and sister to Caenidae. None of the reconstructions supported Caenoidea as sister group of Ephemeroidea or Fossoriae, contradicting Kluge's concept of Fimbriatotergaliae. Ephemerelloidea was supported as a monophyletic group and Ephemerellidae was recovered as sister to all the remaining clades, whose relationships remain problematic (Jacobus & McCafferty, 2006; Ogden *et al.*, 2009).

Conclusion

This study, which extended the taxon sampling of Ogden & Whiting (2005), included morphological data from all major lineages, including some fossil taxa. Ephemeroptera was strongly supported as monophyletic across all analyses. Clades above the familial level that were recovered as monophyletic included Carapacea, Furcatergalia, Fossoria, Pannota, Caenoidea and Ephemerelloidea. Twenty-five families, represented by more than one taxa, were supported (in at least some analyses) as being monophyletic; namely Baetidae, Isonychiidae, Acanthametropodidae, Heptageniidae, Metretopodidae, Ameletidae, Nesameletidae, Oniscigastridae, Siphonuridae, Oligoneuriidae, Prosopistomatidae,

Baetiscidae, Leptophlebiidae, Potamanthidae, Polymitarcyidae, Behningiidae, Ephemeridae, Palingeniidae, Euthyplociidae, Neoephemeridae, Caenidae, Ephemerellidae, Leptohyphidae, Teloganodidae and Tricorythidae. However, some of the other recognized families (e.g. Ameletopsidae and Coloburiscidae) and major lineages (such as Setisura, Pisciforma and Ephemeroidea, among others) were not supported as monophyletic. Potamanthidae was nested outside the other burrowing mayflies, requiring either two unique origins of burrowing behaviour and mandibular tusks, with a loss of tusks in Behningiidae, or an acquisition of burrowing behaviour with a loss in the Pannota. The status of the monotypic families Pseudironidae, Arthropleidae and Dipteromimidae is challenged by these data and analyses, and until more evidence comes to light to support their familial status, perhaps they should be considered lineages within other families. *Siphuriscus* (Siphuriscidae) was supported as sister to all other mayfly clades, followed by branching of the Baetidae and Isonychiidae, confirming the results of the previous ordinal-level analysis using DNA information only (Ogden & Whiting, 2005). These results conflict with McCafferty and Kluge's proposal that Carapacea be placed as sister to all other mayflies. There was relatively strong support for the relationships and general positions among Ephemeroptera taxa, as summarized in Fig. 7. For example, nodal robustness appears to be strong for the lineages Siphonuridae + Oniscigastridae, Carapacea, Furcatergalia, Fossoriae, Pannota, Caenoidea and Ephemerelloidea. On the other hand, some of the relationships remain questionable or weakly supported and new data could alter these hypotheses. For example, branching order for the majority of the Pisciforma, part of the Setisura, and specific positions for the Carapacea and Potamanthidae were not well supported. Additional data and analyses may elucidate the phylogeny of the weakly supported portions of the mayfly phylogeny. The combined phylogeny proposed here challenges some well-established relationships, such as the monophyly of Baetoidea or Setisura. We state that most previous reconstructions were strongly hampered by superficial (external morphology) similarities, which do not always reflect the phylogeny of the order. Homoplasies therefore seem a dominant trait in mayfly morphology (and, consequently, behaviour as well), especially in nymphs.

We hope that morphologists and taxonomists will revisit their data without the historical constraints that previous reconstructions have produced. This first combined analysis may bring some answers to old questions, but it also brings to light new ones.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under DOI reference: DOI 10.1111/j.1365-3113.2009.00488.x.

Table S1 Genbank submission numbers for taxa.

Table S2 Morphological character description.

Table S3 Data matrix.

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