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The problem with "the Paleoptera Problem:" sense and sensitivity

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Abstract

While the monophyly of winged insects (Pterygota) is well supported, phylogenetic relationships among the most basal extant pterygote lineages are problematic. Ephemeroptera (mayflies) and Odonata (dragonflies) represent the two most basal extant lineages of winged insects, and determining their relationship with regard to Neoptera (remaining winged insects) is a critical step toward understanding insect diversification. A recent molecular analysis concluded that Paleoptera (Odonata + Ephemeroptera) is monophyletic. However, we demonstrate that this result is supported only under a narrow range of alignment parameters. We have further tested the monophyly of Paleoptera using additional sequence data from 18SrDNA, 28S rDNA, and Histone 3 for a broader selection of taxa and a wider range of analytical methodologies. Our results suggest that the current suite of molecular data ambiguously resolve the three basal winged insect lineages and do not provide independent confirmation of Odonata + Neoptera as supported via morphological data.

Paleoptera (=Palaeoptera) refers to the grouping of extinct paleodictyopteroids, Ephemeroptera, and Odonata (Hennig, 1981; Kukalova-Peck, 1983, 1985, 1991, 1997; Riek and Kukalova-Peck, 1984). However, the monophyly of this group is still a controversial issue in insect evolution (Beutel and Gorb, 2001; Staniczek, 2000; Wheeler et al., 2001; Whiting et al., 1997). The extant paleopterous insects—dragonflies and damselflies (=Odonata), and mayflies (=Ephemeroptera)—lack the retractor muscle and wing sclerites necessary to fold the wings over the abdomen (Martynov, 1924). The absence of this feature has been suggested as evidence for the group's monophyly. However, this character may simply be symplesiomorphic because the muscles and sclerites allowing insects to fold wings over their abdomen were gained in the neopterous insects (Martynov, 1924). This innovation is presumably correlated with the huge explosion of neopterous species. Despite being one of the most important diversification events in all of evolution, the resolution of the relationships among Ephemeroptera, Odonata, and Neoptera remains ambiguous, and all resolutions of this three-taxon statement have been proposed.

The first hypothesis will be referred to as the *basal Ephemeroptera hypothesis* and it suggests that Ephemeroptera is sister to Odonata + Neoptera (Fürst von Lieven, 2000; Kristensen, 1991; Staniczek, 2000; Wheeler et al., 2001; Whiting et al., 1997). Six morphological characters proposed to support this hypothesis are (1) the anterior articulation of the mandible is a nonpermanent sliding groove and track system in Ephemeroptera, but in other pterygote lineages this articulation is more permanent; (2) subimago stage is present in Ephemeroptera but absent in other pterygotes; (3) tracheation is absent in arch of wing base and in posterior portion of the leg in Ephemeroptera but present in other insects; (4) direct spiracular musculature is absent in Ephemeroptera but present in odonates and neopterans; (5) never more than one tentorial-mandibular muscle is present in Odonata and Neoptera but multiple muscles are present in Ephemeroptera; (6) annulated caudal filament is presumably present in Archaeognatha, Monura, Zygentoma, and Ephemeroptera but absent in the remaining pterygotes; and (7) paired female genital openings are retained in Ephemeroptera and nowhere else among Pterygota. However, with some of these characters, it is unclear whether they are simply autapomorphies of Ephemeroptera or synapomorphies for Odonata + Neoptera.

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The second hypothesis, termed the *Paleoptera hypothesis*, suggests that Ephemeroptera is sister group to Odonata, forming the group Paleoptera (Brodsky, 1994; Hennig, 1981; Kukalova-Peck, 1983, 1985, 1991, 1997; Martynov, 1924; Riek and Kukalova-Peck, 1984). This hypothesis is supported by the following characters: (1) short antennae; (2) fusion of galea and lacinia; (3) lack of the ability to fold back the wings over the abdomen; (4) veinal braces in the wings; (5) separated R and M wing veins; (6) wing fluting; and (7) aquatic larvae. Still, some of these characters (e.g., 1, 3, 7) have been regarded as plesiomorphic (Wheeler et al., 2001; Willmann, 1997).

The third hypothesis places Odonata as sister to Ephemeroptera + Neoptera and will be referred to as the *basal Odonata hypothesis* (Boudreaux, 1979; Matsuda, 1970). This hypothesis is based primarily on the character that direct sperm transfer is synapomorphic for Ephemeroptera + Neoptera. Given that the "apterygotes" and Odonata have indirect sperm transfer, the gonopore-to-gonopore mode could be considered a shared derived character for mayflies and neopterous insects. However, the specific kind of indirect sperm transfer of the odonates appears to be quite different from those of the "apterygotes." Odonate males deposit the sperm from segment 9 to an accessory gland on segment 2. Then, when in tandem (the position where the male grasps the female by the head with his terminalia), the female bends her abdomen down and forward to receive the sperm in her reproductive opening on segment 8. This complicated process does not resemble the indirect sperm transfer of "apterygotes" and is most likely autapomorphic, providing no phylogenetic information at an ordinal level (Beutel and Gorb, 2001).

Due to the disagreement among, and questionable utility of, certain morphological characters, it is important to provide independent data that can corroborate one of these hypotheses to provide a more accurate estimate of phylogeny. We are particularly interested in the sensitivity of molecular topologies to perturbations of parameter values during phylogenetic analysis (Phillips et al., 2000; Wheeler, 2001, 1995). We specifically define robustness as a measure of stability of nodes to fluctuations in parameter values across an analytical landscape. A highly robust node is one that is supported under a wide range of parameter values, in contrast to a poorly supported node that is supported under only one or a few parameter values. We recognize that sensitivity analysis is only one measure of topological robustness and that other measures are currently in vogue (e.g., nonparametric bootstrap, Bremer support, posterior probabilities, etc.) (Archie, 1989; Bremer, 1988; Faith, 1991; Faith and Cranston, 1991; Felsenstein, 1985), each with their own pros and cons (Grant and Kluge, 2003). However, given that the current molecular data used to infer paleopteran phylogeny is primarily ribosomal DNA sequences and that the topologies generated via these sequences are strongly influenced by alignment methodologies, we are interested in addressing the question of whether any analytical method will robustly support one of the three hypotheses listed above under a wide range of parameter values. In approaching the question in this manner, we do not attach any particular significance to congruence among disparate analytical methodologies. We are interested only in determining whether a robust solution exists for the given data under any analytical methodology or whether the molecular data do not discriminate among the hypotheses.

Independent tests (i.e., molecular data) have provided mixed support for the different hypotheses. For instance, Wheeler et al. (2001) published the most extensive formal analysis of ordinal relationships using molecular and morphological information. The 18S rDNA (18S) data and 18S + 28S rDNA (28S) data supported a monophyletic Paleoptera, but the 28S data and the total-evidence analyses (including morphology) supported basal Ephemeroptera. This study, however, did not concentrate sampling on basal pterygotes, so the extent to which these results are influenced by the under sampling of taxa is not clear. In a recent molecular analysis, the relationship among basal pterygotes was specifically tested and the authors conclude that Paleoptera is monophyletic (Hovmöller et al., 2002). However, given the difficult nature of the Paleoptera problem, and some potential flaws in their analytical methodology, we were interested in determining the generality of their conclusion, given additional data and analyses.

The overall objective, therefore, is to determine whether a robust solution to the Paleoptera problem exists given current data and analytical methods. This objective will be specifically examined by two subgoals: (1) test the generality of the claim that the current molecular data support the monophyly of Paleoptera as presented by Hovmöller et al. (2002); (2) provide additional data and analyses to test the sensitivity of the topology to data partitions, cost parameter values, and methods of data analysis.

Materials and methods

Reanalysis of Hovmöller et al. (2002) data

In the Hovmöller et al. (2002) study, sequence data from 18S rDNA and partial 28S rDNA for 18 spp. of Odonata, 8 spp. of Ephemeroptera, 8 spp. of Neoptera, and 2 spp. of Archaeognatha were used to estimate phylogeny. This taxon sampling represents 22% (6 of 27) of the odonate family taxa and 14% (5 of 36) of the mayfly family taxa. No morphological data were incorporated in their analyses, though coded character matrices were available (Beutel and Gorb, 2001;

Wheeler et al., 2001). Reanalysis of their molecular data was performed on each gene separately (18S and 28S) and in a combined analysis. To test the sensitivity of their topology toward alignment parameter values, we imported their sequences into ClustalX (Thompson et al., 1997) and analyzed them under a variety of parameter values. The authors did not report specific alignment parameters, so a wide range of alignment parameters were explored. For all alignments, delay divergent % was set to 30, DNA transition weight was set to 0, and DNA weight matrix was set to ClustalW(1.6), since these are the standard defaults for the program. Gap opening costs were set to the following values: 1, 2, 5, 10, 20, 30, 40, 50, 75, 85, and 100 (Table 1). Gap extension costs either were set to 1 or were equal to the gap opening costs. This resulted in 21 analyses per partition (18S, 28S, and combined), for a total of 63 matrices. These matrices were imported into PAUP*4.0b10 (Swofford, 2002) and analyzed under parsimony, with gaps treated as missing data and as a fifth state character. We executed 100 random additions with TBR branch swapping and strict consensus trees were constructed for each of the 126 analyses (Table 1). This wide selection of parameters appears sufficient to test the sensitivity of the Hovmoller data to varying alignment parameters.

Additional data

To further test resolution among basal pterygote lineages, we generated additional sequence data to more thoroughly represent the taxonomic diversity of these groups. From the Hovmöller et al. (2002) study, we included 13 odonate genera which were not represented in our samples. We decided not to include any of the Hovmöller mayfly sequences, as we have a very extensive sampling of mayfly taxa from nearly all families and have a very good indication of mayfly phylogeny based on these data (T.H. Ogden, unpublished). This allowed us to include sequences that more thoroughly represent the taxonomic diversity of Ephemeroptera. To the Hovmoller taxa, we added 50 more taxa, including 8 additional odonate genera, 7 "apterygote" hexapod spp., 23 genera of mayflies representing 22 families, and 12 taxa within the Polyneoptera to represent the neopteran lineages, for a combined total of 63 taxa (Table 2). This sampling represents 33% of odonate families and 62% of mayfly families. We also included the morphological data matrix coded by Wheeler et al. (2001) for these orders.

Muscle tissue was dissected, incubated, and DNA was extracted following the Qiagen DNeasy protocols. Templates and controls were amplified in a Perkin-Elmer 9700 thermocycler using primers modified for insects. Three genes were targeted for amplification and sequencing: 18S, 28S, and Histone 3 protein coding for the nucleosome (H3). Primer sequences for 18S and 28S are

Table 1
Results of reanalysis of the Hovmöller et al. (2002) data set

Data partition	18S rDNA				28S rDNA				Combined (18S + 28S)			
ClustalX gap costs:	Gap extension = 1		Gap opening = Gap extension		Gap extension = 1		Gap opening = Gap extension		Gap extension = 1		Gap opening = Gap extension	
PAUP treatment of gaps:	Gap = ?	Gap = 5th	Gap = ?	Gap = 5th	Gap = ?	Gap = 5th	Gap = ?	Gap = 5th	Gap = ?	Gap = 5th	Gap = ?	Gap = 5th
Gap opening = 1	Unres	Unres	Unres	Unres	Eph*	Unres	Eph*	Unres	Pal*	Unres	Pal*	Unres
Gap opening = 2	Unres	Unres	Odo*	Unres	Unres	Unres	Eph*	Unres	Pal*	Odo*	Pal*	Odo
Gap opening = 5	Pal*	Unres	Pal*	Unres	Unres	Eph*	Unres	Unres	Pal*	Unres	Pal*	Odo
Gap opening = 10	Pal*	Unres	Odo	Unres	Unres	Unres	Unres	Odo	Pal*	Odo	Odo	Odo
Gap opening = 20	Pal*	Unres	Pal*	Unres	Unres	Unres	Unres	Unres	Pal*	Odo	Pal	Unres
Gap opening = 30	Pal*	Unres	Odo	Unres	Unres	Unres	Unres	Unres	Pal	Odo	Pal	Odo
Gap opening = 40	Pal*	Unres	Odo	Unres	Unres	Unres	Unres	Eph	Pal	Odo	Odo	Odo
Gap opening = 50	Pal	Unres	Odo	Unres	Unres	Eph*	Unres	Eph	Pal	Pal	Odo	Odo
Gap opening = 75	Pal	Unres	Unres	Unres	Unres	Odo*	Eph	Unres	Odo	Odo	Pal	Odo
Gap opening = 85	Pal	Unres	Pal*	Unres	Unres	Odo*	Unres	Eph	Unres	Unres	Pal	Unres
Gap opening = 100	Pal	Unres	Unres	Unres	Unres	Odo*	Unres	Eph	Pal	Pal	Unres	Unres

Unres, analysis supported unresolved topology (this could be a trichotomy or nonmonophyletic Odonata and/or Ephemeroptera); Eph, analysis supported basal Ephemeroptera; Odo, analysis supported basal Odonata; Pal, analysis supported monophyletic Paleoptera.
*Neoptera was not resolved as a monophyletic group.

Table 2
Taxon list and Genbank accession numbers (X = no sequence information)

Order	Family	Genus species	18S rDNA	28S rDNA	H3
Collembola	Hypogastruridae	*Hypogastrura* sp.	AY338691	AY338648	AY338616
Diplura	Campodeidae		AY338692	AY338649	X
Archaeognatha	Machilidae	*Machilsi* sp.	AY338689	AY338646	AY338614
	Machilidae	*Machilis* sp.	AY338690	AY338647	AY338615
Zygentoma	Lepismatidae	*Thermobia* sp.	AY338726	AY338683	AY338644
	Lepidotrichidae	*Tricholepidion* sp.	AY338727	AY338684	AY338645
	Noticoliidae	*Battigrassiella* sp.	AY338728	AY338685	X
Ephemeroptera	Acanthametropodidae	*Analetris eximia*	AY338697	AY338654	AY338620
	Ameletidae	*Ameletus* sp.	AY338712	AY338669	AY338632
	Ameletopsidae	*Chaquihua* sp.	AY338715	AY338672	AY338635
	Ametropodidae	*Ametropus neavei*	AY338700	AY338657	AY338622
	Baetidae	*Baetis* sp.	AY338695	AY338652	AY338619
	Baetiscidae	*Baetisca* sp.	AY338707	AY338664	AY338627
	Behningiidae	*Behningia* sp.	AY338703	AY338660	X
	Caenidae	*Caenis* sp.	AY338710	AY338667	AY338630
	Coloburiscidae	*Coloburiscus humeralis*	AY338706	AY338663	AY338626
	Ephemerellidae	*Drunella coloradensis* AY338694	AY338651	AY338618	
	Ephemeridae	*Hexagenia* sp.	AY121136	AY125276	AY125223
	Euthyplociidae	*Polyplocia* sp.	AY338705	AY338662	AY338625
	Heptageniidae	*Heptagenia* sp.	AY121137	AY125277	AY125224
	Isonychiidae	*Isonychia* sp.	AY338708	AY338665	AY338628
	Leptohyphidae	*Leptohyphes apache*	AY338714	AY338671	AY338634
	Heptageniidae	*Cinygmula* sp.	AY338704	AY338661	AY338624
	Metropodidae	*Metretopus borealis*	AY338698	AY338655	AY338621
	Neoephemeridae	*Neophemera youngi*	AY338702	AY338659	X
	Oligoneuriidae	*Lachlania saskatchewanensis*	AY338701	AY338658	AY338623
	Potamanthidae	*Anthopotamus* sp.	AY338711	AY338668	AY338631
	Pseudironidae	*Pseudiron centralis*	AY338699	AY338656	X
	Rallidentidae	*Rallidens mcfarlanei*	AY338696	AY338653	X
	Siphlonuridae	*Paramaletus columbiae*	AY338713	AY338670	AY338633
Odonata	Aeshnidae	*Anax junius*	AY338719	AY338676	AY338639
	Aeshnidae	*Aeshna juncea*	AF461230	AF461205	X
	Aeshnidae	*Brachytron pratense*	AF4611232	AF461217	X
	Calopterygidae	*Calopteryx aequabilis*	AY338716	AY338673	AY338636
	Calopterygidae	*Heterina americana*	AY338718	AY338675	AY338638
	Coenagrionidae	*Argia vivida*	AY121144	AY125284	AY125229
	Coenagrionidae	*Coenagrion hastulatum*	AF461234	AF461207	X
	Coenagrionidae	*Enallagma cyathigerum*	AF461237	AF461201	X
	Coenagrionidae	*Erythromma najas*	AF461238	AF461209	X
	Coenagrionidae	*Ischnura elegans*	AF461239	AF461215	X
	Coenagrionidae	*Pyrrhosoma nymphula*	AF461241	AF461202	X
	Corduliidae	*Cordulia aenea*	AF461236	AF461210	X
	Corduliidae	*Somatochlora flavomaculata*	AF461242	AF461212	X
	Epiophlebidae	*Epiophlebia superstes*	AF461247	AF461208	X
	Gomphidae	*Ophiogomphus severus*	AY121143	AY125283	AY125228
	Lestidae	*Lestes* sp.	AY338721	AY338677	X
	Libellulidae	*Libellula saturata*	AY338717	AY338674	AY338637
	Libellulidae	*Celithemis eponina*	AF461233	AF461218	X
	Libellulidae	*Leucorrhinia pectoralis*	AF461240	AF461206	X
	Libellulidae	*Sympetrum vulgatum*	AF461246	AF461216	X
	Petaluridae	*Phenes raptor*	AY338720	X	X
Polyneoptera	Acrididae	*Melanoplus* sp.	AY121146	AY125286	AY125231
	Blatellidae	*Supella longipalpa*	AY121130	AY125271	AY125217
	Heteronemiidae	*Sceptrophasma longikawiensis*	AY121166	AY125306	AY125249
	Mantidae	*Tenodera aridifolia*	AY121142	AY125282	AY125227
	Mastotermitidae	*Mastotermes darwinensis*	AY121141	AY125281	X
	Nemouridae	*Malenka californica*	AY338724	AY338680	AY338642
	Notoligotomidae	*Notoligotoma* sp.	AY338693	AY338650	AY338617
	Oligotomidae	*Oligotoma nigra*	AY121134	AY125274	AY125221

Table 2 (*continued*)

Order	Family	Genus species	18S rDNA	28S rDNA	H3
	Styloperlidae	*Cerconychia* sp.	AY338725	AY338681 & AY338682	AY338643
	Tetrigidae	*Paratettix cucullatus*	AY338722	AY338678	AY338640
	Timematidae	*Timema knulli*	AY121162	AY125302	AY125246
	Tridactylidae	*Ellipes minutus*	AY338723	AY338679	AY338641

given in Whiting (2001). Primer sequences for the gene H3 are HexAF: 5′-ATG GCT CGT ACC AAG CAG ACG GC-3′ and HexAR: 5′-ATA TCC TTG GGC ATG ATG GTG AC-3′. 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 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).

Four analytical strategies were employed to examine topological sensitivity (Table 4 and Fig. 1): (1) direct optimization alignment via POY; (2) use of the implied alignment from POY as a multiple alignment for tree reconstruction; (3) alignment in ClustalX using sequences submitted as fragments followed by tree reconstruction; and (4) alignment in ClustalX using sequences submitted as a whole (non-fragmented) followed by tree reconstruction.

Optimization alignment (OA) via POY

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. For the ribosomal genes, a gross alignment was performed by manually aligning the conserved domains across the taxa. The sequences were then sectioned into fragments at the conserved domains. This resulted in six fragments for 18S and nine fragments for 28S. These data were analyzed via OA in the program POY (Gladstein and Wheeler, 1999). POY was implemented on an IBM SP 2 supercomputer [316 Power3 processors @ 375 MHz; 31 Winterhawk nodes (4 processors each); 12 Nighthawk II nodes (16 processors each); 348 GB total memory]. POY command files were as follows: -fitchtrees -maxprocessors 3 -onan -onannum 1 -parallel -noleading -norandomizeoutgroup -impliedalignment

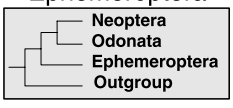
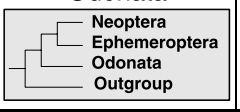
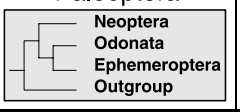
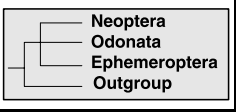
Strategies for sequence alignment	Basal Ephemeroptera 	Basal Odonata 	Monophyletic Paleoptera 	Unresolved 
POY	Mol (1:2:1) Mol (4:4:1) Total (1:1:1) Morphology Mol (2:1:1) 18S (1:1:1)	Mol (1:3:1) Mol (1:4:1) Mol (3:3:1) Mol (3:4:1) Mol (3:1:1) Mol (4:1:1) Mol (10:10:1) Mol (10:1:1) 28S (1:1:1)	Mol (1:1:1) Mol (3:2:1) Mol (4:2:1) Mol (4:3:1) Mol (2:2:1) Mol (2:3:1) Mol (2:4:1) Mol (100:100:1)	Mol (1000:1000:1) Mol (1000:1:1) Mol (100:1:1) H3 (Pre-aligned, 1:1)
POY implied	ML Mol (1:1:1)	MetaPIGA Mol (1:1:1)	Parsimony Mol (1:1:1; gaps = missing and fifth state)	Bayesian Mol (1:1:1)
ClustalX on sequence fragments	MetaPIGA Mol A Parsimony Mol A Parsimony 28S A Total A		ML Mol A Parsimony 18S+28S A	Bayesian Mol A Parsimony 18S A
ClustalX Whole	Parsimony Mol A Parsimony 18S+28S C Total A	Parsimony 18S+28S A	Parsimony 18S C	Parsimony 18S A,B Parsimony 18S+28S B Parsimony Mol B,C Parsimony 28S A,B,C Total B,C

Fig. 1. Summary of topological support for the three hypotheses from all parameters and methods. gap:transversion:transition ratios are indicated in parentheses. ClustalX settings A, B, and C as in Table 5. H3 was submitted to POY as prealigned data and was analyzed with parameters set to unity, gaps and changes = 1.

-sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -random 5 -stopat 25 -multirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 100 -numdriftchanges 30 -driftspr -numdriftspr 10 -drifttbr -numdrifttbr 10 -slop 10 -checkslop 10. Alignments can be found at (http://inbio.byu.edu/faculty/mfw2/whitinglab/).

A variety of alignment cost parameter values were investigated to explore data sensitivity (Table 3). We selected 22 values to explore sensitivity to gap/nucleotide

Table 3
Alignment cost ratios used in POY to explore topological landscape for molecular data and results from these analyses

1:1:1	1:2:1	1:3:1	1:4:1
2:1:1	2:2:1	2:3:1	2:4:1
3:1:1	3:2:1	3:3:1	3:4:1
4:1:1	4:2:1	4:3:1	4:4:1
10:1:1	10:10:1		
100:1:1	100:100:1		
1000:1:1	1000:1000:1		

= BASAL EPHEMEROPTERA
= BASAL ODONATA
= MONOPHYLETIC PALEOPTERA
= UNRESOLVED

The ratio indicates the gap:transversion:transition cost ratio.

Table 4
Four alignment strategies employed to examine topological sensitivity

Strategies	Partitions analyzed	Alignment parameters	Methods employed
Optimization alignment via POY	18S	1:1:1	Parsimony
	28S	1:1:1	Parsimony
	H3	1:1:1	Parsimony
	Molecular	See Table 3	Parsimony
	Total	1:1:1	Parsimony
Implied POY alignment	Molecular	1:1:1	Parsimony, gaps = missing
			Parsimony, gaps = fifth state
			Maximum likelihood
			Bayesian
			MetaPIGA
ClustalX: sequences submitted as fragments	18S	A	Parsimony
	28S	A	Parsimony
	Molecular	A	Parsimony
			Maximum likelihood
			Bayesian
			MetaPIGA
	Total	A	Parsimony
ClustalX: sequences submitted as a whole	18S	A	Parsimony
		B	Parsimony
		C	Parsimony
	28S	A	Parsimony
		B	Parsimony
		C	Parsimony
	18S + 28S	A	Parsimony
		B	Parsimony
		C	Parsimony
	Molecular	A	Parsimony
		B	Parsimony
		C	Parsimony
	Total	A	Parsimony
		B	Parsimony
		C	Parsimony

The data partitions that were analyzed, the specific alignment parameters for each partition, and the methods used under each partition and parameter are indicated in the columns. The ratio of 1:1:1 indicates the gap:transversion:transition cost ratio. The letters A, B, and C coincide to the ClustalX alignment parameter settings in Table 5.

Table 5
ClustalX multiple sequence alignment settings represented as A, B, and C in Table 4

ClustalX setting	Gap opening	Gap extension	Delay divergent %	DNA transition weight	DNA weight matrix
A	1	1	30	0.00	ClustalW(1.6)
B	15	6.66	30	0.50	IUB
C	100	100	30	0.00	ClustalW(1.6)

change ratios (ranging from 1 to 1000) and transition/transversion ratios (ranging from 1 to 1000). Although one could essentially have an infinite number of ratio combinations for these three parameters, we believe that these representative ratios are sufficient to address the goals of this research (Giribet, 2001; Wheeler, 1995). The alignment of the H3 gene was not ambiguous and the sequence data were treated as prealigned and analyzed in unity under parsimony (changes = 1). Results for H3 do not vary from one analytical methodology to the next, because the alignment was stable and thus different alignment methods would have no affect.

Implied POY alignment

We also tested robustness of the data to different methods of tree reconstruction using the implied alignment found in POY (Wheeler, 2003), with costs set to unity to minimize assumptions. We often find that unity for cost parameters is the most optimal parameter configuration for large data sets when implemented in the ILD framework (Kluge, 1989; Mickevich and Farris, 1981; Wheeler and Hayashi, 1998; Wheeler et al., 2001). The implied alignment was analyzed in five ways: (1) under parsimony with gaps treated as missing; (2) under parsimony with gaps treated as a fifth state character; (3) under standard maximum likelihood analysis as implemented in PAUP*4.0b10 (Swofford, 2002); (4) under bayesian analysis as implemented in Mr. Bayes (Huelsenbeck and Ronquist, 2001); and (5) using the metapopulation genetic algorithm executed in the program MetaPIGA (Lemmon and Milinkovitch, 2002) (Table 4). Modeltest (Posada and Crandall, 1998) was used to identify the most ''justified'' model for likelihood and bayesian analyses (Posada and Crandall, 2001). In addition, the implied alignment was also used to calculate nodal support. Nonparametric bootstrap values (500 replications) and partitioned Bremer support values (Baker and DeSalle, 1997) were calculated using the programs PAUP*4.0b10 and TreeRot (Sorenson, 1999).

Sequences submitted as fragments to ClustalX

To test the sensitivity of our results to different alignment algorithms, we chose to investigate performance of the alignment program ClustalX (Thompson et al., 1997). It is important to realize that a direct comparison between parameter values in POY and ClustalX cannot be performed. In other words, there is no parameter set that one can select in POY that will give the ClustalX alignment and vice versa for any complex data set. The first strategy that we evaluated in ClustalX was designed to compare more directly to the results obtained from POY. The fragments were aligned under the ClustalX parameter setting A (Table 5). We believe that these settings most closely resemble the cost ratio of 1:1:1 (gap:transversion:transition) that was used in POY. The alignments from ClustalX were then analyzed under the methods of tree reconstruction as described above. Additionally, 18S, 28S, and 18S + 28S partitions were aligned under setting A and analyzed under parsimony.

Sequences submitted as a whole to ClustalX

In the fourth strategy, each individual gene was submitted to ClustalX as a whole, instead of as fragments sectioned at the conserved domain regions as described above. This was done to compare results using ClustalX with sequences fragmented versus not fragmented, since subdividing sequences into multiple fragments may influence the optimality of the overall alignment (Giribet, 2001). Subdividing sequences into multiple fragments forces a constraint on the alignment search algorithm by never allowing a set of sequences in one fragment to be aligned with those of another fragment. From a practical standpoint, this will generally speed up the alignment process, but introduces the possibility of biasing the overall alignment by a preconceived notion of alignment. The strategy of submitting sequences as a whole was the method used by Hovmöller et al. (2002). Three different sets of alignment parameters (A, B, and C in Table 5) were investigated to produce multiple alignments. All alignments were analyzed under parsimony, with gaps treated as missing.

Results

Reanalysis of Hovmöller et al. (2002)

Hovmöller et al. (2002) reported only topologies for alignments with a gap opening penalty of 75. They state that ''a variety of settings'' were used until the penalty of

75 was selected, but did not provide a rationale for this choice nor discuss results under other parameter values. Our reanalysis of their data suggests that paleopteran monophyly was supported only under a small (23%) subset of analytical parameters (Table 1). The 18S and combined (18S + 28S) data support monophyletic Paleoptera over most of the gap opening values, when the gap extension remains at a value of one and gaps are treated as missing. However, when gaps are treated as a fifth state, the topologies are mostly unresolved or they support basal Odonata. When gap extension equals the gap opening value, with gaps treated as a fifth state, monophyletic Paleoptera is never recovered. The 28S data never support Paleoptera under any combination

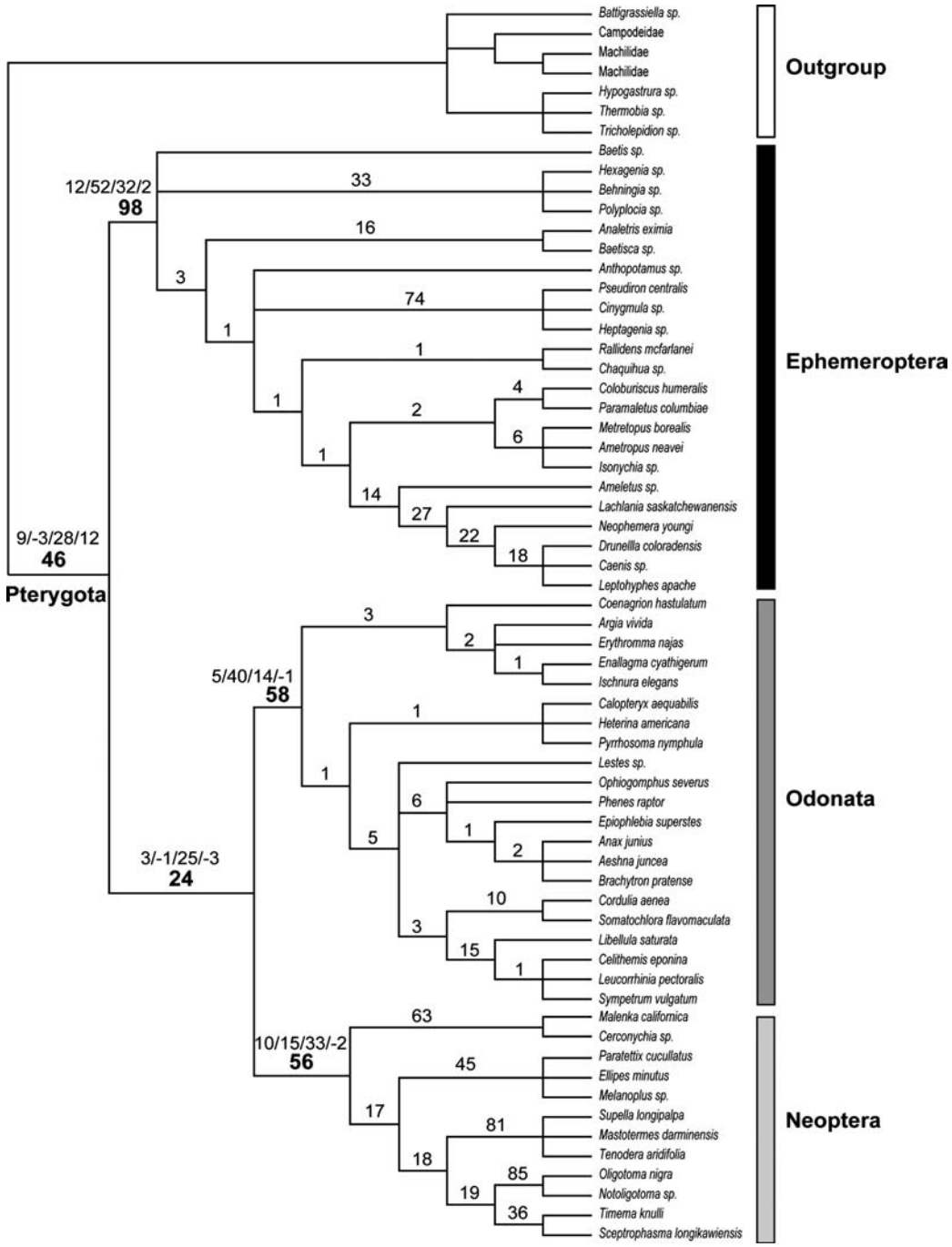


Fig. 2. Total-evidence tree based on 18S + 28S + H3 + morphology under 1:1:1 gap:transversion:transition costs in POY. This analysis produces a single most parsimonious tree (L = 2556; CI = 0.1980, RI = 0.5678) in which Ephemeroptera is basal. Partitioned Bremer values (morphology/18S/28S/H3) for the five basal pterygote nodes are given, and total Bremer values are given for the remaining nodes.

of analytical parameters. These results suggest that the Paleoptera problem has not been robustly solved given the data and analyses presented by Hovmöller et al. (2002).

Optimization alignment

Direct optimization of our expanded data set supports all three hypotheses, as summarized in Fig. 2. Sensitivity analysis suggests that these topologies are very sensitive to alignment cost parameters (Table 3 and Fig. 1). For example, when the transversion weight changes from 1 to 2 to 3 and the gap cost and transition weight remain at 1, each one of the three hypotheses is supported. Similarly, when the gap cost changes from 2 to 3 to 4 and transversion = 4 and transition = 1, all three hypotheses are recovered also. With costs set to unity, the combined molecular data support a monophyletic Paleoptera and the total evidence analysis including morphology supports basal Ephemeroptera (Fig. 2). The partitioned Bremer values for morphology, 18S, 28S, and H3 for the five basal pterygote lineages (Fig. 2) are indicated on the nodes. Support for the node Odonata + Neoptera (=Ephemeroptera basal hypothesis) comes from the 28S and morphological data, with conflicting signal from the 18S and H3 partitions. These results suggest that the monophyly of Paleoptera is highly sensitive to OA cost parameters, even in our expanded data set.

POY implied alignment analyses

The parsimony, maximum likelihood, bayesian, and MetaPIGA analyses on the implied POY alignment support all possible resolutions of the three-taxon statement (Fig. 1). Using the POY implied alignment for the molecular data under parsimony and treating gaps as missing results in a monophyletic Paleoptera. In contrast to the touted claims that model-based methods result in topologies that are highly congruent (Lemmon and Milinkovitch, 2002; Yang and Rannala, 1997), we find that model-based methods also disagree on which hypothesis is best supported. For example, the MetaPIGA analysis supports a basal Odonata, the maximum likelihood supports a basal Ephemeroptera, and the bayesian analysis is unresolved. We want to make it clear that phylogenetic accuracy is not increased by gaining agreement between the results of disparate analytical methodologies. We are interested only in determining whether a robust solution exists for the given data under any analytical methodology or whether the molecular data do not discriminate among the hypotheses.

ClustalX with sequence fragments

The implied alignment generated from POY and the multiple alignment generated by ClustalX were different and produced different topologies. This is not surprising because POY produces alignments using an optimality criterion, whereas ClustalX is algorithmic or progressive in nature (Notredame, 2002). ClustalX alignments are also sensitive with regard to the three hypotheses For instance, when sequences were submitted as fragments for all three genes in a combined molecular analysis, MetaPIGA and parsimony supports basal Ephemeroptera, maximum likelihood supports monophyletic Paleoptera, and bayesian analysis is unresolved (Fig. 1). Likewise, individual gene partitions support different relationships across different analytical methods. For instance, under parsimony the implied POY alignment supports monophyletic Paleoptera, but the ClustalX alignment under parsimony supports basal Ephemeroptera.

ClustalX with whole sequences

Submitting data as whole sequences to ClustalX also results in topological sensitivity. For instance, treating the 18S + 28S data as fragments with ClustalX results in monophyletic Paleoptera under parsimony, but treating these data as whole results in basal Odonata. Moreover, as in the POY sensitivity analyses, the selection of alignment parameters will influence the topology. For example, the alignment of the 18S + 28S data set under parameter condition A recovered a basal Odonata while under parameter C it recovered basal Ephemeroptera (Fig. 1). This further suggests sensitivity of the results to analytical parameters.

Discussion

Is the Paleoptera problem solved? The goal of this study was to determine whether current molecular evidence confirms the monophyly of Paleoptera across multiple parameter landscapes. Our results demonstrate that the particular arrangement of these lineages is extraordinarily sensitive to the current molecular data with regard to alignment methodology, alignment parameters selected within a particular methodology, and method of tree reconstruction. The inclusion of additional data from more taxa and another genetic locus did not help resolve these hypotheses, and sensitivity analyses of these data do not converge on a single solution. Even if one were to reject the notion of sensitivity analysis as a useful measure of robustness and select the values that set parameters to unity, our results demonstrate that the molecular data support a monophyletic Paleoptera under POY, but the ClustalX analysis supports basal Ephemeroptera.

These results suggest that a robust solution to the Paleoptera problem based on molecular data exclusively is more nebulous than suggested by Hovmöller et al.

(2002). However, other relationships on the topology were not as sensitive to parameter perturbations, as many clades are stable across all of the analyses. For instance, the monophyly of Ephemeroptera and Odonata were well-supported under most analyses, and the arrangements of taxa within these groups were also relatively consistent across analyses. For example, within the Odonata the suborders Zygoptera and Anisoptera are consistently recovered. Additionally, the baetid is frequently supported as the basal ephemeropteran lineage and the burrowing mayflies are monophyletic. This suggests that these molecular data are appropriate markers, at least at lower levels in the phylogeny of insects, as has been demonstrated in other analyses (Wheeler et al., 2001; Whiting et al., 2003).

The empirical case presented here underscores the importance of investigating the influence of parameter values on phylogenetic hypotheses. It is not enough to just "plug and chug" during the alignment phase (Grant et al., 2003), relying on default values of the preferred algorithm, since the recovered topology may not be robust to perturbations of the parameter values across all nodes. There may be topologies or nodes that are robust to parameter variation. However, as exemplified by this study, certain important nodes may be very sensitive to methodology. With the plethora of methods available to use in phylogenetic inference, discrimination must be employed to filter out methods that are inferior and that may produce misleading results. Empirical comparisons among alternative methods are useful to investigate methodological performance (Morrison and Ellis, 1997). However, we do not consider congruence among different methodologies to be a suitable measure of robustness because agreement among inferior methods is nebulous at best. We are more concerned with the influence of parameter values within a particular methodology. Even within the same framework, such as parsimony, conflicting topologies were recovered under different methods of alignment. For instance, parsimony (with parameters set to unity) on all molecular data supported monophyletic Paleoptera in POY and basal Ephemeroptera in ClustalX. Moreover, the 18S + 28S data supported monophyletic Paleoptera in ClustalX with fragmented sequences and supported basal Odonata in ClustalX with unfragmented sequences. Hence the different methods (OA in POY or multiple sequence alignment (MSA) in ClustalX) yielded different topologies.

We suggest that there are multiple reasons that OA is superior to MSA when the disparity of sequences results in alignment ambiguity. First, OA heuristically searches across multiple alignments, allowing an optimality criterion to reject nonoptimal solutions, thus freeing itself from the progressive approach which may be biased by the predetermined guide tree (Wheeler, 1995, 2003). Second, OA uses a total evidence approach to infer the topology by including morphology and prealigned data. In our analyses, the inclusion of morphology with the molecular data supported basal Ephemeroptera, as reported in other total-evidence analyses (Wheeler et al., 2001; Terry et al., in prep.), except under the most extreme alignment parameter values. There are many morphological characters that support this relationship (see Wheeler et al., 2001 for detailed treatment of characters). For example both mandibular articulations are fully fixed in Odonata + Neoptera, leg and wing tracheae are connected with the following spiracle, and the terminal medial filament is strongly reduced or absent (Beutel and Gorb, 2001; Staniczek, 2000; Wheeler et al., 2001). Contrary to the position of other authors who argue for partitioned analyses (de Queiroz, 1993; de Queiroz et al., 1995; Simmons and Freudenstein, 2003), we suggest that if total evidence has any merit at all, it must be applied uniformly during alignment and tree reconstruction, and currently POY is the only algorithm that provides a methodology for accomplishing this. Third, in agreement with other authors (Phillips et al., 2000), we find the consistency of using a single criterion throughout the analytical process to be appealing and superior to other methods that rely on a hodgepodge of criteria for alignment and tree reconstruction. Exploration and development of new genes informative at deep levels of evolution, combined with better taxon sampling may eventually lead to a robust solution of the Paleoptera problem.

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