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The Palaeoptera Problem: Basal Pterygote Phylogeny Inferred from 18S and 28S rDNA Sequences

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Monophyly of the pterygote insects is generally accepted, but the relationships among the three basal branches (Odonata, Ephemeroptera and Neoptera) remain controversial. The traditional view, to separate the pterygote insects in Palaeoptera (Odonata + Ephemeroptera) and Neoptera, based on the ability or inability to fold the wings over the abdomen, has been questioned. Various authors have used different sets of morphological characters in support of all three possible arrangements of the basal pterygote branches. We sequenced 18S and 28S rDNA from 18 species of Odonata, 8 species of Ephemeroptera, 2 species of Neoptera, and 1 species of Archaeognatha in our study. The new sequences, in combination with sequences from GenBank, have been used in a parsimony jackknife analysis resulting in strong support for a monophyletic Palaeoptera. Morphological evidence and the phylogenetic implications for understanding the origin of insect flight are discussed.

INTRODUCTION

Insects were the first organisms to evolve self-sustained flight. This event, which may have occurred during the early Devonian (Kukalová-Peck, 1991), has often been cited as a key innovation in insect diversification (e.g., Janzen, 1977; Wootton, 1986; Kingsolver and Koehl, 1994; Wilson, 1996). Daly *et al.* (1978) even made the bold statement that "wings have contributed more to the success of insects than any other structure." Hypotheses about the actual origin of wings, however, are still scenario-based (e.g., Leech and Cady, 1994; Marden and Kramer, 1994; Thomas and Norberg, 1996; Dawkins, 1996) and lack substantial testing.

While there is little doubt that the pterygote insects are a monophyletic group, the relationships among the three basal lineages (Ephemeroptera, Odonata, and Neoptera) have remained controversial and unclear. Traditionally, Ephemeroptera and Odonata have been classified as Palaeoptera (old wings), based on their inability to fold the wings over the abdomen. The remainder of the pterygote insects, who are able to fold their wings over the abdomen due to the presence of auxiliary wing-base sclerites, are placed in the large clade Neoptera (new wings). It may be noted that some representatives of Neoptera, for instance, papilionid Lepidoptera, do not fold their wings, but this is best interpreted as a secondary adaptation.

Whereas the Neoptera is generally accepted as a natural group, the monophyly of Palaeoptera has been

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disputed. The paleontologist Kukalová-Peck (e.g., 1983, 1991, 1997), prefers a monophyletic Palaeoptera, as did Hennig in his later papers (1981). Kristensen (1975), on the other hand, argued for a basal Ephemeroptera but later (Kristensen, 1994, 1995) considered it open an question and stressed the need for a thorough reassessment of all evidence. A different scenario was put forward by Boudreaux (1979), who considered Odonata to be basal and Ephemeroptera and Neoptera to be sister groups. In other words, morphology provides arguments for all three possible phylogenetic topologies at the base of Pterygota.

An early attempt to use molecular information to resolve this controversy was published by Wheeler (1989). Based on evidence from ribosomal DNA using restriction fragment length variation, gene size polymorphism, and direct sequence variation, he found support for the basal position of Ephemeroptera, in agreement with Kristensen (1975). Whiting *et al.* (1997) used rDNA sequences (18S and 28S) and morphology in a landmark phylogenetic study of the insects. Representatives of all pterygote orders as well as apterygote outgroups were included. Palaeoptera was represented by two Odonata and one Ephemeroptera species. However, the different data sets did not agree on basal pterygote relationships. Similar results were obtained in the expanded study by the same authors (Wheeler *et al.* 2001).

Basal pterygote phylogeny remains a challenge. Information from phylogeny is necessary for formulating robust hypotheses about the evolution of insect flight. It is the purpose of the present paper to test basal pterygote phylogenetic hypotheses with more extensive sampling of Palaeoptera and data from complete 18S and partial 28S sequences.

MATERIALS AND METHODS

Taxon Selection

Taxa were chosen to represent the three pterygote subgroups. Where material was available, taxa were chosen to span the variation as far as possible. Ephemeroptera is represented by five families in four suborders. No material from the monogeneric fifth suborder, Carapacea, was available for the study. All three suborders of Odonata are represented. Seven species from two families of Zygoptera, 10 species from three families of Anisoptera and 1 of the 2 species of the suborder Anisozygoptera are included. Four hemimetabolous and four holometabolous insects were selected from Neoptera. 18S and partial 28S rDNA sequences were produced for this study. Other included sequences were retrieved from GenBank (Table 1). For Mecoptera, represented by *Panorpa*, the complete 18S sequence from *Panorpa germanica* was combined with the partial 28S sequence from *Panorpa latipennis*. For all other taxa 18S and 28S sequences represent the same species.

Zygentoma is generally considered to be the sister group of the pterygotes (Hennig, 1981; Kristensen, 1991). Two sequences were available from *Lepisma*, one from *Lepisma* sp. and another from *Lepisma saccharina* (GenBank Accession Nos. AF005458 and X89484). When compared, these sequences are highly divergent from each other. For this reason, we chose to use two species from the nondicondylar insect order Archaeognatha, *Petrobius brevistylis* and *Trigoniophthalmus alternatus*, as outgroups in the final analyses. See Discussion.

DNA Extraction, PCR, and Sequencing

Specimens were preserved in 95% alcohol. For dragonflies and larger mayflies, wing or leg muscle fibers were dissected out and used for extraction. The entire thorax was used from smaller mayflies and apterygote insects. The tissues were rehydrated briefly in distilled water prior to extraction. For most samples, the Qiagen tissue kit (Qiagen) was used. A few samples were extracted using a standard phenol–chloroform–isoamyl alcohol protocol.

The 18S rDNA sequences were amplified as two overlapping segments. PCR and sequencing primers are listed in Table 2. Two different strategies were used: (1) The entire fragment was first amplified with TIM A–TIM B, and two fragments, A and B, were subsequently amplified from the first PCR with primers TIM A–1100R and 600F–TIM B; (2) the overlapping fragments s30–5fk and 400f–1806R were amplified directly. Positions of primers used for PCR are shown in Fig. 1.

An ~600-bp fragment of the 28S rDNA gene was amplified using primers 28SA and 28SBout, corresponding to positions 759–778 and 1315–1338 of the *Drosophila* 28S sequence (part of the *Drosophila* ribosomal rDNA region in GenBank Accession No.

TABLE 1
Names of Terminal Taxa with GenBank Accession Numbers

Taxon		Accession No. 18S	Accession No. 28S	Comment
Archaeognatha				
Machillidae	*Petrobius brevistylis*	AF461258	AF461229	This study
	Trigoniophthalmus alternatus	U65106	U65166	
Ephemeroptera				
Baetidae	*Baetis buceratus*	AF461248	AF461219	This study
	Centroptilum luteoleum	AF461251	AF461221	This study
	Cloeon dipterum	AF461249	AF461220	This study
Heptageniidae	*Leucrocuta aphrodite*	AF461254	AF461224	This study
	Stenonema sp.	AF461252	AF461223	This study
Ephemeridae	*Hexagenia rigida*	AF461253	AF461222	This study
Potamanthidae	*Anthopotamus* sp.	AF461255	AF461226	This study
Caenidae	*Caenis luctuosa*	AF461250	AF461225	This study
Odonata				
Zygoptera				
Coenagrionidae				
	Coenagrion hastulatum	AF461234	AF461207	This study
	Coenagrion sp.[a]	AF461235	AF461213	This study
	Enallagma cyathigerum	AF461237	AF461201	This study
	Erythromma najas	AF461238	AF461209	This study
	Ischnura elegans	AF461239	AF461215	This study
	Pyrrhosoma nymphula	AF461241	AF461202	This study
Lestidae	*Lestes sponsa*	AF461244	AF461204	This study
Anisozygoptera				
Epiophlebiidae	*Epiophlebia superstes*	AF461247	AF461208	This study
Anisoptera				
Aeshnidae	*Aeshna juncea*	AF461231	AF461205	This study
	Aeshna cyanea	AF461230	AF461203	This study
	Brachytron pratense	AF461232	AF461217	This study
Corduliidae	*Cordulia aenea*	AF461236	AF461210	This study
	Somatochlora flavomaculata	AF461242	AF461212	This study
Libellulidae	*Celithemis eponina*	AF461233	AF461218	This study
	Leucorrhinia pectoralis	AF461240	AF461206	This study
	Sympetrum danae	AF461243	AF461211	This study
	Sympetrum sanguineum	AF461245	AF461214	This study
	Sympetrum vulgatum	AF461246	AF461216	This study
Plecoptera				
Nemouridae	*Nemoura cinerea*	AF461257	AF461227	This study
Perlodidae	*Isoperla obscura*	AF461256	AF461229	This study
Orthoptera				
Acrididae	*Melanoplus* sp.	U65115	U65173	
Hemiptera				
Saldidae	*Saldula pallipes*	U65121	U65175	
Hymenoptera				
Ichneumonidae	*Ophion* sp.	U65151	U65193	
Coleoptera				
Tenebrionidae	*Tenebrio molitor*	X07801	X90683	
Megaloptera				
Corydalidae	*Corydalus cognathus*	U65132	U65186	
Mecoptera				
Panorpidae	*Panorpa germanica*	X89493		
	Panorpa latipennis		U65207	

[a] Note that this is either *Coenagrion puella* or *Coenagrion pulchellum*. Material was taken from a nymph of either species, which are indistinguishable at this stage.

TABLE 2
Names and Sequences of 18S and 28S Primers Used in This Study

Primer name	Used for	Primer sequence	Reference
18S Primers			
Tim A	PCR, sequencing	5′-amctggttgatcctgccag-3′	Norén and Jondelius (1999)
Tim B	PCR, sequencing	5′-tgatccatctgcaggttcacct-3′	Norén and Jondelius (1999)
600F	PCR, sequencing	5′-ggtgccagcmgccgcggt-3′	Norén and Jondelius (1999)
1100R	PCR, sequencing	5′-gatcgtcttcgaacctctg-3′	Norén and Jondelius (1999)
18S1F	Sequencing	5′-tacctggttgatcctgccagtag-3′	Norén and Jondelius (1999)
18S30	PCR, sequencing	5′-gcttgtctcaaagattaagcc-3′	Norén (pers. comm.)
18S3F	Sequencing	5′-gttcgattccggagagggagcctg-3′	Norén and Jondelius (1999)
18S3FK	Sequencing	5′-caggctccctctccggaatcgaac-3′	Norén and Jondelius (1999)
18S4F	PCR, sequencing	5′-ccagcagccgcgtaattc-3′	Norén (pers. comm.)
18S4FB	Sequencing	5′-ccagcagccgcggtaattccag-3′	Norén (pers. comm.)
18S4FBK	Sequencing	5′-ctggaattaccgcggctgctgg-3′	Norén (pers. comm.)
18S5F	Sequencing	5′-gcgaaagcatttgccaagaa-3′	Norén and Jondelius (1999)
18S5FK	Sequencing	5′-ttcttggcaaatgctttcgc-3′	Norén and Jondelius (1999)
18S7F	Sequencing	5′-gcaataacaggtctgtgatgc-3′	Norén and Jondelius (1999)
18S7FK	Sequencing	5′-gcatcacagacctgttattgc-3′	Norén and Jondelius (1999)
1806R	PCR, sequencing	5′-ccttgttacgacttttacttcctc-3′	Norén (pers. comm.)
28S primers			
28Sa	PCR, sequencing	5′-gacccgtcttgaaacacgga-3′	Wheeler (pers. comm.)
28Sbout	PCR, sequencing	5′-cccacagcgccagttctgcttacc-3′	Wheeler (pers. comm.)

M21017). A list of the primer sequences used is given in Table 2.

DNA was sequenced using cycle sequencing. Most taxa were sequenced on ABI automatic sequencers (PE Biosystems) using a standard Prism dye terminator cycle sequencing reaction kit (ABI, PE Biosystems). The remaining taxa were sequenced on an ALFexpress DNA Sequencer (Pharmacia-Biotech), using the Amersham Thermo Sequenacse Sequencing kit. Both strands of DNA, except minor parts, were sequenced for most taxa. Where only one strand could be sequenced, the difficult region of the single strand was sequenced at least twice. A schematic of primers used for sequencing is illustrated in Fig. 2.

Fragments were checked for contamination with the BLAST search engine (Altschul *et al.*, 1997). The Staden Package (Staden, 1996) or Sequencher (Gene Codes Corp.) was used for sequence assembly and evaluation.

Sequences were aligned using ClustalX version 1.8 (Thompson *et al.*, 1997). A variety of settings for gap opening penalty were used in a series of trial alignments. For analysis we selected matrices made with a gap opening penalty of 75 for both 18S and 28S. The ends of the matrices were trimmed at conservative positions. In the 28S alignment, a hypervariable region of 222 bases was excised prior to analysis.

The matrices were analyzed with parsimony jackknifing (Farris *et al.*, 1996) using the software XAC (Farris, 1997). One thousand replicates with branch swapping and 10 random additions each were used in all analyses. Branches with a jackknife support of 50% or less were collapsed. The trees were rooted using the

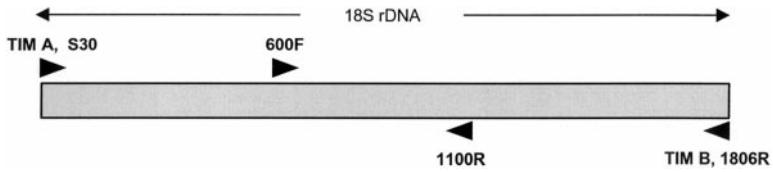

FIG. 1. Position of PCR primers.

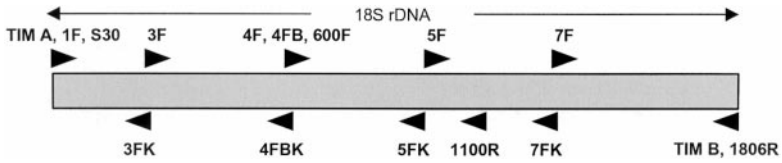

FIG. 2. Position of sequencing primers.

outgroup criterion (Farris, 1972) with *P. brevistylis* and *T. alternatus* as outgroups.

RESULTS

Sequence Alignment

Alignment of the 18S sequences at a gap opening penalty of 75 produced a matrix that was 2127 sites long. The terminal 130 sites were trimmed off prior to analysis. The resulting matrix was 1997 sites long, containing 393 informative characters.

Alignment of the 28S sequences at a gap opening penalty of 75 produced a matrix that was 707 sites long. The first 222 sites and the terminal 146 sites were trimmed off prior to any analysis (see Discussion). The resulting matrix was 338 sites long, containing 43 informative characters.

Matrices have been deposited at TreeBase (http://treebase.org).

18S Tree

The 18S tree (Fig. 3) resolves two well-supported basal branches. Neoptera has a jackknife support of 93%. A monophyletic Palaeoptera is supported by a jackknife value of 86%. Both subgroups of Palaeoptera, Ephemeroptera and Odonata, are stable at 100% support.

Ephemeroptera is split into two well-supported branches, with Baetidae as sister to the remainder of the ephemeropteran taxa. Odonata is split into two weakly supported groups: one clade containing the zygopteran taxa and the other containing a trichotomy of *Epiophlebia*, Aeshnidae, and Libellulidae + Corduliidae.

In Neoptera, the monophyly of the holometabolous insects is supported by a jackknife value of 65%.

28S Tree

In the 28S tree (Fig. 4), only 9 nodes are resolved compared to 26 in the 18S tree. The only well-supported group is Baetidae, at 85%. Other resolved groups are Plecoptera (70%), Ephemeridae + Caenidae (54%), Corduliidae (52%), Heptageniidae (69%), Aeshnidae (52%), Holometabola (59%), and Zygoptera (54%). Pterygota is not found in this tree where nine pterygote taxa end up in the basal polytomy.

Combined Analysis

When the 18S and 28S data sets were combined (Fig. 5), monophyly of Odonata, Ephemeroptera, and Neoptera is well supported. The support value for a monophyletic Palaeoptera increased to 94% compared to 86% in the 18S tree. Monophyly of the holometabolous insects is well supported at 89%.

The hypothesized phylogenetic relationships among Ephemeroptera is consistent with, but less resolved than in the 18S tree. Odonata is split into the Zygoptera and *Epiophlebia*-Anisoptera trichotomy in this tree as well. Aeshnidae, Corduliidae, Libellulidae, and Coenagrionidae are found in the tree.

DISCUSSION

For our 18S study, we initially included Zygentoma, represented by two highly divergent GenBank sequences of *Lepisma* (*Lepisma* sp. AF005458 and *L. saccharina* X89484). By including one at a time in a jackknife analysis (trees not shown), rooted on Archaeognatha, we discovered that they ended up in different parts of the tree. *L. saccharina* was found in an unresolved position outside Pterygota. *Lepisma* sp., on the other hand, was nested within Pterygota, as the poorly supported sister group of Odonata. We chose to exclude

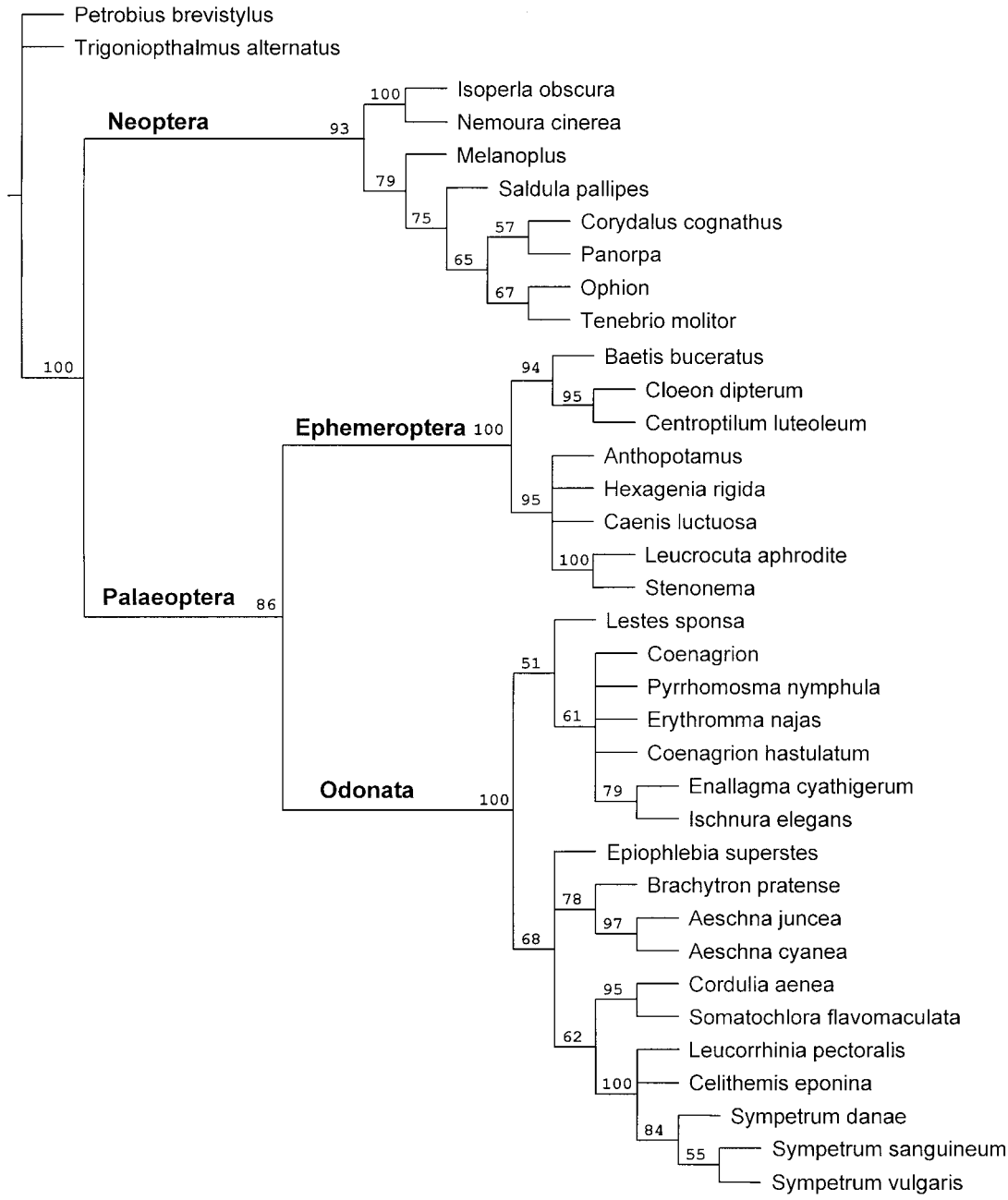

FIG. 3. 185 tree. Numbers on branches on branches indicate jackknife support values.

both *Lepisma* sequences until either one can be confirmed. We think that using Archaeogntha as the outgroup is sufficient for the present purpose of testing Palaeoptera monophyly.

rDNA sequences are often difficult to align as they differ in length. Within Arthropoda, the 18S rDNA gene varies in length between 1350 and 2700 bp (Giribet and Ribera, 2000). Smaller, but substantial, length differences are found among the hexapods. For this study we made several alignments using different parameters. We found that gap opening penalties in the upper range ensured that the insertion of ~150 bp, starting around position 750, in the 18S rDNA of the plecopteran *Isoperla obscura* aligned properly. Alignments us-

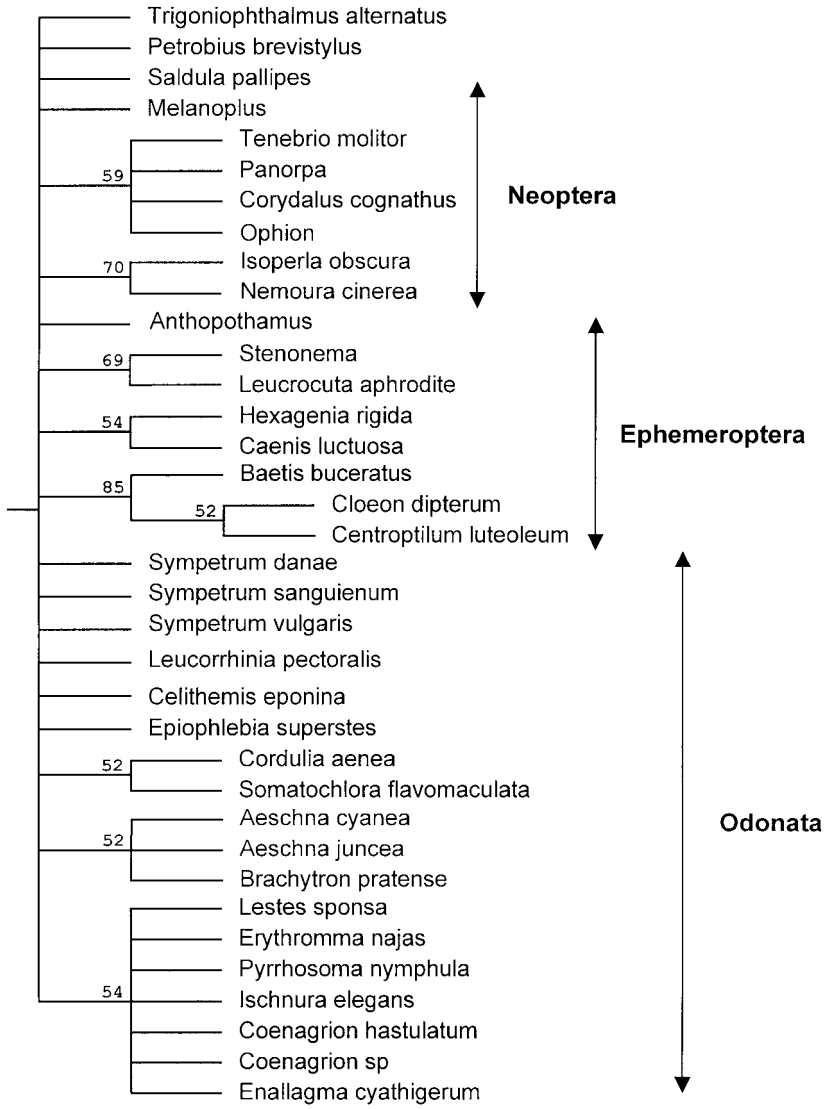

FIG. 4. 285 tree. Numbers indicate jackknife support values.

ing lower range gap opening penalties randomly aligned pieces of flanking regions of other taxa throughout the insertion. The trees resulting from analyses of matrices made with higher gap opening penalties also had generally higher jackknife support.

For this study, we decided to remove a section of the 28S sequences in the final analyses. Regional variations in 28S rDNA were extreme compared to 18S. The fragment used in this study can be divided into a hypervariable and a "rock conservative" region. We tried many different settings for aligning the sequences, but none provided a justifiable alignment for the hypervariable region. In the conservative region, local length differences are small, except for an insertion in Plecoptera.

The combined tree resolves a monophyletic Palaeoptera with high jackknife support (94%). In the 28S tree, neopterans have collapsed into a basal polytomy. Still, the 28S data set does not conflict with the 18S data set, as jackknife support for Palaeoptera has increased from 86% in the 18S tree to 94% for the combined tree. Most of the resolution in the combined tree stems from the 18S data. Nine nodes are found in the 28S tree, 26 in the 18S tree, and 28 in the combined tree. All the

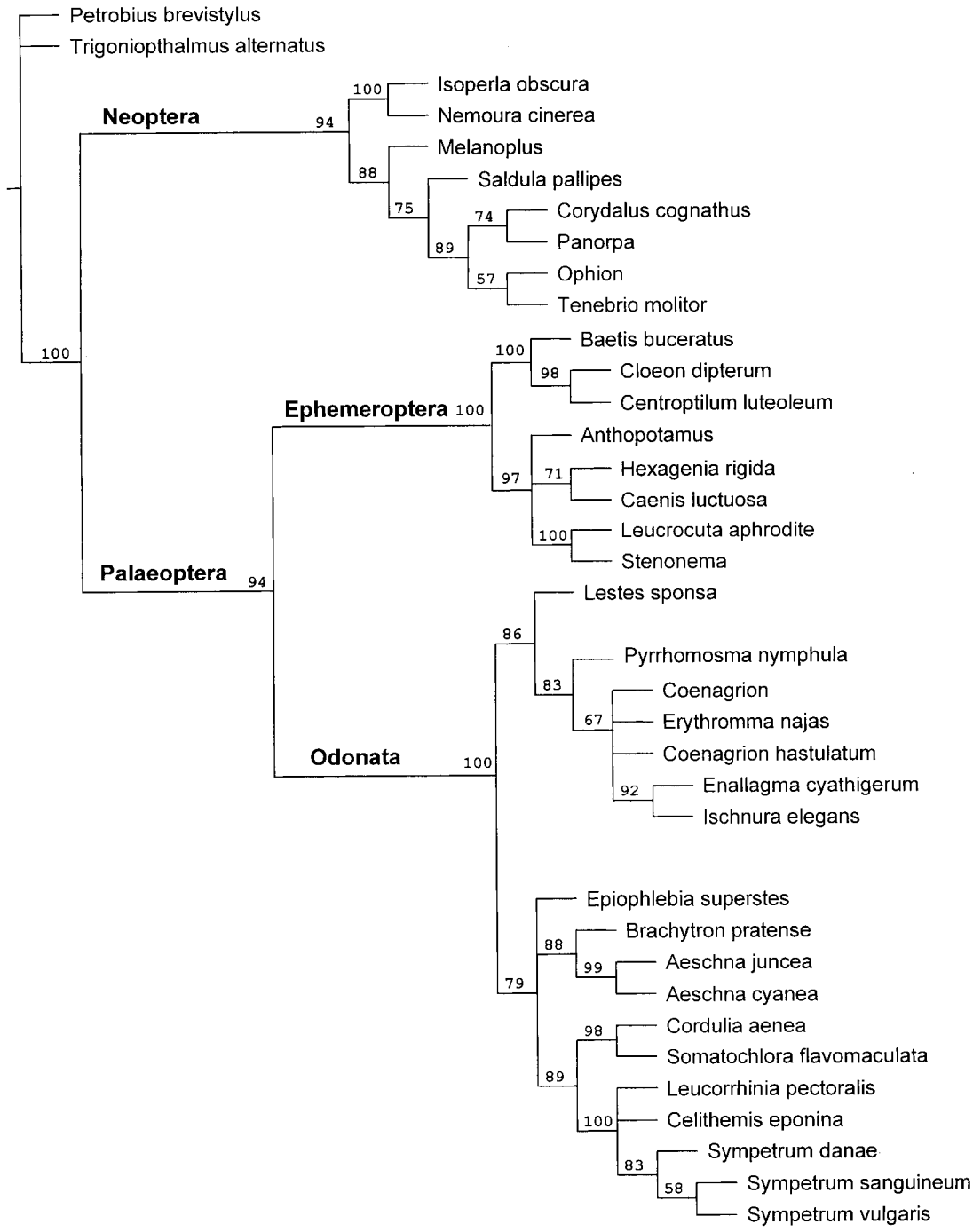

FIG. 5. Combined 185 + 285 tree. Numbers on branches indicale jackknife support values.

nodes found in the 18S tree appear in the combined tree, with increased or unaltered jackknife support.

Groups resolved above the basal dichotomy are generally commonly accepted groups. Holometabola is supported with 89%. Baetidae (100%) and Heptageniidae (100%) are found within Ephemeroptera. Within Odonata, Coenagrionidae (83%), Corduliidae (100%), Aeshnidae (88%), and Libellulidae (100%) are de-

limited. The two groups of Zygoptera sampled, Lestidae and Coenagrionidae, form a clade. This might as well be the result of narrow sampling and should not be taken as support for monophyly of Zygoptera.

In this study, the position of *Epiophlebia* is either in an unresolved clade also containing Anisoptera (18S and combined trees) or in a basal odonate polytomy (28S).

Wheeler *et al.* (2001) published an expanded version of their 1997 study using three data sets: 18S, 28S, and morphology. About 1000 bp of 18S and 400 bp of 28S rDNA were sequenced in a total of 122 18S and 88 28S hexapod sequences. The most-parsimonious trees were presented, with Bremer support values in a separate table. Three species each from Ephemeroptera and Odonata were included. The monophyletic Palaeoptera in the 18S tree was contradicted by the odd basal branchings of the 28S tree. In this tree the basal pterygote dichotomy was between a clade containing Odonata as the sister group of Mantodea + Embioptera and a clade with Ephemeroptera as the sister group of the other insects. No support values were given for this tree, but support was probably very low as Palaeoptera returned in the combined molecular tree. In the morphological tree, Ephemeroptera is basal, and Odonata + Neoptera is supported by six unambiguous character states. However, the interpretation of the characters supporting Odonata + Neoptera follows Kristensen (1975) very closely. Conflicting basal pterygote characters from Kukalová-Peck (1991), Hennig (1981), or Boudreaux (1979) are only briefly discussed.

A monophyletic Palaeoptera is supported by the opinions of Hennig (1981) and Kukalová-Peck (1983, 1991, 1997). Hennig lists three character states as synapomorphies of Palaeoptera: (1) the short bristle-like flagellum of adult antenna; (2) the intercalary veins in the adult wing, which arise between the true longitudinal veins as a result in modifications in the archedictyon; and (3) fusion of galea and lacinia into a single lobe in the nymphal maxilla. Kukalová-Peck lists six characters, focusing on characters *lost* in Palaeoptera. Only one character listed is shared in common with those listed by Hennig: (1) wing vein M always with a basal stem; (2) veins strongly fluted and veinal ridges expressed mostly in only one membrane (dorsal or ventral); (3) thoracic coxal endites eliminated; (4) all pregenital sterna expanded and endites and their original triangular shape lost; (5) cercal coxal endite completely eliminated; and (6) galea and lacinia always fused.

Kukalová-Peck (e.g., 1983, 1991, 1997) supports the aquatic origin hypothesis, with wings evolving from gill pads. The gills on crustacean legs are seen as the origin of the insect wings, implicitly requiring that the gill/wing structure disappeared in apterygote hexapods only to reappear in Pterygota.

Leech and Cady (1994) preferred not to homologize wings to any known structure, instead suggesting that gills were derived from the dorsal edges of the pleurites. Their scenario for wing evolution involves a functional shift from gill pads to wings in fresh water, where the gills served both as respiratory devices and as a means of dispersal by wind. With the extant Palaeoptera, as a monophyletic group, an aquatic wing origin is not supported by phylogeny. Hennig (1981) briefly discusses a terrestrial scenario with wings originating from immobile extensions of the paranota used for gliding flight and motility being acquired secondarily from muscles that originally had other functions. Other terrestrial scenarios involve a function shift from solar panels (Dawkins, 1996; Kingsolver and Koehl, 1985) or controlled falling from plants (Snodgrass, 1958). For a synthetic view of insect wing evolution, see the review by Kingsolver and Koehl (1994).

Boudreaux (1979) and Kristensen (1975, 1991) presented characters supporting alternate basal phylogenies. In Boudreaux's model, Odonata is one of the basal branches, with Ephemeroptera and Neoptera as sister groups. His most convincing synapomorphy is the gonopore to gonopore copulation seen in Ephemeroptera and Neoptera. Mating behavior in Odonata is very specialized, and the indirect sperm transfer via a secondary sexual organ may be a secondary adaptation. Kristensen presents seven characters supporting a basal Ephemeroptera hypothesis, with Odonata as the sister group of Neoptera. Apart from characters dealing with musculation and tracheation, the absence of the subimago is seen as a synapomorphy for Neoptera + Odonata. Ephemeroptera are the only extant insects to have a winged subimago state, molting into the sexually mature imago. The apterygote insects and most other arthropods do not have a final stage, molting even after reaching sexual maturity. Unless this is

seen as a subimaginal state (as coded in the morphological dataset by Whiting *et al.*, 1997; Wheeler *et al.* 2001), the winged subimago is best viewed as an autoapomorphy of Ephemeroptera.

Kristensen (1991) wisely concludes his section on basal pterygote phylogeny by stating that "the problem of the basic dichotomy in extant pterygotes cannot be solved without postulating disturbing homoplasy one way or another." In this study, we have used information from 18S and 28S rDNA from the nuclear genome and found this supporting a monophyletic Palaeoptera. The Palaeoptera problem will have to be further evaluated as information is added from the mitochondrial genome, other nuclear genes, and reanalyses of morphology.

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