Tests of Current Hypotheses of Mayfly (Ephemeroptera) Phylogeny Using Molecular (18s rDNA) Data

LU SUN, ANIKO SABO,[1] M. D. MEYER,[2] R. P. RANDOLPH,[3] LUKE M. JACOBUS,
W. P. MCCAFFERTY, AND VIRGINIA R. FERRIS

Department of Entomology, Purdue University, West Lafayette, IN 47907

Ann. Entomol. Soc. Am. 99(2): 241–252 (2006)

ABSTRACT Partial 18s rDNA sequences from 22 exemplar mayfly species (Ephemeroptera) representing 20 families were analyzed to obtain a best phylogenetic tree for comparison to previous phylogenetic hypotheses. With respect to relationships among the three major groupings, our molecular data support the hypothesis that Pisciforma and Setisura comprise a monophyletic sister group to the Furcatergalia, rather than the hypothesis that Setisura and Furcatergalia comprise a monophyletic group stemming from the Pisciforma. Within Pisciforma, acceptable trees show that Baetidae separates at the base of the Pisciforma clade. The data suggest that Pisciforma is paraphyletic and do not support the grouping of all Southern hemisphere families as a monophyletic group. An evolutionary sequence favored by the data suggests a grouping of Siphlonuridae, Rallidentidae, Nesameletidae, and Ameletidae and a grouping of Oniscigastridae, Ameletopsidae, and Acanthametropodidae. The data support the monophyly of Setisura (Heptageniidae, Arthropleidae, Pseudironidae, Oligoneuriidae, Isonychiidae, and Coloburiscidae). Within Setisura, a bootstrap/jackknife test places the families Heptageniidae, Arthropleidae, and Pseudironidae in one clade at 100% frequency. Also supported are hypotheses that Pseudironidae is a sister group to a Heptageniidae–Arthropleidae group and that a sister relationship exists between the latter two families. Hypotheses that Pseudironidae separated from other Setisura families at an earlier stage and comprises a sister group to a Heptageniidae–Oligonuriidae lineage or that Pseudironidae should be moved out of Setisura are not supported.

KEY WORDS Ephemeroptera, 18s rDNA, phylogeny

EPHEMEROPTERA IS ONE OF the most archaic of extant winged insect orders (Kukalová-Peck 1985). The higher classification of Ephemeroptera has evolved considerably over the past 200 yr to include progressively more higher taxa. However, the use of strict phylogenetic classification is relatively recent (McCafferty 1991b), and cladistic data have been mostly morphological in nature. In this study, we used molecular data to test the various hypotheses outlined below regarding certain aspects of the phylogeny of Ephemeroptera.

Suborder Level Classification of Ephemeroptera. At present, four major groupings are generally recognized in Ephemeroptera under various names. We follow a classification system (Table 1) taken from McCafferty (1991b) and its modifications by Wang and McCafferty (1995), McCafferty (1997, 2004), and McCafferty and Wang (2000). In this classification of four recent suborders, Furcatergalia, which was sometimes previously referred to as Rectracheata (McCafferty 1991b), includes Leptophlebiidae, Behningiidae, all pannote families (Pannota), and all tusked burrower families (Scapphodonta); Setisura includes the flat-headed mayflies and their hypothesized minnowlike mayfly ancestral groups, including all forms that passively filter food with their forelegs; Pisciforma includes all other minnowlike mayflies; and Carapacea includes Baetiscidae and Prosopistomatidae.

Relationship between Carapacea and Other Mayflies. As a distinct monophyletic group supported by series of synapomorphies (e.g., the presence of a larval carapace), the Baetiscidae–Prosopistomatidae relationship was suggested by various researchers (Edmunds and Traver 1954). Kluge (1998) and McCafferty and Wang (2000) showed the group to be basally derived with its sister group represented by all other extant mayflies, which share synapomorphic forewings with the tornus located between the ends of veins CuA and CuP (CuP and A1 terminating in the anal margin, rather than the outer margin, of the wings). McCafferty (1997) first referred to the Baetiscidae–Prosopistomatidae grouping as the suborder Carapacea. In our study using molecular data, all trees

---

[1] Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030.

[2] Department of Biology, Christopher Newport University, Newport News, VA 23606.

[3] Section of Evolution and Ecology, University of California, Davis, CA 95616.

Table 1. McCafferty classification system of extant taxa followed in this study, with families arranged alphabetically within their groupings

Suborder	Infraorder	Superfamily	Family
Carapacea			Baetiscidae
			Prosopistomatidae
Furcatergalia	Lanceolata		Leptophlebiidae
	Palpotarsa		Behningiidae
	Scapphodonta	Potamanthoidea	Potamanthidae
		Euthyplocioidea	Euthyplociidae
		Ephemeroidea	Ephemeridae
			Ichthybotidae
			Palingeniidae
			Polymitarcyidae
	Pannota	Caenoidea	Caenidae
			Neoephemeridae
		Ephemerelloidea	Coryphoridae
			Dicercomyzidae
			Ephemerellidae
			Ephemerythidae
			Leptohyphidae
			Machadorythidae
			Melanemerellidae
			Teloganellidae
			Teloganodidae
			Tricorythidae
			Vietnamellidae
Pisciforma			Acanthametropodidae
			Ameletidae
			Ameletopsidae
			Ametropodidae
			Baetidae
			Dipteromimidae
			Metretopodidae
			Nesameletidae
			Oniscigastridae
			Rallidentidae
			Siphlaenigmatidae
			Siphlonuridae
			Siphluriscidae
Setisura			Arthropleidae
			Coloburiscidae
			Isonychiidae
			Heptageniidae
			Oligoneuriidae
			Pseudironidae

that we modified and tested recognize the hypothesis that Baetiscidae, as a representative of Carapacea, is the most basal clade.

Phylogenetic Hypotheses Regarding Furcatergalia, Setisura, and Pisciforma. Among the suborders Furcatergalia, Setisura, and Pisciforma, which accommodate the majority of mayfly families, Pisciforma and Setisura were considered to be derived together, constituting a sister clade to the Furcatergalia (McCafferty and Edmunds 1979, Landa and Soldán 1985, McCafferty 1991b, Tomka and Elpers 1991). The monophyly of the Furcatergalia and the Pisciforma–Setisura groups were supported by anatomic synapomorphies, including the presence of the ventral anastomosis of tracheae in segments other than 8 and 9 of the abdomen, and the absence of branches of a dorsal trunk of tracheae formed in the head, respectively (Tomka and Elpers 1991). Within the Pisciforma–Setisura clade, no clear evidence has supported the monophyly of Pisciforma, and McCafferty and Edmunds (1979) indicated it was extremely paraphyletic. Monophyly of Setisura, however, is clearly supported by a series of synapomorphies (McCafferty 1991a, Wang and McCafferty 1995, Kluge 1998). An alternative hypothesis regarding the relationships among the three major groupings was given by Kluge et al. (1995) and Kluge (1998, 2004), in which the Pisciforma group (called Tridentiseta) and a Setisura-Furcatergalia group (called Bidentiseta) were considered sister clades, although Kluge admitted that Pisciforma was probably paraphyletic. Kluge's grouping of Setisura and Furcatergalia together was supported by a presumed initial presence of two dentisetae on larval maxillae. Within this grouping, Setisura (or Branchitergaliae) and Furcatergalia (or Furcatergaliae) also were considered monophyletic groups. Soldán and Putz (2000) proposed another hypothesis based on karyotype evidence that generally resembled Kluge's hypothesis. It differed from the latter in the arrangement of lineages within the hypothesized Furcatergalia–Setisura group by treating Scapphodonta, Leptophlebiidae, and Setisura as a monophyletic sister group to Pannota (Fig. 3).

Phylogeny among Major Groups within Furcatergalia. The suborder Furcatergalia is generally thought to be monophyletic, with major clades Leptophlebiidae, Scapphodonta, and Pannota containing >15 families. It has been generally recognized that within the Furcatergalia, Leptophlebiidae is a basal lineage (Kluge 1998), and a sister group to a relatively derived clade that includes a pair of sister groups, Scapphodonta and Pannota (McCafferty and Wang 2000) in addition to a more basal lineage represented by the Behningiidae (McCafferty 2004). Scapphodonta and Pannota are both monophyletic and are supported by a series of morphological synapomorphies (McCafferty and Wang 2000, McCafferty 2004). In our study, we followed this arrangement of branches within Furcatergalia in our construction of hypothesized phylogenetic trees.

Relationships Regarding the Pisciforma–Setisura Grouping. When Ulmer (1920) first introduced suborder concepts to Ephemeroptera, all minnowlike mayflies (except Baetidae) and flatheaded mayflies were placed in the suborder Heptagenioidea. Edmunds and Traver (1954) changed Ulmer's suborders into superfamilies, while adding Baetidae and Oligoneuriidae to Heptagenioidea. Edmunds et al. (1963) considered Isonychiinae and Coloburiscinae as subfamilies of Siphlonuridae (in Heptagenioidea), and Riek (1973) placed them in Oligoneuriidae, which was later followed by McCafferty and Edmunds (1979). Demoulin (1958) proposed Siphlonuroidea for Siphlonuridae and Baetidae to recognize their hypothesized close relationship, and he also separated them from the flatheaded mayflies (Heptageniidae and some Oligoneuriidae), which he placed in Heptagenioidea and Oligoneurioidea. McCafferty and Edmunds (1979) continued to include minnowlike and flatheaded mayflies together in one superfamily but changed the name from Heptagenioidea to Baetoidea; however, Landa and Soldán (1985) used Baetoidea for only minnowlike mayfly families and Heptagenioidea for Heptageniidae and Oligoneuriidae. McCafferty (1990) first considered the Coloburiscidae, Isonychiidae, Oligoneuriidae, and Heptageniidae to constitute the Heptagenioidea, and later further elaborated cladistic evidence for the monophyly of his grouping with numerous synapomorphies (McCafferty 1991a). McCafferty (1991b) proposed suborders Setisura and Pisciforma for Heptagenioidea and Baetoidea, respectively, in which a series of mayfly subfamilies previously placed under Siphlonuridae or Oligoneuriidae were treated as families. Later, Wang and McCafferty (1995) moved Pseudironidae from Siphlonuroidea to Heptagenioidea. However, Kluge (2004) suggested that Pseudironidae should be moved out of Setisura, being instead a pisciform group.

Relationships among Families of Pisciforma. The evolution of the families of Pisciforma still remains largely unclarified. Edmunds (1975), Landa (1973), and Riek (1973) all presented various hypotheses of family evolution that included pisciform taxa based on phenetic interpretations. Landa and Soldán (1985) discussed evolutionary trends of many lineages, which were deduced from a systematic study of mayfly anatomy. Based on internal anatomical data and other morphological evidence of Landa and Soldán (1985), Tomka and Elpers (1991) suggested an evolutionary tree for mayfly families in which most of the clades were supported by presumed synapomorphies. Within the Pisciforma–Setisura grouping, Ametropodidae was thought to branch first, with other families sharing the nerve ganglion of abdominal segment one merging with that of the metathorax. Siphlonuridae (Siphlonuridae and Dipteromimidae in Table 1) and Rallidentidae (Rallidentidae, Nesameletidae, and Ameletidae in Table 1) were thought to be sister groups that branched next, with other families lacking a nerve ganglion in abdominal segment 8. Acanthametropodidae and Ameletopsidae were thought to branch next from the remainder that included Baetidae, Metretopodidae, and Setisura, which supposedly shared paired intercalaries in the forewings parallel with CuA and CuP. Baetidae was treated as a sister group to a Metretopodidae–Heptagenioidea grouping, that shared two-segmented labial palpi. Metretopodidae was considered to be a sister group to Heptagenioidea. However, the conclusions of Landa and Soldán (1985) and Tomka and Elpers (1991) have been suspect because the characters used in their analyses have a propensity for convergence and are inconsistent within the taxa they were said to represent, as have been pointed out, for example, by Kluge et al. (1995) and McCafferty (2004).

Kluge et al. (1995) recognized two superfamilies in Pisciforma: Baetoidea (including Baetidae and Siphlaenigmatidae) and Siphlonuroidea (including the rest of the Pisciforma families). These authors also divided Siphlonuroidea into a Northern hemisphere group of families that included Siphlonuridae, Dipteromimidae, Ameletidae, Metretopodidae, Acanthametropodidae, and Ametropodidae, and a Southern hemisphere group of families that included Oniscigastridae, Nesameletidae, Rallidentidae, and Ameletopsidae and suggested that the Southern hemisphere group was possibly monophyletic.

Relationships among Families of Setisura. Various researchers have believed that Setisura represented an evolutionary branch derived from Pisciforma mayflies that became adapted to flowing water habitats by way of passive filter feeding (Sinitshenkova 1984, Edmunds and McCafferty 1988, McCafferty 1991a). The determination of which families represent the intermediate evolutionary links is most important for establishing a phylogenetic classification. Within Setisura, McCafferty (1990, 1991a) hypothesized an evolutionary sequence from the primitive forms, including Coloburiscidae, Isonychiidae, and Oligoneuriidae, to the more derived Heptageniidae based on morphological evidence from fossil and extant mayflies. Wang and McCafferty (1995) also hypothesized that Pseudironidae was relatively highly derived within Setisura, representing a sister group to the Heptageniidae–Arthropleidae group, all of which represented the most apotypic clade in the

Table 2. Mayfly taxa sequenced for this study

Family	Species	Collection locale
Acanthametropodidae	*Analetris eximia* Edmunds	Canada: Saskatchewan
Ameletidae	*Ameletus celer* McDunnough	USA: Montana
Ameletopsidae	*Chiloporter* sp.	Chile: Region IX
Ametropodidae	*Ametropus neavei* McDunnough	Canada: Saskatchewan
Arthropleidae	*Arthroplea bipunctata* (McDunnough)	USA: Michigan
Baetidae	*Baetis bicaudatus* Dodds	USA: Montana
	Callibaetis ferrugineus (Walsh)	USA: Montana
Baetiscidae	*Baetisca lacustris* McDunnough	USA: Montana
Caenidae	*Caenis youngi* Roemhild	USA: Montana
Coloburiscidae	*Coloburiscoides giganteus* Tillyard	Australia: Australian Capital Territory
Ephemeridae	*Ephemera simulans* Walker	USA: Indiana
Heptageniidae	*Epeorus grandis* (McDunnough)	USA: Montana
	Heptagenia diabasia Burks	USA: Nebraska
Isonychiidae	*Isonychia rufa* McDunnough	USA: Indiana
Leptophlebiidae	*Paraleptophlebia memorialis* (Eaton)	USA: Montana
Metretopodidae	*Metretopus borealis* (Eaton)	Canada: Northwest Territory
Nesameletidae	*Ameletoides lacusalbinae* Tillyard	Australia: New South Wales
Oligoneuriidae	*Lachlania talea* Allen & Cohen	Honduras: Olancho
Oniscigastridae	*Tasmanophlebia* sp.	Australia: New South Wales
Polymitarcyidae	*Tortopus primus* (McDunnough)	USA: Indiana
Pseudironidae	*Pseudiron centralis* McDunnough	USA: Wisconsin
Siphlonuridae	*Siphlonurus columbianus* McDunnough	USA: Montana

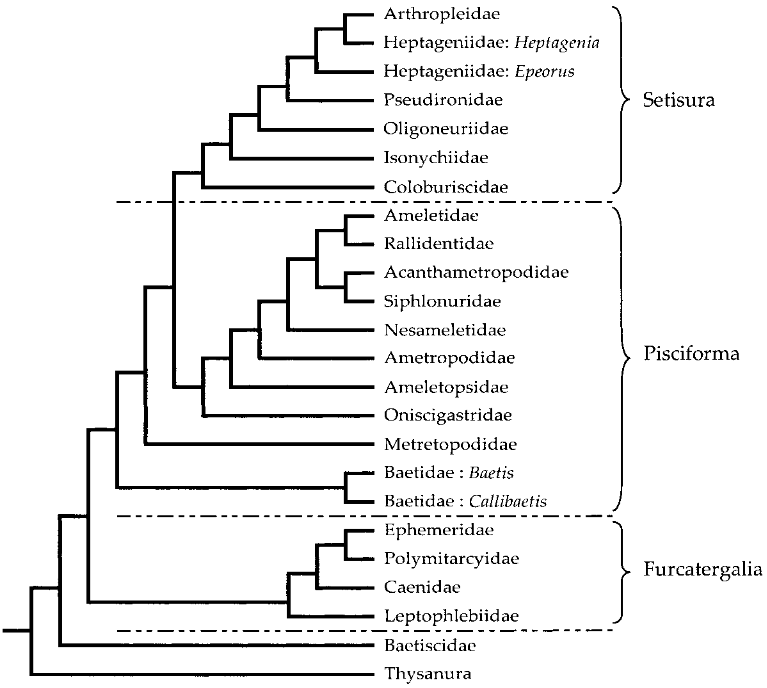
suborder. Alternative evolutionary relationships of Pseudironidae and other Setisura families were suggested by Tomka and Elpers (1991), in which Pseudironidae was thought to separate from other Setisura at an earlier stage and was a sister taxon to a hypothesized Heptageniidae–Oligoneuriidae lineage. Also, as mentioned above, Kluge (2004) did not consider Pseudironidae to be a member of Setisura.

Materials and Methods

Specimens. We sequenced partial 18s rDNA from 22 mayfly species belonging to 20 families as shown in Table 2. Specimens were collected between 1982 and 2002, preserved in 70% ethanol, and deposited in the Purdue Entomological Research Collection (PERC). The homologous sequence of one species of Rallidentidae, *Rallidens mcfarlanei* Penniket (GenBank accession no. AY338696), was incorporated into our analyses. Therefore, all Pisciforma and Setisura families were included in this study except Siphlaenigmatidae, Siphluriscidae, and Dipteromimidae. These three families are thought to have close relationships with Baetidae, Acanthametropodidae (or possibly Nesameletidae), and Siphlonuridae, respectively, based on morphological evidence (Edmunds and Koss 1972, Edmunds 1973, Meyer et al. 2003, Zhou and Peters 2003). We used one species of Thysanura (*Lepisma* sp., GenBank accession no. AF005458) to serve as the non-Ephemeroptera outgroup in our molecular analyses.

Methods for Obtaining and Manipulating DNA. We dissected and rinsed in Tris-EDTA buffer (TE, pH 7.5) pieces of muscle from the thorax or abdomen of a single specimen of each mayfly species. We used a Kontes grinder (Kontes Glass, Vineland, NJ) to grind the tissue in 25 μl of Molecular Grinding Resin (Genotech, St. Louis, MO) in a 1.5-ml microcentrifuge tube, which was immediately used or frozen at −20°C for later use. Total genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA) according to manufacturer's recommendations. The mixtures were incubated overnight at 56°C and then were boiled and centrifuged as directed by the manufacturer. The supernatant was stored at 4°C for use as templates for polymerase chain reaction (PCR). Primers (Ferris et al. 2004) for the ≈620-bp fragment of the 18s rDNA were (forward) 5′-AGGGCAAGTCTGGTGCCAGC-3′ and (reverse) 5′-TTTCAGCTTTGCAACCATAC-3′. The amplified fragment was cloned into the pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* strain JM109 (Promega). Colony cultures containing inserts of the expected size, as determined by PCR, were used to make Wizard Plus plasmid preparations (Promega). Automatic sequencing of the plasmid preparations was done at the Purdue Genomics Facility. Both strands of DNA from two to four clones were sequenced for each insect isolate.

Methods for Phylogenetic Analysis, Tree Building, and Testing. Sequences were aligned using Clustal X (Thompson et al. 1997) with default parameters (gap open = 15, gap extend = 6.66). Phylogenetic analysis was performed with PAUP* 4.0b10 (Swofford 2002), under Parsimony criteria, by using the heuristic search with *Lepisma* sp. assigned as an outgroup. We used MacClade 4.01 (Maddison and Maddison 2000) to create trees reflective of hypotheses for the phylogeny of Ephemeroptera based on morphological data discussed above. Hypotheses were compared individually to the best tree based on molecular data by using the Shimodaira–Hasegawa (S/H) test as implemented in PAUP. In this test, when $P \geq 0.05$, trees were considered not significantly different from the original best tree, and the hypotheses represented by these trees were not rejected. When $P < 0.05$, the trees were rejected. In addition, we manipulated these trees in PAUP and MacClade to obtain additional shorter trees



Tree 1

Fig. 1. Tree 1: an acceptable tree that supports the hypothesis of relationships among the three major groupings of mayflies, in which Pisciforma and Setisura comprise a monophyletic sister group to Furcatergalia.

with better S/H scores if possible for the hypothesis being tested. For example, we frequently searched partial trees containing only some of the groups to obtain optimal arrangements for them.

Results and Discussion

Relationships among Setisura, Pisciforma, and Furcatergalia. The first hypothesis with respect to relationships among the three major groupings is that Pisciforma and Setisura comprise a monophyletic sister group to Furcatergalia. In the test of this hypothesis, the best tree was modified in the following ways: Baetiscidae was moved to the base of the clade that represented all extant Ephemeroptera. Above the Baetiscidae branch, all Furcatergalia taxa were placed at the base of the three major lineages to form one clade, which was a sister group to a hypothesized Pisciforma–Setisura clade. Within Furcatergalia, arrangements of families followed hypotheses that were described above: Letophlebiidae separated at an earlier stage at the base of the lineage; the sister groups, Pannota (represented by Caenidae) and Scaphodonta (represented by Ephemeridae and Polymitarcyidae) were placed at the top. In the Pisciforma–Setisura clade, Setisura was considered to be monophyletic. To obtain the tree with the highest possible score, a partial tree that included only data of the Pisciforma taxa plus Coloburiscidae was inferred using the Heuristic Search under Parsimony in PAUP with Thysanura as the outgroup. The family Coloburiscidae, hypothesized as the most primitive Setisura mayfly form (McCafferty 1991a), served as a positional indicator of the origin of the Setisura suborder within Pisciforma. Taxa of Pisciforma were arranged in MacClade according to this partial tree. The remaining lineages of Setisura were added on the Coloburiscidae branch and were placed in the search for the shortest total length function in MacClade. When the first hypothesis and other assumptions described above were followed, the tree with the highest possible score is shown in Fig. 1. This tree was not significantly different from the best molecular tree based on the S/H test score. Therefore, our data supported the first hypothesis regarding the relationships among the three major groupings of mayflies, represented by the framework of McCafferty (1991b).

The alternative hypothesis is that the suborders Setisura and Furcatergalia comprise a monophyletic sister group. However, the ancestral nature of this group has not been clearly inferred in this hypothesis. Therefore, we assumed that there are two possibilities: the hypothesized ancestor could be either a Setisura-like form, or a Furcatergalia-like form. To obtain the best tree score, the above-mentioned possibilities were examined separately in two trees. In the first tree, the ancestor of the hypothesized grouping of Setisura and Furcatergalia was represented by Coloburiscidae, which was considered to be the most primitive form in Setisura, whereas in the second tree it was repre-

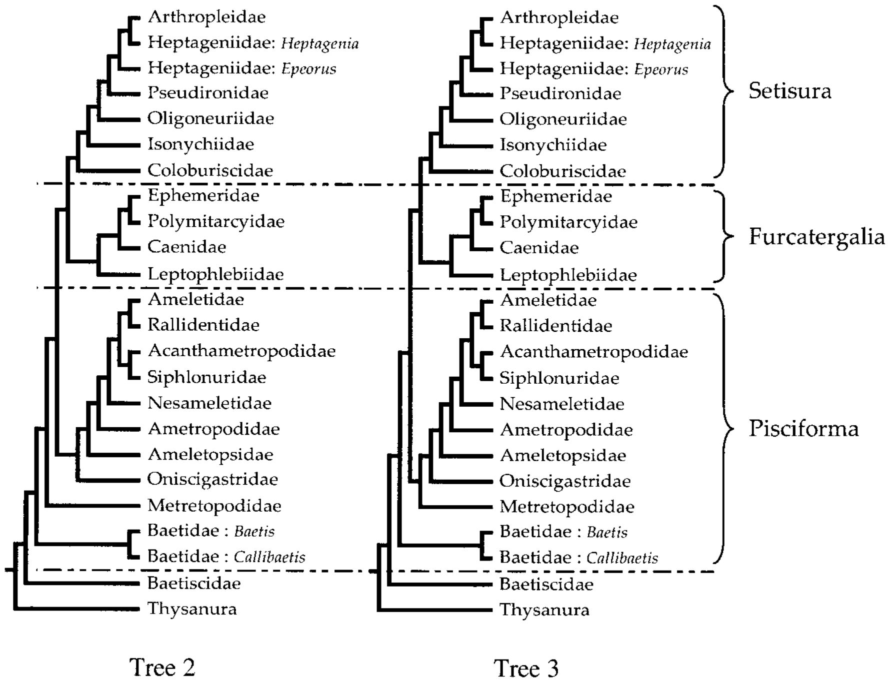
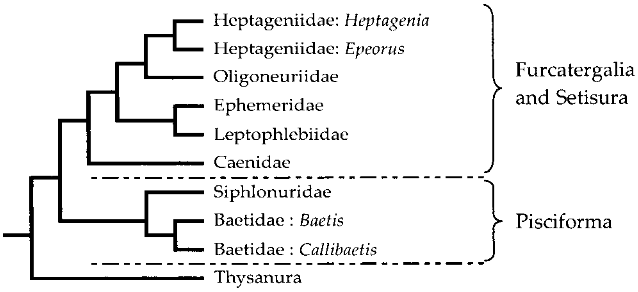


Fig. 2. Trees 2 and 3: best trees following the second hypothesis regarding relationships among the three major groupings of mayflies, in which Furcatergalia and Setisura comprise a monophyletic group stemming from Pisciforma. Tree 2: a tree built on the assumption that the ancestor of the hypothesized Furcatergalia–Setisura lineage is a Setisura-like form. Tree 3: a tree built on the assumption that the ancestor of the hypothesized Furcatergalia–Setisura lineage is a Furcatergalia-like form. Both trees were rejected by the S/H test.

sented by Leptophlebiidae, which was considered to be the most primitive form in Furcatergalia. After placing Baetiscidae at the base of the entire Ephemeroptera clade, we inferred using the Heuristic Search under Parsimony a partial tree that included only data of Pisciforma taxa plus Coloburiscidae, with Thysanura as the outgroup. Next, taxa of Pisciforma were arranged above Baetiscidae in MacClade as indicated by the partial tree. The rest of the lineages of Setisura and Furcatergalia were then added on the Coloburiscidae branch. Taxa of these two suborders were placed in two respective clades as sister groups. In each of the clades, families and genera were arranged in the same way as for tree 1 (Fig. 1). The tree created is shown in Fig. 2 (tree 2). Second, a partial tree including only data of all Pisciforma taxa plus Leptophlebiidae was inferred using the Heuristic Search under Parsimony with Thysanura as the outgroup. Another complete tree was constructed based on data from this partial tree in the same way as that described above, except that the hypothesized Furcatergalia–Setisura clade was added on the Leptophlebiidae branch instead of the Coloburiscidae branch (Fig. 2, tree 3). Trees two and three were compared with the best tree based on molecular data (using the S/H test as implemented in PAUP), and both trees were significantly different from the best tree. As a further test of the possibility that Furcatergalia and Setisura comprise a monophyletic group, we made further searches for the shortest total length in MacClade by using the taxa of the Furtcatergalia–Setisura group of trees 2 and 3. However, all trees generated using this method were rejected by the S/H tests. Therefore, our molecular data did not support the second hypothesis, represented by the framework of Kluge (1998, 2004).

We also examined the hypothesis given by Soldán and Putz (2000) based on karyotype evidence. Trees 2 and 3 (Fig. 2) were modified by moving Ephemeridae, Polymitarcyidae, and Leptophlebiidae from Furcatergalia to Setisura as suggested by Soldán and Putz (2000). The S/H test showed these trees to be significantly different from the best tree. We also performed the Heuristic Search under Parsimony criteria and obtained a best tree based on the molecular data for only the families studied by Soldán and Putz (2000) (except Potamanthidae and Ephemerellidae) with Thysanura as the outgroup. We used MacClade to create another tree that included the same mayfly families (Baetidae, Siphlonuridae, Heptageniidae, Oligoneuriidae, Leptophlebiidae, Ephemeridae, and Caenidae) following the arrangement of Soldán and Putz (2000) (Fig. 3). The S/H test indicated a significant difference between this tree and the best tree based on molecular data. Therefore, the framework suggested by Soldán and Putz (2000) was not supported by our molecular data.



Tree 4

Fig. 3. Tree 4: a tree created including limited families following the hypothesis given by Soldán and Putz (2000), in which Setisura, tusked burrowers, and Leptophlebiidae were considered to form a monophyletic group. This tree was rejected by the S/H test when compared with the best tree based on our molecular data of the same families.

According to our results, Setisura evolved independently from a siphlonurid-like ancestor within Pisciforma and did not share a direct common ancestor with Furcatergalia, which originated independently within Ephemeroptera. Because the Pisciforma–Setisura (Pisciforma–Heptagenioidea) clade is monophyletic, subordinal status could be assigned to this entire group. As such, Setisura would be subsumed under Pisciforma, as per McCafferty (1997).

Status of Pisciforma. Additional trees were generated in MacClade to examine the status of Pisciforma, sensu stricto. These trees were modified from tree 1 (Fig. 1) by adjusting the positions of Pisciforma families, which included Acanthametropodidae, Ameletidae, Ameletopsidae, Ametropodidae, Baetidae, Metretopodidae, Nesameletidae, Oniscigastridae, Rallidentidae, Siphlaenigmatidae, and Siphlonuridae.

We first explored the possibility that Pisciforma is monophyletic. A partial tree was obtained by including data of all Pisciforma taxa (with Thysanura as outgroup) by using the Heuristic Search under Parsimony. We then selected the partial tree with the best score and modified tree 1 (Fig. 1) by placing all taxa of Pisciforma in one clade that followed the arrangement indicated by the best partial tree. Baetidae is at the base of the Pisciforma clade as in tree 1 (Fig. 1), whereas all other families (Siphlonuridae-like forms) form a sister clade (Fig. 4, tree 5). The S/H test indicated that the new tree was significantly different from the best tree based on molecular data. We also explored the possibility that all Siphlonuridae-like families comprised a monophyletic group (Siphlonuroidea) when a paraphyletic Pisciforma was divided into two superfamilies: Baetoidea (represented in our test by Baetidae) and Siphlonuroidea (represented by all other Pisciforma families). Another tree was constructed based on tree 5, in which all Pisciforma (excluding Baetidae) were placed in one clade as a sister group to Setisura. This tree (Fig. 4, tree 6) also was rejected by the S/H test, and therefore our molecular data suggest that Pisciforma is paraphyletic. If it is divided into two superfamilies, Siphlonuroidea in itself is also a paraphyletic group.

We then examined the possibility that Siphlonurioidea included a monophyletic Southern hemisphere group comprised of Oniscigastridae, Nesameletidae, Rallidentidae, and Ameletopsidae as suggested by Kluge et al. (1995). Tree 1 was modified so that the four families comprised one clade. We used MacClade to find an arrangement with the shortest total length within this clade and tentatively added it at the original position of each of these families to explore all possibilities (e.g., Fig. 4, tree 7). However, all such trees were rejected by the S/H test, and our molecular data suggest that all Southern hemisphere Siphlonuroidea families are not likely to be derived from a single common ancestor.

Following the assumption that Pisciforma is paraphyletic, a series of trees was constructed to test the phylogeny of this group as hypothesized by Tomka and Elpers (1991) as shown in tree 8 (Fig. 4). Because the family Oniscigastridae was not included in the Tomka and Elpers study, we obtained another tree that included molecular data for all our taxa except Oniscigastridae by using the Heuristic Search under Parsimony. This best molecular tree was compared with a series of trees created to examine the various aspects of the hypothesis. As suggested by Tomka and Elpers (1991), we first treated Metretopodidae as a sister group to Setisura. A partial tree was obtained by including data of all Pisciforma taxa (with Thysanura as outgroup) based on molecular data using the Heuristic Search under Parsimony in PAUP. Tree 1 (Fig. 1) was modified in MacClade, with the families of Pisciforma arranged as indicated by the partial tree obtained previously, whereas Setisura was added on the Metretopodidae branch as a sister group to the latter. The S/H test showed the resulting tree to be significantly different from the best tree.

Next, we examined the possibility that Setisura, Baetidae, and Metretopodidae comprise a monophyletic group. Another partial tree that included all Pisciforma taxa except Metretopodidae was obtained based on molecular data using PAUP. Arrangements of these families in tree 1 (Fig. 1) were modified as indicated in this new partial tree, and Metretopodidae and Setisura were added on the Baetidae branch. The

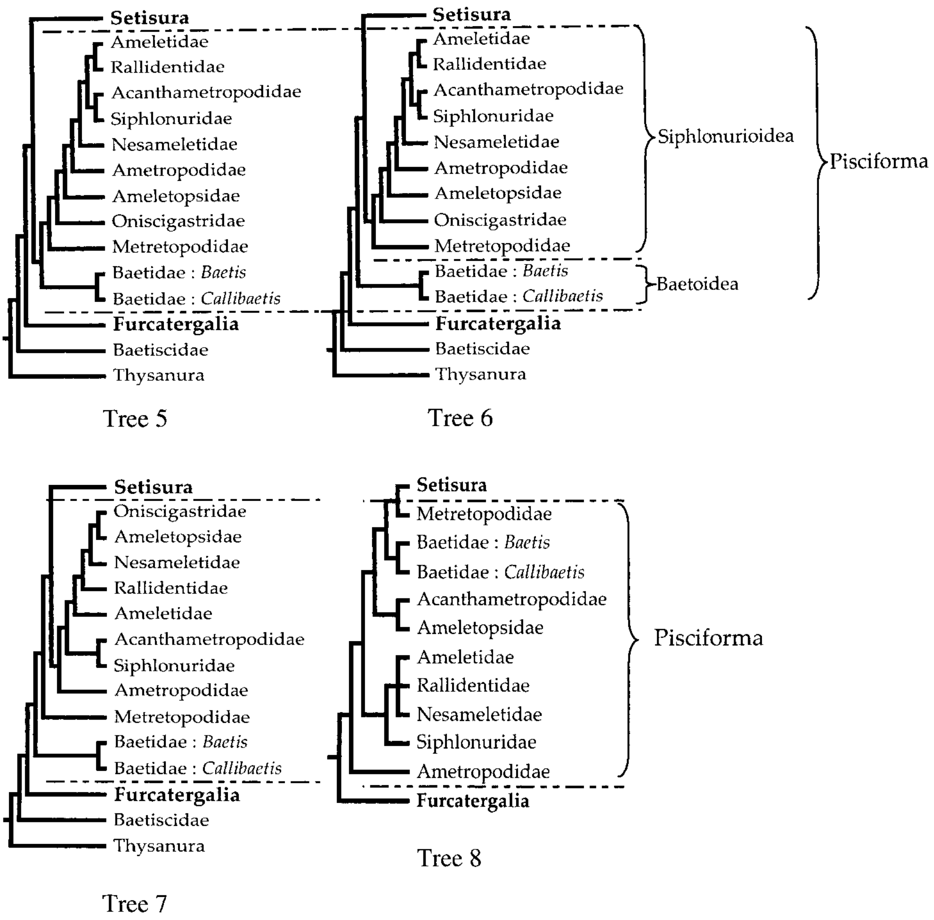
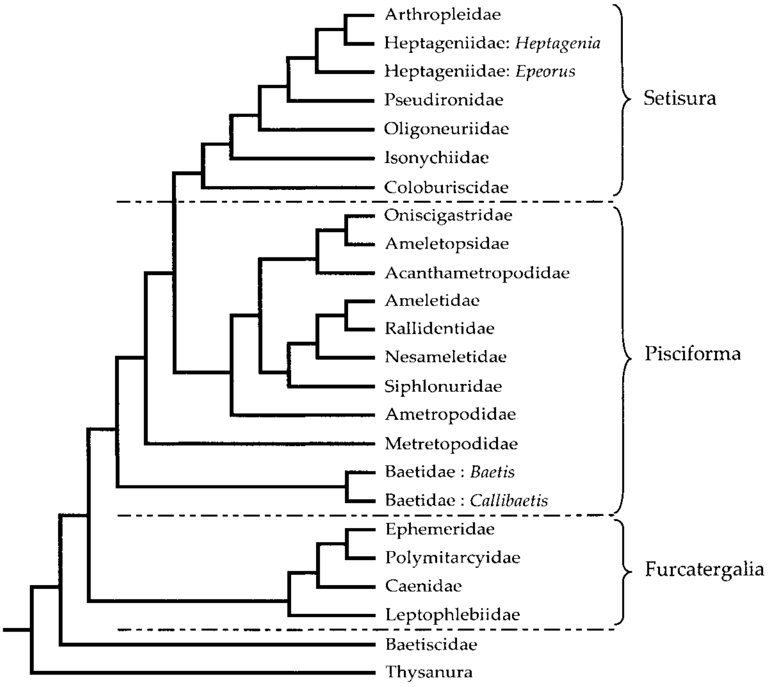


Fig. 4. Trees 5–8. Tree 5: the best tree following the assumption that Pisciforma is a monophyletic group. Tree 6: the best tree following the assumption that Pisciforma could be divided into two separate monophyletic groups, Baetoidea and Siphlonuroidea. Tree 7: an example of trees following the assumption that Pisciforma included a monophyletic Southern hemisphere family group. Tree 8: a hypothesized tree in which the arrangement of Pisciforma families follows the hypothesis given by Tomka and Elpers (1991), where Nesameletidae and Ameletidae were genera under Rallidentidae. All of these trees were rejected by the S/H test.

clade comprised by these three groups also was placed in the search-above-branch function to obtain the highest possible score. This tree also was rejected by the S/H test.

We then constructed another tree to examine the possibility that Setisura, Baetidae, Metretopodidae, Acanthametropodidae, and Ameletopsidae comprise a monophyletic group as suggested by Tomka and Elpers (1991). Because they suggested that Acanthametropodidae and Ameletopsidae were a sister group and formed a most primitive clade in this group, we placed each of two families at the base of this hypothesized group. The first partial tree included only Acanthametropodidae plus the rest of the Pisciforma taxa. The arrangements of these families were modified in tree 1 following this partial tree, and then a group of taxa including Ameletopsidae, Baetidae, Metretopodidae, and Setisura was added to the Acanthametropodidae branch as a monophyletic group and placed in the search for the shortest total length function in MacClade. The second partial tree included the same taxa as in the first partial tree except that Acanthametropodidae was replaced by Ameletopsidae. Accordingly, the group of interest was added on the Ameletopsidae branch in MacClade. Both these trees were shown by the S/H test to be significantly different from the best tree.

We examined whether Ametropodidae could be the most primitive form of the Pisciforma–Setisura lineage, as suggested by Tomka and Elpers (1991), by placing the family at the base of the clade that included all Pisciforma–Setisura families in tree 1 (Fig. 1), and using the search for the shortest total length function in MacClade on all taxa above the Ametropodidae branch. The tree generated was rejected according to the S/H test. Therefore, we conclude that several characteristics treated as synapomorphies by Tomka and Elpers (1991) in their hypothesized phylogeny of the Setisura–Pisciforma group are the result of homoplasy. These character-



Tree 9

Fig. 5. Tree 9: an acceptable tree based on tree 1 following one hypothesis given by Landa and Soldán (1985), in which Ameletopsidae and Oniscigastridae are sister groups, and two hypotheses given by Tomka and Elpers (1991): 1) Present Siphlonuridae, Rallidentidae, Nesameletidae, and Ameletidae (of Table 1) comprise a monophyletic group; and 2) Ameletopsidae and Acanthametropodidae are sister groups (when Oniscigastridae was absent).

istics include the presence of two-segmented labial palps, the presence of paired intercalaries parallel with CuA and CuP on forewings, the absence of a nerve ganglion in abdominal segment 8, and the merging of the nerve ganglion of abdominal segment 1 and that of the metathorax.

We tested two additional aspects of the Tomka and Elpers (1991) hypothesis. In their study, Siphlonuridae and Rallidentidae (Rallidentidae, Nesameletidae, and Ameletidae in Table 1) were thought to comprise a monophyletic group, although no synapomorphy was provided. They also suggested that Acanthametropodidae and Ameletopsidae are sister groups supported by anatomic evidence. The trees that we created that reflected these hypotheses could not be rejected based on the S/H tests. We further added Oniscigastridae back to this tree. Because the family was not included in the Tomka and Elpers (1991) study, we followed the hypothesis given by Landa and Soldán (1985), in which Ameletopsidae and Oniscigastridae were considered to be sister groups. Thus, in our tree Acanthametropodidae was placed at the base of a clade comprised by the two families (Fig. 5, tree 9). The S/H test indicated that this new tree was not significantly different from the best tree. Therefore, these particular aspects of the Tomka and Elpers (1991) hypothesis are acceptable.

On the best phylogenetic tree based on molecular data of the families of the Setisura–Pisciforma group (with Thysanura as the outgroup), placement of Baetidae at the base of this clade was supported by a 100% bootstrap score. This suggested that Baetidae (the superfamily Baetoidea, including Baetidae and possibly Siphlaenigmatidae) separated at a very early stage from other lineages of Pisciforma, leaving a paraphyletic superfamily Siphlonuroidea. The combination of the rest of the Pisciforma families plus all the Setisura families into one monophyletic group had a bootstrap support of 52%, as did the arrangement of Rallidentidae and Ameletidae as a sister group in Pisciforma.

Evolution of Setisura. At the outset of our study, the suborder Setisura was considered by many to be a monophyletic group that included the families Heptageniidae, Arthropleidae, Pseudironidae, Oligoneuriidae, Coloburiscidae, and Isonychiidae (Wang and McCafferty 1995, Kluge 1998). Our data strongly supported the hypothesis that Heptageniidae, Arthropleidae, and Pseudironidae form a monophyletic group. These three families (represented by four sequences) were exclusively placed in one clade at 100% bootstrap frequency on the best tree based on molecular data that included all our mayfly sequences. As discussed above, our S/H test results indicated that it was acceptable to include the families Oligoneuriidae, Coloburiscidae, and Isonychiidae in Setisura, thus supporting the monophyly of this suborder that was based

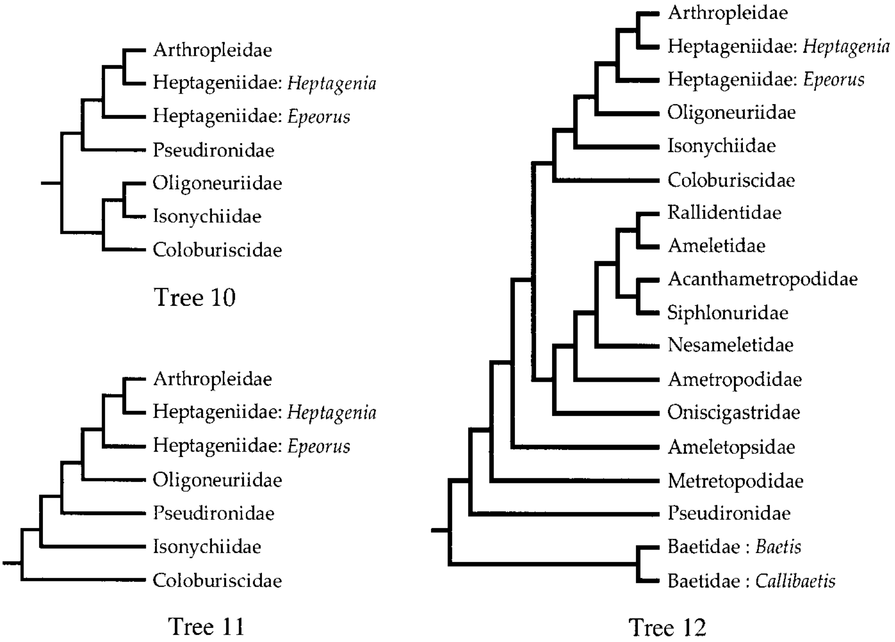
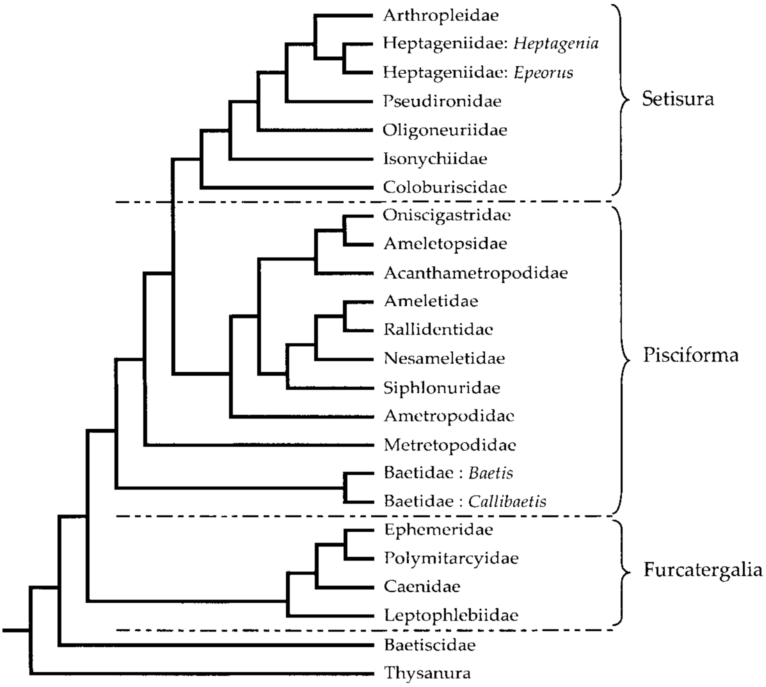


Fig. 6. Trees 10–12. Tree 10: an alternative hypothesis regarding the evolution of Setisura lineages, in which Coloburiscidae, Isonychiidae, and Oligoneuriidae were placed in one clade. Tree 11: an alternative evolutionary hypothesis of Setisura, in which Pseudironidae is a sister group to a hypothesized Oligonuriidae–Heptageniidae–Arthropleidae lineage. Tree 12: a parsimonious arrangement of Pisciforma and Setisura lineages based on the hypothesis that Pseudironidae is a part of Pisciforma, not Setisura. All of these trees were rejected by the S/H test.

on morphological evidence, as per the framework of Wang and McCafferty (1995).

The sequence of evolution of the Setisura lineages shown in tree 1 (Fig. 1) and tree 9 (Fig. 5) was a result of the search for the shortest total length function in MacClade, and favored the hypothesis given by McCafferty (1991a) based on morphological evidence, regarding the origins of Setisura and relationships among Coloburiscidae, Isonychiidae, Oligoneuriidae, and Heptageniidae. McCafferty (1991a) also had observed that two of the 36 apomorphies he studied occurred exclusively in the families Coloburiscidae, Isonychiidae, and Oligoneuriidae, all possessing apomorphic maxillary gills and filtering setae on the forelegs. McCafferty (1991a) hypothesized that these were gained synapomorphies at the base of Setisura that were subsequently lost in the apotypic family Heptageniidae. Nonetheless, an alternative arrangement of lineages in a phylogeny might be to place these three families exclusively in a single clade to represent a primitive lineage of Setisura. We constructed a tree modified from our tree 1 (Fig. 1), in which such a clade was recognized (Fig. 6, tree 10). The rest of the Setisura families, Pseudironidae, Arthropleidae, and Heptageniidae, were placed in another clade as a sister group to the former. This grouping of the three lineages was supported with the 100% bootstrap frequency and also was supported by morphological synapomorphies noted by McCafferty (1991a) and Wang and McCafferty (1995). Each of these clades was then placed in the search for the shortest total length function in MacClade. The S/H test indicated that this tree was significantly different from the best tree and therefore not acceptable based on our molecular data. Thus, our molecular evidence suggested that Coloburiscidae, Isonychiidae, and Oligoneuriidae separated from the rest of Setisura at different times and do not comprise a monophyletic group, and the explanation for the two incongruent characters, as given by McCafferty (1991a), is highly probable, which also was supported by data from ecology and behavior (McCafferty 1991a).

Our data favored the hypothesis by Wang and McCafferty (1995) regarding the relationship between Pseudironidae and the Heptageniidae–Arthropleidae grouping (Figs. 1 and 4). To examine the acceptability of alternative hypotheses regarding sequence of evolution of these families, we created additional trees. Tree 1 (Fig. 1) was modified by moving Pseudironidae to the base of the clade comprised of Heptageniidae, Arthropleidae, and Oligoneuriidae (Fig. 6, tree 11) as suggested by Tomka and Elpers (1991). To examine the hypothesis given by Kluge (2004), who suggested that Pseudironidae should be moved out of Setisura, a partial tree that included only Pseudironidae, Coloburiscidae, and all the Pisciforma families was inferred using the Heuristic Search under Parsimony in PAUP, with Thysanura as the outgroup. The complete tree was constructed by modifying tree 1 (Fig. 1), in which the Pseudironidae and Pisciforma taxa were arranged as indicated by the new partial tree; the Setisura taxa (except Pseudironidae) were



Tree 13

Fig. 7. Tree 13: a tree modified from tree 9 with Arthropleidae a sister group to Heptageniidae. This tree was accepted by the S/H test.

added on the Coloburiscidae branch and placed in another search for the shortest total length in MacClade (Fig. 6, tree 12). Both tree 11 and tree 12 (Fig. 6) were rejected by the S/H tests. Therefore, our molecular data support the hypothesis that Heptageniidae, Arthropleidae, and Pseudironidae comprise a clade separate from Oligoneuriidae within the Heptagenioidea.

Wang and McCafferty (1995) discussed the sister group relationship between Heptageniidae and Arthropleidae, which was supported by morphological synapomorphies. We tested this hypothesis by modifying tree 9 (Fig. 5) to make another tree in which Heptageniidae genera *Heptagenia* and *Epeorus* were placed in one clade to represent the family; Arthropleidae was treated as a sister group to the Heptageniidae clade (Fig. 7, tree 13). The S/H test indicated no significant difference from the best tree. Therefore, our data support a sister group relationship between Heptageniidae and Arthropleidae.

Our molecular data support tree 13 (Fig. 7) regarding the phylogeny of Ephemeroptera. Other trees are probably also acceptable, especially with respect to evolution among Pisciforma families. Uncertainty in the reconstruction of a phylogenetic tree for mayflies probably stems from their long evolutionary history, being an ancient group of winged insects. Despite the often-demonstrated highly conserved nature of 18S ribosomal DNA (Ferris et al. 2004), variations in rates of molecular change in the rRNA gene may occur over very long time spans. These variations can lead to errors in estimates of phylogenetic relationships in the same way that long time spans may obscure pathways of changes in morphological characters. To further clarify the phylogeny of Ephemeroptera, more research with both morphological and molecular data sets is needed.

Acknowledgments

We thank J. Webb (Purdue University, West Lafayette, IN) for collecting part of the mayfly specimens. We also thank J. S. Yaninek (Purdue University) and the Department of Entomology for supporting this project, which was initiated during a Molecular Systematics course in which we participated.

References Cited

Demoulin, G. 1958. Nouveau schema de classification des Archodonates et des Ephemeropteres. Bull. Inst. R. Sci. Nat. Belg. 39: 1–18.

Edmunds, G. F., Jr. 1973. Some critical problems of family relationships in the Ephemeroptera, pp. 145–154. *In* W. L. Peters and J. G. Peters [eds.], Proceedings of the First International Conference on Ephemeroptera. E. J. Brill, Leiden, The Netherlands.

Edmunds, G. F., Jr. 1975. Phylogenetic biogeography of mayflies. Ann. Mo. Bot. Gard. 62: 251–263.

Edmunds, G. F., Jr., and R. W. Koss. 1972. A review of the Acanthametropodinae with a description of a new genus

(Ephemeroptera: Siphlonuridae). Pan-Pac. Entomol. 48: 136–144.

Edmunds, G. F., Jr., and W. P. McCafferty. 1988. The mayfly subimago. Annu. Rev. Entomol. 33: 509–529.

Edmunds, G. F., Jr., and J. R. Traver. 1954. An outline of a reclassification of the Ephemeroptera. Proc. Entomol. Soc. Wash. 56: 236–240.

Edmunds, G. F., Jr., R. K. Allen, and W. L. Peters. 1963. An annotated key to the nymphs of the families and subfamilies of mayflies (Ephemeroptera). Univ. Utah Biol. Ser. 13: 1–49.

Ferris, V. R., A. Sabo, J. G. Baldwin, M. Mundo-Ocampo, R. N. Inserraa, and S. Sharma. 2004. Phylogenetic relationships among selected Heteroderoidea based on 18S and ITS ribosomal DNA. J. Nematol. 36: 202–206.

Kluge, N. J. 1998. Phylogeny and higher classification of Ephemeroptera. Zoosyst. Ross. 7: 255–269.

Kluge, N. J. 2004. The phylogenetic system of Ephemeroptera. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kluge, N. J., D. Studemann, P. Landolt, and T. Gonser. 1995. A reclassification of Siphlonuroidea (Ephemeroptera). Bull. Soc. Entomol. Suisse 68: 103–132.

Kukalová-Peck, J. 1985. Ephemeroid wing venation based upon new gigantic Carboniferous mayflies and basic morphology, phylogeny, and metamorphosis of pterygote insects (Insecta, Ephemerida). Can. J. Zool. 63: 933–955.

Landa, V. 1973. A contribution to the evolution of the order Ephemeroptera based on comparative anatomy, pp. 155–159. *In* W. L. Peters and J. G. Peters [eds.], Proceedings of the First International Conference on Ephemeroptera. E. J. Brill, Leiden, The Netherlands.

Landa, V., and T. Soldán. 1985. Phylogeny and higher classification of the order Ephemeroptera: a discussion from the comparative anatomical point of view. Academia, Cesk. Akad. Ved., Praha, Czech Republic.

Maddison, D. R., and W. P. Maddison. 2000. MacClade 4: analysis of phylogeny and character evolution, version 4.0. Sinauer, Sunderland, MA.

McCafferty, W. P. 1990. Chapter 2. Ephemeroptera, pp. 20–50. *In* D. A. Grimaldi [ed.], Insects from the Santana Formation, Lower Cretaceous, of Brazil. Bull. Am. Mus. Nat. Hist. 195.

McCafferty, W. P. 1991a. The cladistics, classification, and evolution of the Heptagenioidea, pp. 87–101. *In* J. Alba-Tercedor and A. Sanchez-Ortega [eds.], Overview and strategies of Ephemeroptera and Plecoptera. The Sandhill Crane Press, Inc., Gainesville, FL.

McCafferty, W. P. 1991b. Toward a phylogenetic classification of the Ephemeroptera (Insecta): a commentary on systematics. Ann. Entomol. Soc. Am. 84: 343–360.

McCafferty, W. P. 1997. Ephemeroptera, pp. 89–117. *In* R. W. Poole and P. Gentili [eds.], Nomina Insecta Nearctica, a check list of the Insects of North America. vol. 4: non-holometabolous orders. Entomological Information Services, Rockville, MD.

McCafferty, W. P. 2004. Higher classification of the burrowing mayflies (Ephemeroptera: Scapphodonta). Entomol. News 115: 84–92.

McCafferty W. P., and G. F. Edmunds, Jr. 1979. The higher classification of the Ephemeroptera and its evolutionary basis. Ann. Entomol. Soc. Am. 72: 5–12.

McCafferty, W. P., and T.-Q. Wang. 2000. Phylogenetic systematics of the major lineages of pannote mayflies (Ephemeroptera: Pannota). Trans. Am. Entomol. Soc. 126: 9–101.

Meyer, M. D., W. P. McCafferty, and E. L. Silldorff. 2003. Relationships of *Edmundsius* Day (Ephemeroptera: Siphlonuridae), with notes on early instar larvae and eggs. Pan-Pac. Entomol. 79: 112–118.

Riek, E. F. 1973. Classification of the Ephemeroptera, pp. 160–178. *In* W. L. Peters and J. G. Peters [eds.], Proceedings of the First International Conference on Ephemeroptera. E. J. Brill, Leiden, The Netherlands.

Sinitshenkova, N. D. 1984. The Mesozoic mayflies (Ephemeroptera) with special reference to their ecology, pp. 61–66. *In* V. Landa, T. Soldán, and M. Toner [eds.], Proceedings of the Fourth International Conference on Ephemeroptera, Czechoslovak Academy of Sciences, Prague, Czech Republic.

Soldán, T., and M. Putz. 2000. Karyotypes of some central European mayflies (Ephemeroptera) and their contribution to phylogeny of the order. Acta Soc. Zool. Bohem. 64: 437–445.

Swofford, D. L. 2002. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0b10. Sinauer, Sunderland, MA.

Thompson, J. D., T. J. Gibons, F. Plewnik, F. J. Mougin, and D. G. Higgins. 1997. The Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by qualitative analysis tools. Nucleic Acids Res. 25: 4876–4882.

Tomka, I., and C. Elpers. 1991. Problems in the phylogeny of the Ephemeroptera, pp. 115–134. *In* J. Alba-Tercedor and A. Sanchez-Ortega [eds.], Overview and strategies of Ephemeroptera and Plecoptera. The Sandhill Crane Press, Inc., Gainesville, FL.

Ulmer, G. 1920. Neue Ephemeropteren. Arch. Naturges. (1919) 85 (Abt. A, Heft 11): 1–80.

Wang, T.-Q., and W. P. McCafferty. 1995. Relationships of the Arthropleidae, Heptageniidae, and Pseudironidae (Ephemeroptera: Heptagenioidea). Entomol. News 106: 251–256.

Zhou, C.-F., and J. G. Peters. 2003. The nymph of *Siphluriscus chinensis* and additional imaginal description: a living mayfly with Jurassic origins (Siphluriscidae new family: Ephemeroptera). Fla. Entomol. 86: 345–352.

Received 24 March 2005; accepted 6 December 2005.