

EVOLUTION AND PHYLOGENY OF BASAL WINGED INSECTS
WITH EMPHASIS ON MAYFLIES (EPHEMEROPTERA)

By

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ABSTRACT

EVOLUTION AND PHYLOGENY OF BASAL WINGED INSECTS WITH EMPHASIS ON MAYFLIES (EPHEMEROPTERA)

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Doctor of Philosophy

Ephemeroptera (mayflies) is a monophyletic group of semi-aquatic pterygote insects, comprising 3083 species, 376 genera, and 37 described families and are present on all continents, excluding Antarctica, being associated with freshwater and brackish water habitats. The order 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. The purpose of this dissertation is to use molecular and morphological data, in order to investigate the position of the order Ephemeroptera among other insect orders, the higher-level relationships among the major lineages of mayflies, and a detailed analysis of the family Ephemerellidae.

Ephemeroptera has been considered by many to be sister to Odonata + Neoptera although alternate hypotheses have been suggested. Data from three molecular loci ambiguously resolve basal pterygote relationships, however, total evidence analysis (combined molecular and morphological data) strongly supports the position of mayflies

as sister to all other extant pterygotes. These results and methodologies were recently criticized, and, therefore, the response to the author is included following the manuscript.

The phylogenetic relationships among mayfly families is debatable and in some groups unknown. Prior studies have produced phylogenies based on morphological characters mixed with intuition. The first molecular phylogeny for the Order Ephemeroptera is presented. The analyses include 31 of the 37 families, representing ~24% of the genera. The suborders Furcatergalia and Carapacea are supported as monophyletic while Setisura and Pisciforma are not supported as monophyletic. The evolution of the wings, mandibular tusks, burrowing lifestyle, and fishlike body are investigated. Topological sensitivity analysis is 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.

The Pannote family Ephemerellidae is comprised of 16 genera and over 300 species and is distinguished from other mayfly families by the absence of the second pair of abdominal gills. The position of Ephemerellidae relative to other closely related pannote mayflies is unclear as are the relationships of the genera within the family. The combined molecular and morphological analyses resulted in a monophyletic Ephemerellidae as sister to the other ephemerelloid families. The subfamily Ephemerellidae was supported as monophyletic, while Timpanoginae had conflicting results.

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The problem with “the Paleoptera Problem:” sense and sensitivity

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Abstract

While the monophyly of winged insects (Pterygota) is well supported, phylogenetic relationships among the most basal extant pterygote lineages are problematic. Ephemeroptera (mayflies) and Odonata (dragonflies) represent the two most basal extant lineages of winged insects, and determining their relationship with regard to Neoptera (remaining winged insects) is a critical step toward understanding insect diversification. A recent molecular analysis concluded that Paleoptera (Odonata + Ephemeroptera) is monophyletic. However, we demonstrate that this result is supported only under a narrow range of alignment parameters. We have further tested the monophyly of Paleoptera using additional sequence data from 18SrDNA, 28S rDNA, and Histone 3 for a broader selection of taxa and a wider range of analytical methodologies. Our results suggest that the current suite of molecular data ambiguously resolve the three basal winged insect lineages and do not provide independent confirmation of Odonata + Neoptera as supported via morphological data.

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Paleoptera (=Palaeoptera) refers to the grouping of extinct paleodictyopteroids, Ephemeroptera, and Odonata (Hennig, 1981; Kukalova-Peck, 1983, 1985, 1991, 1997; Riek and Kukalova-Peck, 1984). However, the monophyly of this group is still a controversial issue in insect evolution (Beutel and Gorb, 2001; Staniczek, 2000; Wheeler et al., 2001; Whiting et al., 1997). The extant paleopterous insects—dragonflies and damselflies (=Odonata), and mayflies (=Ephemeroptera)—lack the retractor muscle and wing sclerites necessary to fold the wings over the abdomen (Martynov, 1924). The absence of this feature has been suggested as evidence for the group's monophyly. However, this character may simply be symplesiomorphic because the muscles and sclerites allowing insects to fold wings over their abdomen were gained in the neopterous insects (Martynov, 1924). This innovation is presumably correlated with the huge explosion of neopterous species. Despite being one of the most important diversification events in all of evolution, the resolution of the relationships among Ephemeroptera, Odonata, and Neoptera remains ambiguous, and all resolutions of this three-taxon statement have been proposed.

The first hypothesis will be referred to as the *basal Ephemeroptera hypothesis* and it suggests that Ephemeroptera is sister to Odonata + Neoptera (Fürst von Lieven, 2000; Kristensen, 1991; Staniczek, 2000; Wheeler et al., 2001; Whiting et al., 1997). Six morphological characters proposed to support this hypothesis are (1) the anterior articulation of the mandible is a nonpermanent sliding groove and track system in Ephemeroptera, but in other pterygote lineages this articulation is more permanent; (2) subimago stage is present in Ephemeroptera but absent in other pterygotes; (3) tracheation is absent in arch of wing base and in posterior portion of the leg in Ephemeroptera but present in other insects; (4) direct spiracular musculature is absent in Ephemeroptera but present in odonates and neopterans; (5) never more than one tentorial-mandibular muscle is present in Odonata and Neoptera but multiple muscles are present in Ephemeroptera; (6) annulated caudal filament is presumably present in Archaeognatha, Monura, Zygentoma, and Ephemeroptera but absent in the remaining pterygotes; and (7) paired female genital openings are retained in Ephemeroptera and nowhere else among Pterygota. However, with some of these characters, it is unclear whether they are simply autapomorphies of Ephemeroptera or synapomorphies for Odonata + Neoptera.

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The second hypothesis, termed the *Paleoptera hypothesis*, suggests that Ephemeroptera is sister group to Odonata, forming the group Paleoptera (Brodsky, 1994; Hennig, 1981; Kukalova-Peck, 1983, 1985, 1991, 1997; Martynov, 1924; Riek and Kukalova-Peck, 1984). This hypothesis is supported by the following characters: (1) short antennae; (2) fusion of galea and lacinia; (3) lack of the ability to fold back the wings over the abdomen; (4) veinal braces in the wings; (5) separated R and M wing veins; (6) wing fluting; and (7) aquatic larvae. Still, some of these characters (e.g., 1, 3, 7) have been regarded as plesiomorphic (Wheeler et al., 2001; Willmann, 1997).

The third hypothesis places Odonata as sister to Ephemeroptera + Neoptera and will be referred to as the *basal Odonata hypothesis* (Boudreaux, 1979; Matsuda, 1970). This hypothesis is based primarily on the character that direct sperm transfer is synapomorphic for Ephemeroptera + Neoptera. Given that the “apterygotes” and Odonata have indirect sperm transfer, the gonopore-to-gonopore mode could be considered a shared derived character for mayflies and neopterous insects. However, the specific kind of indirect sperm transfer of the odonates appears to be quite different from those of the “apterygotes.” Odonate males deposit the sperm from segment 9 to an accessory gland on segment 2. Then, when in tandem (the position where the male grasps the female by the head with his terminalia), the female bends her abdomen down and forward to receive the sperm in her reproductive opening on segment 8. This complicated process does not resemble the indirect sperm transfer of “apterygotes” and is most likely autapomorphic, providing no phylogenetic information at an ordinal level (Beutel and Gorb, 2001).

Due to the disagreement among, and questionable utility of, certain morphological characters, it is important to provide independent data that can corroborate one of these hypotheses to provide a more accurate estimate of phylogeny. We are particularly interested in the sensitivity of molecular topologies to perturbations of parameter values during phylogenetic analysis (Phillips et al., 2000; Wheeler, 2001, 1995). We specifically define robustness as a measure of stability of nodes to fluctuations in parameter values across an analytical landscape. A highly robust node is one that is supported under a wide range of parameter values, in contrast to a poorly supported node that is supported under only one or a few parameter values. We recognize that sensitivity analysis is only one measure of topological robustness and that other measures are currently in vogue (e.g., nonparametric bootstrap, Bremer support, posterior probabilities, etc.) (Archie, 1989; Bremer, 1988; Faith, 1991; Faith and Cranston, 1991; Felsenstein, 1985), each with their own pros and cons (Grant and Kluge, 2003). However, given that the current molecular data used to infer paleopteran phylogeny is primarily ribosomal

DNA sequences and that the topologies generated via these sequences are strongly influenced by alignment methodologies, we are interested in addressing the question of whether any analytical method will robustly support one of the three hypotheses listed above under a wide range of parameter values. In approaching the question in this manner, we do not attach any particular significance to congruence among disparate analytical methodologies. We are interested only in determining whether a robust solution exists for the given data under any analytical methodology or whether the molecular data do not discriminate among the hypotheses.

Independent tests (i.e., molecular data) have provided mixed support for the different hypotheses. For instance, Wheeler et al. (2001) published the most extensive formal analysis of ordinal relationships using molecular and morphological information. The 18S rDNA (18S) data and 18S+28S rDNA (28S) data supported a monophyletic Paleoptera, but the 28S data and the total-evidence analyses (including morphology) supported basal Ephemeroptera. This study, however, did not concentrate sampling on basal pterygotes, so the extent to which these results are influenced by the under sampling of taxa is not clear. In a recent molecular analysis, the relationship among basal pterygotes was specifically tested and the authors conclude that Paleoptera is monophyletic (Hovmöller et al., 2002). However, given the difficult nature of the Paleoptera problem, and some potential flaws in their analytical methodology, we were interested in determining the generality of their conclusion, given additional data and analyses.

The overall objective, therefore, is to determine whether a robust solution to the Paleoptera problem exists given current data and analytical methods. This objective will be specifically examined by two subgoals: (1) test the generality of the claim that the current molecular data support the monophyly of Paleoptera as presented by Hovmöller et al. (2002); (2) provide additional data and analyses to test the sensitivity of the topology to data partitions, cost parameter values, and methods of data analysis.

Materials and methods

Reanalysis of Hovmöller et al. (2002) data

In the Hovmöller et al. (2002) study, sequence data from 18S rDNA and partial 28S rDNA for 18 spp. of Odonata, 8 spp. of Ephemeroptera, 8 spp. of Neoptera, and 2 spp. of Archaeognatha were used to estimate phylogeny. This taxon sampling represents 22% (6 of 27) of the odonate family taxa and 14% (5 of 36) of the mayfly family taxa. No morphological data were incorporated in their analyses, though coded character matrices were available (Beutel and Gorb, 2001;

Wheeler et al., 2001). Reanalysis of their molecular data was performed on each gene separately (18S and 28S) and in a combined analysis. To test the sensitivity of their topology toward alignment parameter values, we imported their sequences into ClustalX (Thompson et al., 1997) and analyzed them under a variety of parameter values. The authors did not report specific alignment parameters, so a wide range of alignment parameters were explored. For all alignments, delay divergent % was set to 30, DNA transition weight was set to 0, and DNA weight matrix was set to ClustalW(1.6), since these are the standard defaults for the program. Gap opening costs were set to the following values: 1, 2, 5, 10, 20, 30, 40, 50, 75, 85, and 100 (Table 1). Gap extension costs either were set to 1 or were equal to the gap opening costs. This resulted in 21 analyses per partition (18S, 28S, and combined), for a total of 63 matrices. These matrices were imported into PAUP*4.0b10 (Swofford, 2002) and analyzed under parsimony, with gaps treated as missing data and as a fifth state character. We executed 100 random additions with TBR branch swapping and strict consensus trees were constructed for each of the 126 analyses (Table 1). This wide selection of parameters appears sufficient to test the sensitivity of the Hovmoller data to varying alignment parameters.

Additional data

To further test resolution among basal pterygote lineages, we generated additional sequence data to more thoroughly represent the taxonomic diversity of these groups. From the Hovmöller et al. (2002) study, we included 13 odonate genera which were not represented in our samples. We decided not to include any of the Hovmöller mayfly sequences, as we have a very extensive sampling of mayfly taxa from nearly all families and have a very good indication of mayfly phylogeny based on these data (T.H. Ogden, unpublished). This allowed us to include sequences that more thoroughly represent the taxonomic diversity of Ephemeroptera. To the Hovmoller taxa, we added 50 more taxa, including 8 additional odonate genera, 7 “apterygote” hexapod spp., 23 genera of mayflies representing 22 families, and 12 taxa within the Polyneoptera to represent the neopteran lineages, for a combined total of 63 taxa (Table 2). This sampling represents 33% of odonate families and 62% of mayfly families. We also included the morphological data matrix coded by Wheeler et al. (2001) for these orders.

Muscle tissue was dissected, incubated, and DNA was extracted following the Qiagen DNeasy protocols. Templates and controls were amplified in a Perkin-Elmer 9700 thermocycler using primers modified for insects. Three genes were targeted for amplification and sequencing: 18S, 28S, and Histone 3 protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are

Table 1
Results of reanalysis of the Hovmöller et al. (2002) data set

Data partition	18S rDNA			28S rDNA			Combined (18S + 28S)		
	ClustalX gap costs:			ClustalX gap costs:			ClustalX gap costs:		
	PAUP treatment of gaps:	Gap extension = 1	Gap extension = Gap	PAUP treatment of gaps:	Gap extension = 1	Gap extension = Gap	PAUP treatment of gaps:	Gap extension = 1	Gap extension = Gap
Gap opening = 1	Unres	Unres	Unres	Eph*	Unres	Unres	Pal*	Unres	Unres
Gap opening = 2	Unres	Unres	Unres	Unres	Unres	Unres	Pal*	Odo*	Odo
Gap opening = 5	Pal*	Unres	Unres	Unres	Eph*	Unres	Pal*	Unres	Odo
Gap opening = 10	Pal*	Unres	Unres	Unres	Unres	Odo	Pal*	Odo	Odo
Gap opening = 20	Pal*	Unres	Unres	Unres	Unres	Pal*	Pal*	Odo	Unres
Gap opening = 30	Pal*	Unres	Unres	Unres	Unres	Odo	Pal	Odo	Odo
Gap opening = 40	Pal*	Unres	Unres	Unres	Unres	Odo	Pal	Odo	Odo
Gap opening = 50	Pal	Unres	Unres	Unres	Eph*	Odo	Pal	Odo	Odo
Gap opening = 75	Pal	Unres	Unres	Unres	Odo*	Unres	Odo	Odo	Odo
Gap opening = 85	Pal	Unres	Unres	Unres	Odo*	Pal*	Unres	Unres	Unres
Gap opening = 100	Pal	Unres	Unres	Unres	Odo*	Unres	Pal	Unres	Unres

Unres, analysis supported unresolved topology (this could be a trichotomy or nonmonophyletic Odonata and/or Ephemeroptera); Eph, analysis supported basal Ephemeroptera; Odo, analysis supported basal Odonata; Pal, analysis supported monophyletic Paleoptera.
* Neoptera was not resolved as a monophyletic group.

Table 2
Taxon list and Genbank accession numbers (X = no sequence information)

Order	Family	Genus species	18S rDNA	28S rDNA	H3
Collembola	Hypogastruridae	<i>Hypogastrura</i> sp.	AY338691	AY338648	AY338616
Diplura	Campodeidae		AY338692	AY338649	X
Archaeognatha	Machilidae	<i>Machilis</i> sp.	AY338689	AY338646	AY338614
	Machilidae	<i>Machilis</i> sp.	AY338690	AY338647	AY338615
Zygentoma	Lepismatidae	<i>Thermobia</i> sp.	AY338726	AY338683	AY338644
	Lepidotrichidae	<i>Tricholepidion</i> sp.	AY338727	AY338684	AY338645
	Notocoliidae	<i>Battigrassiella</i> sp.	AY338728	AY338685	X
Ephemeroptera	Acanthametropodidae	<i>Analetris eximia</i>	AY338697	AY338654	AY338620
	Ameletidae	<i>Ameletus</i> sp.	AY338712	AY338669	AY338632
	Ameletopsidae	<i>Chaquihua</i> sp.	AY338715	AY338672	AY338635
	Ametropodidae	<i>Ametropus neavei</i>	AY338700	AY338657	AY338622
	Baetidae	<i>Baetis</i> sp.	AY338695	AY338652	AY338619
	Baetiscidae	<i>Baetisca</i> sp.	AY338707	AY338664	AY338627
	Behningiidae	<i>Behningia</i> sp.	AY338703	AY338660	X
	Caenidae	<i>Caenis</i> sp.	AY338710	AY338667	AY338630
	Coloburiscidae	<i>Coloburiscus humeralis</i>	AY338706	AY338663	AY338626
	Ephemerellidae	<i>Drunella coloradensis</i>	AY338651	AY338618	AY338694
	Ephemeridae	<i>Hexagenia</i> sp.	AY121136	AY125276	AY125223
	Euthyplociidae	<i>Polyplocia</i> sp.	AY338705	AY338662	AY338625
	Heptageniidae	<i>Heptagenia</i> sp.	AY121137	AY125277	AY125224
	Isonychiidae	<i>Isonychia</i> sp.	AY338708	AY338665	AY338628
	Leptohyphidae	<i>Leptohyphes apache</i>	AY338714	AY338671	AY338634
	Heptageniidae	<i>Cinygmula</i> sp.	AY338704	AY338661	AY338624
	Metropodidae	<i>Metretopus borealis</i>	AY338698	AY338655	AY338621
	Neophemeridae	<i>Neophemera youngi</i>	AY338702	AY338659	X
	Oligoneuriidae	<i>Lachlania saskatchewanensis</i>	AY338701	AY338658	AY338623
	Potamanthidae	<i>Anthopotamus</i> sp.	AY338711	AY338668	AY338631
	Pseudironidae	<i>Pseudiron centralis</i>	AY338699	AY338656	X
	Rallidentidae	<i>Rallidens mcfarlanei</i>	AY338696	AY338653	X
	Siphonuridae	<i>Paramaetus columbiae</i>	AY338713	AY338670	AY338633
Odonata	Aeshnidae	<i>Anax junius</i>	AY338719	AY338676	AY338639
	Aeshnidae	<i>Aeshna juncea</i>	AF461230	AF461205	X
	Aeshnidae	<i>Brachytron pratense</i>	AF4611232	AF461217	X
	Calopterygidae	<i>Calopteryx aequabilis</i>	AY338716	AY338673	AY338636
	Calopterygidae	<i>Heterina americana</i>	AY338718	AY338675	AY338638
	Coenagrionidae	<i>Argia vivida</i>	AY121144	AY125284	AY125229
	Coenagrionidae	<i>Coenagrion hastulatum</i>	AF461234	AF461207	X
	Coenagrionidae	<i>Enallagma cyathigerum</i>	AF461237	AF461201	X
	Coenagrionidae	<i>Erythromma najas</i>	AF461238	AF461209	X
	Coenagrionidae	<i>Ischnura elegans</i>	AF461239	AF461215	X
	Coenagrionidae	<i>Pyrrhosoma nymphula</i>	AF461241	AF461202	X
	Corduliidae	<i>Cordulia aenea</i>	AF461236	AF461210	X
	Corduliidae	<i>Somatochlora flavomaculata</i>	AF461242	AF461212	X
	Epiophlebiidae	<i>Epiophlebia superstes</i>	AF461247	AF461208	X
	Gomphidae	<i>Ophiogomphus severus</i>	AY121143	AY125283	AY125228
	Lestidae	<i>Lestes</i> sp.	AY338721	AY338677	X
	Libellulidae	<i>Libellula saturata</i>	AY338717	AY338674	AY338637
	Libellulidae	<i>Celithemis eponina</i>	AF461233	AF461218	X
	Libellulidae	<i>Leucorrhinia pectoralis</i>	AF461240	AF461206	X
	Libellulidae	<i>Sympetrum vulgatum</i>	AF461246	AF461216	X
Petaluridae	<i>Phenes raptor</i>	AY338720	X	X	
Polyneoptera	Acrididae	<i>Melanoplus</i> sp.	AY121146	AY125286	AY125231
	Blatellidae	<i>Supella longipalpa</i>	AY121130	AY125271	AY125217
	Heteronemiidae	<i>Sceptrophasma longikawiensis</i>	AY121166	AY125306	AY125249
	Mantidae	<i>Tenodera aridifolia</i>	AY121142	AY125282	AY125227
	Mastotermitidae	<i>Mastotermites darwinensis</i>	AY121141	AY125281	X
	Nemouridae	<i>Malenka californica</i>	AY338724	AY338680	AY338642
	Notoligotomidae	<i>Notoligotoma</i> sp.	AY338693	AY338650	AY338617
	Oligotomidae	<i>Oligotoma nigra</i>	AY121134	AY125274	AY125221

Table 2 (continued)

Order	Family	Genus species	18S rDNA	28S rDNA	H3
	Styloperlidae	<i>Cerconychia</i> sp.	AY338725	AY338681 & AY338682	AY338643
	Tetrigidae	<i>Paratettix cucullatus</i>	AY338722	AY338678	AY338640
	Timematidae	<i>Timema knulli</i>	AY121162	AY125302	AY125246
	Tridactylidae	<i>Ellipes minutus</i>	AY338723	AY338679	AY338641

given in Whiting (2001). Primer sequences for the gene H3 are HexAF: 5'-ATG GCT CGT ACC AAG CAG ACG GC-3' and HexAR: 5'-ATA TCC TTG GGC ATG ATG GTG AC-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).

Four analytical strategies were employed to examine topological sensitivity (Table 4 and Fig. 1): (1) direct optimization alignment via POY; (2) use of the implied alignment from POY as a multiple alignment for tree reconstruction; (3) alignment in ClustalX using sequences submitted as fragments followed by tree reconstruction; and (4) alignment in ClustalX using

sequences submitted as a whole (non-fragmented) followed by tree reconstruction.

Optimization alignment (OA) via POY

Sequences were initially assembled in Sequencher 4.0 (Genecodes, 1999). The protein coding H3 gene was manually 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 sequences were then sectioned into fragments at the conserved domains. This resulted in six fragments for 18S and nine fragments for 28S. These data were analyzed via OA in the program POY (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 follows: -fitchtrees -maxprocessors 3 -onan -onannum 1 -parallel -noleading -norandomizeoutgroup -impliedalignment

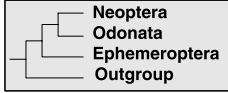
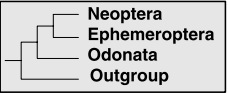
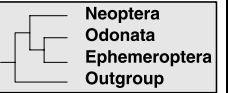
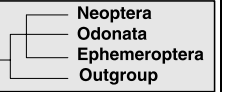
Strategies for sequence alignment	Basal Ephemeroptera	Basal Odonata	Monophyletic Paleoptera	Unresolved
				
POY	Mol (1:2:1) Mol (4:4:1) Total (1:1:1) Morphology Mol (2:1:1) 18S (1:1:1)	Mol (1:3:1) Mol (1:4:1) Mol (3:3:1) Mol (3:4:1) Mol (3:1:1) Mol (4:1:1) Mol (10:10:1) Mol (10:1:1) 28S (1:1:1)	Mol (1:1:1) Mol (3:2:1) Mol (4:2:1) Mol (4:3:1) Mol (2:2:1) Mol (2:3:1) Mol (2:4:1) Mol (100:100:1)	Mol (1000:1000:1) Mol (1000:1:1) Mol (100:1:1) H3 (Pre-aligned, 1:1)
POY implied	ML Mol (1:1:1)	MetaPIGA Mol (1:1:1)	Parsimony Mol (1:1:1; gaps = missing and fifth state)	Bayesian Mol (1:1:1)
ClustalX on sequence fragments	MetaPIGA Mol A Parsimony Mol A Parsimony 28S A Total A		ML Mol A Parsimony 18S+28S A	Bayesian Mol A Parsimony 18S A
ClustalX Whole	Parsimony Mol A Parsimony 18S+28S C Total A	Parsimony 18S+28S A	Parsimony 18S C	Parsimony 18S A,B Parsimony 18S+28S B Parsimony Mol B,C Parsimony 28S A,B,C Total B,C

Fig. 1. Summary of topological support for the three hypotheses from all parameters and methods. gap:transversion:transition ratios are indicated in parentheses. ClustalX settings A, B, and C as in Table 5. H3 was submitted to POY as prealigned data and was analyzed with parameters set to unity, gaps and changes = 1.

-sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -random 5 -stopat 25 -multirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 100 -numdriftchanges 30 -driftspr -numdriftspr 10 -drifttbr -numdrifttbr 10

-slop 10 -checkslop 10. Alignments can be found at (<http://inbio.byu.edu/faculty/mfw2/whitinglab/>).

A variety of alignment cost parameter values were investigated to explore data sensitivity (Table 3). We selected 22 values to explore sensitivity to gap/nucleotide

Table 3

Alignment cost ratios used in POY to explore topological landscape for molecular data and results from these analyses

1:1:1	1:2:1	1:3:1	1:4:1	<input type="checkbox"/> = BASAL EPHEMEROPTERA <input type="checkbox"/> = BASAL ODONATA <input type="checkbox"/> = MONOPHYLETIC PALEOPTERA <input type="checkbox"/> = UNRESOLVED
2:1:1	2:2:1	2:3:1	2:4:1	
3:1:1	3:2:1	3:3:1	3:4:1	
4:1:1	4:2:1	4:3:1	4:4:1	
10:1:1	10:10:1			
100:1:1	100:100:1			
1000:1:1	1000:1000:1			

The ratio indicates the gap:transversion:transition cost ratio.

Table 4

Four alignment strategies employed to examine topological sensitivity

Strategies	Partitions analyzed	Alignment parameters	Methods employed
Optimization alignment via POY	18S	1:1:1	Parsimony
	28S	1:1:1	Parsimony
	H3	1:1:1	Parsimony
	Molecular	See Table 3	Parsimony
	Total	1:1:1	Parsimony
Implied POY alignment	Molecular	1:1:1	Parsimony, gaps = missing Parsimony, gaps = fifth state Maximum likelihood Bayesian MetaPIGA
ClustalX: sequences submitted as fragments	18S	A	Parsimony
	28S	A	Parsimony
	Molecular	A	Parsimony Maximum likelihood Bayesian MetaPIGA
	Total	A	Parsimony
	ClustalX: sequences submitted as a whole	18S	A
B			Parsimony
C			Parsimony
28S		A	Parsimony
		B	Parsimony
		C	Parsimony
18S + 28S		A	Parsimony
		B	Parsimony
		C	Parsimony
Molecular		A	Parsimony
		B	Parsimony
		C	Parsimony
Total		A	Parsimony
		B	Parsimony
		C	Parsimony

The data partitions that were analyzed, the specific alignment parameters for each partition, and the methods used under each partition and parameter are indicated in the columns. The ratio of 1:1:1 indicates the gap:transversion:transition cost ratio. The letters A, B, and C coincide to the ClustalX alignment parameter settings in Table 5.

Table 5
ClustalX multiple sequence alignment settings represented as A, B, and C in Table 4

ClustalX setting	Gap opening	Gap extension	Delay divergent %	DNA transition weight	DNA weight matrix
A	1	1	30	0.00	ClustalW(1.6)
B	15	6.66	30	0.50	IUB
C	100	100	30	0.00	ClustalW(1.6)

change ratios (ranging from 1 to 1000) and transition/transversion ratios (ranging from 1 to 1000). 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). The alignment of the H3 gene was not ambiguous and the sequence data were treated as prealigned and analyzed in unity under parsimony (changes = 1). Results for H3 do not vary from one analytical methodology to the next, because the alignment was stable and thus different alignment methods would have no affect.

Implied POY alignment

We also tested robustness of the data to different methods of tree reconstruction using the implied alignment found in POY (Wheeler, 2003), with costs set to unity to minimize assumptions. We often find that unity for cost parameters is the most optimal parameter configuration for large data sets when implemented in the ILD framework (Kluge, 1989; Mickevich and Farris, 1981; Wheeler and Hayashi, 1998; Wheeler et al., 2001). The implied alignment was analyzed in five ways: (1) under parsimony with gaps treated as missing; (2) under parsimony with gaps treated as a fifth state character; (3) under standard maximum likelihood analysis as implemented in PAUP*4.0b10 (Swofford, 2002); (4) under bayesian analysis as implemented in Mr. Bayes (Huelsenbeck and Ronquist, 2001); and (5) using the metapopulation genetic algorithm executed in the program MetaPIGA (Lemmon and Milinkovitch, 2002) (Table 4). Modeltest (Posada and Crandall, 1998) was used to identify the most “justified” model for likelihood and bayesian analyses (Posada and Crandall, 2001). In addition, the implied alignment was also used to calculate nodal support. Nonparametric bootstrap values (500 replications) and partitioned Bremer support values (Baker and DeSalle, 1997) were calculated using the programs PAUP*4.0b10 and TreeRot (Sorenson, 1999).

Sequences submitted as fragments to ClustalX

To test the sensitivity of our results to different alignment algorithms, we chose to investigate performance of the alignment program ClustalX (Thompson et al., 1997). It is important to realize that a direct

comparison between parameter values in POY and ClustalX cannot be performed. In other words, there is no parameter set that one can select in POY that will give the ClustalX alignment and vice versa for any complex data set. The first strategy that we evaluated in ClustalX was designed to compare more directly to the results obtained from POY. The fragments were aligned under the ClustalX parameter setting A (Table 5). We believe that these settings most closely resemble the cost ratio of 1:1:1 (gap:transversion:transition) that was used in POY. The alignments from ClustalX were then analyzed under the methods of tree reconstruction as described above. Additionally, 18S, 28S, and 18S + 28S partitions were aligned under setting A and analyzed under parsimony.

Sequences submitted as a whole to ClustalX

In the fourth strategy, each individual gene was submitted to ClustalX as a whole, instead of as fragments sectioned at the conserved domain regions as described above. This was done to compare results using ClustalX with sequences fragmented versus not fragmented, since subdividing sequences into multiple fragments may influence the optimality of the overall alignment (Giribet, 2001). Subdividing sequences into multiple fragments forces a constraint on the alignment search algorithm by never allowing a set of sequences in one fragment to be aligned with those of another fragment. From a practical standpoint, this will generally speed up the alignment process, but introduces the possibility of biasing the overall alignment by a preconceived notion of alignment. The strategy of submitting sequences as a whole was the method used by Hovmöller et al. (2002). Three different sets of alignment parameters (A, B, and C in Table 5) were investigated to produce multiple alignments. All alignments were analyzed under parsimony, with gaps treated as missing.

Results

Reanalysis of Hovmöller et al. (2002)

Hovmöller et al. (2002) reported only topologies for alignments with a gap opening penalty of 75. They state that “a variety of settings” were used until the penalty of

75 was selected, but did not provide a rationale for this choice nor discuss results under other parameter values. Our reanalysis of their data suggests that paleopteran monophyly was supported only under a small (23%) subset of analytical parameters (Table 1). The 18S and combined (18S + 28S) data support monophyletic Paleoptera over most of the gap opening values, when the

gap extension remains at a value of one and gaps are treated as missing. However, when gaps are treated as a fifth state, the topologies are mostly unresolved or they support basal Odonata. When gap extension equals the gap opening value, with gaps treated as a fifth state, monophyletic Paleoptera is never recovered. The 28S data never support Paleoptera under any combination

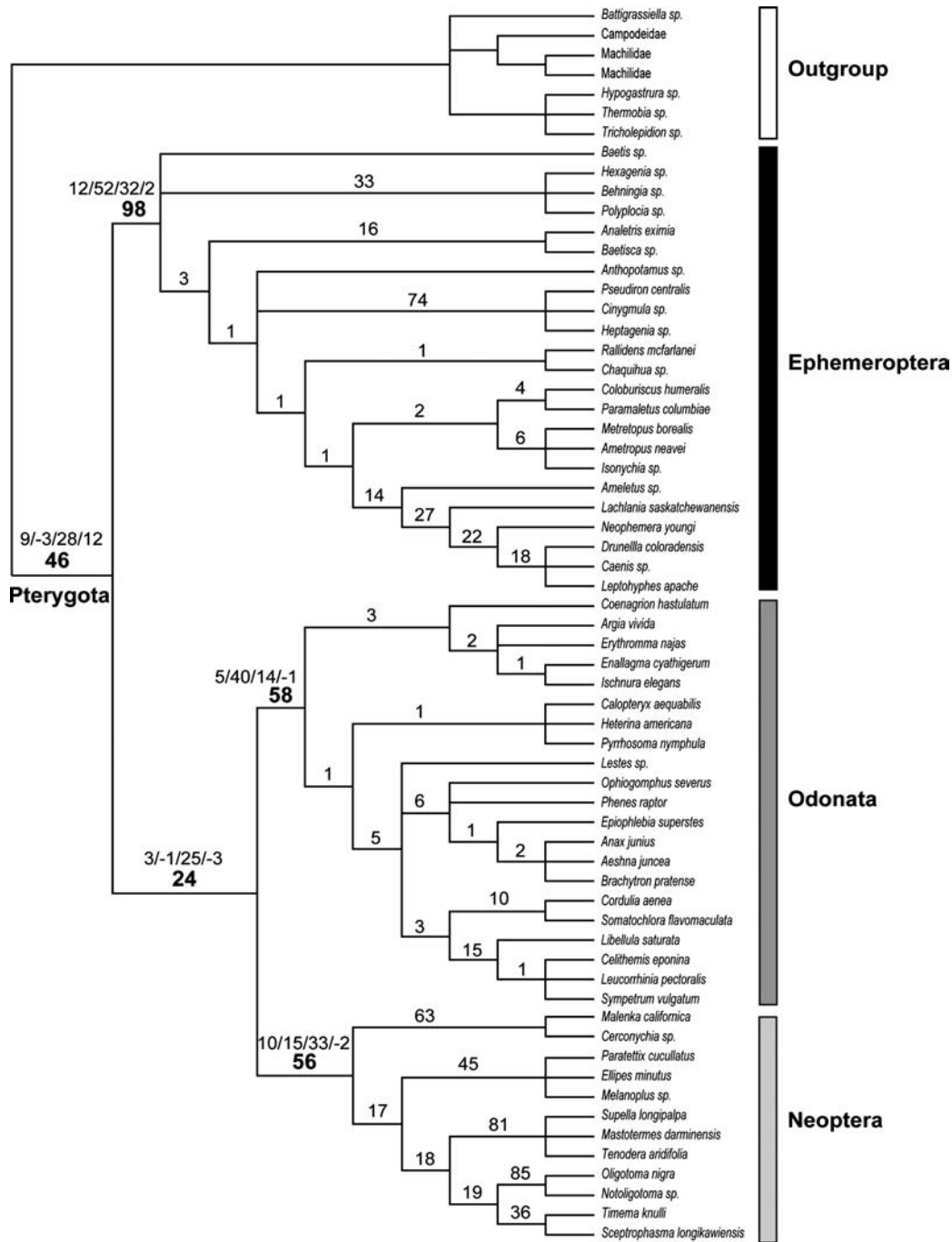


Fig. 2. Total-evidence tree based on 18S + 28S + H3 + morphology under 1:1:1 gap:transversion:transition costs in POY. This analysis produces a single most parsimonious tree (L = 2556; CI = 0.1980, RI = 0.5678) in which Ephemeroptera is basal. Partitioned Bremer values (morphology/18S/28S/H3) for the five basal pterygote nodes are given, and total Bremer values are given for the remaining nodes.

of analytical parameters. These results suggest that the Paleoptera problem has not been robustly solved given the data and analyses presented by Hovmöller et al. (2002).

Optimization alignment

Direct optimization of our expanded data set supports all three hypotheses, as summarized in Fig. 2. Sensitivity analysis suggests that these topologies are very sensitive to alignment cost parameters (Table 3 and Fig. 1). For example, when the transversion weight changes from 1 to 2 to 3 and the gap cost and transition weight remain at 1, each one of the three hypotheses is supported. Similarly, when the gap cost changes from 2 to 3 to 4 and transversion = 4 and transition = 1, all three hypotheses are recovered also. With costs set to unity, the combined molecular data support a monophyletic Paleoptera and the total evidence analysis including morphology supports basal Ephemeroptera (Fig. 2). The partitioned Bremer values for morphology, 18S, 28S, and H3 for the five basal pterygote lineages (Fig. 2) are indicated on the nodes. Support for the node Odonata + Neoptera (=Ephemeroptera basal hypothesis) comes from the 28S and morphological data, with conflicting signal from the 18S and H3 partitions. These results suggest that the monophyly of Paleoptera is highly sensitive to OA cost parameters, even in our expanded data set.

POY implied alignment analyses

The parsimony, maximum likelihood, bayesian, and MetaPIGA analyses on the implied POY alignment support all possible resolutions of the three-taxon statement (Fig. 1). Using the POY implied alignment for the molecular data under parsimony and treating gaps as missing results in a monophyletic Paleoptera. In contrast to the touted claims that model-based methods result in topologies that are highly congruent (Lemmon and Milinkovitch, 2002; Yang and Rannala, 1997), we find that model-based methods also disagree on which hypothesis is best supported. For example, the MetaPIGA analysis supports a basal Odonata, the maximum likelihood supports a basal Ephemeroptera, and the bayesian analysis is unresolved. We want to make it clear that phylogenetic accuracy is not increased by gaining agreement between the results of disparate analytical methodologies. We are interested only in determining whether a robust solution exists for the given data under any analytical methodology or whether the molecular data do not discriminate among the hypotheses.

ClustalX with sequence fragments

The implied alignment generated from POY and the multiple alignment generated by ClustalX were different

and produced different topologies. This is not surprising because POY produces alignments using an optimality criterion, whereas ClustalX is algorithmic or progressive in nature (Notredame, 2002). ClustalX alignments are also sensitive with regard to the three hypotheses. For instance, when sequences were submitted as fragments for all three genes in a combined molecular analysis, MetaPIGA and parsimony supports basal Ephemeroptera, maximum likelihood supports monophyletic Paleoptera, and bayesian analysis is unresolved (Fig. 1). Likewise, individual gene partitions support different relationships across different analytical methods. For instance, under parsimony the implied POY alignment supports monophyletic Paleoptera, but the ClustalX alignment under parsimony supports basal Ephemeroptera.

ClustalX with whole sequences

Submitting data as whole sequences to ClustalX also results in topological sensitivity. For instance, treating the 18S + 28S data as fragments with ClustalX results in monophyletic Paleoptera under parsimony, but treating these data as whole results in basal Odonata. Moreover, as in the POY sensitivity analyses, the selection of alignment parameters will influence the topology. For example, the alignment of the 18S + 28S data set under parameter condition A recovered a basal Odonata while under parameter C it recovered basal Ephemeroptera (Fig. 1). This further suggests sensitivity of the results to analytical parameters.

Discussion

Is the Paleoptera problem solved? The goal of this study was to determine whether current molecular evidence confirms the monophyly of Paleoptera across multiple parameter landscapes. Our results demonstrate that the particular arrangement of these lineages is extraordinarily sensitive to the current molecular data with regard to alignment methodology, alignment parameters selected within a particular methodology, and method of tree reconstruction. The inclusion of additional data from more taxa and another genetic locus did not help resolve these hypotheses, and sensitivity analyses of these data do not converge on a single solution. Even if one were to reject the notion of sensitivity analysis as a useful measure of robustness and select the values that set parameters to unity, our results demonstrate that the molecular data support a monophyletic Paleoptera under POY, but the ClustalX analysis supports basal Ephemeroptera.

These results suggest that a robust solution to the Paleoptera problem based on molecular data exclusively is more nebulous than suggested by Hovmöller et al.

(2002). However, other relationships on the topology were not as sensitive to parameter perturbations, as many clades are stable across all of the analyses. For instance, the monophyly of Ephemeroptera and Odonata were well-supported under most analyses, and the arrangements of taxa within these groups were also relatively consistent across analyses. For example, within the Odonata the suborders Zygoptera and Anisoptera are consistently recovered. Additionally, the baetid is frequently supported as the basal ephemeropteran lineage and the burrowing mayflies are monophyletic. This suggests that these molecular data are appropriate markers, at least at lower levels in the phylogeny of insects, as has been demonstrated in other analyses (Wheeler et al., 2001; Whiting et al., 2003).

The empirical case presented here underscores the importance of investigating the influence of parameter values on phylogenetic hypotheses. It is not enough to just “plug and chug” during the alignment phase (Grant et al., 2003), relying on default values of the preferred algorithm, since the recovered topology may not be robust to perturbations of the parameter values across all nodes. There may be topologies or nodes that are robust to parameter variation. However, as exemplified by this study, certain important nodes may be very sensitive to methodology. With the plethora of methods available to use in phylogenetic inference, discrimination must be employed to filter out methods that are inferior and that may produce misleading results. Empirical comparisons among alternative methods are useful to investigate methodological performance (Morrison and Ellis, 1997). However, we do not consider congruence among different methodologies to be a suitable measure of robustness because agreement among inferior methods is nebulous at best. We are more concerned with the influence of parameter values within a particular methodology. Even within the same framework, such as parsimony, conflicting topologies were recovered under different methods of alignment. For instance, parsimony (with parameters set to unity) on all molecular data supported monophyletic Paleoptera in POY and basal Ephemeroptera in ClustalX. Moreover, the 18S + 28S data supported monophyletic Paleoptera in ClustalX with fragmented sequences and supported basal Odonata in ClustalX with unfragmented sequences. Hence the different methods (OA in POY or multiple sequence alignment (MSA) in ClustalX) yielded different topologies.

We suggest that there are multiple reasons that OA is superior to MSA when the disparity of sequences results in alignment ambiguity. First, OA heuristically searches across multiple alignments, allowing an optimality criterion to reject nonoptimal solutions, thus freeing itself from the progressive approach which may be biased by the predetermined guide tree (Wheeler, 1995, 2003). Second, OA uses a total evidence approach to infer the

topology by including morphology and prealigned data. In our analyses, the inclusion of morphology with the molecular data supported basal Ephemeroptera, as reported in other total-evidence analyses (Wheeler et al., 2001; Terry et al., in prep.), except under the most extreme alignment parameter values. There are many morphological characters that support this relationship (see Wheeler et al., 2001 for detailed treatment of characters). For example both mandibular articulations are fully fixed in Odonata + Neoptera, leg and wing tracheae are connected with the following spiracle, and the terminal medial filament is strongly reduced or absent (Beutel and Gorb, 2001; Staniczek, 2000; Wheeler et al., 2001). Contrary to the position of other authors who argue for partitioned analyses (de Queiroz, 1993; de Queiroz et al., 1995; Simmons and Freudenstein, 2003), we suggest that if total evidence has any merit at all, it must be applied uniformly during alignment and tree reconstruction, and currently POY is the only algorithm that provides a methodology for accomplishing this. Third, in agreement with other authors (Phillips et al., 2000), we find the consistency of using a single criterion throughout the analytical process to be appealing and superior to other methods that rely on a hodgepodge of criteria for alignment and tree reconstruction. Exploration and development of new genes informative at deep levels of evolution, combined with better taxon sampling may eventually lead to a robust solution of the Paleoptera problem.

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CONTRIVED DATASET: A REPLY TO KJER

Poor Taxon Sampling, Poor Character Sampling, and Non-Repeatable Analyses of a Contrived Dataset do not Provide a More Credible Estimate of Insect Phylogeny: A Reply to Kjer

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USA

Phylogenetic relationships among insect orders have generated a great deal of interest and controversy over the past few years. From the early work of Hennig (1969), to the descriptive work of Kristensen (1991, 1995, 1999) to the most recent work which combines molecules with morphology (Whiting et al., 1997; Wheeler et al., 2001), recovering ordinal level relationships for the vast diversity of insect groups has been a challenge.

Recently Kjer (2004) presented an analysis of insect ordinal relationships based exclusively on a single source of information: 18S rDNA sequence data. Kjer downloaded a small subset of the available sequences from GenBank, manually aligned the sequences with a model of secondary structure as a guide, excluded major regions of the molecule, and reconstructed a topology using Bayesian methods. Kjer (2004) claims that his analysis results in a more “credible” phylogeny for the insect orders which is claimed to be more congruent with “traditional ideas of insect ordinal relationships based on morphology,” and strongly criticizes our previous phylogenetic results (Whiting et al., 1997; Wheeler et al., 2001; Ogden and Whiting, 2003) as being the outcome of flawed analytical methodology. He uses this tree to argue for the superiority of manual alignment over algorithmic methods of sequence alignment in general, and Direct Optimization (Wheeler, 1996) in particular, and wishes to dispel the myth that there are “large molecular data sets supporting the conclusions of Wheeler et al. (2001)”. However, Kjer (2004) only uses a subset of the data that are currently available for insect ordinal phylogeny, misrepresents our analyses, and omits other analyses we have

published on insect ordinal phylogeny. In our estimation, Kjer does a poor job of representing the current state of affairs in insect ordinal phylogenetics.

EMPIRICAL ISSUES

The first important point to recognize is that Kjer (2004) does not present any new evidence, but relies on a contrived matrix assembled from a subset of the 18S sequences available on GenBank. His “laboratory’s interest in Paleoptera” appears to be limited to downloading subsets of 18S sequences for Odonata and Ephemeroptera, as he brings no new information to the table, and actually neglects important taxa in his analysis for which 18S sequence data are freely available. Given that he presents no additional data, it seems odd to criticize us for not having “large molecular data sets” underlying our results, particularly since these data sets (Whiting et al., 1997; Wheeler et al., 2001), constituted some of the largest data to date addressing ordinal relationships. It is unclear to us why a treatment of published data but with fewer taxa, fewer loci, and absent morphological information represents an advance on the primary literature.

Kjer presents his paper as if only a single methodology (Direct Optimization) has been applied to questions in insect interordinal phylogeny by our group and he is generally anachronistic and inaccurate in discussing our analyses and results. While Direct Optimization (DO) is currently our favored methodology (for reasons discussed below), in fairness it is important to recognize that multiple methods have been applied to different molecular and morphological datasets as these methods have been developed.

For instance, Whiting et al. (1997) presented a multiple alignment for two genes (18S and 28S) that was derived from a multiple alignment program (MALIGN), not a DO program. These multiple alignments were published in the original paper as well as posted on the Systematic Biology website, and these “homology statements” have been widely reanalyzed in a variety of contexts (Huelsenbeck, 1997; Huelsenbeck, 1998; Hwang et al., 1998; Whiting, 2002c). It is ironic that Kjer criticizes us for not using Bayesian analysis in our 1997 paper, when Bayesian analysis was not made available until 2001 (Huelsenbeck and Ronquist, 2001). Likewise, it is inaccurate to insinuate that our data sets were not made generally available to the public allowing others to examine them in terms of a “hypothesis of homology”. In 2002, Whiting edited a volume on holometabolan relationships in which Kjer (Kjer et al., 2002) contributed results from his work on Trichoptera (referenced in Kjer, 2004) and Whiting (Whiting, 2002b) contributed results from a reanalysis of 182 18S sequences for insects (not referenced in Kjer, 2004). In this analysis, POY was only used as a tool to generate multiple alignments, with the conserved portions of the alignment being used to resolve interordinal relationships. This paper discusses the pitfalls of using 18S as a single marker for interordinal relationships with some specific examples of where 18S fails, but Kjer does not discuss this paper nor places his results within the context of our larger analysis. In 2003, we published a paper placing stick insects among polyneopterous insects (Whiting et al., 2003) using three molecular markers (18S, 28S, and H3) and multiple methods of tree reconstruction including DO, parsimony, Bayesian, and likelihood analysis. This paper provided additional insight into ordinal relationships among the basal Neoptera, but was also not referenced by Kjer. In 2003, we published an

extensive analysis of Paleoptera using the three molecular markers above plus morphology, and comparing ClustalX (Thompson et al., 1997) versus DO methods of alignment, and multiple methods of tree reconstruction. Kjer (2004) cites this paper but ignores the underlying data (he fails to include these sequences in his analysis), and dismisses our finding that the current arsenal of molecular data does not provide a robust solution to the Paleoptera problem, by stating this is a case where “the methods failed, not the data.” This simplistic vision makes for a convenient argument – by dismissing DO, one can dismiss all of our prior work on insect phylogeny – but Kjer has failed to provide a complete appraisal of the data available for insect ordinal relationships and has ignored other analyses that would complicate his findings.

A WEALTH OF DATA

What data are available for inferring insect ordinal phylogeny? Given Kjer’s description, a person unfamiliar with insect systematics would fail to recognize the wealth of data available from multiple sources. According to Kjer (2004), from a molecular standpoint there is only 18S data and a small fragment of 28S (d3 region, ~300 bp); the latter molecule he insists is useless for interordinal phylogeny. As of November 2003, there were 1849 18S rDNA sequences of at least 637 bp (the shortest length included by Kjer) representing 1504 hexapod species. But there were also 1638 sequences of 28S of at least 637 bp for insects, and in many cases these are nearly complete sequences (2500bp) for a wide diversity of insects. Moreover, previous studies have demonstrated that 28S carries much more signal than 18S for interordinal relationships (Whiting, 2002a; Ogden and Whiting, 2003; Svenson and Whiting, 2004;

Whiting and Whiting, 2004), and we find the same pattern for our ongoing studies on Odonata, Ephemeroptera, Grylloblattodea, Plecoptera, Siphonaptera, Thysanoptera, and other insect groups. It is unfortunate that Kjer did not also download 28S rDNA data to provide a molecular result that is more comparable to our own, and to truly demonstrate that his methods of data analysis produce more “credible” results.

Kjer also fails to discuss the morphological matrix of 275 characters that was coded for all insect orders, originally in Whiting et al. (1997) and more comprehensively by Wheeler et al. (2001). The value in this matrix is that we brought together, for the first time, character descriptions given by Kristensen, Boudreaux, Hennig, and other workers, provided additional characters ourselves, and coded these across all of the insect orders. Coding matrices is vastly superior to simple narratives, which consist of character descriptions in a subset of which are never rigorously evaluated, since it allows the formal assessment of congruence and allows the direct combination of this evidence with molecular data. This matrix has been subsequently expanded and revised by other workers (Beutel and Gorb, 2001) who have understood the value of explicit character coding. It is thus ironic that Kjer should make claims about producing a topology more congruent with “traditional ideas” while providing no indication of what these traditional views might be nor how he assessed relative congruence, and all the while ignoring other assembled data matrices which speak to the issue directly.

TAXON SAMPLING:

The taxon sampling strategy of Kjer was to obtain “as complete a sampling of nonholometabolous insects as possible, while also limiting the size of the taxon sample” with an “extended sampling of Odonata and Ephemeroptera.” For the other orders “randomly selected divergent taxa were used.” He is thus left with the challenge of including sufficient taxa to capture insect ordinal diversity, but not so many as to overwhelm his “labor intensive” process of manual alignment. This highlights a fundamental problem with his methodology: as one gathers more data, the ability to manually align the information becomes logistically more difficult, less objective, and less repeatable. While the proponents of manual alignment claim to handle effectively only around 100 sequences, how does one deal with hundreds and even thousands of sequences, when you can’t fit them all on a single computer screen? Recognizing the vast diversity represented by insect evolution, Kjer is forced to always deal with woefully inadequate sampling of taxa with no prospects ever of significantly increasing the size of the data set. Clearly it has been demonstrated that taxon sampling is crucial in phylogenetic studies (Pollock et al., 2002; Zwickl and Hillis, 2002).

How well does Kjer represent insect diversity with the GenBank data available to him? His analysis includes 132 sequences. This seems like a broad, unless one views the data available on GenBank to him at the time of submission. Of the 1849 18S rDNA sequences available, he included roughly only 8% of the available species, but more critically omitted some important taxa that have been shown to play a pivotal role in insect phylogeny. For instance, he inexplicably omits the order Thysanoptera, whose position in the Paraneoptera has been difficult to establish with 18S data alone. He

similarly excludes the order Neuroptera, by far the most diverse clade within Neuropteroidea, even though 12 sequences were available. He purposely omits Strepsiptera by arguing that other workers have demonstrated that 18S does not provide adequate signal for the placement of Strepsiptera. However, he fails to recognize that the references he cites to argue this point (Huelsenbeck, 1998; Hwang et al., 1998) rely exclusively on the alignment published by Whiting et al. (1997), and thus he avoids an opportunity to demonstrate the superiority of his methods. He omits *Timema*, a basal stick insect whose inclusion is critical in polarizing groups within Phasmida and linking it with Embiidina (Whiting et al., 2003). He omits *Nannochorista*, a basal mecopteran that may warrant ordinal status (Whiting, 2002a), and is important for establishing the monophyly of Antliophora. If his method of analysis were truly superior, then why omit the orders which have been the greatest challenge to place, and why omit taxa whose inclusion have been shown to be critical towards understanding patterns of insect diversification?

The sampling of Kjer within orders is quite poor. For Coleoptera, he includes two sequences (out of a possible 598), which appear as sister group on his tree. He then launches into a diatribe and claims that the paraphyletic Coleoptera as published by Whiting et al. (1997) was due to a contaminated 18S sequence. But he fails to cite the paper (Whiting, 2002c) which discusses these possible contaminants and also demonstrates that regardless of analytical methodology, Coleoptera is always paraphyletic under 18S, and that their reported monophyly (based on molecular data) is an artifact of inadequate taxon sampling (Caterino et al., 2002; Whiting, 2002c; Whiting,

2002b). Within Phasmida, he included only 2 of the 14 major lineages available. Even within Odonata and Ephemeroptera, the two groups he claims to have sampled the most densely, he only included 63% and 34% of the available sequences respectively. We also find his claim of randomly selecting taxa within each order to be suspect, given the avoidance of sequences generated by our group (even if this means neglecting key taxa such as those discussed above). For instance, within Phasmida, there are 44 sequences, 41 of which we generated in a previous study (Whiting, 2003), and Kjer “randomly selected” 2 sequences generated in different studies. The same pattern is seen for Ephemeroptera, Odonata, and other groups. A chi-squared goodness of fit test indicates that it is highly improbable that this sample is random for these groups ($p < 0.05$). Moreover, his methodology eliminates a major portion of the data, and in this case he eliminated regions he designated as “unalignable” which accounted for more than 50% of the possible parsimony informative sites given a ClustalX alignment. The Kjer (2004) data matrix is contrived and fails to take into account all of the complexity of the data available. While we agree that a limited taxon size may be required by the “labor intensive” manual alignment process, we do not think that 8% of available sequences, with half of the information removed, is adequate to the task.

BASAL PTERYGOTES – GROUP OF EMPHASIS

Kjer’s emphasis concerning investigation of the relationships of the basal pterygotes prompts greater examination of the results from his methodology. The secondary structure manual alignment and Bayesian analysis of Kjer supports *Tricholepedion gertschi* rendering Pterygota nonmonophyletic in 62% of the trees. He

suggests that this placement “should not be taken seriously” based on the results of other analyses. We similarly suggest that his result of Odonata as sister to (Ephemeroptera + Neoptera) should not be taken seriously, due to additional evidence and analyses that are available (Kristensen, 1991; Whiting et al., 1997; Fürst von Lieven, 2000; Staniczek, 2000; Wheeler et al., 2001; Ogden and Whiting, 2003). It is not surprising that any one partition and any one methodology recover a particular arrangement for the basal pterygotes as was shown in Ogden and Whiting (2003). We demonstrated that, for these relationships, the current suite of molecular data, treated as partitions or simultaneously, did not come to a robust solution across various alignment and tree reconstruction methods such as DO, ClustalX, Parsimony, Likelihood, Bayesian, and MetaPIGA. Nevertheless, a combined analysis, which included morphological characters, strongly supported a robust topology recovering Ephemeroptera as sister to remaining pterygotes under the various methods. Kjer stated that in this case “the methods failed, not the data.” This statement is incorrect because the analyses clearly depicted that any unresolved or non-robust nodes resulted from a lack of signal from the data or conflict (homoplasy), not from tree reconstruction methodology error. Ironically, his topology suggests that Anisoptera is not monophyletic. However, this is not because the method of Bayesian analysis failed, but because of “lack of change on terminal branches” or in other words, insufficient molecular autapomorphies from the reduced Kjer 18S matrix. We agree that the relationships recovered in his analysis among odonate taxa were unexpected and are in contradiction to morphological analyses (Rehn, 2003). Likewise, the placement of the roach *Periplaneta* as sister group to Mantodea rather than to the Isoptera + *Cryptocercus* clade (Maekawa et al., 1999; Lo et al., 2000), the placement of

Orthoptera as sister group to Holometabola + Paraneoptera (Hennig, 1969; Boudreaux, 1979; Kristensen, 1991; Kristensen, 1995; Kristensen, 1999), the placement of Phasmida in a clade separate from Embiidina (Matsuda, 1970; Rahle, 1970; Flook and Rowell, 1998; Whiting et al., 2003), and the placement of *Stenoperla* in a clade separate from *Zelanoperla* (Zwick, 2000), disagree with previous hypotheses and are in fact contradictory to “traditional ideas of insect ordinal relationships”. His monophyletic Mecopterida (which he calls “Remaining Panorpids”) and the Coleoptera + “Neuropteroidea” clade have never been supported in a molecular analysis that includes a broad sample of taxa (Whiting, 2002c; Whiting, 2002b) and are artifacts of his poor taxon sampling and data exclusion and is a contrived result. Thus, his analysis of a subset of 18S sequences does not seem “credible” on all nodes, when compared to previous works, and he has provided no test of the accuracy of his method (simulation studies or comparisons to “known” phylogenies), and should be cautiously used to explore “vexing questions” in insect evolution

A SPECIFIC EXAMPLE

A clear example of the pitfalls of simple narratives emerges from Kjer’s discussion of the support for a basal placement of Odonata. Kjer discusses the “interesting scenario” of direct sperm transfer as a synapomorphy for (Ephemeroptera + Neoptera). We also coded this character in our morphological data matrix (Wheeler et al., 2001) and used it in our analysis of Paleoptera as discussed above (Ogden and Whiting, 2002). He omits the discussion of morphological characters that contradict

Odonata as sister to (Ephemeroptera + Neoptera), and neglects any discussion of characters in any explicit sense at all. For example seven morphological characters, ignored by Kjer, support Ephemeroptera as sister to (Odonata + Neoptera): (1) the anterior articulation of the mandible is a non-permanent sliding groove and track system in Ephemeroptera, but in other pterygote lineages this articulation is more permanent; (2) subimago stage present in Ephemeroptera but absent in other pterygotes; (3) tracheation absent in arch of wing base and in posterior portion of the leg in Ephemeroptera, but present in other insects; (4) direct spiracular musculature absent in Ephemeroptera but present in odonates and neopterans; (5) never more than one tentorial-mandibular muscle in Odonata and Neoptera, but multiple muscles are present in Ephemeroptera; (6) annulated caudal filament presumably present in Archaeognatha, Monura, Zygentoma, and Ephemeroptera but absent in the remaining pterygotes; and (7) paired female genital openings retained in Ephemeroptera and nowhere else among Pterygota (Kristensen, 1991; Whiting et al., 1997; Fürst von Lieven, 2000; Staniczek, 2000; Wheeler et al., 2001). We agree that his “hypothesis is speculative, coming from a single gene” plus one morphological character, but emphasize that studies which take into account all available data – by coding the data in a formal matrix -- contradict his conclusions. Kjer’s simple narratives are misleading and not fair to the body of data at hand.

ANALYTICAL ISSUES

Systematics endeavors to achieve objective knowledge through hypothesis testing. How useful is Kjer’s methodology for furthering insect molecular systematics?

Repeatability and Objectivity: Kjer's method violates the criteria of repeatability and objectivity in that it is not a transparent and explicit analytical procedure. Kjer's method does not allow other investigators to repeat the experiment and test claimed results.

Manual alignments generally lack any explicit discussion of how they are generated or the reasoning behind the chosen hypotheses of homology, and will be irreproducible and highly prone to bias, except in the most trivial of cases (Giribet et al., 2002). Kjer claims that manual alignment is repeatable by stating, "Anyone can repeat the analyses performed here by downloading the data and using the alignment." But certainly this is not repeatability *of alignment* in any useful scientific sense, since it is the methodology that produces the alignment that must be replicated to make the alignment procedure repeatable, and not the subsequent analysis of a fixed alignment. Following his logic, any tree reconstruction method is repeatable – no matter how bizarre it may be -- if one can download and examine the results from a website. Availability for download is not the hallmark of repeatability; independent investigators using a prescribed set of rules and arriving at the same end point is. Manual alignment could only be deemed repeatable if raw sequences, not the alignment, were downloaded (ideally, with the taxa names stripped, in order to blind the bias of the investigator) and identical alignments were reproduced time and time again.

Locating Alignment Boundaries: Kjer claims that his method allows one to "locate the boundaries of unalignable regions according to repeatable criteria," and cites his earlier paper (Kjer, 1997) on amphibians. The Kjer method locates these boundaries by

“delimiting unalignable regions flanked by hydrogen-bonded stems” (Kjer, 2004), but provides no explanation of how this is actually accomplished, but he does reference his 1997 paper for a description of how this “repeatable” criterion is used. However, his 1997 paper provides no description of this criterion, but it does reference his 1995 paper (on frogs) which has an appendix with “Instructions on applying structural information to raw data” (Kjer, 1995). This appendix describes steps that allow one to take sequences and apply structural symbols to indicate hypothesized conserved stem and loop regions. However, this description lacks adequate information to explain how these regions are identified in the first place, how the boundaries are established, and relies more on intuition than algorithm (see his step 4). There is insufficient information in this appendix to code this methodology into any sort of automated algorithm, such that different workers would repeatedly find the same boundaries between alignable and unalignable data. Algorithmic approaches do already exist, in some form, for determining such boundaries (Castresana, 2000; Pei and Grishin, 2001).

Secondary Structure: Kjer argues that ribosomal secondary structure provides an explicit, repeatable, and objectively defensible basis for performing manual alignments.

However, several points should be further considered. First, secondary structure does not actually solve the problem of nucleotide homology. At best, it places constraints by establishing putative limits between loops and stems, but the nucleotides within each of those units must still be homologized (Giribet et al., 2002). Second, determination of secondary structure is not nearly as simple and unambiguous as many studies suggest (Durbin et al., 1998). Indeed, in phylogenetic studies, secondary structure is typically

inferred by aligning with a sequence of “known” secondary structure, although the basis of that knowledge remains uncertain in many cases. Kjer appropriately recognizes several potential problems with secondary structure manual alignments such as “slippage”, “bulges”, “nonconserved stems”, and regions where the placements of nucleotides “remain arbitrary”, among others (Kjer, 1995; Kjer, 1997). Third, although it might be reasonable to expect selective pressures to apply to secondary structure interactions (that is, requirements of compensatory changes), it is unclear just how relevant those interactions are compared to selective pressures applied at other structural levels. Fourth, although functional constraint plays a role in preserving the pattern of shared ancestry, there is no necessary connection between functional considerations, including secondary structure, and the concept of homology, which refers strictly to the historical identity of objects related through shared transformation events. Kjer (1995) claimed without evidence that, “structural features are more highly conserved than are nucleotides, and therefore structures are a better indication of homology than are nucleotides.” Recently, it has been shown that protein coding nucleotide sequences, while less conserved than the amino acids, were found to have a much greater phylogenetic signal (Kallersjo et al., 1999; Simmons et al., 2004). Therefore conserved structural features are not necessarily a better indication of homology (see Homology section below).

Goodness of fit measure: The most obvious thing that is lacking from the Kjer method is any sort of goodness of fit measure for a given alignment relative to a specific model of secondary structure. Aside from the issue of the applicability of his “custom arthropod

rRNA secondary structural model” (which is never described) across all of insect diversity, if this methodology is to be useful, it must have some way of taking two different multiple alignments, comparing them head to head, and determining which one best fits a given secondary structure model. Kjer has never presented a metric that would allow an investigator to objectively “challenge and upgrade these hypotheses”, and it is unclear the specific criterion Kjer would use to demonstrate that one alignment is a better match to a model than another alignment. Kjer states that his hypothesized alignment “will be periodically updated”, but provides no way of determining if the new “updated” hypothesis is actually a superior alignment based on any sort of measurable criterion. This simply underscores the fact that his methodology uses neither an algorithmic criterion (such as ClustalX) or an optimality criterion (such as POY or MALIGN), but rather is dependent on some sort of intuition that lacks description and defies quantification.

Epistemological coherence: For scientific inferences to be valid, we believe they must be methodologically, theoretically, and philosophically consistent. Empirical investigations must be firmly rooted in notions of evidence and inference, and they must describe and defend what is done, what is assumed, and why. These requirements, although crucial in science, are compromised by procedures such as the Kjer method of manual alignment of sequences even in reference to secondary structure. Furthermore, how can the nonobjective homology decision of manual alignment be carried over in a logically consistent framework to the tree reconstruction phase? For example, if the manual aligner really feels that a set of bases ought to be homologous, should a higher weight

then be given to that character during tree reconstruction? Clearly there is no way to maintain an epistemological coherence throughout the entire process of manual alignment and tree reconstruction.

Practicality: While manual alignment falls short in these basic principles of scientific systematics, one can also question the practicality of manual alignment in the genomics age. No automated approach to manual alignment is available and may never be, because the explicit rules of decision making have never been specifically articulated to allow for automation. Thus, manual methods used to align just one gene for a relatively small data set, would be futile for assembling the tree of life, particularly for insects, where data sets undoubtedly will reach to thousands of terminals for multiple genes in the near future. The issue of practicality most likely played a role in the paucity of 18S sequences selected by Kjer.

Homology: Cladograms imply statements of homology. Alternative cladograms might have alternative optimal homology statements and content. Features are homologous when their origins can be traced to a unique transformation on the branch of a cladogram leading to their most recent common ancestor. There can be no notion of homology without reference to a cladogram (albeit implicitly) and no choice among cladograms without statements of homology. So although Kjer suggests, “homology statements are found in alignments”, a cladogram is necessary to legitimize or test those generated statements. Although homology assessment often involves a two-stage procedure of first submitting each hypothesis of homology to a round of separate tests and then submitting

the surviving, constrained set of hypotheses to the test of character congruence (that is, “static” homology assessment), this separation is neither a methodological nor epistemological necessity. POY embodies the concept of dynamic homology (Wheeler, 2001; Wheeler, 2003) in which the test of character congruence is applied to the entire, unconstrained set of hypotheses of homology, thereby allowing entire transformation series to be discovered on the basis of a single optimality criterion. That is, dynamic homology employs the same procedure to discover both the character (in the traditional sense) and the character-state transformations within the character. Since the same optimality criterion is employed in both cladogram assessment and homology assessment, the globally optimal explanation of the observed variation is achieved by the minimum-cost (or most likely, under a likelihood optimality criterion) cladogram-plus-homology-scheme combination. Kjer incorrectly states that POY does not produce an alignment, and therefore does not allow assessment of homology. Recognizing that alignments may be useful as visual representations of nucleotide homology, POY can produce an implied alignment by taking the dynamic homologies established through direct optimization and tracing them back through the cladogram, linking the unaligned sequence positions through the respective transformation series. Thus, an implied alignment is really just a means of visualizing nucleotide transformation series, and the optimal set of nucleotide homologies for a given data set is topology and parameter specific. Dynamic homology is a powerful conceptual approach to the study of highly simplified data types, such as DNA and amino acid sequences or simple morphological structures like annelid segments, where structural or developmental evidence that could allow a defensible choice among competing hypotheses of homology is either non-

existent or unavailable.

In summary, manual alignment is not repeatable, not objective, not epistemologically coherent, and not a practical method in the genomics age. Even if one does not agree with DO as a method of tree reconstruction, surely it is clear that an automated, optimality criterion driven approach is more appropriate to test hypotheses of homology, than one which is deeply rooted in intuition and relies on a methodology that cannot be repeated by any other worker.

CONCLUSIONS

In our previous papers, we have demonstrated the importance of taxon sampling in higher level phylogeny, the use of multiple molecular markers, the formal combination of morphology with molecules, and we have argued for the need to make all stages of analysis as objective, transparent, and reproducible as possible (Whiting et al., 1997; Wheeler et al., 2001; Ogden and Whiting, 2003). With his paper, Kjer argues for the opposite in each case. He has done an inadequate job of sampling taxa, has myopically focused on a single molecular marker while ignoring other data sources, has eliminated a major portion of the data using a non-repeatable methodology, and champions an opaque, inefficient, and non-reproducible method of data analysis. His “credible” topology is suspect at many levels, and he has done a poor job of summarizing the current data available for inferring insect ordinal relationships.

There certainly are merits in exploring the influence of any particular partition such as 18S for deciphering insect ordinal relationships. Likewise, there are merits in trying to use secondary structure within the context of an alignment algorithm. The past two decades of systematic research have demonstrated the importance of optimality criteria and have focused on algorithms that allow the discrimination among multiple hypotheses. While systematists are known to argue over what is the most appropriate criterion and how it should best be evaluated, there is a general consensus that a quantifiable approach is vastly superior to an intuitive or authoritarian approach. Kjer fails to find any way to algorithmically describe his procedure such that it could be reproduced by other researchers, he has never performed any experiments to demonstrate repeatability, and never presented any metric to test alternative alignment hypotheses and compare relative accuracy. Moreover, since the Kjer method cannot be readily applied to large data sets, the prospects of it becoming a vital tool in these days of phylogenomics are increasingly dim. We would consider the widespread adoption of his methods as a major setback towards a full and robust understanding of insect ordinal phylogeny. Fortunately, such an adoption will not happen, since the method is wholly unreproducible. We further argue that the most robust estimate of ordinal level phylogenetic relationships comes from using all the available data in a robust and repeatable phylogenetic analysis framework.

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Title: 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. 15 families were supported as being monophyletic, 5 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.

KeyWords: Ephemeroptera; Mayflies; Molecular phylogeny; Evolution of flight; Origin of wings; Pterygota; Mandibular tusks; Sensitivity analysis; Direct optimization

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; Kukalova-Peck, 1997; Martynov, 1924; Matsuda, 1970; Riek and Kukalova-Peck, 1984). Recently, it was shown that, while data from three 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; Kukalova-Peck, 1978; Kukalova-Peck, 1983; Kukalova-Peck, 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.

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; Eaton, 1883-1888; Eaton, 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

1970's (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 following 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; Hennig, 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 (figure 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 (figure 1b). Retracheata was defined by: (1) ventral tracheal anastomoses 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. 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 sub groups 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 90's. Kluge's 2004 topology (figure 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 figure 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; McCafferty, 1997), and from personal communication with McCafferty.

The most recent systems for McCafferty and Kluge (Kluge, 2004) are mostly congruent (figure 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 (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 (2 families) and Ephemerelloidea (8 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 Setisura mostly apply for this grouping, and Kluge (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 fishlike body form and swimming movement. Kluge recognizes that most included taxa present 3 dentisetae with exceptions in *Ameletus*, *Metreletus*, and *Acanthametropus*, yet he also concluded that his Tridentisata 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 paper 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.

Materials and Methods

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 (*Hypogastrura*). 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 in order 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 Leptohephidae 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, 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 3 protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are given in Whiting (2001). 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' AAAC TACGATTAGATACCCTATTAT 3'; 12Sbi: 5' AAGAGCGACGGGCGATGTGT 3'. Primers for 16S rDNA are: 16Sa: 5' GCCTGTTTATCAAAAACAT 3'; 16Sb: 5' CTCCGGTTTGAACTCAGATCA 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.

Phylogenetic Analyses

Sequences were initially assembled in Sequencher® 4.0 (Genecodes, 1999). The protein coding H3 gene was manually 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 7 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 base pairs, 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 -numslaveprocesses 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 -treefuse -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 (Figure 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 -holdmaxtrees 25 -seed -1 -slop 5 -checkslop 5 -multirandom -replicates 1 -treefuse -fuselimit 5 -fusemingroup 5 -fusemaxtrees 25 -noestimateparamsfirst -noestimatep -noestimateq -freqmodel f5 -invariantsitesadjust -gammaclasses 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.

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 figure 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 (figure 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 (figure 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* (Neoephemeridae), *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 (figure 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.

Discussion

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 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 (figure 6d) or the likelihood tree (figure 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 (figure 4a). *Centroptilum*, which was recovered as the next lineage in Baetidae (figure 3), also presents highly movable gills. On the other hand, the position of the next clade, Isonychiidae, which was well supported (Bs = 24, bt = 100) but not very 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*, *Macdunnoa*), suggesting that movable gills may be a

plastic character. Therefore, these data unambiguously support or refute the mayfly gill to wing hypothesis.

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 Prosopistomatidae and Baetiscidae. McCafferty's Pisciforma (from figure 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 (figure 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 (figure 6h).

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).

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.

Furcatergalia

The Suborder Furcatergalia was strongly supported as monophyletic (Bs = 29 bt = 100), and was present in four parameter sets. The positions of many of the families were not supported as previously proposed (compare figure 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 Potamanthidae outside the rest of Ephemeroidea and because Behningiidae nested within this clade. The support values and parameter landscape for node 48 (Bs = 28 and bt = 100) indicate evidence for the nonmonophyly of burrowing mayflies. Additionally, the likelihood phylogram does not support their monophyly (figure 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 (figure 6f). Furthermore, these data strongly support nesting Behningiidae within the tusked burrowing mayflies as sister to the genus *Tortopus* (Polymitarciidae). Thus, a secondary loss of the mandibular tusks occurred in the behningiids, while the burrowing lifestyle was retained (figure 5c). The

reconstruction on the likelihood tree would suggest two origins with a loss in Behningiidae as well (figure 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 (figure 5) differs substantially from the topology in figure 3, especially among the higher level relationships. In fact, except for the superfamilies Caenoidea and Ephemerelloidea, none of the proposed higher level groupings (above family level) were recovered in the likelihood tree.

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 (figure 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 (figure 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*), Polymitarcidae (*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, Metropodidae (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 non-monophyletic 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). The positions of the borrowing mayflies representing the family Ephemeridae did not support its monophyly. Clearly, a better sampling is needed to more robustly test these hypotheses. Behningiidae, while not supported as monophyletic in the 1:1:1 topology, was recovered in 89% of the parameter sets and the likelihood topology (figure 5).

Within Pannota, The monophyly of Caenidae was fairly stable being present in 78% of the parameter sets (figure 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 (figure 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 (figure 5). Because of the placement of *Hyrtanella* in the 1:1:1 topology (figure 3), Leptohyphidae was recovered as nonmonophyletic, but across all parameter sets it was recovered as monophyletic in 94% of the analyses (figure 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 some 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 is also true 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 (Bremers, 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 (figure 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 sensitivity 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.

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 4 others (Behningiidae, Caenidae, Ephemerellidae, and Leptohyphidae) were recovered in a large portion of the parameter landscapes supporting their monophyly as well; 5 families were supported as nonmonophyletic, although 2 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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Figure Legend:

Figure 1. (a) Topology representing the higher classification system of McCafferty and Edmunds (1979).
(b) Topology summarizing the phylogenetic and classification system of McCafferty (1991b).

Figure 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.

Figure 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)

Figure 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 figure 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.

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

Figure 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 *Polyplacia* (top right), and lateral view of tuskless *Dolania* (Behningiidae); d & g) presence of highly movable gills (in blue) on parsimony and likelihood topologies; e & h) presence of fishlike body form and swimming behavior (in purple) on parsimony and likelihood topologies; c & 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.

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

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, Ephemerythidae, 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)

Table 2. Taxon list and genbank accession numbers. (Genbank numbers to be supplied upon acceptance)

Order	Family	Genus	Species	12S	16S	18S	28S	H3
Collembola	Hypogastruridae	<i>Hypogastrura</i>	sp.	-	-	AY338691	AY338648	AY338616
Diplura	Campodeidae			-	-	AY338692	AY338649	-
Archaeognatha	Machilidae	<i>Machilis</i>	sp.	-	-	AY338689	AY338646	AY338614
Zygentoma	Lepidotrichidae	<i>Tricholepidion</i>	sp.	-	-	AY338727	AY338684	AY338645
	Lepismatidae	<i>Thermobia</i>	sp.	-	-	AY338726	AY338683	AY338644
Ephemeroptera	Acanthametropodidae	<i>Analetris</i>	<i>eximia</i>	-	-	AY338697	AY338654	AY338620
	Ameletidae	<i>Ameletus</i>	sp.	-	-	AY338712	AY338669	AY338632
	Ameletopsidae	<i>Ameletopsis</i>	<i>perscitus</i>	-	-	-	-	-
	Ameletopsidae	<i>Chaquihua</i>	sp.	-	-	AY338715	AY338672	AY338635
	Ameletopsidae	<i>Chilopter</i>	sp.	-	-	-	-	-
	Ameletopsidae	<i>Mirawara</i>	sp.	-	-	-	-	-
	Ametropodidae	<i>Ametropus</i>	<i>neavei</i>	-	-	AY338700	AY338657	AY338622
	Arthropleidae	<i>Arthroplea</i>	<i>bipunctata</i>	-	-	-	-	-
	Baetidae			-	-	-	-	-
	Baetidae	<i>Baetis</i>	sp.	-	-	AY338695	AY338652	AY338619
	Baetidae	<i>Callibaetis</i>	sp.	-	-	-	-	-
	Baetidae	<i>Centropitulum</i>	<i>luteolum</i>	-	-	-	-	-
	Baetidae	<i>Dipheter</i>	sp.	-	-	-	-	-
	Baetidae	<i>Jubabaetis</i>	sp.	-	-	-	-	-
	Baetidae	<i>Platybaetis</i>	<i>probus</i>	-	-	-	-	-
	Baetidae 2			-	-	-	-	-
	Baetiscidae	<i>Baetisca</i>	<i>lacustris</i>	-	-	-	-	-
	Baetiscidae	<i>Baetisca</i>	sp.	-	-	AY338707	AY338664	AY338627
	Behningiidae	<i>Behningia</i>	sp.	-	-	AY338703	AY338660	X
	Behningiidae	<i>Dolania</i>	<i>americana</i>	-	-	-	-	-
	Caenidae	<i>Brachycercus</i>	<i>harrisella</i>	-	-	-	-	-
	Caenidae	<i>Caenis</i>	sp.	-	-	AY338710	AY338667	AY338630
	Caenidae	<i>Callistina</i>	<i>panda</i>	-	-	-	-	-
	Caenidae	Genus Y	sp.	-	-	-	-	-
	Caenidae	<i>Madecocercus</i>	sp.	-	-	-	-	-
	Caenidae	<i>Tasmanocaenis</i>	sp.	-	-	-	-	-
	Caenidae	<i>Tricorythodes</i>	sp.	-	-	-	-	-
	Coloburiscidae	<i>Coloburiscoides</i>	sp.	-	-	-	-	-
	Coloburiscidae	<i>Coloburiscus</i>	<i>humeralis</i>	-	-	AY338706	AY338663	AY338626
	Coloburiscidae	<i>Murphyella</i>	sp.	-	-	-	-	-
	Dipteromimidae	<i>Dipteromimus</i>	sp.	-	-	-	-	-
	Ephemerellidae	<i>Attenella</i>	<i>margarita</i>	-	-	-	-	-
	Ephemerellidae	<i>Caudatella</i>	<i>hystrix</i>	-	-	-	-	-
	Ephemerellidae	<i>Drunella</i>	<i>doddsi</i>	-	-	-	-	-
	Ephemerellidae	<i>Ephemerella</i>	sp.	-	-	-	-	-
	Ephemerellidae	<i>Hyrtanella</i>	sp.	-	-	-	-	-
	Ephemeridae	<i>Hexagenia</i>	sp.	-	-	AY121136	AY125276	AY125223
	Ephemeridae	<i>Plethogenesia</i>	sp.	-	-	-	-	-
	Euthyplociidae	<i>Euthyplocia</i>	<i>hecuba</i>	-	-	-	-	-
	Euthyplociidae	<i>Polyplacia</i>	sp.	-	-	AY338705	AY338662	AY338625
	Euthyplociidae	<i>Probosciodoplocia</i>	sp.	-	-	-	-	-
	Heptageniidae	<i>Afronurus</i>	<i>peringueyi</i>	-	-	-	-	-
	Heptageniidae	<i>Atopopus</i>	sp.	-	-	-	-	-
	Heptageniidae	<i>Cinygma</i>	sp.	-	-	-	-	-
	Heptageniidae	<i>Cinygmina</i>	sp.	-	-	-	-	-
	Heptageniidae	<i>Cinygmula</i>	sp.	-	-	AY338704	AY338661	AY338624
	Heptageniidae	<i>Ecdyonurus</i>	<i>dispau</i>	-	-	-	-	-
	Heptageniidae	<i>Epeorus</i>	sp.	-	-	-	-	-
	Heptageniidae	<i>Heptagenia</i>	sp.	-	-	AY338709	AY338666	AY338629
	Heptageniidae	<i>Leucrocuta</i>	<i>maculipennis</i>	-	-	-	-	-
	Heptageniidae	<i>Macdunnoa</i>	<i>hipawinia</i>	-	-	-	-	-
	Heptageniidae	<i>Rhithrogena</i>	sp.	-	-	-	-	-
	Heptageniidae	<i>Stenonema</i>	sp.	-	-	-	-	-
	Ichthybotidae	<i>Ichthybotus</i>	<i>hudsoni</i>	-	-	-	-	-
	Isonychiidae	<i>Isonychia</i>	sp.	-	-	AY338708	AY338665	AY338628
	Isonychiidae	<i>Isonychia</i>	sp.	-	-	-	-	-
	Leptohyphidae	<i>Allenhyphes</i>	<i>flinti</i>	-	-	-	-	-
	Leptohyphidae	<i>Leptohyphes</i>	<i>zalope</i>	-	-	AY338714	AY338671	AY338634
	Leptohyphidae	<i>Traverhyphes</i>	<i>indicator</i>	-	-	-	-	-
	Leptohyphidae	<i>Yaurina</i>	<i>mota</i>	-	-	-	-	-
	Leptophlebiidae			-	-	-	-	-
	Leptophlebiidae	<i>Adenophlebia</i>	<i>peringueyella</i>	-	-	-	-	-
	Leptophlebiidae	<i>Austrophlebiodes</i>	sp.	-	-	-	-	-
	Leptophlebiidae	<i>Choroterpes</i>	sp.	-	-	-	-	-
	Leptophlebiidae	<i>Meridialaris</i>	<i>diguillina</i>	-	-	-	-	-
	Leptophlebiidae	<i>Paraleptophlebia</i>	sp.	-	-	-	-	-
	Leptophlebiidae	<i>Penaphlebia</i>	sp.	-	-	-	-	-
	Leptophlebiidae	<i>Thraulodes</i>	sp.	-	-	-	-	-
	Lestidae	<i>Lestes</i>	sp.	-	-	AY338721	AY338677	-
	Metropodidae	<i>Metretopus</i>	<i>borealis</i>	-	-	AY338698	AY338655	AY338621
	Metropodidae	<i>Siphloplecton</i>	<i>interlineatum</i>	-	-	-	-	-
	Neophemeridae	<i>Neophemera</i>	<i>youngi</i>	-	-	AY338702	AY338659	-
	Nesameletidae	<i>Nesameletus</i>	<i>ornatus</i>	-	-	-	-	-
	Oligoneuriidae	<i>Elassoneuria</i>	sp.	-	-	-	-	-
	Oligoneuriidae	<i>Homoeoneuria</i>	<i>alleni</i>	-	-	-	-	-
	Oligoneuriidae	<i>Lachlania</i>	<i>dominguez</i>	-	-	-	-	-
	Oligoneuriidae	<i>Lachlania</i>	<i>saskatchewanensis</i>	-	-	AY338701	AY338658	AY338623
	Oligoneuriidae	<i>Oligoneuriella</i>	<i>rhenana</i>	-	-	-	-	-
	Oniscigastridae	<i>Oniscigaster</i>	<i>distans</i>	-	-	-	-	-

	Oniscigastridae	<i>Siphonella</i>	sp.	-	-	-	-	-
	Oniscigastridae	<i>Tasmanophlebia</i>	sp.	-	-	-	-	-
	Polymitarcidae	<i>Tortopus</i>	sp.	-	-	-	-	-
	Potamanthidae	<i>Anthopotamus</i>	sp.	-	-	<u>AY338711</u>	<u>AY338668</u>	<u>AY338631</u>
	Potamanthidae	<i>Rhoenanthus</i>	sp.	-	-	-	-	-
	Potamanthidae	<i>Stygifloris</i>	sp.	-	-	-	-	-
	Prosopistomatidae	<i>Prosopistoma</i>	sp.	-	-	-	-	-
	Prosopistomatidae	<i>Prosopistoma</i>	<i>wouterae</i>	-	-	-	-	-
	Pseudironidae	<i>Pseudiron</i>	<i>centralis</i>	-	-	<u>AY338699</u>	<u>AY338656</u>	-
	Rallidentidae	<i>Rallidens</i>	<i>mcfarlanei</i>	-	-	<u>AY338696</u>	<u>AY338653</u>	-
	Siphlaenigmatidae	<i>Siphlaenigma</i>	<i>janae</i>	-	-	-	-	-
	Siphonuridae	<i>Ameletoides</i>	sp.	-	-	-	-	-
	Siphonuridae	<i>Edmundsius</i>	<i>agilis</i>	-	-	-	-	-
	Siphonuridae	<i>Metamoniis</i>	sp.	-	-	-	-	-
	Siphonuridae	<i>Parameletus</i>	<i>columbiae</i>	-	-	<u>AY338713</u>	<u>AY338670</u>	<u>AY338633</u>
	Siphonuridae	<i>Siphonurus</i>	sp.	-	-	-	-	-
	Tricorythidae	<i>Callistina</i>	<i>panda</i>	-	-	-	-	-
	Tricorythidae	<i>Tricorythodes</i>	sp.	-	-	-	-	-
Odonata	Aeshnidae	<i>Oplanaeschna</i>	sp.	-	-	-	-	-
	Coenagrionidae	<i>Hesperagrion</i>	sp.	-	-	-	-	-
	Diphlebiidae	<i>Diphlebia</i>	<i>coerulescens</i>	-	-	-	-	-
	Epiophlebiidae	<i>Epiophlebia</i>	<i>superstes</i>	-	-	<u>AF461247</u>	<u>AF461208</u>	-
	Gomphidae	<i>Phyllogomphoides</i>	sp.	-	-	-	-	-
	Isostictidae	<i>Labidosticta</i>	<i>vallisi</i>	-	-	-	-	-
	Libellulidae	<i>Erythemis</i>	sp.	-	-	-	-	-
	Megapodagrionidae	<i>Griseargiolestes</i>	<i>olbesens</i>	-	-	-	-	-

Table 3. Ephemeroptera specific primers for 28S rDNA. These primers were used in conjunction with the Whiting (2001) 28S rDNA primers.

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

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 (figure 3). SA% = number of parameter sets monophyletic divided by 36 (total number of parameter sets). PIC = the number of parsimony informative characters.

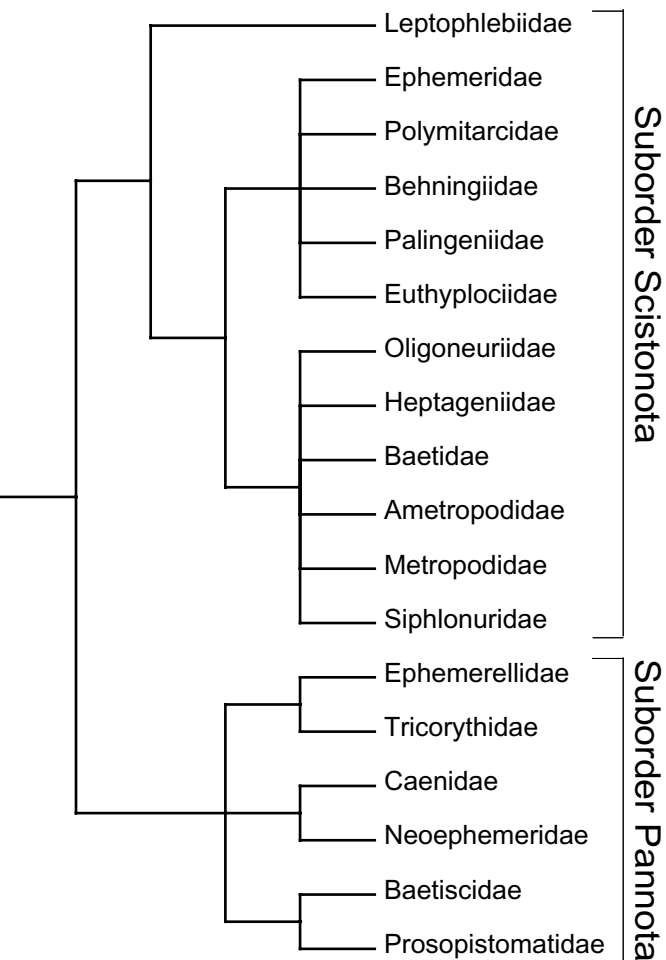
Node	Total Bremer	Bremer 12S	Bremer 16S	Bremer 18S	Bremer 28S	Bremer H3	bootstrap	SA%	Taxon level
1	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	113	0	24	27	71	-9	100	2.78%	Higher
3	72	0	20	13	42	-3	100	2.78%	Higher
4	73	12	10	2	44	5	100	80.56%	Higher
5	127	0	0	85	42	0	100	83.33%	Higher
6	40	0	10.5	18	12.5	-1	100	5.56%	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
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		0.671502591	1.241643836	1.151391162	1.004567144	0.575362319			

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

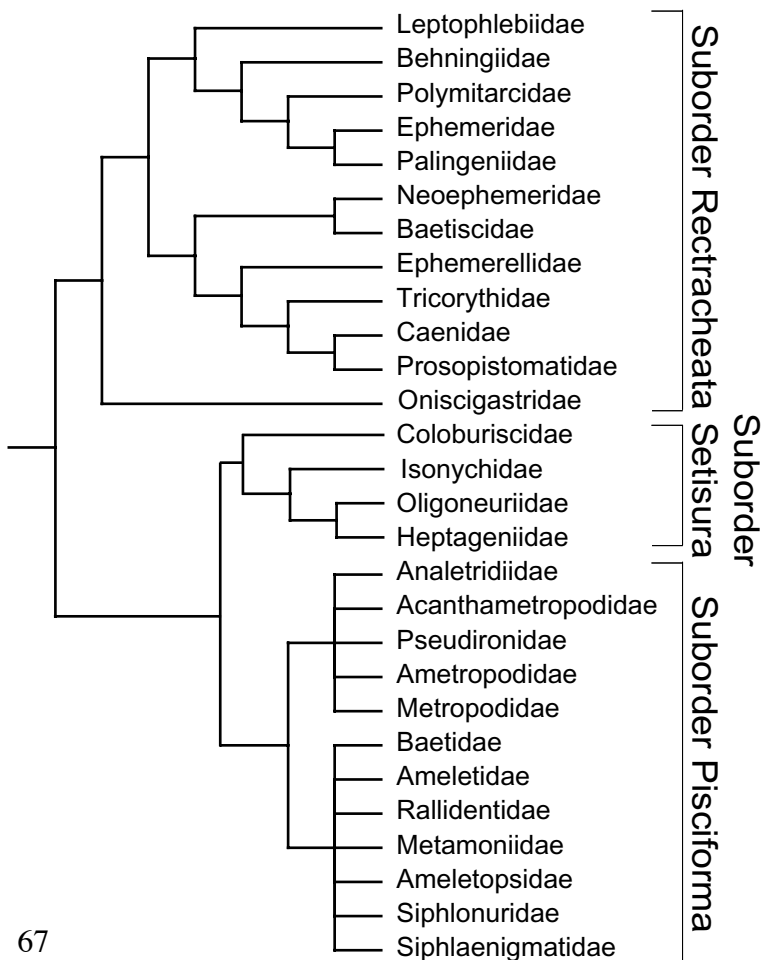
	Total Bremer	Bremer 12S	Bremer 16S	Bremer 18S	Bremer 28S	Bremer H3	bootstrap	SA%
"Lower" Total sum	1278	103	198.5	301.3	627.9	47.3		23.4722
"Lower" Average		8.1%	15.5%	23.6%	49.1%	3.7%	94.00%	48.90%
"Higher" Total sum	1691	156.2	254.7	402.2	845.8	32.1		977.78%
"Higher" Average		9.2%	15.1%	23.8%	50.0%	1.9%	94.85%	24.44%

McCafferty and Edmund's 1979 System



a.

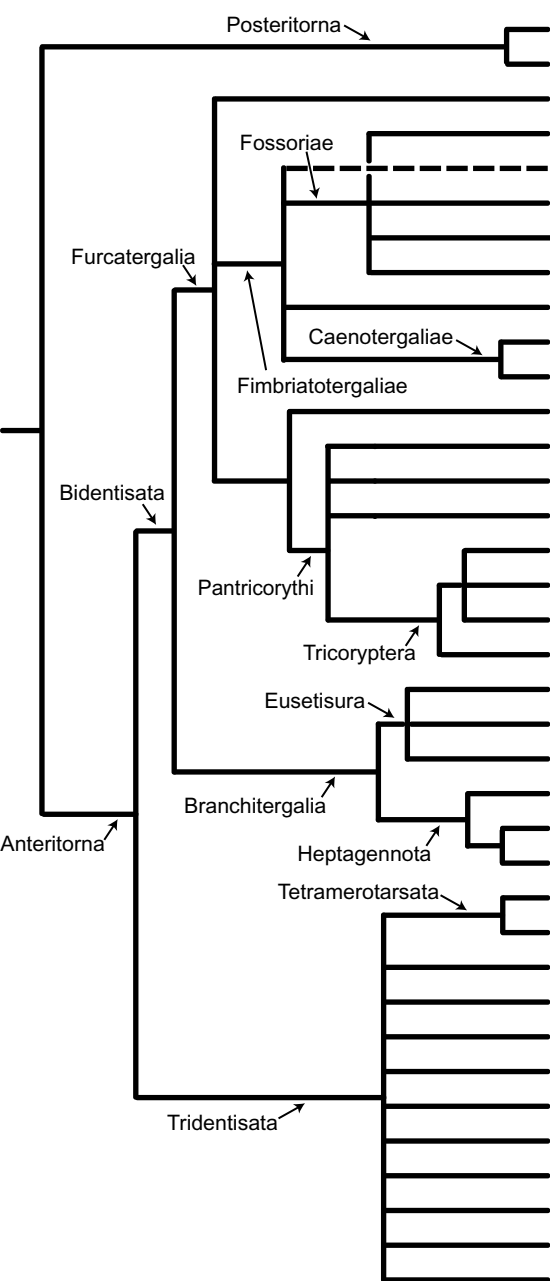
McCafferty's 1991 System



67

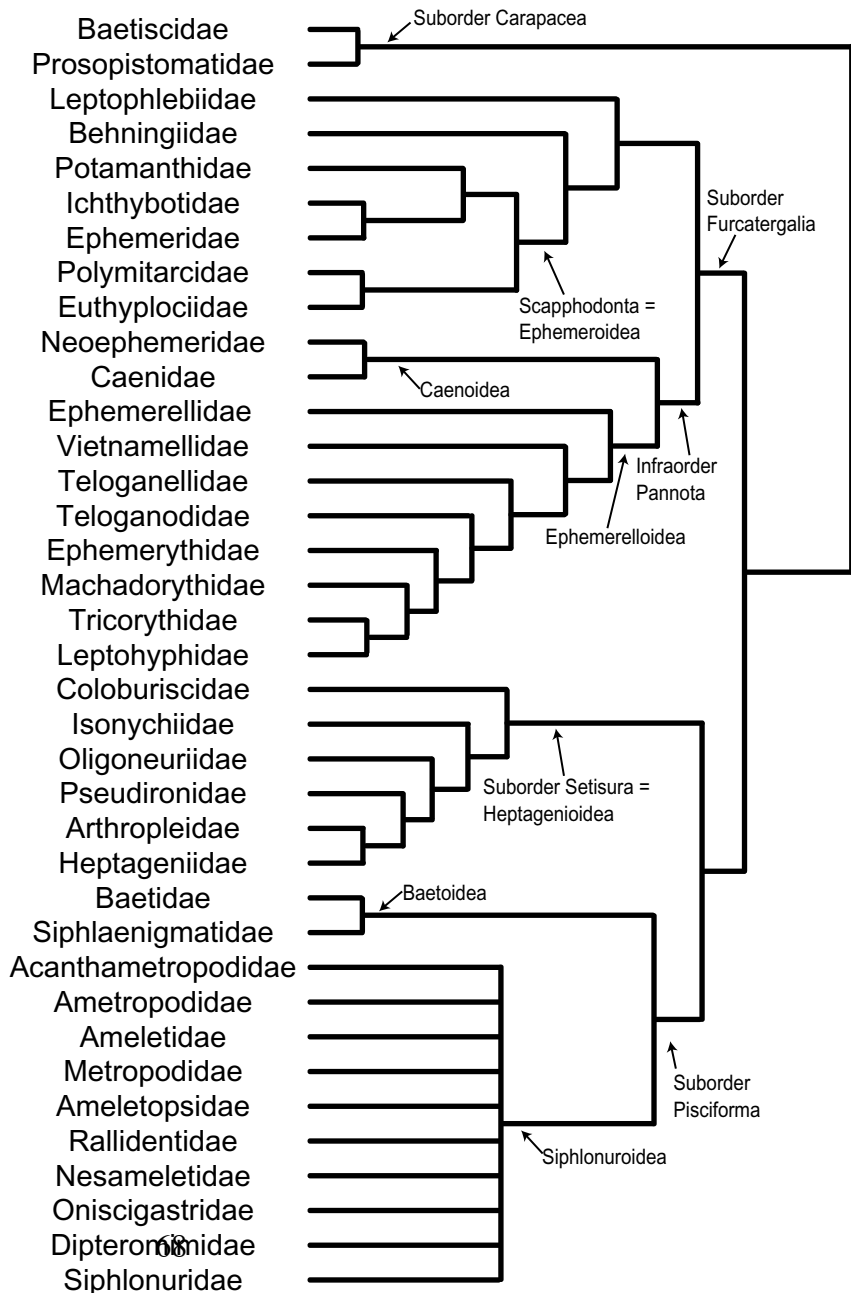
b.

Figure 2 Kluge's system

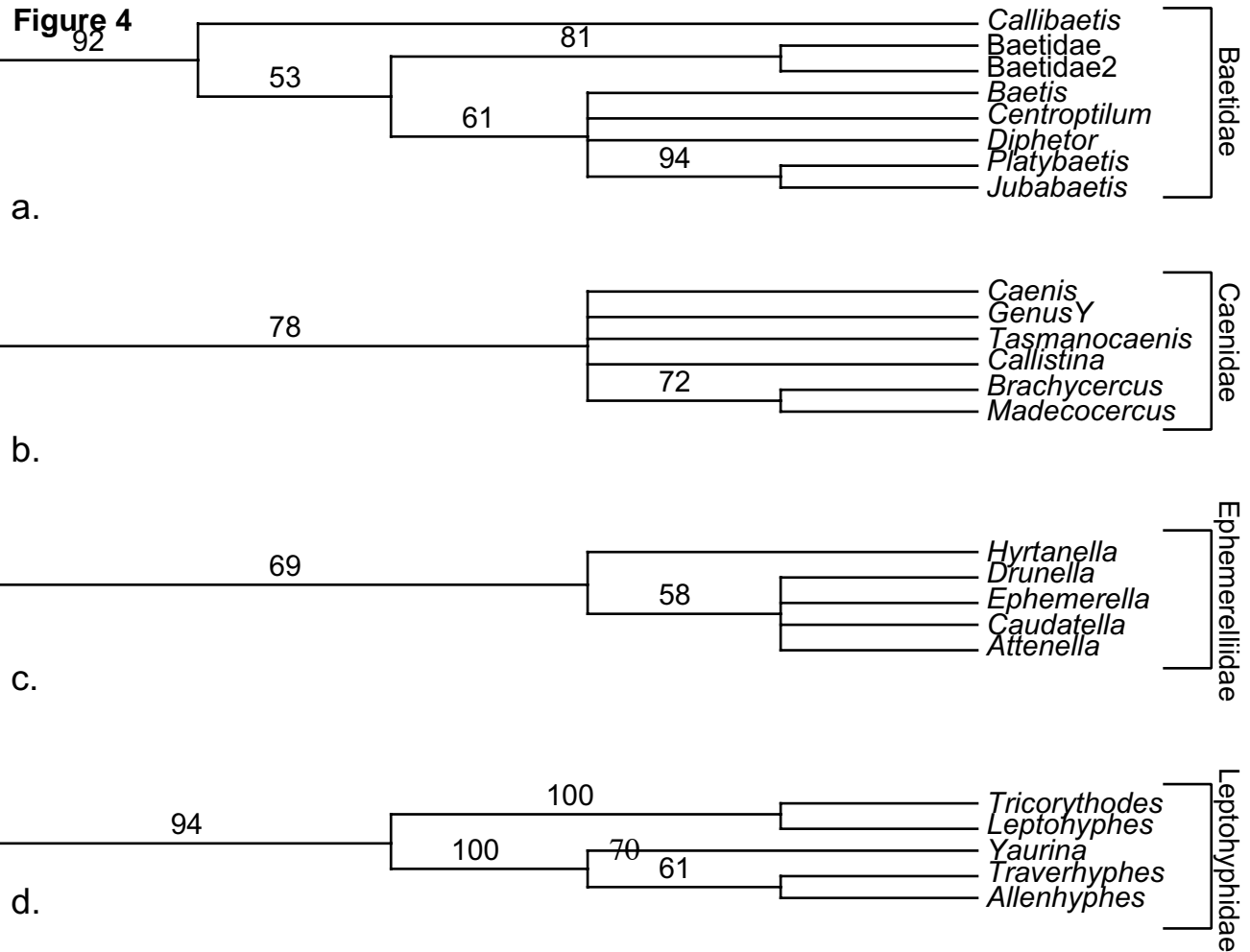


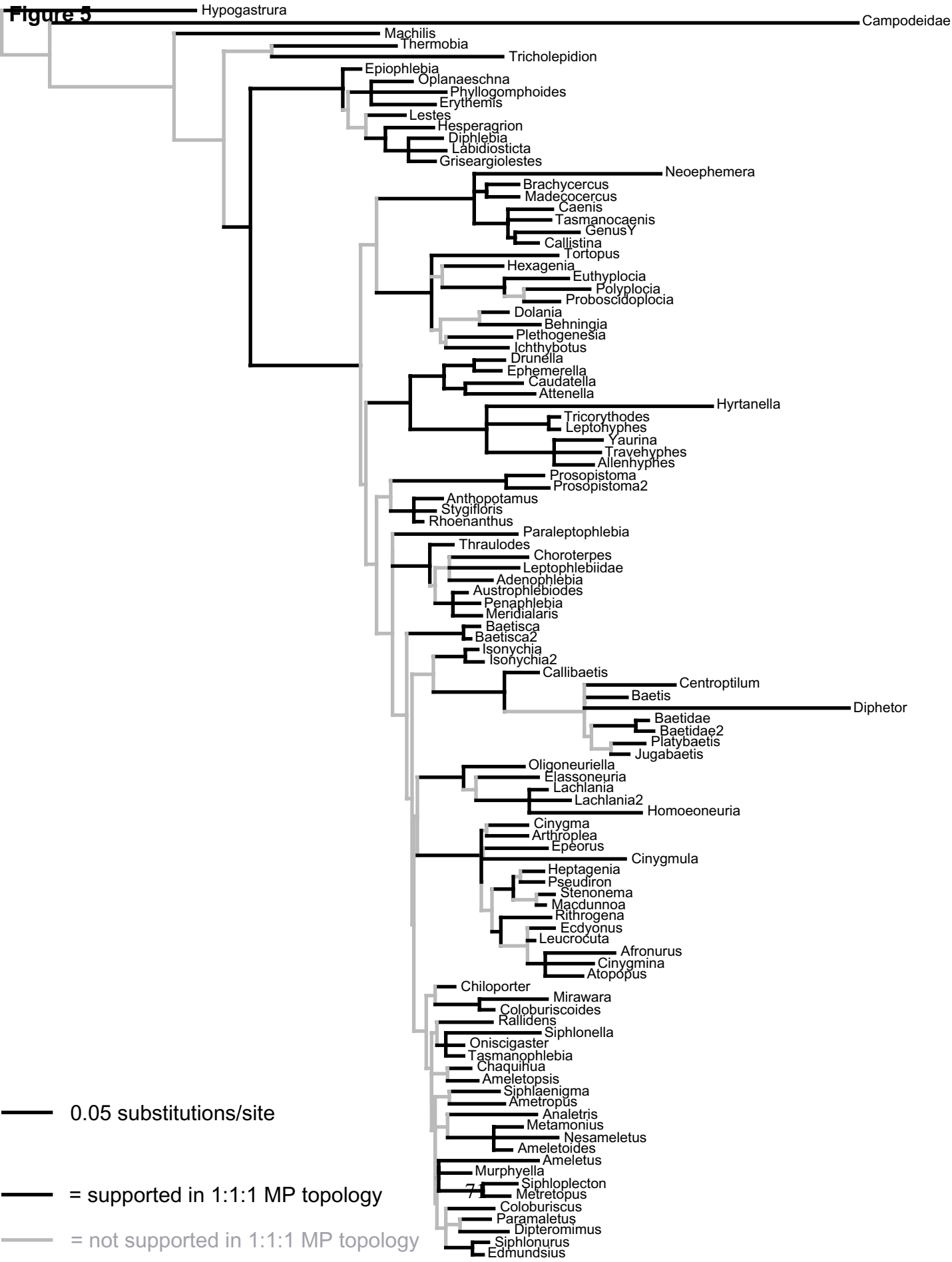
a.

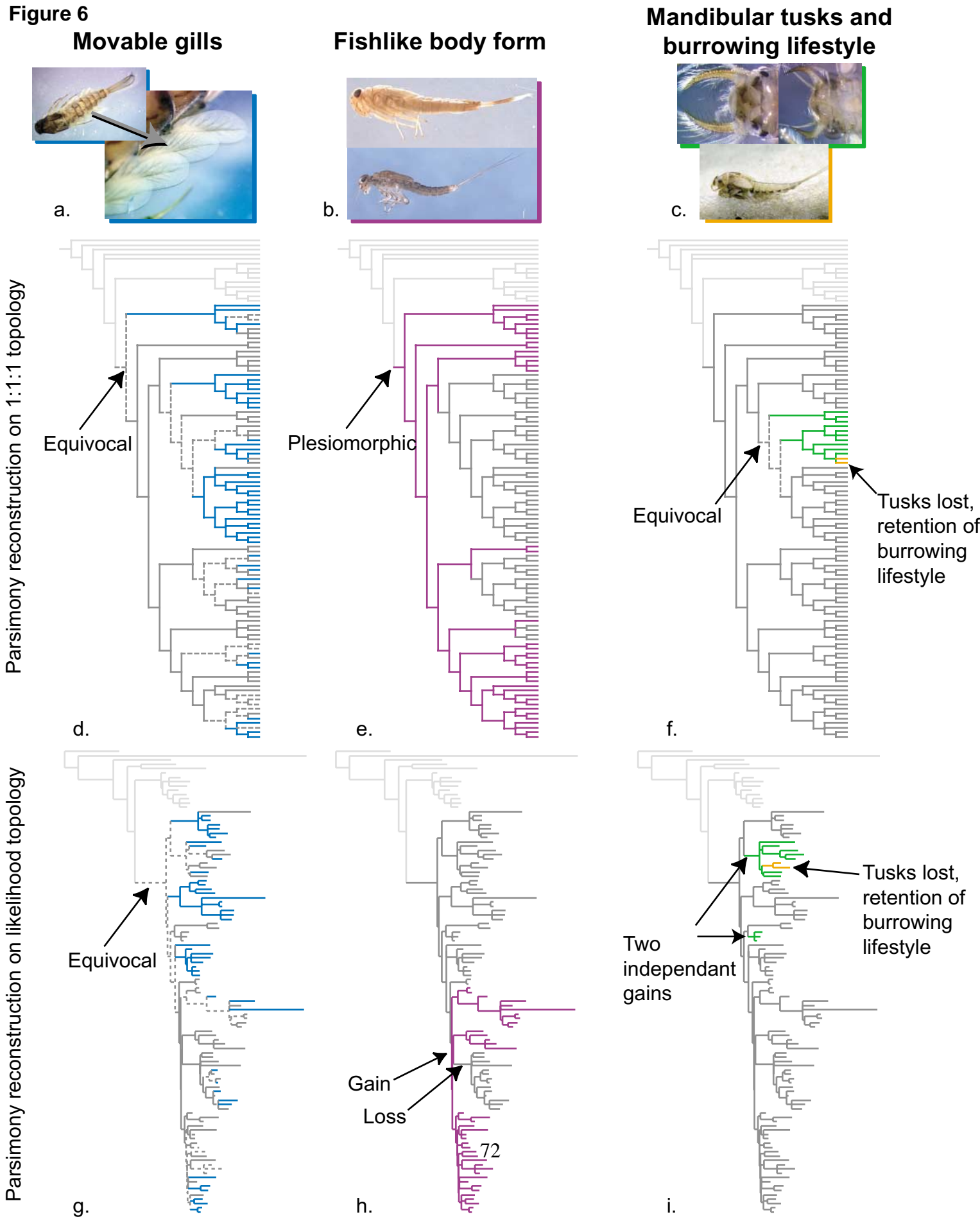
McCafferty's system



b.







Combined molecular and morphological phylogeny of Ephemerellidae (Ephemeroptera)
and its position within Pannota

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Abstract

This study represents the first combined molecular and morphological analysis for pannota mayflies with emphasis on the family Ephemerellidae. The phylogeny was constructed based on DNA sequence data from 3 nuclear (18S rDNA, 28S rDNA, histone H3), 2 mitochondrial (12S rDNA, 16S rDNA) genes, and 46 morphological characters via direct optimization. Taxon sampling for Ephemerellidae included 16 of the 18 described extant genera. The superfamily Ephemerelloidea was strongly supported as monophyletic and sister to the Caenoidea. The family Ephemerellidae was supported as monophyletic and as sister to the other ephemerelloid lineages. Timpanoginae was not supported as monophyletic under the parameter set with 1:1:1 gap:transversion:transition cost ratio, but was supported as monophyletic in portions of the sensitivity parameter landscape (32%) with *Attenella* nested as sister to (*Eurylophella* + *Dentatella*). Ephemerellinae was well supported as monophyletic consisting of two main lineages. *Drunella* and *Cincticostella* were recovered as the only genus groups, represented with multiple specimens, as monophyletic. *Ephemerella*, the largest genus of Ephemerellidae, was supported as grossly paraphyletic.

KeyWords: Ephemeroptera; Mayflies; Molecular phylogeny; Pannota; Ephemerellidae

Introduction

The taxonomic history of the mayfly family Ephemerellidae (Insecta: Ephemeroptera) is long and complex. (Klapálek, 1909) first recognized Ephemerellidae as a family, but the concept dates back to Eaton's (Eaton, 1883-1888) section VI of *Ephemerella* Walsh. The concept of the family Ephemerellidae has changed much in subsequent years (Edmunds et al., 1963; Allen, 1965; Allen, 1980; Allen, 1984). During the last two decades, several genera have been removed from the Ephemerellidae, due to emphasis on the need for taxonomic classifications to reflect phylogenetic relationships in Ephemeroptera (McCafferty, 1991), and familial status has been given to many of these lineages. For example, genera of the family Teloganodidae were originally placed in Ephemerellidae, but the group was later elevated to familial status based primarily on maxillae palpi and gill characters (McCafferty and Wang, 1997). Similarly, *Teloganella* Ulmer, *Austremerella* Riek, and *Vietnamella* Tshernova, once placed within Ephemerellidae, have also been elevated to familial status (Teloganellidae and Vietnamellidae; the latter family having priority over Austramerellidae)(McCafferty and Wang, 1995; Wang and McCafferty, 1995; McCafferty and Wang, 2000). Finally, the genus *Melanemerella* Ulmer, which has been included in several families, including Ephemerellidae (Edmunds et al., 1963; Allen and Edmunds, 1965), was recently placed in the monotypic family Melanemerellidae (Molineri and Domínguez, 2003). Only in the case of Molineri and Domínguez (2003) were these taxonomic changes supported by formal phylogenetic analysis.

Currently, the family Ephemerellidae belongs to the suborder Furcatergalia and infraorder Pannota. The pannote mayflies are characterized by the presence of

forewingpads fused basally over one-half their length, while still remaining externally recognizable as wingpads, unlike the complete thorax fusion in the Carapacea (families Prosopistomatidae and Baetiscidae). Pannota is comprised of two superfamilies: Caenoidea (Caenidae + Neoephemeridae) and Ephemerelloidea (Ephemerellidae, Vietnamellidae, Teloganellidae, Teloganodidae, Melanemerellidae, Ephemerythidae, Machadorythidae, Tricorythidae, and Leptohyphidae). The phylogenetic position of the family Ephemerellidae relative to other ephemerelloid mayfly families has been unclear.

Figure 1 compares the congruence from four recent studies that examined relationships within Pannota (McCafferty and Wang, 2000; Molineri and Domínguez, 2003; Kluge, 2004; Ogden and Whiting, 2005). All were based on morphological data except for Ogden (2005), which was based exclusively on DNA sequence information. Although McCafferty and Wang (2000) and Kluge (2004) did not perform formal quantitative phylogenetic analysis, they did suggest numerous apomorphies supporting the hypothesized lineages. Only the analysis of Molineri and Domínguez does not suggest Ephemerellidae as sister to the remaining ephemerelloid lineages, due to the placement of *Lithogloea* Barnard. The sampling of Ogden & Whiting (2005) lacks many of the families that are, in most cases, monogeneric families from the Old World, and material had not yet been sequenced.

Within the family Ephemerellidae, there are approximately 300 species placed in two subfamilies: Ephemerellinae and Timpanoginae (McCafferty and Wang, 2000; Brittain and Sartori, 2003). According to current classification (Hong, 1979; McCafferty, 2000; McCafferty and Wang, 2000; McCafferty et al., 2003; Sartori, 2004) the family Ephemerellidae includes the following twenty genera: *Attenella* Edmunds, *Caudatella*

Edmunds, *Caurinella* Allen, *Cincticostella* Allen, *Crinitella* Allen and Edmunds, *Dannella* Edmunds, *Dentatella* Allen, *Drunella* Needham, *Ephacerella* Paclt, *Ephemerella* Walsh, *Eurylophella* Tiensuu, *Hyrtnella* Allen and Edmunds, *Kangella* Sartori, *Philolimnias* Hong [fossil], *Serratella* Edmunds, *Teloganopsis* Ulmer, *Timpanoga* Needham, *Torleya* Lestage, *Turfanerella* Demoulin [fossil], and *Uracanthella* Belov. Five subgenera are recognized for the genus *Drunella*: *Drunella*, *Eatonella* Needham, *Myllonella* Allen, *Tribrochella* Allen, and *Unirhachella* Allen. Two subgenera (*Cincticostella* and *Rhionella* Allen) are recognized under the genus *Cincticostella* (Allen, 1980). Utilizing a nonranking classification system, (Kluge, 1997; Kluge, 2004) two generic group names have been proposed under *Ephemerella*, *sensu lato*: *Amurella* Kluge and *Notacanthella* Kluge.

Most systematic studies of Ephemerellidae have been restricted to fauna within certain geographical bounds, including, for example, North America (Allen and Edmunds, 1962; Allen and Edmunds, 1963; Allen and Edmunds, 1965), Korea (Yoon and Kim, 1981; Yoon and Bae, 1988), Taiwan (Kang and Yang, 1995), China (You and Gui, 1995), Europe (Studemann et al., 1995), and Japan (Ishiwata, 2000; Ishiwata, 2001; Ishiwata, 2003). This approach to the taxonomy and phylogeny of the group has inherent problems (Edmunds, 1959; McCafferty, 1991; Studemann and Landholt, 1997). Furthermore, relationships of Ephemerellidae have been difficult to deduce, due in part to a preponderance of hypothetically plesiomorphic characters (McCafferty and Wang, 2000), undocumented morphological variability, poorly delimited species and generic boundaries (Jacobus and McCafferty, 2003a; Jacobus and McCafferty, 2003b), and the apparently arbitrary assignment of some species in genera (Studemann and Landholt,

1997; Thomas et al., 1999). This latter problem is illustrated well by the taxonomic history of *Uracanthella punctisetae* (Matsumura). The synonymy of this widespread Asian species includes binomial combinations with the genera *Drunella* Needham, *Ephemerella* Walsh, and *Serratella* Edmunds (Torres et al., 1993; Ishiwata, 2001). Differing hypotheses of relationships of species and genus groups within the subfamily Timpanoginae have been proposed (Allen, 1977; McCafferty, 1977; McCafferty, 1978; McCafferty and Wang, 1994; McCafferty, 2000; Kluge, 2004). Kluge (2004) also proposed relationships for some of the generic groups of the subfamily Ephemerellinae, but otherwise, relationships within this subfamily have been studied little.

The purpose of this paper is to investigate phylogenetic relationships within the family Ephemerellidae, and to test the hypothesis that Ephemerellidae is sister to the other ephemerelloid clades, based on morphological and molecular data. Specifically, we address: (1) Are Ephemerelloidea and Ephemerellidae supported as monophyletic groups? (2) What is the position of the family Ephemerellidae in relation to other Ephemerelloidea? (3) What are the relationships among the major lineages of Ephemerellidae?

Materials and Methods

Taxon Sampling (Table 1)

Taxonomic sampling for molecular work consisted of 48 total exemplars. We included 32 species of Ephemerellidae representing 16 of the 18 extant genera. From the families constituting Ephemerelloidea, we included 7 genera within Leptohiphidae, 3

genera within Telagonoidae, and 2 genera within Tricorythidae. The tree was rooted to *Paraleptophlebia* Lestage (Leptophlebiidae). Morphological characters were extracted from two previous studies. All 32 characters from Molineri and Dominguez (2003) were selected. From McCafferty and Wang (2000) characters 1, 3, 4, 5, 6, 7, 10, 12, 15, 27, 30, 37, 40, and 45; ambiguous and duplicate character (in relation to Molineri and Dominguez) were not included. These selected characters were combined into a matrix in MacClade (Maddison and Maddison, 2000). Only the families Teloganellidae, Melanemerellidae, Vietnamellidae, Ephemerythidae, and Machadorythidae were not represented by any molecular data. These are, for the most part, monogeneric families from the Old World, and fresh material has not yet been acquired for molecular analysis.

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 H3 protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are given elsewhere (Whiting, 2001; Ogden and Whiting, 2003). 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 nearly 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.

Phylogenetic Analyses

Sequences were initially assembled in Sequencher® 4.0 (Genecodes, 1999). The protein coding H3 gene was manually 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 12 fragments for 18S and 13 fragments for 28S. Fragment 11 from 28S (corresponding to part of region D7a) contained a highly length-variable insertion region and was excluded because the sequence fragments were judged non-homologous. These data were analyzed via direct optimization in the program POY version 3.0 (Gladstein and Wheeler, 1999). Some taxa had missing data in one or more of the DNA fragments given to POY to align, as indicated in Table 1. 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] or on a cluster [RackSaver (Verari Systems) RS-1100V-66XT: 65 nodes, dual Opteron 240 1.4 GHz processor configuration with each node having 512 MB of RAM]. POY command files were as follow: -fitchtrees -noleading -norandomizeoutgroup -sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop

5 -checkslop 10 -buildspr -buildmaxtrees 2 -stopat 25 -treefuse -fuselimit 10 -
fusemingroup 5 -fusemaxtrees 100 -ratchetspr 2 -ratchettbr 2 -checkslop 10 -seed -1.

A variety of cost parameter values were investigated to explore data sensitivity (Figure 2). We selected 25 values to explore sensitivity to gap/nucleotide change ratios (ranging from 1 to 10) and transition/transversion ratios (ranging from 1 to 10). 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 (Wheeler, 1995; Giribet, 2001). 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.

Results

The direct optimization analysis of the 1:1:1 (gap:tv:ts) ratio parameter set resulted in 2 equally parsimonious trees with a length (cost) of 9529. The implied alignment consisted of 6301 characters, with 1496 parsimony informative sites. Partitioned Bremer, total Bremer, bootstrap, and sensitivity analysis percent values are reported in Table 2 for corresponding numbered nodes on the tree (figure 2). The relative Bremer support (total Bremer for the partition/total Bremer for all partitions) for each partition shows that 38.2% of the signal comes from the 18S data set, 28S contributes

36.8%, 16S contributes 12.3%, and 12S contributes 8.6%. The other two partitions, morphology and H3, contribute considerably less with the remaining 2.4% and 1.6% of the relative Bremer support (Table 2).

The superfamily Ephemeroidea was strongly supported as monophyletic and sister to the Caenoidea with a Bremer support value (Bs) of 22 and bootstrap value (bt) of 100. Monophyly of Leptohiphidae was highly robust (recovered in every parameter set) and well supported (Bs = 32 and bt = 100). Similarly, Tricorythidae was recovered as monophyletic (Bs = 75 and bt = 100). Teloganodidae was not recovered as monophyletic in the 1:1:1 topology (figure 2), however, monophyly was supported in 48% of the analyses across the sensitivity analysis parameter landscape (figure 3). Ephemerellidae was well supported (Bs = 29 and bt = 100) as monophyletic and as sister to a clade containing the other ephemerelloid lineages in this analysis. Timpanoginae was not recovered as monophyletic in the 1:1:1 parameter set topology (figure 2), however, 32% of the sensitivity parameter sets did support monophyly of this subfamily (figure 3). The subfamily Ephemerellinae was highly supported as monophyletic (Bs = 13 and bt = 96) and relatively robust being present in four of the parameter sets. The *Hyrtanella*, *Torleya*, *Uracanthella*, and *Teloganopsis* assemblage (node 22) was very stable and highly supported. The sister group relationships of (*Caurinella idahoensis* + *Ephemerella maculata*), (*Cincticostella elongatula* + *C. insolita*), (*Ephemerella* + *E. septentrionalis*), and (*Drunella coloradensis* + *D. pelosa*) were also very stable throughout the parameter landscape.

Discussion

The position of Ephemerellidae as sister to a clade comprised of (Teloganodidae + (Tricorythidae + Leptohyphidae)) confirms the hypotheses (figure 1) of Kluge (2004), McCafferty & Wang (2000), and Ogden and Whiting (2005). *Lithogloea* was not included in this analysis, and therefore the results of Molineri and Dominguez (2003) of a paraphyletic Teloganodidae could not be tested.

Our 1:1:1 parameter set results conflict with the current subfamilial system by supporting a nonmonophyletic Timpanoginae (Bs = 13 and bt = 96). However, sensitivity analysis is useful because it allows one to examine the effect, prior to alignment, of different cost parameter sets on relationships of interest. Thus, the information from the eight parameter sets supporting Timpanoginae (figure 3) would have never been recovered without sensitivity analysis, and the conclusion of nonmonophyly would be undisputed. Thus, we can, with some confidence, suggest that there is support for a monophyletic Timpanoginae.

If we accept a monophyletic Timpanoginae (figure 3), the supported relationships conflict with previous hypotheses (McCafferty, 1977; McCafferty and Wang, 1994; McCafferty, 2000), in that *Attenella* is supported as sister to (*Eurylophella* + *Dentatella*). This is in contrast to the proposal that *Attenella* be placed sister to a clade containing the four other genera (McCafferty and Wang, 1994). Therefore, McCafferty and Wang's characters 3, 4 and 5 would either have to be mapped twice on the clades (*Dannella* + *Timpanoga*) and (*Eurylophella* + *Dentatella*) or would have a single origin on the Timpanoginae node with a reversal on the *Attenella* branch.

Ephemerellinae is structured into two major lineages (node 20 and 26). Although Node 20 is not well supported (Bs = 2 and bt = 67), it is present in 28% of the parameter sets. However, the remaining topological structure within this lineage (nodes 21-25) is highly supported (Bs > 10 and bt > 95) and robust (present in > 50% of parameter sets). The historically problematic taxon *Uracanthella punctisetae* is clearly not closely related to the genera *Drunella*, *Ephemerella*, or *Serratella* as might be inferred from its synonymy (Torres et al., 1993; Ishiwata, 2001). Rather, *U. punctisetae* along with another *Uracanthella* and *Teloganopsis* form a robustly supported clade (Bs = 51 and bt = 100) sister to (*Hyrtanella* + *Torleya*), which is also a well supported group (Bs = 32 and bt = 100). This relationship calls into question the validity of the genus *Teloganopsis*. There is good nodal support (node 27) for the basal placement of *Caudatella* relative to the remaining Ephemerellinae. However the support for the relationships within these remaining lineages is weak and sensitive to parameter perturbation (low Bs, bt, and parameter set % recovery along backbone). Notwithstanding, the genus *Drunella*, is supported as monophyletic with a Bremer value of 9, bootstrap value of 93, and present in 12% of the landscape. Within *Drunella*, the *D. trispina* clades are not supported as sister, which is surprising considering that both specimens are from Japan. The genus *Cincticostella* was robustly recovered as monophyletic in all but one of the parameter sets. *Serratella* and *Ephemerella* were not supported as monophyletic, with the latter being the “trash bag” genus for Ephemerellidae.

The relative contribution of morphology, based on Bremer values, appears to be negligible. For example the average score is 0.52 and the Bs contribution is 2.4% of the total Bs. However, the Bs score for nodes 15-18 and 20-44 is 0 because the characters

taken from the two literature sources did not provide any characters below the subfamilial level. Nevertheless, an adjusted contribution value (calculated as the BS divided by the number of parsimony informative characters) suggests that morphology is the second most important contributor to overall tree support.

It is clear that portions of the tree are well supported and robust, such as many of the higher level nodes (1, 2, 3, 4, 10, 14, and 26 among others). Whereas, other parts of the topology lack support and robustness (nodes along the Ephemerellinae backbone or within Timpanoginae) and relationships are more likely to be falsified in future studies, decreasing the confidence in these nodes. Overall, this study represents an important attempt to combine available morphological and molecular data in order to examine the position of Ephemerellidae within the Ephemerelloidea and relationships within the family.

Conclusions

Direct optimization was used to reconstruct the phylogeny based on DNA sequence data from 3 nuclear (18S rDNA, 28S rDNA, histone H3), 2 mitochondrial (12S rDNA, 16S rDNA) genes and a 46 character morphological matrix. Taxon sampling included 35 exemplars representing 16 of the 18 described ephemerellid genera. The superfamily Ephemerelloidea was strongly supported as monophyletic and sister to the Caenoidea with a Bremer support value of 22, bootstrap value of 100, and present in 96% of the sensitivity analysis parameter sets. The family Ephemerellidae was well supported (Bremer support value = 29 and bootstrap value = 100) as monophyletic and as sister to the other ephemerelloid lineages. Timpanoginae not supported as monophyletic in the

1:1:1 parameter set, but was supported as monophyletic in portions of the sensitivity parameter landscape (32%) with *Attenella* nested as sister to (*Eurylophella* + *Dentatella*). EphemereLLinae was well supported (Bremer support value = 13 and bootstrap value = 96) and present in a 52% of the sensitivity analyses. Two main lineages were identified, with the genus *Caudatella* as basal in one of these. *Drunella* and *Cincticostella* was recovered as monophyletic

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Figure Legend:

Figure 1. 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 2. 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 > 95, respectively.

Figure 2. This topology shows a number of relationships (nodes with a % score above branch), that while not present in figure 1 (1:1:1 parameter set), the relationships were recovered across a large portion of the parameter landscape. The % numbers above each node represent the percentage of the parameter sets support the particular clade.

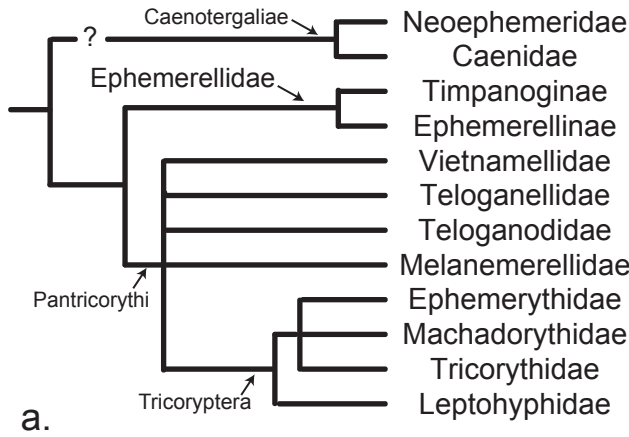
Table 1. Taxon list and Genbank accession numbers. Subgenera are indicated in parentheses, following the genus. Genbank numbers to be provided upon acceptance.

Family	Genus	Species	12S rDNA	16S rDNA	18S rDNA	28S rDNA	histone H3
Leptophlebiidae	<i>Paraleptophlebia</i>	<i>vaciva</i>					
Neophemeridae	<i>Neophemera</i>	<i>youngi</i>					
Caenidae	<i>Caenis</i>	<i>sp.</i>					
	<i>Callistina</i>	<i>panda</i>					
Leptohyphidae	<i>Allenhyphes</i>	<i>flinti</i>					
	<i>Haplohyphes</i>	<i>baritu</i>					
	<i>Leptohyphes</i>	<i>zalope</i>					
	<i>Traverhyphes</i>	<i>indicator</i>					
	<i>Trichorythopsis</i>	<i>chiriguano</i>					
	<i>Tricorythodes</i>	<i>sp.</i>					
	<i>Yaurina</i>	<i>mota</i>					
Teloganodidae	<i>Teloganodes</i>						
	<i>Lestagella</i>	<i>penicillata</i>					
	<i>Manohyphella</i>	<i>sp.</i>					
	<i>Teloganodes</i>	<i>tristis</i>					
Tricorythidae	<i>Spinirythus</i>	<i>sp.</i>					
	<i>Tricorythus</i>	<i>sp.</i>					
Ephemerellidae	<i>Attenella</i>	<i>margarita</i>					
	<i>Caudatella</i>	<i>hystrix</i>					
	<i>Caurinella</i>	<i>idahoensis</i>					
	<i>Cincticostella</i> (<i>Rhionella</i>)	<i>insolta</i>					
	<i>C.</i> (<i>Cincticostella</i>)	<i>elongatula</i>					
	<i>Dannella</i>	<i>provonshai</i>					
	<i>Dentatella</i>	<i>coxalis</i>					
	<i>Drunella</i> (<i>Myllonella</i>)	<i>coloradensis</i>					
	<i>D.</i> (<i>Eatonella</i>)	<i>doddsii</i>					
	<i>D.</i> (<i>Drunella</i>)	<i>pelosa</i>					
	<i>D.</i> (<i>Drunella</i>)	<i>spinifera</i>					
	<i>D.</i> (<i>Tribrochella</i>)	<i>trispina</i>					
	<i>D.</i> (<i>Tribrochella</i>)	<i>trispina</i>					
	<i>D.</i> (<i>Unirhachella</i>)	<i>tuberculata</i>					
	<i>Ephacerella</i>	<i>longicaudata</i>					
	<i>Ephemerella</i>	<i>sp.</i>					
	<i>Ephemerella</i>	<i>atagosana</i>					
	<i>Ephemerella</i>	<i>berneri</i>					
	<i>Ephemerella</i>	<i>cornuta</i>					
	<i>Ephemerella</i>	<i>maculata</i>					
	<i>Ephemerella</i>	<i>needhami</i>					
	<i>Ephemerella</i>	<i>septentrionalis</i>					
	<i>Eurylophella</i>	<i>verisimilis</i>					
	<i>Hyrtenella</i>	<i>sp.</i>					
	<i>Serratella</i>	<i>sp.</i>					
	<i>Serratella</i>	<i>serrata</i>					
	<i>Serratella</i>	<i>teresa</i>					
<i>Teloganopsis</i>	<i>sp.</i>						
<i>Timpanoga</i>	<i>hecuba</i>						
<i>Torleya</i>	<i>major</i>						
<i>Uracanthella</i>	<i>sp.</i>						
<i>Uracanthella</i>	<i>punctisetae</i>						

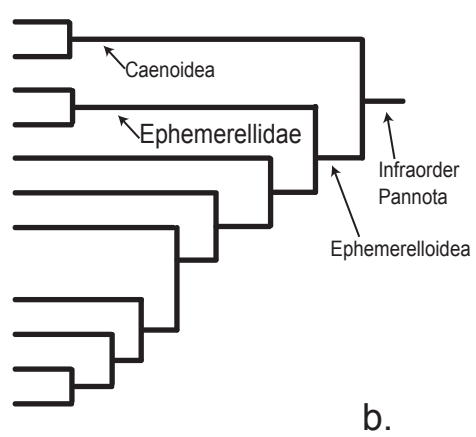
Table 2. Bremer support (Bs) values, bootstrap values, sensitivity analysis percent (SA%) score for each node on the 1:1:1 topology (figure 2). SA% = number of parameter sets monophyletic divided by 25 (total number of parameter sets). PIC = the number of parsimony informative characters.

Node	Morphology	Bremer 12S	Bremer 16S	Bremer 18S	Bremer 28S	Bremer H3	Total Bremer	bootstrap	SA%
1	2	0	0	2	20	0	24	100	24%
2	3	0	0	35	52	0	90	100	100%
3	1	2	0	17	3	-1	22	100	96%
4	1	3	-3	10	17	2	30	100	48%
5	0	4	12	17	10	1	44	100	100%
6	6	7	7	0.5	2.5	3	26	100	44%
7	-1	-1	9	6	2	0	15	98	44%
8	2	4	0	39	30	0	75	100	56%
9	0	14.5	28	18.5	29	0	90	100	100%
10	4	5	8	9	6	0	32	100	100%
11	3	17	0	3	9	-1	31	100	100%
12	0	8	11	3	0	5	27	100	100%
13	0	12	8	14	11	0	45	100	100%
14	3	6	0	13	7	0	29	100	52%
15	0	4.5	0.5	0	13.5	0.5	19	98	28%
16	0	25	0.5	8.5	24	1	59	100	100%
17	0	3	0	2	1	0	6	86	8%
18	0	0	0	0	0	0	0	54	8%
19	1	6	0	0	6	0	13	96	16%
20	0	1	0	0	5	-4	2	67	28%
21	0	2	1	-1	16	-4	14	97	56%
22	0	2	2	19	13	-4	32	100	100%
23	0	4	0	32.5	13.5	1	51	100	100%
24	0	13	0	6	5	2	26	100	100%
25	0	4	-3	14	10	-2	23	100	100%
26	0	2	5	-3	3	6	13	96	52%
27	0	3	0	12	12	-1	26	100	8%
28	0	-1.5	0.5	6	3	0	8	86	8%
29	0	-4	1	5	2	-1	3	59	100%
30	0	-2.5	1	4.5	2	-2	3	71	8%
31	0	-9	1	5	2	2	1	<50	16%
32	0	-9	1	5	2	2	1	<50	8%
33	0	-13	1	10	2	2	2	72	96%
34	0	2.5	3.2	-0.5	2	2.8	10	93	8%
35	0	-9	3.7	11	8	4.3	18	100	8%
36	0	-5.8	1	7.8	0	1	4	73	4%
37	0	-1	0	2	1	1	3	74	8%
38	0	-5.8	1	7.8	0	1	4	71	100%
39	0	-8	3	6	8	0	9	93	12%
40	0	-8	1	5	4	0	2	52	4%
41	0	1	4	8	4	-2	15	100	16%
42	0	-4.7	4.3	5.7	1.7	0	7	91	16%
43	0	-4	3	0	3	-2	0	67	4%
44	0	13	1.5	0.5	5	2	22	100	80%
Total	23	82.2	117.2	363.8	350.2	15.6	952		
Average	0.52	1.87	2.66	8.27	7.96	0.35		86.2%	51.5%
Bs contribution	2.4%	8.6%	12.3%	38.2%	36.8%	1.6%			
Total # PIC	37	170	250	301	630	108			
Total Bs/PIC	0.62	0.48	0.47	1.21	0.56	0.14			

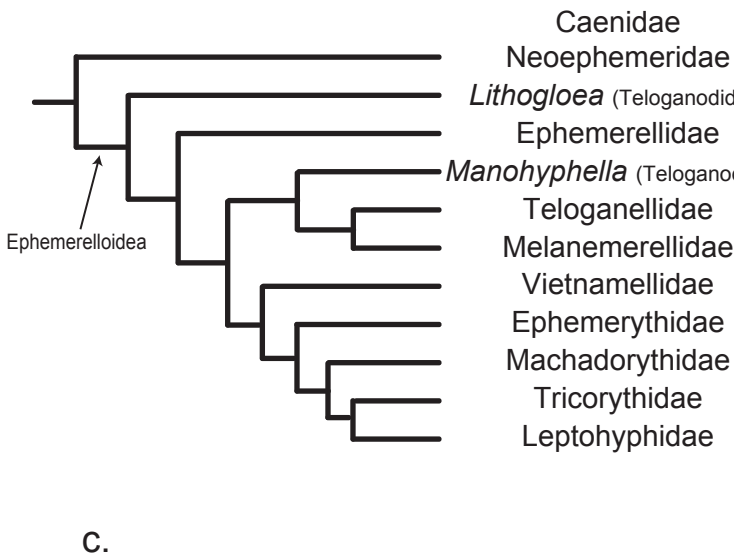
Kluge (2004) hypothesis



McCafferty & Wang (2000) hypothesis



Molineri & Dominguez (2003) hypothesis



Ogden & Whiting (2005) hypothesis

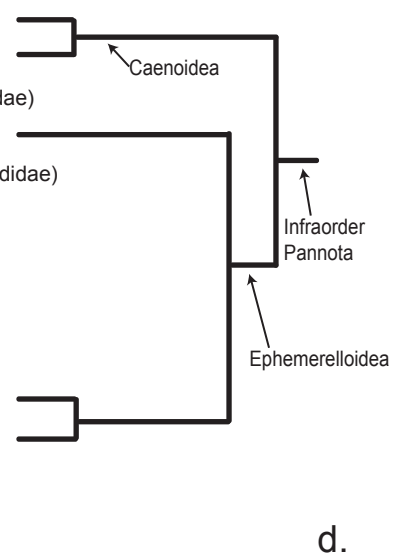


Figure 1

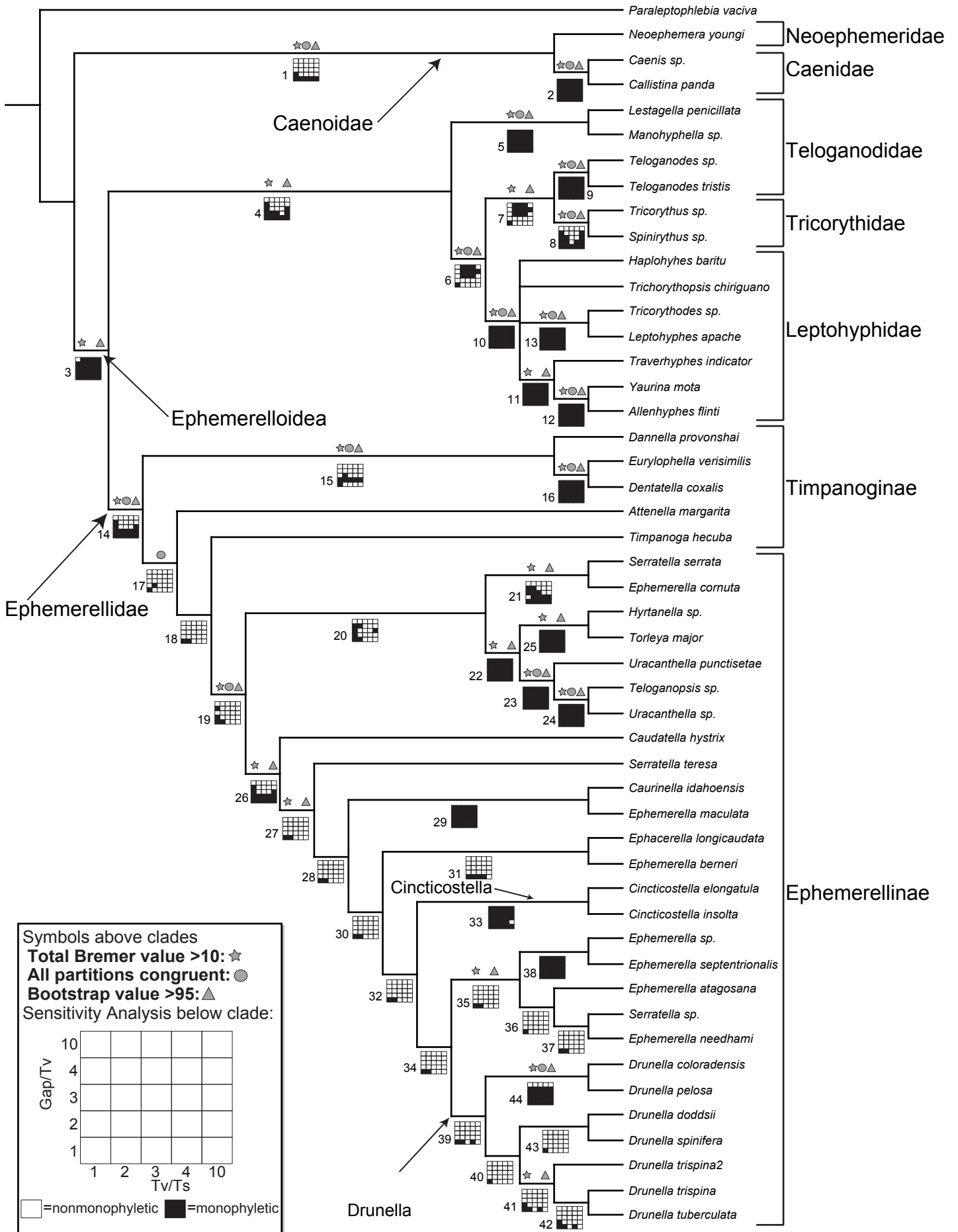


Figure 2

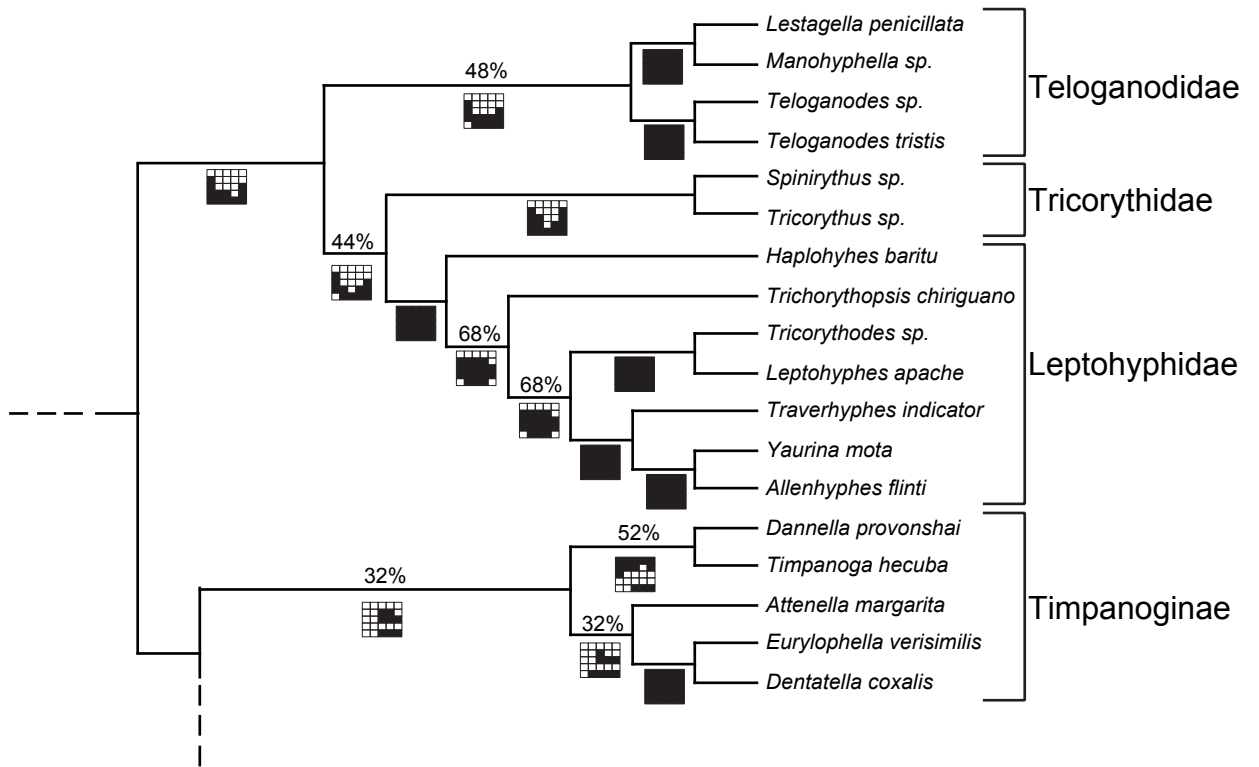


Figure 3