

Mayfly Phylogenomics: Initial Evaluation of Anchored Hybrid Enrichment Data for the order Ephemeroptera

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Abstract

This study was presented at the International Conference on Ephemeroptera and Plecoptera held in Aracruz, Brazil from June 3–8, 2018. Higher-level relationships among major mayfly lineages remains controversial and previous molecular and morphological data do not robustly support the current classification scheme and many of the proposed branching orders (Ogden *et al.* 2009a). This project represents the largest phylogenetic analysis for mayflies to date. Over 440 targeted genomic protein coding regions (exons) were generated using a novel hybrid enrichment probes set. The dataset, analyzed as amino acids, allowed testing for the monophyly of many of the proposed higher-level groupings. The results from this work were congruent with the lineages Carapacea, Caenoidea, and Ephemerelloidea, but the other higher-level lineages were not supported as monophyletic. This study was an initial evaluation of the anchored hybrid enrichment dataset for uses in deep level mayfly systematics, and a more comprehensive analysis is forthcoming. Still, this study supports the conclusion that more taxa and data, especially phylogenomic data, should result in more robust trees for future studies in mayfly phylogenetics and systematics.

Keywords: phylogeny, systematics, mayflies, evolution

Introduction

This study is a synthesis of the research that was presented at the International Conference on Ephemeroptera and Plecoptera held in Aracruz, Brazil from June 3–8, 2018. It is an initial analysis of next generation data, generated using anchored hybrid enrichment methods, and describes their usefulness in deep level mayfly systematics. A larger and more comprehensive analysis is forthcoming.

Recently, Sartori & Brittain (2015) summarized that there were 3,328 described species, in 441 genera, in 41 families within Ephemeroptera. These numbers were slightly different from a previous study (Barber-James *et al.* 2013) that indicated there were 3,269 species, in 442 genera, in 42 families. The difference in the number of families between the two studies was that Sartori and Brittain did not consider Chromarcyidae to be its own family, rather that *Chromarcys* belongs to the family Oligoneuriidae. The mayflies, as an order, are consistently supported as a monophyletic group of pterygote insects (Ogden & Whiting 2005; Ogden *et al.* 2009). Many recent molecular and morphological analyses support their sister group relationship to Odonata constituting Palaeoptera (Regier *et al.* 2010; Ishiwata *et al.* 2011; Sasaki *et al.* 2013; Blanke *et al.* 2013; Thomas *et al.* 2013; Misof *et al.* 2014), although some studies also support Metapterygota – Ephemeroptera

sister to Odonata + Neoptera (Ogden & Whiting 2003, Zhang *et al.* 2008; Klass 2009) and Chiasmomyria – Odonata sister to Ephemeroptera + Neoptera (Misof *et al.* 2007; Simon *et al.* 2009; Lin *et al.* 2010; Wan *et al.* 2012; Meusemann *et al.* 2010; Regier *et al.* 2010; Simon & Hadrys 2013; Li *et al.* 2014).



FIGURE 1. Phylogenetic tree from Bayesian analysis. The numbers below the nodes represent posterior probabilities. * = non-monophyletic Ephemeridae.

A history of the classification of Ephemeroptera was reviewed previously (Ogden & Whiting 2005; Ogden *et al.* 2009a), and here we offer additional review, comments, and highlights of the major clades and problematic relationships. The number of families over the years changed and trees depicting relationships were only sometimes included (Table 1). Eaton (1883–1888) was the first to discuss some relationships for the entire order, but it is unclear how many families were proposed and no tree was included. In the 1900s the number of families ranged from as low as 14 and as high as 28, although Edmunds (1972) depicted a tree that included 36 subfamilies as terminals. Today, most of these proposed subfamilies are now considered to be families. Most recent analyses or reviews propose the number of families to be in the high thirties to the low

forties. It is important to point out that only one formal cladistic (or any other formal approach) analysis, that included all major lineages, has been carried out (Ogden *et al.* 2009a).

TABLE 1. Summary of the studies that have proposed phylogenetic relationships for the entire order Ephemeroptera (or close to the entire order).

Author(s)	Year	# of proposed families	Tree figure proposed	Type of Analysis or Review
Eaton	1883–1888	Unclear	No	Review
Ulmer	1920	14	No	Review
Edmunds & Traver	1954	Unclear	No	Review
Demoulin	1958	20	No	Review
Edmunds	1962	19	Yes	Intuitive
Landa	1969	16	No	Review
Tshernova	1970	23	No	Review
Edmunds	1972	36 (subfamilies)	Yes	Intuitive
Edmunds	1973	Unclear	Partial Trees	Intuitive
Koss	1973	20	Yes	Intuitive
Landa	1973	25	Yes	Intuitive
Riek	1973	14	Yes	Intuitive
Koss & Edmunds	1974	21	Yes	Intuitive
McCafferty & Edmunds	1979	19	Yes	Intuitive
Landa and Soldan	1985	21	No	Review
Tomka & Elpers	1991	25	Yes	Intuitive
McCafferty	1991	28	Partial Trees	Intuitive
Brittain & Sartori	2003	37	No	Review
Kluge	2004	Unclear	Yes	Intuitive
Ogden & Whiting	2005	Not all families sampled	Yes	Formal, Molecular data
Sun <i>et al.</i>	2006	Not all families sampled	Yes	Formal, Molecular data
Ogden <i>et al.</i>	2008	Not all families sampled	Yes	Formal, Molecular data
Ogden <i>et al.</i>	2009a	38	Yes	Formal, Molecular and Morphological data
Barber-James <i>et al.</i>	2013	42	No	Review
Sartori & Brittain	2015	41	No	Review
Gao <i>et al.</i>	2018	Not all families sampled	Yes	Formal, Molecular data
Ye <i>et al.</i>	2018	Not all families sampled	Yes	Formal, Molecular data
Cai <i>et al.</i>	2018	Not all families sampled	Yes	Formal, Molecular data
Wu <i>et al.</i>	2018	Not all families sampled	Yes	Formal, Molecular data

Ogden and Whiting's (2005) figure 2 summarized the hypotheses of McCafferty & Kluge (Kluge 2004), indicating that they were mostly congruent with each other. McCafferty's hypothesis was a compilation based partially on cladistic analyses for the Pannota (McCafferty & Wang 2000; Jacobus & McCafferty 2006) and Setisura (McCafferty 1991a; Wang & McCafferty 1995), and from published trees (McCafferty 1991b; McCafferty 1997). The molecular only (Ogden & Whiting 2005; Ogden *et al.* 2008) and combined analyses (Ogden *et al.* 2009a) have shown some support for the monophyly of Carapacea, Furcatergalia, Fossoria,

Pannota, Caenoidea and Ephemerelloidea. However, other major lineages, such as Setisura, Pisciforma, Siphonuroidea, Baetoidea, and Ephemerioidea, among others, were not supported as monophyletic. Potamanthidae was supported as sister to the other burrowing mayflies + Pannota, however, Miller *et al.* (2018) have recently shown strong support for a monophyletic burrowing mayfly group that includes the families Ephemeridae, Euthyplociidae, Ichthybotidae, Polymitarcyidae, Palingeniidae, Potamanthidae, and Behningiidae. The status of the monogeneric families Pseudironidae, Arthropleidae and Dipteromimidae has been challenged (Ogden & Whiting 2005; Ogden *et al.* 2009a). The 2009 analysis proposed that *Siphuriscus* (Siphuriscidae) was supported as sister to all other mayfly clades.

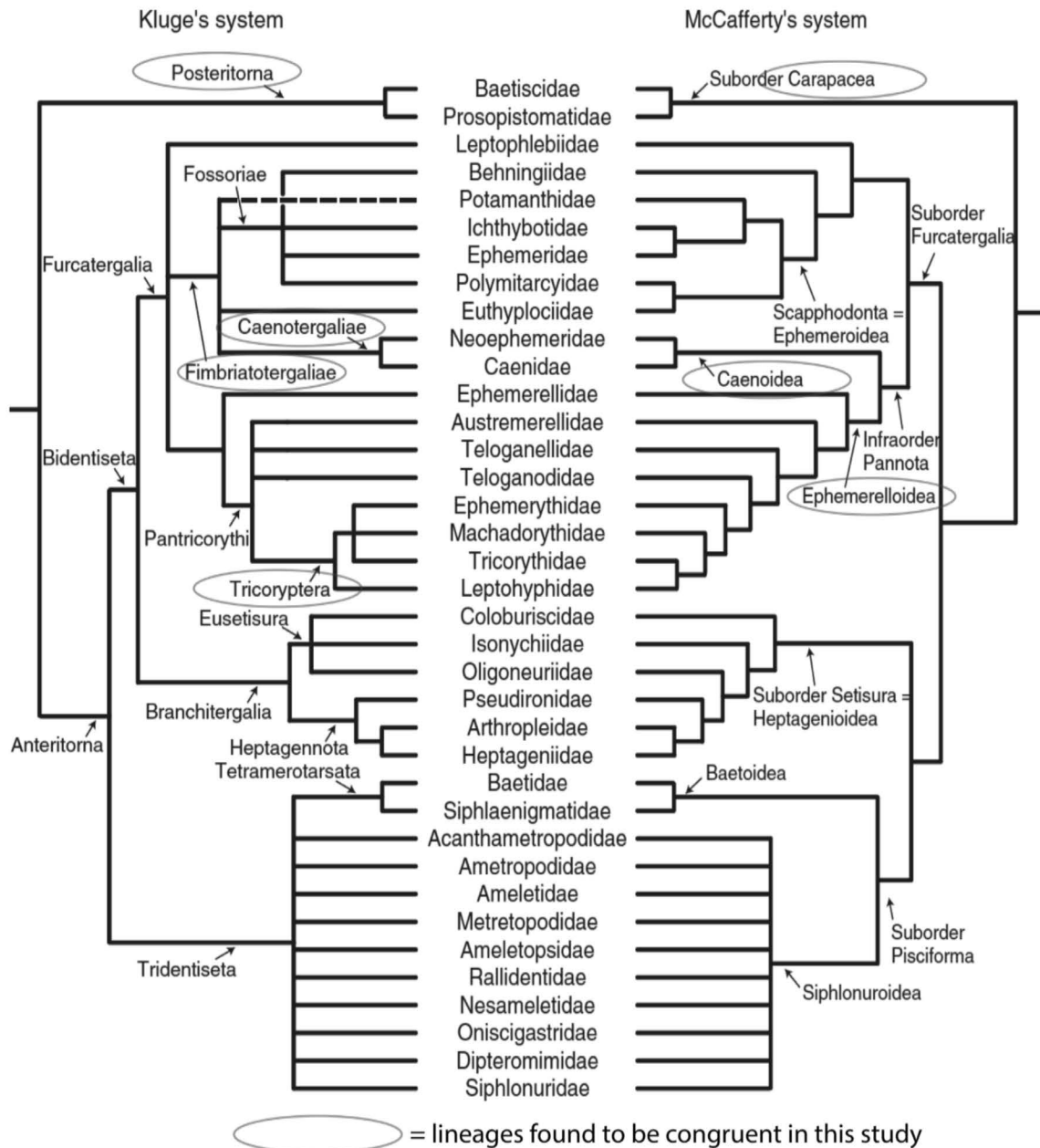


FIGURE 2. This figure is modified from Ogden *et al.* (2005) figure 2. The circles indicate the higher-level groups that were not supported as monophyletic in this analysis (compare to Figure 1).

Aside from the three Ogden *et al.* analyses (Ogden & Whiting 2005; Ogden *et al.* 2008; and Ogden *et al.* 2009a) and the Sun *et al.* (2008) analysis, there have been relatively few formal systematic studies that have contributed to our knowledge of the relationships among mayfly families. For example, morphological cladistic analyses have focused mainly on the pannote mayflies (Molineri & Dominguez 2003; Jacobus & McCafferty 2006; Souto *et al.* 2019 unpublished). For molecular data, there have been some recent contributions. The analysis of the burrowing mayflies (Miller *et al.* 2018) that concluded that Euthyplociidae was sister to Behningiidae, Ichthybotidae sister to Palingeniidae + nonmonophyletic Ephemeridae, with Potamanthidae as sister to all the burrowing lineages. Multiple analyses using mitochondrial genomes to reconstruct familial relationships have recently been published (Gao *et al.* 2018; Ye *et al.* 2018; Cai *et al.* 2018; Wu *et al.* 2018); however all of these include only a subset of the mayfly families and therefore suffer from a lack of taxon sampling.

There are more examples of molecular phylogenetic research being done at the family level and below. Some examples include studies on Dipteromimidae (Tojo & Matsukawa 2003; Takenaka & Tojo 2019), Baetidae (Monaghan *et al.* 2005; Williams *et al.* 2006; Stahls & Savolainen 2008; Gattolliat & Monaghan 2010; Sroka 2012; Pereira-da-Conceicao *et al.* 2012; Rutschmann *et al.* 2014; Vuataz *et al.* 2016), Leptophlebiidae (O'Donnell & Jockusch 2008), Heptageniidae (Webb *et al.* 2007; Vuataz *et al.* 2011; Vuataz *et al.* 2013; Yanai *et al.* 2017), Ephemerellidae (Alexander *et al.* 2009; Ogden *et al.* 2009b), Ephemeridae (Hwang *et al.* 2013). Many genus or species level studies are also contributing relevant information and data; for example, *Ameletus* (Theissinger 2011), *Ephoron* (Sekine *et al.* 2013), *Rhithrogena* (Vuataz *et al.* 2016), *Baetis rhodani* (Williams *et al.* 2006), and *Baetis alpinus* (Leys *et al.* 2016) among others.

The current suite of molecular data for mayflies has not resulted in a robust phylogeny particularly along the deep ancestral “backbone nodes” of the tree (Ogden *et al.* 2009a). Therefore, it seems imperative to generate more data from homologous region across the genomes from representatives of the major lineages of mayflies. Data generation has changed in the last two decades in both speed and cost. For example, it may have cost around one billion dollars to generate the first human genome and it took about 8 years to accomplish this task. However, we are now in the age of human genomes being generated for less than a thousand dollars with a data generation time of one to two days. As a comparison for mayflies, the Ogden *et al.* (2009a) analysis was a five gene by 96 taxon dataset, with an alignment of ~5,800 nucleotides. If you estimate that it took about 20 sequences per taxa and about \$10 per sequence (including all the plastics, reagents, and sequencing costs—and this may be a very conservative estimate), it would have cost \$19,200 to generate the data for this dataset. It also took about four years (of a PhD students time) to generate that data. As compared to new phylogenomic datasets, for example Ogden and collaborators have been working on a dataset containing around 450 “loci” for over 200 taxa, and an alignment with more than 9.5 million sites. Using the next generation targeted sequence approach, the cost of generating the data is estimated at around \$20,000 for kits and sequencing, and once genomic samples were prepared and submitted, it only took about a week to generate the actual sequence data. Therefore, the tools and approaches of next generation sequencing has brought mayfly evolution and phylogenomics into a new era.

This paper has two main objectives: 1) present a simplified workflow of generating targeted capture sequence data for Ephemeroptera; and 2) present preliminary analyses of the phylogenomic dataset. These results were initially presented at the conference in Brazil, in advance of the more robust and complete analysis that will be published elsewhere (Ogden *et al.*, in prep).

Materials and Methods

Taxon sampling

Samples were chosen due to tissue availability, DNA quality, and sample that maximized the breadth of diversity. There were more than 300 possible sample species in the Ogden Lab genomic tissue library, and deciding which samples should be sequenced first was based on two main factors: the quality of genomic DNA, and the species potential to maximize diversity and breadth across the order. Ultimately, the dataset consisted of 105 species, 8 outgroup taxa and 97 ingroup taxa (Table 2). Among the ingroup, thirty-five families were represented, with multiple genera from the more speciose families. The recognized families (lineages) Prosopistomatidae, Vietnamellidae, Teloganellidae, Ephemerythidae, Melanemerellidae, Coryphoridae, Diceromyzidae, and Machadorythidae were not sampled due to lack of specimens producing quality DNA. The outgroup was represented by the orders Odonata, Archaeognatha, and Zygentoma.

TABLE 2. Taxonomic sampling. The number of loci captured for each taxon is indicated.

Family	Genus	species	Loci Captured
Acanthametropodidae	<i>Analetris</i>	<i>eximia</i>	421
Ameletidae	<i>Ameletus</i>	<i>sp</i>	167
Ameletidae	<i>Ameletus</i>	<i>sp</i>	422
Ameletopsidae	<i>Ameletopsis</i>	<i>perscitus</i>	431
Ameletopsidae	<i>Chaquihua</i>	<i>sp</i>	441
Ameletopsidae	<i>Mirawara</i>	<i>sp</i>	435
Ametropodidae	<i>Ametropus</i>	<i>neavei</i>	429
Arthropleidae	<i>Arthroplea</i>	<i>bipunctata</i>	431
Baetidae	<i>Acerpenna</i>	<i>pygmaea</i>	347
Baetidae	<i>Afroptilum</i>	<i>sp</i>	412
Baetidae	<i>Baetis</i>	<i>tricaudatus</i>	403
Baetidae	<i>Baetis</i>	<i>bicaudatus</i>	407
Baetidae	<i>Baetis</i>	<i>sp</i>	415
Baetidae	<i>Baetis</i>	<i>tricaudatus</i>	434
Baetidae	<i>Callibaetis</i>	<i>ferrugineus</i>	403
Baetidae	<i>Centroptilum</i>	<i>luteolum</i>	399
Baetidae	<i>Liebebiella</i>	<i>sp</i>	62
Baetiscidae	<i>Baetisca</i>	<i>sp</i>	414
Behningiidae	<i>Behningia</i>	<i>sp</i>	71
Behningiidae	<i>Dolania</i>	<i>americana</i>	267
Caenidae	<i>Brachycercus</i>	<i>harrisella</i>	215
Caenidae	<i>Caenis</i>	<i>sp</i>	237
Caenidae	<i>Tasmanocoenis</i>	<i>sp</i>	286
Coloburiscidae	<i>Coloburiscoides</i>	<i>sp</i>	442
Coloburiscidae	<i>Coloburiscus</i>	<i>humeralis</i>	435
Coloburiscidae	<i>Murphyella</i>	<i>sp</i>	438
Dipteromimidae	<i>Dipteromimus</i>	<i>sp</i>	432
Ephemerellidae	<i>Caudatella</i>	<i>hystrix</i>	137
Ephemerellidae	<i>Drunella</i>	<i>doddsii</i>	219
Ephemerellidae	<i>Drunella</i>	<i>coloradensis</i>	303
Ephemerellidae	<i>Drunella</i>	<i>coloradensis</i>	443
Ephemerellidae	<i>Ephemerella</i>	<i>inermis</i>	442
Ephemerellidae	<i>Eurylophella</i>	<i>verisimilis</i>	154
Ephemerellidae	<i>Eurylophella</i>	<i>sp</i>	442
Ephemerellidae	<i>Serratella</i>	<i>serrata</i>	418
Ephemerellidae	<i>Timpanoga</i>	<i>hecuba</i>	414
Ephemerellidae	<i>Torleya</i>	<i>major</i>	259
Ephemeridae	<i>Ephemera</i>	<i>simulans</i>	433
Ephemeridae	<i>Ephemera</i>	<i>sp</i>	441

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TABLE 2. (Continued)

Family	Genus	species	Loci Captured
Ephemeridae	<i>Ephemera</i>	<i>danica</i>	448
Ephemeridae	<i>Hexagenia</i>	<i>sp</i>	441
Euthyplociidae	<i>Polyplocia</i>	<i>sp</i>	432
Euthyplociidae	<i>Probosciodoplocia</i>	<i>sp</i>	381
Heptageniidae	<i>Afromurus</i>	<i>peringueyi</i>	134
Heptageniidae	<i>Atopopus</i>	<i>sp</i>	136
Heptageniidae	<i>Cinygma</i>	<i>sp</i>	401
Heptageniidae	<i>Ecdyonurus</i>	<i>dispar</i>	432
Heptageniidae	<i>Epeorus</i>	<i>longimanus</i>	426
Heptageniidae	<i>Heptagenia</i>	<i>sp</i>	431
Heptageniidae	<i>Rhithrogena</i>	<i>sp</i>	431
Heptageniidae	<i>Rhithrogena</i>	<i>robusta</i>	435
Heptageniidae	<i>Stenonema</i>	<i>mediopunctatum</i>	434
Ichthybotidae	<i>Ichthybotus</i>	<i>hudsoni</i>	442
Isonychiidae	<i>Isonychia</i>	<i>bicolor</i>	429
Isonychiidae	<i>Isonychia</i>	<i>kiangsinensis</i>	442
Isonychiidae	<i>Isonychia</i>	<i>sp</i>	444
Isonychiidae	<i>Isonychia</i>	<i>sp</i>	444
Leptohyphidae	<i>Allenhyphes</i>	<i>flinti</i>	61
Leptohyphidae	<i>Leptohyphes</i>	<i>apache</i>	143
Leptohyphidae	<i>Tricorythodes</i>	<i>sp</i>	296
Leptophlebiidae	<i>Austrophlebiodes</i>	<i>sp</i>	419
Leptophlebiidae	<i>Choroterpes</i>	<i>sp</i>	416
Leptophlebiidae	<i>Euthraulus</i>	<i>sp</i>	349
Leptophlebiidae	<i>Habroleptoides</i>	<i>confusa</i>	404
Leptophlebiidae	<i>Meridialaris</i>	<i>diguillina</i>	413
Leptophlebiidae	<i>Paraleptophlebia</i>	<i>sp</i>	187
Leptophlebiidae	<i>Paraleptophlebia</i>	<i>vaciva</i>	193
Leptophlebiidae	<i>Paraleptophlebia</i>	<i>vaciva</i>	442
Leptophlebiidae	<i>Penaphlebia</i>	<i>sp</i>	440
Metropodidae	<i>Metretopus</i>	<i>borealis</i>	397
Metropodidae	<i>Siphloplecton</i>	<i>interlineatum</i>	432
Neophemeridae	<i>Neophemera</i>	<i>youngi</i>	115
Nesameletidae	<i>Ameletoides</i>	<i>sp</i>	420
Nesameletidae	<i>Metamonius</i>	<i>sp</i>	442
Nesameletidae	<i>Nesameletus</i>	<i>ornatus</i>	420
Oligoneuriidae	<i>Lachlania</i>	<i>sp</i>	280
Oligoneuriidae	<i>Oligoneuriella</i>	<i>rhenana</i>	415
Oniscigastridae	<i>Oniscigaster</i>	<i>distans</i>	441

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TABLE 2. (Continued)

Family	Genus	species	Loci Captured
Oniscigastridae	<i>Siphonella</i>	<i>sp</i>	435
Oniscigastridae	<i>Tasmanophlebia</i>	<i>sp</i>	440
Palingeniidae	<i>Palingenia</i>	<i>longicauda</i>	138
Palingeniidae	<i>Plethogenesisia</i>	<i>sp</i>	435
Polymitarciidae	<i>Ephoron</i>	<i>sp</i>	444
Potamanthidae	<i>Anthopotamus</i>	<i>sp</i>	428
Potamanthidae	<i>Potamanthus</i>	<i>luteus</i>	187
Potamanthidae	<i>Rhoenanthus</i>	<i>sp</i>	44
Potamanthidae	<i>Stygifloris</i>	<i>sp</i>	87
Pseudironidae	<i>Pseudiron</i>	<i>centralis</i>	410
Rallidentidae	<i>Rallidens</i>	<i>mcfarlanei</i>	358
Siphlaenigmatidae	<i>Siphlaenigma</i>	<i>janae</i>	390
Siphonuridae	<i>Parameletus</i>	<i>columbiae</i>	400
Siphonuridae	<i>Parameletus</i>	<i>columbiae</i>	441
Siphonuridae	<i>Siphonurus</i>	<i>sp</i>	445
Siphuriscidae	<i>Siphuriscus</i>	<i>sp</i>	340
Teloganodidae	<i>Manohypheila</i>	<i>sp</i>	305
Tricorythidae	<i>Spinirithus</i>	<i>sp</i>	195
Outgroups / Order			
Odonata	<i>Ladona</i>	<i>fulva</i>	448
Odonata	<i>Calopteryx</i>	<i>splendens</i>	285
Odonata	<i>Cordulegaster</i>	<i>boltonii</i>	389
Odonata	<i>Epiophlebia</i>	<i>superstes</i>	403
Archeoagnatha	<i>Machilis</i>	<i>hrabei</i>	331
Archeoagnatha	<i>Meinertellus</i>	<i>cundinamarcensis</i>	309
Zygentoma	<i>Thermobia</i>	<i>domestica</i>	338
Zygentoma	<i>Tricholepidion</i>	<i>gertschi</i>	370
Zygentoma	<i>Atelura</i>	<i>formicaria</i>	373
Total Loci			37257

DNA extraction

DNA was extracted from the specimens and tested for quality and quantity by running a small sample of the DNA out on a gel and using Qubit fluorometric quantification. When the amount of tissue was limited for a specimen and a greater concentration of DNA was needed, the genome was amplified with Qiagen REPLI-g Mini kit (Valencia, CA, U.S.A.) using the standard protocol.

Hybrid enrichment probe design for protein coding loci

It was necessary to design a mayfly and outgroup taxa specific probe kit. The methods for hybrid enrichment probe design have been detailed in Miller *et al.* (2018). In summary, we used a Mayfly, *Ephemera danica* (GenBank: AYNC00000000.1, i5K Consortium 2013), and a dragonfly, *Ladona fulva* (GenBank: GCA_000376725.2, i5K Consortium 2013) as the initial basis of comparisons to identify orthologous exons across the genomes of the two insects. One must decide on a number of thresholds and parameters when

designing a loci probe kit and we followed the single hit and genome mapping orthology criteria of Breinholt *et al.* (2017). We excluded hits that were smaller than 120 base pairs. This initial screening returned hundreds of possible loci. We then screened these against 15 transcriptomes: *Baetis bicaudatus* (Suvorov *et al.* 2016), *Baetis tricaudatus*, *Rhithrogena robusta*, *Ephemerella inermis*, *Drunella coloradensis*, *Epeorus longimanus* (Suvorov *et al.* 2016), *Isonychia kiangsinensis* (Nanakai University, Tianjin, China), *Ephemera* sp. (Nanakai University, Tianjin, China), *Baetis* sp. (Misof *et al.* 2014), *Eurylophella* sp. (Misof *et al.* 2014), *Isonychia bicolor* (Misof *et al.* 2014), *Ameletus* sp., *Drunella doddsii*, *Leptohyphes apache*, and *Paraleptophlebia* sp. Also included in the screening were genomes from *Parametetus columbiae* and *Paraleptophlebia vaciva*, which were sequenced in the Ogden lab. The screening resulted in a reduced number of orthologs across all the taxa and we filtered for the exons with the highest taxa representation and an average pairwise identity above 65%. This resulted in 503 total exons. Once we defined the exons that we aimed to capture, 120 bp probes were laid down at 2x coverage across the sequences for each taxon and locus in the reference set. Probes that were 95% identical were collapsed to centroids using the USEARCHcluster_fast algorithm (Edgar 2010). After this entire bioinformatic process the result was a total of 22,846 probes that would be generated and included in the probe kit. This set of probes, referred to as the Mayfly_1 probe kit (Available from the Dryad Digital Repository: <http://datadryad.org>, <https://doi.org/10.5061/dryad.cj262df>), was synthesized as a Custom SureSelect probes from Agilent Technologies (Santa Clara, CA). We recommend that in the immediate future, additional mayfly targeted dataset use this probe kit in order to continue to have maximum homology coverage for more comprehensive analyses.

Library prep for protein coding loci

Library preparation, hybridization enrichment, and sequencing were done at RAPiD Genomics (Gainesville, FL) for the selected 79 ephemeropteran taxa to be captured (the data for the outgroups and other mayflies were mined from genomic and transcriptomic data). The libraries were constructed by random mechanical shearing of DNA to an average length of 300 bp followed by an end-repair reaction and ligation of an adenine residue to the 3' end of the blunt-end fragments. The ligation of the residues allowed for barcoded adapter ligation and PCR-amplification of the library. The Sure Select XT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library protocol was followed for solution-based target enrichment of a pool of 16 libraries using Custom SureSelect probes. Illumina HiSeq 3000 was used to generate 100 bp, paired-end reads.

Assembly and data cleanup for protein coding loci

The anchored phylogenomics pipeline of Breinholt *et al.* (2017) was used to clean and assemble loci. Illumina paired-end data were cleaned with Trim Galore! vers. 0.4.0 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) which allowed a minimum read size of 30 bp and trimmed bases with a Phred score below 20. The iterative baited assembly of Breinholt *et al.* (2017) was used to assemble the loci for each taxon (Mayfly_1 sequences used as baits for IBA assembly are available from the Dryad). These loci were screened for orthology using single hit and orthology location mapping to the *Ephemera danica* genome using the Breinholt *et al.* (2017) methodology. After orthology assessment, data were analyzed for contamination using the Breinholt *et al.* (2017) protocol to remove nearly identical sequences from different families and genera. Sequence data from the transcriptomes and genomes used in the probe design were included in the data set. Lastly, we filtered loci to have at least 70% of the taxa we sequenced with hybrid enrichment across all the data generated for Ephemeroptera. This resulted in a slightly reduced dataset consisting of 448 exons or protein coding loci. Not all taxa captured all 448 loci, therefore, we excluded 16 mayfly taxa that captured less than 44 loci, leaving 97 mayflies as part of the ingroup (Table 2). Even though some taxa captured low numbers of loci, the average loci represented by all taxa in the dataset was around 354 loci. We consider this to be an impressive and robust dataset that should help elucidate the phylogeny of the group.

Phylogenetic analyses

Each protein coding locus was aligned separately with MAFFT using default parameters and a strict consensus was made of any assembled isoforms using FASconCAT-G. All protein coding loci were concatenated together into a supermatrix using FASconCAT-G. We suspected that the third position might be saturated and therefore not informative for this level of phylogenetic analysis (Miller *et al.* 2018). Therefore, we translated the DNA sequences into amino acid sequences using seaview version 4.6.2. (Gouy *et al.* 2010; Galtier *et al.* 1996) in order to create an amino acid dataset.

The amino acid supermatrix was analyzed in a Bayesian framework using MrBayes (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) and the Jones+invgamma model. MrBayes was run for 3,000,000 and 2,000,000 generations and the first 25% of the sample from the cold chain was discarded as the burnin. The final split-frequencies was .017.

Results

The alignments for the AA datasets consisted of 30,558 amino acid sites. The topology (Figure 1) strongly supports Ephemeroptera as a monophyletic group and that the family Siphuriscidae, represented by the genus *Siphuriscus*, is the sister group to all other mayflies. Baetidae is the next branch to split off and thus sister to the remaining families. The family Baetiscidae is the third lineage to branch off and is sister to the rest of the families. The tree then splits into two large branches. The upper branch has Coloburiscidae splitting off first, then Leptophlebiidae, and then Oligoneuriidae. The remaining groups branch into three main lineages: 1) the Ephemerelloidea (containing Teloganodidae, Tricorythidae, Leptohyphidae, and Ephemerellidae), 2) the Caenoidea (containing the families Neophemeridae and Caenidae), and 3) the burrowing mayflies (containing the families Potamanthidae, Polymitarcyidae, Behningiidae, Euthyplociidae, Ichthybotidae, Palingeniidae, and Ephemeridae). The lower branch contains the Acanthametropodidae + Ametropodidae as sister to two main groups. The first group supports Metretopodidae as sister to the Heptageniidae + Isonychiidae. And the second group supports a lineage containing Ameletopsidae as sister to Rallidentidae + Siphlaenigmatidae + Oniscigastriidae, and a final lineage with Nesameletidae as sister to Ameletidae + Siphonuridae.

Discussion and Conclusions

This study takes a big step forward in mayfly phylogenetics. This study is congruent with a few of the lineages proposed by McCafferty and Kluge hypotheses (Figure 2), however many of the higher-level classifications were not supported. From the McCafferty system, the suborders Furcatergalia, Setisura (=Heptagenoidea), Pisciforma, and suborder Pannota were not supported as monophyletic. The superfamilies Ephemeroidea, Baetoidea, and Siphonuroidea were not supported as monophyletic. From the Kluge system, the taxa Anteritorna, Bidentiseta, Tridentiseta, Furcatergalia, Fossoriae, Pantricorythi, Eusetisura, Branchitergalia, and Tetramerotarsata were not supported as monophyletic. This study contradicts the Ogden hypothesis (2009a) in that it strongly supports a monophyletic burrowing mayflies (Potamanthidae + other burrowers, including Behningiidae) similar to Miller *et al.*'s (2018) conclusion. The phylogenomic data have clearly recovered a more well supported view of the higher-level relationships across mayflies, even though some relationships still lack strong support.

While this study was more exploratory and preliminary in nature as it was carried out for the mayfly meeting that occurred in Brazil, it allowed us to put in context the history of ideas surrounding the higher-level relationships of the lineages in Ephemeroptera. The results indicate that many of the problematic relationships are being better resolved, with higher support, when large amounts of molecular data are generated and analyzed. It can be concluded that, future phylogenomic approaches will prove useful for elucidating more parts of the phylogenetic tree for mayflies.

This study represents the largest phylogenetic study carried out for Ephemeroptera to date. Analysis of the amino acid dataset reconstructed a much better supported tree for the order, especially for deep nodes (higher level relationships) of the tree, than previous studies. This allowed testing of many of the putatively established higher-level groups, most of which were not supported as monophyletic. Clearly phylogenomic data can help to elucidate phylogenetic relationships that were difficult to reconstruct because of inaccuracies due to lack of data, lack of taxonomic sampling, homoplasy and noise, and perhaps many other issues. Still, there were some relationships that this phylogenomic dataset was unable to robustly estimate, but the notion that more taxa and data should equal a more robust tree seems to be a good general conclusion, especially for mayflies.

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