Combined molecular and morphological phylogeny of Ephemerellinae (Ephemerellidae: Ephemeroptera), with remarks about classification

T. HEATH OGDEN1,2,5, JONATHAN T. OSBORNE2, LUKE M. JACOBUS3,4, & MICHAEL F. WHITING2

1Department of Biological Sciences, Idaho State University
2Department of Integrative Biology, Brigham Young University
3Department of Entomology, Purdue University
4Department of Biology, Indiana University
5Corresponding author. E-mail: ogdet@isu.edu

Abstract

This study represents the first combined molecular and morphological analysis for the mayfly family Ephemerellidae (Ephemeroptera), with a focus on the relationships of genera and species groups of the subfamily Ephemerellinae. The phylogeny was constructed based on DNA sequence data from 3 nuclear (18S rDNA, 28S rDNA, histone H3) and 2 mitochondrial (12S rDNA, 16S rDNA) genes, and 23 morphological characters. Taxon sampling for Ephemerellidae included exemplars from all 25 extant genus groups and additional representatives from those genera with the highest diversity. Ephemerellidae appears to consist of three major clades. Ephemerella, the largest genus of Ephemerellidae, and Serratella were not supported as monophyletic, and each had representatives in two of the three major clades. However, the genera Drunella and Cincticostella were supported as monophyletic. Lineages strongly supported as monophyletic include a grouping of the Timpanoginae genera Timpanogosa, Dannella, Dentatella and Euryphella, and groupings of the Ephemerellinae genera Torleya, Hyrtanella and Crinitella and the genera Kangella, Uracanthella and Teloganopsis. The placement of the Timpanoginae genus Attenella fell within Ephemerellinae, based on molecular and combined data, but it grouped with other Timpanoginae based on morphological data alone. Further study and analysis of Ephemerellidae morphology is needed, and classification should be revised, if it is to reflect phylogenetic relationships.

Key words: Ephemeroptera; Mayflies; Molecular phylogeny; Pannota; Ephemerellidae

Introduction

The systematics of the mayfly family Ephemerellidae (Insecta: Ephemeroptera) has a long and complex history. Klapálek (1909) first recognized Ephemerellidae as a family group, but the concept dates back to Eaton’s (Eaton, 1883–1888) section VI of Ephemerella Walsh. Allen (1965, 1980, 1984) and Edmunds et al. (1963) revised the composition of the family. During the last two decades, Ephemerellidae has been refined further as part of an effort to have taxonomic classifications that reflect phylogenetic hypotheses (McCafferty, 1991). The families Austremerellidae, Melanemerellidae, Philolimniidae, Teloganellidae, Teloganodidae and Viet- namellidae each contain genera that have been removed from Ephemerellidae as part of this effort (McCafferty & Wang, 1997; McCafferty & Wang, 2000; Jacobus & McCafferty, 2006).

Currently, the family Ephemerellidae is classified as part of the infraorder Pannota of the suborder Fur-catergalia. Based on current classifications (Hong, 1979; McCafferty, 2000; McCafferty, Jacobus, & Wang, 2003; McCafferty & Wang, 2000; Sartori, 2004) the family Ephemerellidae includes the following genera: Attenella Edmunds, Caudatella Edmunds, Caurinella Allen, Cincticostella Allen, Clephemera Lin [fossil], Crinitella Allen and Edmunds, Dannella Edmunds, Dentatella Allen, Drunella Needham, Ephacerella Paclt,
Ephemerella Walsh, Eurylophella Tiensuu, Hyrtanella Allen and Edmunds, Kangella Sartori, Serratella Edmunds, Teloganopsis Ulmer, Timpanoga Needham, Torleya Lestage, Turfanerella Demoulin [fossil], and Uracanthella Belov. Five subgenera are recognized for the genus Drunella: Drunella s.s., Eatonella Needham, Myllonella Allen, Tribrochella Allen, and Unirhachella Allen. Two subgenera (Cincticostella s.s. and Rhionella Allen) are recognized under the genus Cincticostella (Allen, 1980), and Ephemerella contains the subgenera Ephemerella s.s., Amurella Kluge and Notacanthella Kluge (Kluge 2004). These genus groups represent approximately 300 nominal species and are classified into two subfamilies: Ephemerellinae and Timpanogninae (Brittain & Sartori, 2003; McCafferty, 2000).

McCafferty (2000) revised the classification of Timpanogninae to include the tribes Attenellini (Attenella), Timpanogini (Danella + Timpanoga) and Eurylophellini (Dentatella + Eurylophella). All other genera comprise the subfamily Ephemerellinae. Ephemerellinae contains the monogeneric tribe Hyrtanellini Allen and the large, diverse tribe Ephemerellini s.s. (Jacobus and Sartori 2004). The taxonomic placement of the two fossil genera, Clephemera and Turfanerella, is uncertain (McCafferty, 1990; Kluge, 2004), and placement in Ephemerellidae is questionable.

Most studies of Ephemerellidae have been geographically restricted, and include, for example, reviews of North America (Allen & Edmunds, 1962, 1963, 1965), Korea (Yoon & Bae, 1988; Yoon & Kim, 1981), Taiwan (Kang & Yang, 1995), China (You & Gui, 1995), Europe (Jacob, 1993; Studemann, Landholt, & Tomka, 1995), and Japan (Ishiwata, 2000, 2001, 2003). A regional, piecemeal, approach to the systematics of this group has inherent problems, such as those indicated by (Edmunds, 1959; McCafferty, 1991; Studemann & Landholt, 1997). Relationships within Ephemerellinae have been unclear due to this geographic approach. As a result of the poor systematics, the “genera show unstable and fluctuating characters and often mosaic-like occurrence of characters as well” (Landa et al., 1982). Furthermore, a preponderance of hypothetically pleiomorphic morphological characters (McCafferty & Wang, 2000), poorly delimited species and generic boundaries (Jacobus & McCafferty, 2003a, 2003b), and the apparently arbitrary assignment of some species to genera (Studemann & Landholt, 1997; Thomas, Masselot, & Brulin, 1999) also have hampered the formulation of phylogenetic hypotheses. This latter problem of assigning species to genera is illustrated well by the taxonomic history of the species, Uracanthella punctisetae (Matsumura), which has been associated with five different generic groups, depending on the time and place it was studied. The synonymy of this widespread Asian species (Tong and Dudgeon, 2000; Ishiwata, 2001; Beketov and Kluge, 2003) includes binomial combinations with the genera Drunella, Ephemerella, Serratella and Uracanthella (Ishiwata, 2001). Zaika (2000) listed the species as belonging to Torleya, but as a subgenus of Ephemerella.

More exploration has focused on relationships of species groups and genus groups of Timpanognae (Allen, 1977; Kluge, 2004; McCafferty, 1977, 1978, 2000; McCafferty & Wang, 1994) than Ephemerellinae, due in part to these groups being more geographically restricted, more easily recognized and in most cases, less diverse. Kluge (2004) discussed relationships of some of the generic groups of the subfamily Ephemerellinae; Studemann and Landolt (1997) explored egg morphologies; and Landa et al. (1982) detailed the internal anatomies of some genera. Otherwise, this subfamily has not been studied comprehensively on a global scale.

The purpose of this paper is to provide the first global investigation of phylogenetic relationships of the subfamily Ephemerellinae, based on newly generated molecular data and the existing published morphological characters utilized for distinguishing genera. Modifications of existing classification schemes are beyond the scope of the current study and will be addressed elsewhere. At this time, we address specifically: (1) What are the phylogenetic relationships between the major lineages of extant Ephemerellinae? (2) Does the current classification reflect these relationships? (3) Is the current suite of morphological characteristics used for identifying genera useful for recognizing phylogenetic relationships?
Material and methods

Taxon Sampling (Table 1 and 2)

Taxonomic sampling consisted of 33 exemplars from the global Ephemerellidae fauna. We included five species from Timpanoginae representing each of the five extant genera (Attenella, Timpanoga, Dannella, Dentatella, and Eurylophella) to test the monophyly of Ephemerellinae. All 13 extant genera of Ephemerellinae were represented by exemplar species. Additionally, we included exemplar species representing each of the subgenera of the genera Cincticostella, Drunella and Ephemeralella. We attempted to utilize type species of nominal genera and subgenera whenever fresh material was available; otherwise, we used presumably closely related species from the same biogeographic region as the type species. We note that the Ephemeralella subgenus Amarella, which has an eastern Palearctic type species, is represented by its eastern Nearctic representative, Ephemeralella septemtrionalis (McDunnough), per Kluge (2004). The genus Eurylophella, which has a western Palearctic type species, is represented by an eastern Nearctic species, Eurylophella verisimilis (McDunnough). We also included additional species, representing morphological diversity within the larger genera Drunella, Ephemeralella, and Serratella.

The morphological characters that we studied were selected from among those traditionally used to differentiate between major species groups (Allen, 1980; Jacobus & McCafferty, 2004a, 2006; Jacobus & Sartori, 2004; Kluge, 2004), and these were coded into a morphology matrix (Table 1). The specimens from which tissue was extracted for molecular analyses were used to code morphological characters, whenever possible; however, additional conspecific specimens often were used to verify character states from other metamorphic stages and some character states that were obliterated by the removal of tissue. Occasionally, a literature source was consulted; if so, this is indicated below. Our matrix was built using MacClade (Maddison & Maddison, 2005), and it includes the following characters from the egg (E), larva (L) and male adult (A) stages. Characters with multiple states were coded as unordered. Some character states were not applicable to all taxa studied. These included the relative development of gills 3 and dorsal abdominal spines. The relative development of gills 4 was coded only for those taxa for which gills 4 are the most anterior gills. If a character state could not be coded, a dash (−) was inserted into the data matrix.

List of characters

1 (E). Number of polar caps [zero=0; one=1; two=2].
5 (L). Maxillary palp robust [0], reduced [1] or absent [2].
6 (L). Forefemur enlarged [1] or not enlarged [0].
8 (L). Dorsal thoracic spines present [1] or absent [0].
9 (L). Prothorax greatly expanded and projected anteriorly [1] or not developed in this way [0].
10 (L). Mesothoracic notum with [1] or without [0] sharp anterolateral spines.
11 (L). Abdominal friction disc present [1] or absent [0].
12 (L). Abdomen with dorsal spine(s) absent [0], single [1] or paired [2].
13 (L). Paired abdominal spines, if present, large and stout [1], or weakly developed [0].
14 (L). Gills 1 present [0] or absent [1].
15 (L). Gills 3 present [0] or absent [1].
16 (L). Gills 3, when present, imbricate [0] or operculate [1].
18 (L). Gills 6 ventral lamella cleft present [0] or absent [1].
20 (A). Genital forceps segment 3 elongate [1] or ovoid [0].
22 (A). Penes with dorsal projection absent [0], present laterally [1] or present along proximal ridge of gonopore [2].
23 (A). Penes lobes elongate and with deep medial cleft [1] or with another morphology [0].

**TABLE 1. Morphological Matrix.**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attenella</td>
<td>margarita</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudatella</td>
<td>hystrix</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Caurinella</td>
<td>idahoensis</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cincticostella</td>
<td>elongatula</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cincticostella</td>
<td>insolta</td>
<td>?</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Subgenera in parentheses*
Molecular data
(table 2)

**TABLE 2.** Taxon list and Genbank accession numbers. Subgenera are indicated in parentheses, following the genus. NA = data was not generated

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>12S rDNA</th>
<th>16S rDNA</th>
<th>18S rDNA</th>
<th>28S rDNA</th>
<th>histone H3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attenella</td>
<td>margarita</td>
<td>FJ442997</td>
<td>FJ443027</td>
<td>FJ443048</td>
<td>FJ443075</td>
<td>NA</td>
</tr>
<tr>
<td>Caudatella</td>
<td>hystrix</td>
<td>FJ442998</td>
<td>AY749787</td>
<td>AY749866</td>
<td>AY749962</td>
<td>AY749719</td>
</tr>
<tr>
<td>Caurinella</td>
<td>idahoensis</td>
<td>FJ442999</td>
<td>FJ443028</td>
<td>FJ443049</td>
<td>FJ443076</td>
<td>FJ443101</td>
</tr>
<tr>
<td>Cincticostella</td>
<td>elongatula</td>
<td>FJ443000</td>
<td>NA</td>
<td>FJ443050</td>
<td>FJ443077</td>
<td>FJ443102</td>
</tr>
<tr>
<td>Cincticostella (Rhionella)</td>
<td>insolta</td>
<td>NA</td>
<td>FJ443029</td>
<td>FJ443051</td>
<td>FJ443078</td>
<td>FJ443103</td>
</tr>
<tr>
<td>Crinitella</td>
<td>coheri</td>
<td>NA</td>
<td>NA</td>
<td>FJ443052</td>
<td>NA</td>
<td>FJ443104</td>
</tr>
<tr>
<td>Dannella</td>
<td>provonshai</td>
<td>FJ443001</td>
<td>FJ443030</td>
<td>FJ443053</td>
<td>FJ443079</td>
<td>FJ443105</td>
</tr>
<tr>
<td>Dentatella</td>
<td>coxalis</td>
<td>FJ443002</td>
<td>FJ443031</td>
<td>FJ443054</td>
<td>FJ443080</td>
<td>FJ443106</td>
</tr>
<tr>
<td>Drunella</td>
<td>ishiyamana</td>
<td>FJ443007</td>
<td>FJ443035</td>
<td>FJ443057</td>
<td>FJ443083</td>
<td>FJ443109</td>
</tr>
<tr>
<td>Drunella</td>
<td>pelosa</td>
<td>FJ443005</td>
<td>FJ443033</td>
<td>FJ443055</td>
<td>FJ443081</td>
<td>FJ443107</td>
</tr>
<tr>
<td>Drunella</td>
<td>spinifera</td>
<td>FJ443006</td>
<td>FJ443034</td>
<td>FJ443056</td>
<td>FJ443082</td>
<td>FJ443108</td>
</tr>
<tr>
<td>Drunella (Eatonella)</td>
<td>doddsi</td>
<td>FJ443004</td>
<td>AY749756</td>
<td>AY749836</td>
<td>AY749915,</td>
<td>AY749916,</td>
</tr>
<tr>
<td></td>
<td>coloradensis</td>
<td>FJ443003</td>
<td>FJ443032</td>
<td>AY338694</td>
<td>AY338651</td>
<td>AY338618</td>
</tr>
<tr>
<td>Drunella (Myllonella)</td>
<td>trispina</td>
<td>FJ443008</td>
<td>FJ443036</td>
<td>FJ443058</td>
<td>FJ443084</td>
<td>NA</td>
</tr>
<tr>
<td>Drunella (Tribrochella)</td>
<td>tuberculata</td>
<td>FJ443009</td>
<td>FJ443037</td>
<td>FJ443059</td>
<td>FJ443085</td>
<td>FJ443110</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>longicaudata</td>
<td>FJ443010</td>
<td>FJ443038</td>
<td>FJ443060</td>
<td>FJ443086</td>
<td>FJ443111</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>atagosana</td>
<td>FJ443011</td>
<td>FJ443039</td>
<td>FJ443061</td>
<td>FJ443087</td>
<td>FJ443112</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>berneri</td>
<td>FJ443012</td>
<td>FJ443040</td>
<td>FJ443062</td>
<td>FJ443088</td>
<td>FJ443113</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>cornutus</td>
<td>FJ443013</td>
<td>FJ443041</td>
<td>FJ443063</td>
<td>FJ443089</td>
<td>NA</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>excrucians</td>
<td>NA</td>
<td>NA</td>
<td>AY749838</td>
<td>AY749918,</td>
<td>AY749970</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>maculata</td>
<td>FJ443014</td>
<td>NA</td>
<td>FJ443064</td>
<td>FJ443090</td>
<td>FJ443114</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>needhami</td>
<td>FJ443015</td>
<td>FJ443042</td>
<td>FJ443065</td>
<td>FJ443091</td>
<td>FJ443115</td>
</tr>
<tr>
<td>Ephemerella</td>
<td>septentrionalis</td>
<td>FJ443016</td>
<td>NA</td>
<td>FJ443066</td>
<td>FJ443092</td>
<td>NA</td>
</tr>
<tr>
<td>Ephemerella (Notacanthella)</td>
<td>sp.</td>
<td>FJ443020</td>
<td>FJ443044</td>
<td>FJ443068</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Eurylophella</td>
<td>verisimilis</td>
<td>FJ443017</td>
<td>FJ443043</td>
<td>FJ443067</td>
<td>FJ443093</td>
<td>FJ443116</td>
</tr>
<tr>
<td>Hyrtanella</td>
<td>pascalae</td>
<td>FJ443018</td>
<td>AY749815</td>
<td>AY749891</td>
<td>AY750012</td>
<td>AY749742</td>
</tr>
<tr>
<td>Kangella</td>
<td>brocha</td>
<td>FJ443019</td>
<td>NA</td>
<td>NA</td>
<td>FJ443094</td>
<td>FJ443117</td>
</tr>
<tr>
<td>Serratella</td>
<td>serrata</td>
<td>FJ443021</td>
<td>FJ443045</td>
<td>FJ443069</td>
<td>FJ443095</td>
<td>FJ443118</td>
</tr>
<tr>
<td>Serratella</td>
<td>teresa</td>
<td>FJ443022</td>
<td>NA</td>
<td>FJ443070</td>
<td>FJ443096</td>
<td>FJ443119</td>
</tr>
<tr>
<td>Teloganopsis</td>
<td>media</td>
<td>FJ443023</td>
<td>FJ443046</td>
<td>FJ443071</td>
<td>FJ443097</td>
<td>FJ443120</td>
</tr>
<tr>
<td>Timpanoga</td>
<td>hecuba</td>
<td>FJ443024</td>
<td>NA</td>
<td>FJ443072</td>
<td>FJ443098</td>
<td>NA</td>
</tr>
<tr>
<td>Torleya</td>
<td>major</td>
<td>FJ443025</td>
<td>FJ443047</td>
<td>FJ443073</td>
<td>FJ443099</td>
<td>FJ443121</td>
</tr>
<tr>
<td>Uracanthella</td>
<td>punctisetae</td>
<td>FJ443026</td>
<td>NA</td>
<td>FJ443074</td>
<td>FJ443100</td>
<td>FJ443122</td>
</tr>
</tbody>
</table>

*Subgenera in parentheses*
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 (12S), and histone H3 protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are given elsewhere (Ogden & Whiting, 2003; Ogden & Whiting, 2005; Wheeler, Whiting, Wheeler, & Carpenter, 2001). 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 2. Specimens vouchers are deposited in the Insect Genomics Collection (IGC) of Brigham Young University. Collection data for the specimens used in the analysis is available online at http://whitinglab.byu.edu/Ephemeroptera/datasets.htm.

Materials examined

The materials examined for morphological data are deposited with the following institutional collections: The Natural History Museum, London, England [BMNH]; Brigham Young University, Provo, Utah [BYU]; California Academy of Sciences, San Francisco, California [CAS]; C. P. Gillette Museum of Arthropod Diversity, Colorado State University, Ft. Collins, Colorado [CSUC]; Hokkaido University, Sapporo, Japan [EIHU]; Florida A&M University, Tallahassee, Florida [FAMU]; Iowa State University, Ames, Iowa [ISUI]; Musée de Zoologie, Lausanne, Switzerland [MZL]; Purdue University Entomological Research Collection, West Lafayette, Indiana [PERC]; the Royal Ontario Museum, Toronto, Ontario, Canada [ROME]; Seoul Women’s University, Seoul, Korea [SWU]; and Virginia Polytechnic Institute and State University, Blacksburg, Virginia [VPIC]. Some material was collected as part of the All Taxa Biodiversity Inventory of Great Smoky Mountains National Park (Sharkey, 2001) and as part of biotic inventories of Thailand (Panrong, Buathong, & Sites, 2002; Sites, Wang, Perkam, & Hubbard, 2001).

*Attenella margarita* (Needham): USA, Montana, Odell Cr, 1-VIII-1981, 21 male adults [PERC]; New Mexico, Rio Costilla at 7S boundary, 17-VIII-1989, GZ Jacobi, one larva [PERC]; Wyoming, Teton Co, Snake R at Moose, Grand Teton National Park, 18-VIII-1985, GF Edmunds, Jr., three larvae (eggs dissected) [PERC].


*Caurinella idahoensis* Allen: USA, Idaho, Idaho Co, Bridge Cr at Hoodoo Lake Rd (FR360), 46°21’53”N, 114°38’11”W (WGS84), 1708m elev, 29-VII-2002, WP McCafferty, LM Jacobus, three male adults, two female adults, one male subimago, two female subimagos, associated exuviae (alates emerged 9-VIII through 17-VIII), four larvae [PERC]; same data, one male adult, one set larval exuviae [CSUC]. Literature consulted: Jacobus and McCafferty (2004a: Fig. 1).

Cincticostella insolta (Allen): Thailand, Mae Hong Son Prov, Namtok Maw Pang, 19°22’N, 98°22’E, 850m, 14-X-2002, CMU team, one larva [ISUI]. Egg and male adult unknown.

Crinitella coheri (Allen and Edmunds): Nepal, Palung, 5850’, 17-IV-1957, El Coher, two larvae (holotype; paratype, mouthparts on slide) [PERC]; Thailand, Mae Hong Son, River Nam Lang, Soppong, 3–27/IV/2003, Braasch, four larvae, three male adults, one female subimago [PERC]; Mae Hong Son Prov, Namtok Mae Surin NP, Nam Mae Surin, above falls, 18°56’N, 98°04’E, 1220 m, 15-X-2002, GW Courtney, one larva [ISUI]. Literature consulted: Jacobus and Sartori (2004: Fig. 20).


Drunella (Mylonella) coloradensis (Dodds): USA, Utah, Salt Lake Co, Mill Cr., Mill Creek Canyon, WL Peters, K Terry, three male adults [PERC]; Montana, Glacier NP, Avalanche Cr at Avalanche Campground, 10-IX-1958, RW Baumann, two male subimagos, four larvae [PERC]. Literature consulted: Studemann and Landolt (1997: Figs 52 and 53).

Drunella (Eatonella) doddsii (Needham): USA, Montana, Missoula Co, Lolo Cr, 0.6 mi NE Lolo Pass, 46°38’37”N, 114°34’44”W (WGS84), 28-VII-2002, WP McCafferty, LM Jacobus, two larvae [PERC]; Utah, Utah Co, Aspen Grove Cr, VI-1965, GF Edmunds, one male adult [PERC]. Literature consulted: Studemann and Landolt (1997: Fig. 54), Allen and Edmunds (1962: Figs. 28–33).

Drunella ishiyamana Matsumura: Japan, Ishiyama, 7-VIII-1903, one male adult (D. ishiyamana type) [EIHU]; Japan, Nara, Kawaki, Unokawa, Nakai Stream, 8-VI-2002, T Fujitani, seven larvae [PERC]; Japan, Tochigi Pref., Yudaki Falls. 7km north of Lake Chuzenji, 12-VII-2002, Terry & Jarvis, DNA holotype EP204, larvae [BYU]. Literature consulted: Okazaki (1984: Fig. 26); identified as D. cryptomeria.

Drunella pelosa (Mayo): USA, Idaho, Lemhi Co, Salmon R below jet Panther Cr, ca 8.5 mi W of Shoup, Salmon NF, 6–16-VII-1964, one male adult [PERC]; California, Los Angeles Co, Elizabeth Lake, 16-V-1951, one larva (mouthparts on slide) [PERC]. Egg unknown.

Drunella (Drunella) spinifera (Needham): USA, Montana, Missoula Co, Lolo Cr, 0.6 mi NE of Lolo Pass, 46°38’37”N, 114°34’44”W (WGS84), 28-VII-2002, WP McCafferty, LM Jacobus, 13 larvae [PERC]; Washington, Pierce Co, Hillside stream, 1.5 mi N Hwy 706 Westside Rd, Mt Rainier NP, 14-VII-2003, Konradieff & Schmidt, one male adult (reared), associated larval exuviae [CSUC]. Egg unknown.

Drunella (Tribrochella) trispina (Ueno): Japan, Nara, Higashi-Yoshino, Omata, Omata Stream, Kuramae Bridge, 5-V-2002, T Fujitani, three larvae [PERC]. Literature consulted: Okazaki (1984: Fig. 50). Male adult unknown [adult described as trispina by Ueno (1931) is actually that of sachalinensis (Ishiwata, 2001)].

Drunella (Unirhachella) tuberculata (Morgan): USA, North Carolina, Swain Co, Great Smoky Mountains NP, Kanati Fork, 0.3 km upstream from Newfound Gap Rd (US441), Kenati Fork Trailhead at 35°35’14”N, 83°21’48”W (NAD27), 26-IX-2003, LM&PD Jacobus, one larva [PERC]. South Carolina, Oconee Co, E Fk Chattooga R, Rt 107, 1 km S of North Carolina state line, 35°N, 83°04’W, 3-X-1997, S Spichiger, one male adult (genitalia on slide), associated exuviae (reared) [PERC]. Literature consulted: Jacobus and McCafferty (2004b).


Ephemera atagosana Imanishi: Japan, Honshu, Nara, Higashi-yoshino, Omata, Omata Stream, Kuramae Bridge, 5-V-2001, T Fujitani, one larva [PERC]; Tokyo, Ange R, Hachiouji-shi, 30-IV-1985, S Ishiwata, one male adult [PERC]. Korea, Jeollabuk-do, Muju, Deokyusan (Mt), 2-V-1983, YJ Bae, one female adult
(reared; eggs dissected) [SWU-EPH-1606]; Donggureung, Namyangju, 10-V-1994, YJ Bae, one male adult, associated exuviae [SWU-EPH-1614].

*Ephemerella berneri* Allen and Edmunds: USA, Virginia, Henry Co, Smith R at Rt674, 26-IV-1980, emerged 7-V-1980, Kondratieff, one male adult, two female adults, associated exuviae (eggs dissected) [VPIC].

*Ephemerella cornutus* Gose: Japan, Nara, Kawakami, Unokawa, Nakai-keikoku, 5-V-2002, T Fujitani, one larva [PERC]; Nara, Kawakami, Unokawa, Nakai Stream, 8-VI-2002, T Fujitani, four male adults [PERC].

*Ephemerella maculata* Traver: USA, California, Napa Co, Hopper Cr, 2-V-2000, larvae (eggs dissected) [PERC]; San Luis Obispo Co, blacklight at Tassajara Cr, 7 mi. north of San Luis Obispo, 6-VI-1971, JD Pinto, one male adult [PERC]. Literature consulted: Smith (1935: Fig. 28) described the egg as having no polar cap; examination of eggs dissected from larvae (Fig. 7) reveals that his observation was incorrect.

*Ephemerella needhami* McDunnough: USA, Indiana, Elkhart Co, Elkhart R, 1 mi southeast of Millersburg at bridge on gravel road, 21-V-1972, WP McCafferty, AV Provonsha, one male adult, associated exuviae [PERC]; Bartholomew Co, White Cr, private farm crossing south of Co Rd 930 South, 39°03’02”N, 85°58’01”W, 19-IV-2005, LM Jacobus, JM Webb, MW Jacobus, one larva [PERC]; Michigan, Arenac Co, Omer, Rifle R at public access site, 44°02’41”N, 83°51’20”W, 24-VI-2002, LM Jacobus, BL Hass, subimago (eggs dissected), associated exuviae [PERC].

*Ephemerella* (*Amurella*) *septentrionalis* McDunnough: USA, Massachusetts, Amherst, 3-VI-1939, JR Traver, eight male adults, eight female adults [PERC]; Tennessee, Blount Co, Abrams Cr at Cades Cove Loop Rd, Great Smoky Mountains NP, stream bank (flowing water), 35°35’36”N, 83°50’42”W (NAD27), 16-VI-2003, LM&BLH Jacobus, one larva [PERC]. Literature consulted: Smith (1935).


*Serratella serrata* (Traver): USA, California, Napa Co, Garnett Cr, 1-V-2000, larvae (dissected for eggs) [PERC]; San Mateo Co, San Gregorio Cr, 17-VI-1950, WC Day, three male adults, two female adults, one larva [CAS].

*Teloganopsis media* Ulmer: Malaysia (east), Sabah, Liwagu R at Liwagu Cave, southeast of Kinabalu NP Headquarters, 1525 m, S7, 14–15-VIII-1972, WL & JG Peters, one larva [FAMU]; Liwagu R at bridge, Ranau, 335 m, S4e, 11–16-VIII-1972, GF & CG Edmunds, four male adults, two male subimagos, associated subimago exuviae [PERC]; Sungai Moyog, 3 mi E of Penampung, 27-IX-1978, GF & CH Edmunds, three male subimagos, three female subimagos (eggs dissected) [PERC].

Torleya major (Klapálek): Germany, Stream Fulda, D-36110 Schlitz/Pfordt, 16-IV-2002, R Lieske, ten larvae (some gills removed) [PERC]; Simmerbach (a confluence of the Nahe River, a left hand tributary of the River Rhine), 3-V-2003 (reared to 18-V-2003), A Haybach, three male adults, two female adults (all reared), five subimago exuviae [PERC]. Literature consulted: Studemann et al. (1995: Figs. 30-32); Studemann and Landolt (1997: Figs. 20 and 21).


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. Multiple sequence alignment was performed on the ribosomal genes in MUSCLE (Edgar, 2004a, 2004b) using default settings. Some taxa had missing data, as indicated (Table 2), in one or more of the genes that were submitted to MUSCLE for alignment.

The aligned molecular data were combined with the morphological data and were analyzed under different approaches. First, partitioned Baysian estimation analysis was performed in MrBayes 3 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using mixed models, in order to include the morphological partition. 1,500,000 generations were sampled every 1000 generations. The first 1,000,000 generations were excluded (as the burn in). Two separate runs were performed. Posterior probabilities were taken from the trees that resulted, post burn in, from the two MrBayes runs. Furthermore, the combined dataset was analyzed in PAUP* 4.0b10 (Swofford, 2002) under maximum parsimony, with gaps treated as missing and as a fifth state character. Parsimony settings consisted of multiple random additions (100 for combined datasets and 10,000 random additions for morphological dataset) with TBR swapping. Strict and 50% consensus trees were examined. Bootstrap and Bremer values were calculated for the topology resulting from the combined dataset parsimony analysis with gaps treated as missing. The Bootstrap analyses consisted of 1000 replicates with 20 random additions per replicate. Bremer values were computed via Treerot (Sorenson, 1999) and PAUP*. The subfamily Timpanoginae were used as outgroup taxa for rooting purposes. Additionally, a few exploratory analyses were done with species from families closely related to the Ephemerellidae to ensure that rooting using only Timpanoginae taxa would not be an issue, and in all cases, Ephemerellinae was confirmed to be monophyletic.

Additionally, analyses were carried out in PAUP* under maximum parsimony, with gaps treated as missing, for subset data partitions such as: mitochondrial data, nuclear data, and each partition individually. The original dataset file and results will be available at the website http://whitinglab.byu.edu/Ephemeroptera/data-sets.htm.

The molecular dataset resulting from the MUSCLE alignment was also analyzed with MODELTEST (Posada & Crandall, 1998) to identify the most “justified” model for likelihood settings. The model selected (GTR+G+I) and the following parameters were implemented in the PAUP block: Lset Base=(0.2295 0.2449 0.2896) Nst=6 Rmat=(0.6376 2.1573 1.6472 0.7677 3.5978) Rates=gamma Shape=0.4507 Pinvar=0.4058. A maximum likelihood search was then performed in PAUP*.
Results

The MUSCLE alignments for each gene consisted of 274 characters for 12S, 478 characters for 16S, 1,882 characters for 18S, and 2,395 characters for 28S. The aligned characters for the ribosomal genes (5,029 total), the H3 protein coding gene (367 nucleotides long), and the morphological data (23 characters) combined for a total of 5,419 characters. 1,067 of these characters were parsimony informative. The partitioned Bayesian analysis of the combined data resulted in a fairly resolved topology (Figure 1). Congruence with some of the other analyses performed is represented on this tree by the use of squares colored in gray if the node is supported with the other approaches. Four additional topologies are given in Figure 2. For maximum parsimony (MP), the combined data analysis resulted in 1 most parsimonious tree (Fig. 2a), with a length of 5437. The maximum likelihood (ML) analysis of the molecular data resulted in one most likely topology (score = 31976.70842) (Fig. 2b). Maximum parsimony analysis of molecular data resulted in 6 most parsimonious trees with a length of 5357; the majority consensus of these trees is given as Figure 2c. The morphological data analysis resulted in many most parsimonious trees (our settings retained 4,895) with a length of 63; the majority consensus of these trees is given as Figure 2d.

**FIGURE 1.** Combined data (molecular and morphology) mixed model Bayesian analysis topology. The tree represents the 50% consensus of all of the Bayesian topologies that were selected outside of the “burn in”. For each node the five squares represent sensitivity of the data to different approaches of analysis. The first square is colored gray when the Bayesian posterior probability was greater than 90. The second square is grayed when the node is supported in the maximum likelihood (ML) tree. The third square is grayed when the node is supported in the maximum parsimony (MP) tree. The fourth square is grayed to represent a greater than 50% bootstrap value. The fifth square is grayed when the node is supported in the maximum parsimony analysis of the morphological data.
Discussion

Although we were not specifically testing the monophyly of Timpanoginae in this study, molecular evidence suggests that this group may not be monophyletic as previous hypothesized (Kluge, 2004; McCafferty, 1977, 2000; McCafferty & Wang, 1994). Based on our molecular evidence, *Attenella* is supported as nesting within Ephemerellinae, in contrast to the hypothesis that *Attenella* is sister to a clade containing the four other Timpanoginae genera (Kluge, 2004; McCafferty & Wang, 1994). Moreover, the relationships within this subfamily differ in that *Dannella* was recovered as sister to (*Dentatella + Eurylophella*) and not as sister to *Timpanoga*. However, the monophyletic grouping of *Timpanoga, Dannella, Dentatella* and *Eurylophella* is consistent with the recent phylogenetic hypotheses reviewed above. Because of the inclusion of *Attenella*, the subfamily Ephemerellinae was not recovered as monophyletic in any analysis containing molecular data.

The branching order along the backbone of our trees and of some of the major clades is not very well supported in general, and thus some relationships remain difficult to assess. However, a few major clades were recovered with strong support. These include the clade of *Timpanoga, Dannella, Dentatella* and *Eurylophella* mentioned above, which was recovered in all five depicted analyses; within this clade, the grouping of *Dentatella* and *Eurylophella* was recovered consistently. Furthermore, the lineage containing (*Torleya + Hyrтанella*) and (*Kangella + Uracanthella + Teloganopsis*) was strongly supported as monophyletic with high congruence across the different analyses, and *Ephemerella cornutus* grouped with *Serratella serrata* consistently. This clade is similar to Kluge’s (2004) *Torleya/g1* grouping. *Uracanthella, Teloganopsis* and *Kangella* form a robustly supported clade present in all 5 depicted analyses. Larvae of these genera lack paired spines on abdominal terga and have dense fields of setae at the apex of the maxilla.

A large clade containing *S. teresa* and corresponding. Roughly to Kluge’s (2004) *Ephemerella/fg4* grouping is present in four of the depicted analyses (>90 Bayesian, ML, MP, MP >50% bootstrap) but not supported by the MP morphology analysis (see legend in figure 1). With the exception of *Ephemerella septentrionalis*, each taxon has the ventral lamellae of gills 6 with the lateral lobes “fused together, so its ventral lobe is integral, not bifurcate” (Kluge, 2004).

The sampled species of the genus *Drunella* were supported as a monophyletic group (>90 Bayesian, ML, MP analyses). Each of these *Drunella* species has male adults with elongate genital forceps segment 3 and larvae with the forefemur enlarged and/or marginally denticulate and with a felt of long setae on the abdominal sternum (Jacobus & McCafferty, 2004b). The subgenus *Drunella*, following the concept of Allen (1980), was not supported as monophyletic in any analysis. These results corroborate the decision of Jacobus and McCafferty (2004b) not to recognize Allen’s (1980) subgeneric groups. No analysis indicated that *Cincticostella* is not monophyletic.

The genera *Serratella* and *Ephemerella* were not supported as monophyletic in any analysis, which was not unexpected, because *Ephemerella* has been essentially the default genus for newly described species (e.g., Kluge, Zhou, Jacobus, & McCafferty, 2004), and both genera are poorly defined. Also, some authors have chosen not to recognize the genus *Serratella* (e.g., Ishiwata, 2001). However, some *Ephemerella* species were supported as constituting a monophyletic clade by all but the exclusively morphological analysis.

Conclusions

This study is the first combined data phylogenetic analysis of the family Ephemerellidae, which appears to consist of three major clades (Figs. 1; 2a,b). The current classification of the family does not reflect the relationships indicated by our research. Timpanoginae was found to be nonmonophyletic because *Attenella* nested well within the subfamily Ephemerellinae. Within Timpanoginae (excluding *Attenella*), *Dannella* is highly supported as sister to (*Eurylophella + Dentatella*). The subfamily Ephemerellinae was not supported as monophyletic only due to the placement of *Attenella*. The large tribe Ephemerellini was not recovered as mono-
phylectic, due to the placement of *Hyrtanella*, the type genus of the monogeneric tribe Hyrtanellini, within one of its major branches. Our results suggest a broader concept of Hyrtanellini and a more restricted concept of Ephemerellini may be necessary. Of the large, diverse genera for which we included multiple exemplars, the genera *Drunella* and *Cincticostella* were recovered as monophyletic based on our sampling, while *Ephemerella* and *Serratella* were nonmonophyletic.

**FIGURE 2.** Four additional trees resulting from analyses. A. Most Parsimonious tree from maximum parsimony analysis of combined data (one most parsimonious topology; Length = 5437). B. Most likely tree from maximum likelihood analysis of molecular data (Score = 31976.70842). C. Majority-rule consensus of most parsimonious trees from maximum parsimony analysis of molecular data (6 most parsimonious trees found; Length = 5357). D. Majority-rule consensus of most parsimonious trees from maximum parsimony analysis of morphological data (Length = 63).
The contribution of the diagnostic morphological characters to the combined analysis appears to be limited, as, for example, only five nodes of our Bayesian tree were consistent with those recovered from analysis of only the morphological data. In general, the morphological characters currently used to diagnose genera do not allow for a reflection of monophyletic groupings, as evidenced by the nature of *Ephemerella* and *Serratella* and the placements of *Attenella* and *Ephemerella (Amurella)* _septentrionalis_ in our tree. *Attenella* has been considered part of the Timpanoginae, rather than Ephemerellinae, and Kluge (2004) included _E. septentrionalis_ in his Torleya/g1 group, rather than Ephemerella/fg4, where it falls here.

The placement of *Attenella* in our molecular and combined data trees was surprising, given that it has been considered sister to the Timpanoginae genera _Timpanoga, Eurylophella, Dannella_ and _Dentatella_ based on each of these genera lacking lamellate gills on abdominal segment 3.

Considering that the branching order of major clades is weakly supported by our molecular data, further study and analysis of Ephemerellidae morphology is needed. A more complete sampling of taxa and morphological characters, especially new characters for differentiating species groups, may offer additional evidence, that when combined with molecular data, will be important for resolving the phylogeny of Ephemerellidae. The supraspecific classification of Ephemerellinae clearly requires further evaluation and possible revision, especially if it is to reflect monophyletic groupings of species and genera.

**Acknowledgments**

We thank M. Sartori, J-L. Gattolliat, Sartori Lab, W. P. McCafferty, McCafferty Lab, A. Haybach, FAMU group, J. Peters, T. Fujitani, B. O’Donnell, G. Courtney, D. Lenat, Y. J. Bae, D. Braasch, D. Goodger, S. Hiner, S. Ishiwata, N. Kluge, B. Kondratieff, W. Pulawski, S. Spichiger, J. Voshell, K. Yoshizawa and R. Wiseman for providing specimens and other advice on the project; Whiting Lab for valuable discussion and assistance. G. Hass helped build a rearing apparatus for Michigan field studies. Examination of some specimens via Scanning Electron Microscopy was made possible by Chia-Ping Huang, Debra Sherman and Lu Sun (West Lafayette, Indiana). DNA sequences are deposited in GenBank under accession numbers. Discover Life In America, Inc. and the U. S. National Park Service provided funding and facilities for some associated fieldwork. This work was supported in part by NSF grants 0206505 (MFW and THO), and DEB-0120718 (MFW). Furthermore, this material is based upon work supported under a National Science Foundation Graduate Research Fellowship and USEPA fellowship 91601701-0 to LMJ.

**References**


