



## Emerging trends in molecular systematics and molecular phylogeny of mayflies (Insecta: Ephemeroptera)

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**Abstract:** Current trends are reviewed in the molecular systematics and phylogeny of the Ephemeroptera (mayflies), an ancient monophyletic lineage of pterygote insects. Theories of mayfly origins are analyzed, followed by a discussion of higher classification schemes in light of recent developments in molecular systematics. Ephemeroptera evolution is a classic example of ancient rapid radiation, presenting challenges for phylogenetic analysis. The utility of combined studies of morphological and molecular data is substantiated with examples and the role of molecular systematics in unraveling the taxonomy of cryptic species complexes is highlighted. The importance of DNA barcoding in mayfly taxonomy is discussed in the light of recent progress, and future contributions of genetics to the study of taxonomy, ecology and evolution in mayflies are discussed.

**Keywords:** Cryptic species, DNA barcoding, Ephemeroptera, molecular phylogeny, molecular systematics.

## INTRODUCTION

The order Ephemeroptera presently encompasses over 3000 species and 400 genera, constituting at least 42 described families (Barber-James et al. 2008). The Ephemeroptera (mayflies) are an archaic lineage of insects, dating back to the late Carboniferous or early Permian periods, some 290 mya (Brittain & Sartori 2003). They occupy freshwater and brackish water habitats across the world, with the exception of Antarctica. The nymphs are immature stages inhabiting lentic and lotic waters. The imago or adults are terrestrial; they lack mouth parts and do not feed, relying on nutritional build up during immature stages. They have an ephemeral lifespan of a day or two and their only function is reproduction. The presence of a subimago with functional wings at the penultimate moult is unique to pterygote insects. The winged stages of Ephemeroptera, as with Odonata (dragonflies and damselflies) and the extinct Palaeodictyoptera, cannot fold their wings horizontally over the abdomen as neopterans can.

This article briefly reviews current trends in the molecular systematics and phylogeny of the Ephemeroptera and discusses how combined analysis of morphological and molecular data can be used to fine tune phylogenetic conclusions.

## MAYFLY ORIGINS

The phylogenetic position of Ephemeroptera within the winged insects

(Pterygota) is hotly debated by systematists, and significant disagreement still exists in morphological and molecular studies. The first complete mitochondrial genome of a heptageniid mayfly, *Parafronurus youi* was sequenced using a long PCR-based approach by Zhang et al. (2008). In their analysis, the basal Ephemeroptera hypothesis (Ephemeroptera versus (Odonata + Neoptera)) was supported. This result also received strong support by the nucleotide and amino acid datasets from mitochondrial protein-coding genes with BI and ML analyses. Zhang et al. (2008) tentatively concluded that mitochondrial genomes can answer the difficult question of the basic relationships among the winged insects. Ephemeroptera evolution is a classic example of “ancient rapid radiation of insects” presenting challenges for phylogenetic analysis because such radiations take place over short periods of time and allow few distinctive phylogenetic markers to accumulate among lineages.

The Ephemeroptera, Odonata and Neoptera present a challenging phylogenetic tree shape, regardless of their true relationships, because the first pterygotes may have emerged up to 400 mya, but the earliest representatives of their extant descendants is much younger than the first emergence of the lineage whose relationships are in question (Whitfield & Kjer 2008).

## MOLECULAR SYSTEMATICS AND HIGHER CLASSIFICATION

The original subordinal classification of McCafferty & Edmunds (1979), based mostly on thoracic morphology and wing pad position, comprised the holophyletic suborder Pannota and the paraphyletic suborder Schistonota indicating the retention of certain plesiomorphic (ancestral) traits. It was realized that monophyly derived from synapomorphy (shared derived characters) should be the driving force behind any taxonomic classification (Hennig 1966, 1979; Farris 1979). Later, McCafferty (1991) proposed 3 different suborders (Pisciforma, Setisura and Rectracheata) and traced phylogenetic relationships within and among the suborders. Concurrent to McCafferty's work, Kluge (1988, 1998) independently proposed two suborders for Ephemeroptera. His suborder Furcatergalia is equivalent to McCafferty's Rectracheata, except the exclusion of Oniscigastridae from Furcatergalia. The other suborder proposal (Kluge 1988) was

Costatergalia, which is equal to McCafferty's (1991) Pisciforma + Setisura + Oniscigastridae. Topological comparison of Kluge's system and McCafferty's system of mayfly classification is presented in Fig. 1, after Ogden et al. (2009). In contrast to previous hypotheses based on morphological observations, the relationships inferred from the molecular data (Ogden & Whiting 2005) were congruent in some cases, but incongruent in others. In their analysis, the groups, Furcatergalia, Pannota, Carapacea, Ephemerelloidea and Caenoidea and 15 families were supported as monophyletic. On the other hand, Setisura, Pisciforma, Baetoidea, Siphonuroidea, Ephemeroidea, Heptagenioidea and five families (having more than one taxon represented) were not supported as monophyletic.

However, evidence supports the notion that combined data (morphology + molecular data) analysis provides a more robust estimate of phylogenetic relationships. The study of Ogden et al. (2009) represents the first formal morphological and combined (morphological and molecular) phylogenetic analyses of the order Ephemeroptera. Taxonomic sampling comprised 112 species in 107 genera, including 42 recognized families (all major lineages of Ephemeroptera). Morphological data consisted of 101 morphological characters. Molecular data were acquired from DNA sequences of 12S, 16S, 18S, 28S and H3 genes. The Asian genus *Siphuriscus* (Siphuriscidae) was supported as sister to all other mayflies. The lineages Carapacea, Furcatergalia, Fossoriae, Pannota, Caenoidea and Ephemerelloidea were supported as monophyletic. However, some recognized families (for example, Ameletopsidae and Coloburiscidae) and major lineages (such as Setisura, Pisciforma and Ephemeroidea among others) were not supported as monophyletic, mainly due to convergences within nymphal characters (Ogden et al. 2009).

## EFFICACY OF COMBINED MORPHOLOGICAL AND MOLECULAR PHYLOGENY AND SYSTEMATICS - EXAMPLES FROM THE EPHEMEROPTERA

It is quite obvious that most previous reconstructions of phylogeny and classification were strongly hampered by superficial external morphological similarities, which do not always reflect the true phylogeny of the

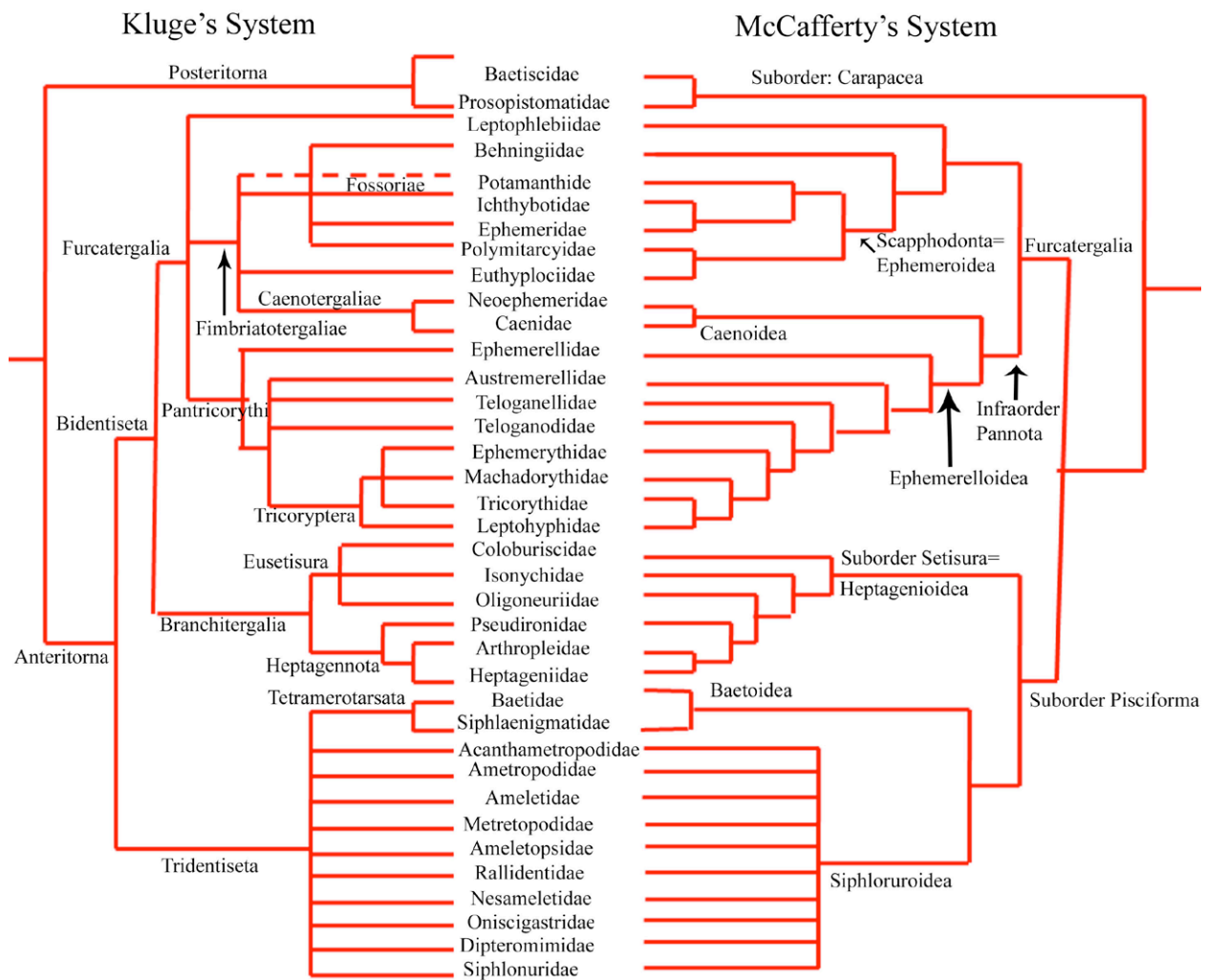


Figure 1. Topological comparison of Kluge's system and McCafferty's system of mayfly classification (after Ogden et al. 2009).

Order. Homoplasies therefore seem a dominant trait in mayfly morphology and behaviour, especially in nymphs (Ogden et al. 2009) and combined analysis may solve many riddles. Apart from the outstanding recent contribution of Ogden et al. (2009) on these lines towards evolving a new paradigm in mayfly phylogeny, some families notably, Leptophlebiidae (O'Donnell & Jockusch 2008), Baetidae (Gattolliat et al. 2008) and Ephemerellidae (Ogden et al. 2009) have received considerable attention regarding intrafamilial relationships, in which molecular phylogenetic tools were extensively employed.

Using two nuclear genes (the D2 + D3 region of 28S ribosomal DNA and histone H3) and maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI), O'Donnell and Jockusch (2008) inferred the evolutionary relationships of

69 leptophlebiids sampled from six continents and representing 30 genera plus 11 taxa of uncertain taxonomic rank from Madagascar and Papua New Guinea. Although they did not recover monophyly of the Leptophlebiidae, monophyly of two of the three leptophlebiid subfamilies, Habrophlebiinae and Leptophlebiinae, was recovered with moderate to strong support in most analyses. The Atalophlebiinae was rendered paraphyletic as a result of the inclusion of numbers of Ephemerellidae or the Leptophlebiinae clade. For the species-rich Atalophlebiinae, four groups of taxa were recovered with moderate to strong branch support: (i) an endemic Malagasy clade, (ii) a Paleoaustral group, a pan-continental cluster with members drawn from across the Southern Hemisphere, (iii) the *Choroterpes* group uniting fauna from North America, southeast Asia and Madagascar and (iv) a

group uniting three new world genera, *Thraulodes*, *Farrodes* and *Traverella*.

Gattolliat et al. (2008) reconstructed the first comprehensive molecular phylogeny of the Afrotropical Baetidae. They sequenced a total of ca. 2300 bp from nuclear (18S) and mitochondrial (12S and 16S) gene regions from 65 species belonging to 26 genera. They used three different approaches of phylogeny reconstruction viz., direct optimization, maximum parsimony and maximum likelihood. The molecular reconstruction indicated the Afrotropical Baetidae require a global revision at a generic as well as suprageneric level.

The investigation of Ogden et al. (2009) represented the 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 three nuclear (18S rDNA, 28S rDNA, histone H3) and two mitochondrial (12S rDNA, 16S rDNA) genes, and 23 morphological characters. *Ephemerella*, the largest genus of Ephemerellidae, and *Serratella* were not supported as monophyletic lineages. Strongly supported as monophyletic include a grouping of the Timpanoginae genera *Timpanoga*, *Dannella*, *Dentatella* and *Eurylophella*, and groupings of the Ephemerellinae genera *Torleya*, *Hyrtanella* and *Crinitella* and the genera *Kangella*, *Uracanthella* and *Teloganopsis*. Further study and analysis of Ephemerellidae morphology is needed, and classification should be revised, if it is to reflect true phylogenetic relationships (Ogden et al. 2009).

## MOLECULAR SYSTEMATICS AND CRYPTIC SPECIES COMPLEX

Genetic studies have highlighted cryptic diversity in many well-known taxa including aquatic insects, with the general implication that there are more species than are currently recognized. *Baetis rhodani* Pictet are among the most widespread, abundant and ecologically important of all European mayflies (Ephemeroptera), and used widely as biological indicators of stream quality. Traditional taxonomy and systematics have never fully resolved differences among suspected cryptic species in the *B. rhodani* complex because morphological characters alone

do not allow reliable distinction. This is particularly true among larvae, the life-stage used most widely in biomonitoring studies. Williams et al. (2006) assessed the molecular diversity of this complex in one of the largest such studies of cryptic species in the order Ephemeroptera to date. Phylogenies were constructed using data from the mitochondrial cytochrome oxidase subunit I (COI) gene. Two monophyletic groups were recovered consisting of one major haplogroup and a second clade of six smaller but distinct haplogroups. Haplogroup divergence ranged from 0.2–3 % (within) to 8–19 % (among) with the latter values surpassing maxima typically reported for other insects, and provided strong evidence for cryptic species in the *B. rhodani* complex. However, the taxonomic status of these seven haplogroups remains to be defined clearly.

The potential implications of cryptic species in the *B. rhodani* complex on current and future ecological studies are particularly far-reaching given the large number of studies carried out on what now appears to be several possible distinct taxa. These results have wider relevance since cryptic species have been detected in other aquatic insects (Jackson & Resh 1998), and the presence and diversity of several taxa are widely used as biological indicators (Mason 1996). The presence of cryptic species also has ramifications for the assessment of biodiversity in general, and the ability to account for them in future studies emphasizes the need to correlate genetic differences from multi-locus data, with identifiable morphological characters and/or other factors including physiology.

## DNA BARCODING AND MAYFLY TAXONOMY

The tool of DNA barcoding shows great potential for use by those studying the systematics of many Ephemeroptera species groups. One example of the utility of barcoding is the verification of stage associations, especially those not made by careful rearing. Recent revisionary study, on the family Ephemerellidae Klapalek provides an illustration. The species concept of *Ephemerella altana* Allen, a western Nearctic taxon, had been based on a larva belonging to the genus *Ephemerella* Walsh and an adult of *Serratella* Edmunds. Had barcoding technology been available at the time of *E. altana*'s discovery and description, it potentially could have shown that this association was



erroneous. Furthermore, barcoding could have helped to resolve the species identities of the larva and adult. Based on traditional specimen comparisons, the larva is thought to be that of the transcontinental species, *Ephemerella excrucians* Walsh, and the adult to be that of the western species, *Serretella micheneri* (Traver). *Ephemerella excrucians* exhibits an amazing amount of morphological variability throughout its wide geographic range, which begs the question of whether the current species concept might contain various cryptic lineages that are unrecognizable by traditional, morphological means. Barcoding technology could be used to study various populations, including those from type localities, and could provide a guideline for decisions about species identities and boundaries (Zhou et al. 2008). Zhou et al. (2009) have made a pioneering attempt to generate a DNA barcode reference library for three insect orders- Ephemeroptera, Plecoptera and Trichoptera at one site in the Canadian subarctic. This study has demonstrated that DNA barcoding holds great promise as a tool for rapid biodiversity assessment in unknown faunas. A very close correspondence was observed between morphospecies as determined by taxonomic experts and barcode clusters designated using a standard sequence threshold. Several cases of proposed splitting may reflect cryptic species.

DNA barcodes of stream mayflies will improve descriptions of community structure and water quality for both ecological and bioassessment purposes (Sweeney et al. 2011). Rapid assessment of biodiversity will aid the selection of sites of special conservation value and will help to focus the efforts of taxonomists in revising and characterizing the diversity of life (Zhou et al. 2009).

## CONCLUSIONS

Future perspectives on systematics and phylogeny of Ephemeroptera using recent molecular tools are highlighted below:

- Four sequencing markers which are well-surveyed and informative across a range of divergences viz., the mitochondrial COI and 16S genes, and the nuclear 18S and EF-1 $\alpha$  genes are suggested as standards for comparison for insect molecular systematic studies (Caterino et al. 2000).

- Species delineation continues to be one of the

primary applications of genetic techniques. Application of the Generalized Mixed Yule-coalescent (GMYC) model to species circumscription using single-locus DNA appears rewarding (Pons et al. 2006; Fontaneto et al. 2007). This approach has been applied successfully to 64 species of mayflies in Madagascar (Monaghan et al. 2009).

- Investigators of the demographic history of closely related populations or species can use several nuclear DNA sequences to test specific hypotheses of how past geological events influence observed patterns (e.g. Knowles et al. 2007). These techniques allow one to test *a priori* hypotheses rather than post hoc conclusions from patterns (Monaghan & Sartori 2009).

- Wilcock et al. (2005) demonstrated very well how the combination of ecological and genetic research, applied to several parts of the life cycle, can greatly advance our understanding of how populations function in nature.

- Routine sampling of population- and species- level genetic diversity, combined with coalescent-based methods of species delineation has great potential to become a standard procedure for the study of poorly known taxonomic groups like Ephemeroptera (Gatolliat & Monaghan 2010).

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