

Relaxed Phylogenetics and the Palaeoptera Problem: Resolving Deep Ancestral Splits in the Insect Phylogeny

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Abstract.—The order in which the 3 groups of winged insects (the Pterygota) diverged from their common ancestor has important implications for understanding the origin of insect flight. But despite this importance, the split between the Odonata (dragonflies and damselflies), Ephemeroptera (mayflies), and Neoptera (the other winged orders) remains very much unresolved. Indeed, previous studies have obtained strong apparent support for each of the 3 possible branching patterns. Here, we present a systematic reinvestigation of the basal pterygote split. Our results suggest that outgroup choice and limited taxon sampling have been major sources of systematic error, even for data sets with a large number of characters (e.g., in phylogenomic data sets). In particular, a data set of 113 taxa provides consistent support for the Palaeoptera hypothesis (the grouping of Odonata with Ephemeroptera), whereas results from data sets with fewer taxa give inconsistent results and are highly sensitive to minor changes in data and methods. We also focus on recent methods that exploit temporal information using fossil calibrations, combined with additional assumptions about the evolutionary process, and so reduce the influence of outgroup choice. These methods are shown to provide more consistent results, for example, supporting Palaeoptera, even for data sets that previously supported other hypotheses. Together, these results have implications for understanding insect origins and for resolving other problematic splits in the tree of life. [Bayesian phylogenetics; BEAST; Chiasmomyaria; Metapterygota; Pterygota.]

The evolution of flight allowed the winged insects, the Pterygota, to become one of the most diverse and successful groups on the planet (Grimaldi and Engel 2005). In 1924, Martynov divided the Pterygota into 2 parts: defining the Neoptera, or “new winged” insects, on the presence of complex structural elements that enable the wings to be folded back over the abdomen (Martynov 1924), and by elimination, the Palaeoptera, or “old winged” insects, which lack these structures. There are 3 supraordinal divisions of the Palaeoptera, namely, the dragonflies and damselflies (order Odonata), the mayflies (order Ephemeroptera), and the extinct (Carboniferous–Permian) superorder Palaeodictyopteroidea. Since then, while the monophyly of the Neoptera has been almost universally accepted (Martynov 1924; Hennig 1953, 1969; Kristensen 1991; Grimaldi and Engel 2005), the monophyly of the Palaeoptera and its extant taxonomic range (Odonata + Ephemeroptera) has been the subject of much debate. Indeed, different researchers have argued for all 3 possible branching patterns between the Odonata, Ephemeroptera, and Neoptera (Fig. 1). A clear resolution of these relationships is necessary for inferring the characteristics of the ancestral pterygotan insect and is thus crucial for understanding the origin of winged insect flight (Kingsolver and Koehl 1994).

Unfortunately, the application of cladistic methodology to large morphological data sets has been unable to resolve the Palaeoptera problem, and different authors have identified multiple synapomorphies that

support the monophyly of the Palaeoptera (Fig. 1a; Martynov 1924; Hennig 1969; Kukalová-Peck 1983, 1991, 2008; Bechly 1996; Haas and Kukalová-Peck 2001), the Chiasmomyaria (Fig. 1b; Boudreaux 1979; Carle 1982a, 1982b), and the Metapterygota (Fig. 1c; Hennig 1953; Kristensen 1991; Staniczek 2000; Wheeler et al. 2001; Grimaldi and Engel 2005; Beutel and Gorb 2006; Willkommen and Hörnschemeyer 2007). In some respects, the disagreements among these studies are unsurprising because of the lack of a suitable outgroup for many characters. This is particularly true of wing venation characters, which are a major source of characters for proponents of all 3 hypotheses.

Other similar cases have been resolved by the introduction of molecular data. For example, the monophyly of the Ecdysozoa (Aguinaldo et al. 1997); the close relationship between Crustacea and Hexapoda (Friedrich and Tautz 1995; Boore et al. 1998; Mallatt et al. 2004; Regier et al. 2005); or the position of Isoptera within Dictyoptera (Inward et al. 2007). However, concerning the basal pterygote relationships, molecular studies have shown no more agreement than have morphological approaches. Significant molecular support has been offered for the Palaeoptera (Giribet and Ribera 2000; Wheeler et al. 2001; Hovmöller et al. 2002; Kjer et al. 2006; Regier et al. 2010), Chiasmomyaria (Kjer 2004; Mallatt and Giribet 2006; Misof et al. 2007; Simon et al. 2009), and Metapterygota (Wheeler 1989; Ogden and Whiting 2003; Zhang et al. 2008) hypotheses. Even recent studies with large “phylogenomic” data sets (Zhang et al. 2008; Simon

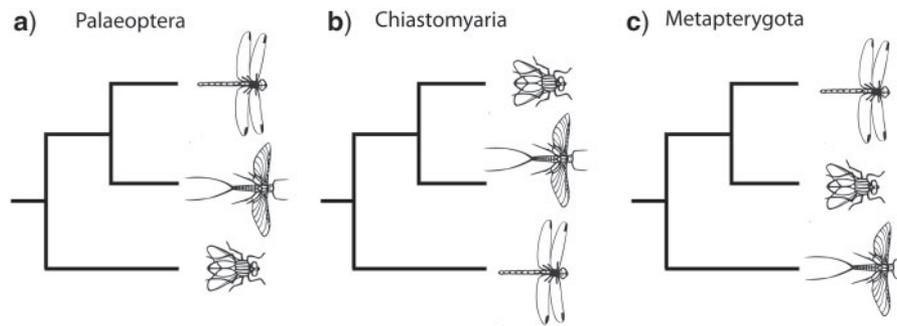


FIGURE 1. The 3 hypothesized divisions within the Pterygota.

et al. 2009; Meusemann et al. 2010; Regier et al. 2010) have failed to resolve the issue.

These striking disagreements may reflect a real biological phenomenon: the rapid divergence of all 3 groups from their common ancestor in the distant past. The possibility of a rapid radiation in the evolution of the early winged insects is supported by the almost simultaneous appearance in the lowermost Upper Carboniferous of fossils identified as stem members of the 3 extant pterygotan lineages, and a complete lack of fossils assignable to any common stem (Carpenter 1992; Rasnitsyn and Quicke 2002; Grimaldi and Engel 2005). There are also well-characterized examples from other taxa, where phylogenetic disagreement and simultaneous appearance in the fossil record are combined with the signature of incomplete lineage sorting, strongly implying that 3 groups diverged in rapid succession (e.g., the 3 mammalian orders that comprise the Paenungulata [Nishihara et al. 2005]).

Whether or not it leads to genuinely conflicting signal among characters, ancient rapid radiation can also lead to weak phylogenetic signal, which may make analyses more susceptible to systematic error (i.e., more sensitive to small inadequacies in the data or methods employed [Rokas and Carroll 2006; Baurain et al. 2007; Whitfield and Lockhart, 2007; Whitfield and Kjer 2008]). In molecular phylogenies, 3 types of inadequacies are particularly important.

First, inadequate taxon sampling, particularly when there is substantial variation in patterns of molecular evolution, can allow a few anomalous taxa to strongly influence the results and can also lead to the phenomenon of long-branch attraction (Felsenstein 1978; Hedtke et al. 2006). Nevertheless, many studies of deep pterygote relationships have included only single members of Odonata and Ephemeroptera. Problems of limited taxonomic sampling are particularly acute when it comes to the choice of outgroup (Bergsten 2005; Shavit et al. 2007; Rota-Stabelli and Telford 2008), especially because many apterygotes have very fast rates of molecular evolution (Whitfield and Kjer 2008).

Second, systematic bias may arise from the data itself. The use of phylogenetically uninformative sequence data, that is sequences without a suitable level of divergence, has been shown to affect topological

reconstruction (Townsend 2007; Lopez-Giraldez and Townsend 2011; Townsend and Leuenberger 2011). Questionable alignment methods are also problematic: automated alignment algorithms without manual adjustment may seriously compromise phylogenetic inference. For example, Ogden and Whiting (2003) argued that the results of Hovmöller et al. (2002) could be attributed to questionable automated alignments generated by CLUSTAL (Ogden and Whiting 2003). The same problems can apply to algorithms such as POY, which performs alignment and topological estimation simultaneously (Kjer et al. 2007; Yoshizawa 2010).

Finally, systematic bias can arise from the choice of phylogenetic methods and their models. The use of parsimony has been prevalent in studies of the pterygotan splits (Giribet and Ribera 2000; Wheeler et al. 2001; Hovmöller et al. 2002; Ogden and Whiting 2003), but this method is particularly susceptible to long-branch-attraction problems (Felsenstein 1978; Hendy and Penny 1989). Even with explicitly model-based methods, model misspecification can lead to error. For example, artifactual groupings can arise from a failure to account for nonstationary base composition (Delsuc et al. 2003; Phillips and Penny 2003). In the Pterygota, GC content does indeed vary greatly (e.g. Hassanin 2006; Jørgensen et al. 2006), particularly for mitochondrial genes encoded on different strands in different species (Hassanin et al. 2005; Jones et al. 2007; Masta et al. 2009). There are also issues with Bayesian methods that assign zero prior probability to polytomies. This can lead to artifactually strong support for arbitrary groupings when there is little signal in the data (Lewis et al. 2005; Susko 2008). Importantly, all of the aforementioned sources of systematic bias can apply—and even be exacerbated—in large “phylogenomic” data sets (Phillips et al. 2004; Delsuc et al. 2005; Jeffroy et al. 2006; Rokas and Carroll 2006; Simon et al. 2009).

This study investigates the Palaeoptera problem, making a particular attempt to address potential sources of error discussed above. First, we use wide taxonomic sampling across the Palaeoptera, targeting as many genes for as many taxa as possible, with a supermatrix approach. Second, we use explicit tests and corrections for base compositional bias, employing RY recoding to remove any Guanine-Cytosine (GC) heterogeneity and

TABLE 1. Dating information for BEAST analyses

	Node	Fossil and deposit locality	Age (Ma)	Reference	Prior distribution
Root	Crustacea–Hexapoda split	<i>Klausmuelleria salopiensis</i> from Comley Limestones, Shropshire, UK	Lower Cambrian, 543–510	Benton et al. (2009) and Siveter et al. (2001)	Exponential, mean=8, offset=510
1	Collembola	<i>Rhyniella praecursor</i> (springtail) from Rhynie chert, Scotland	Devonian: (Pragian–Emsian), 412.3–391.9	Grimaldi and Engel (2005) and refs therein; Palaeobiology database: http://paleodb.org/ Grimaldi and Engel (2005) and references therein	Lognormal, mean=30, log(SD)=0.6, offset=391.9
2	Pterygota	Griffenflies (Protodonata) and Protorthopterans (ancestral Neoptera) from Commentry and Montceau-les-Mines deposits, France; <i>Lithoneura lameerei</i> (Protoephemeroptera) from Mazon Creek, Illinois	Members of all groups present by Late Carboniferous, 318–299		Normal, mean=369.0, SD=22.5
3	Orthoptera	<i>Raphogla rubra</i> (oldest representative of modern Ensifera), Lodeve Basin, France	Late Permian, 260–251	Bethoux et al. (2002), in Grimaldi and Engel (2005)	Gamma, shape=2.0, scale=13.5, offset=251.0
4	Dictyoptera	<i>Baissatermes lapideus</i> (Isoptera) and <i>Baissomantis</i> sp. (Mantids) from Zaza formation of Baissa, Siberia, Russia	Early Cretaceous, 145–99.6	Engel et al. (2007) and Gratshev and Zherikhi (1994)	Gamma, shape=2.0, scale=33.4, offset=99.6
5	Plecoptera	Lemmatophoridae, Liomopteridae, Probnidae families: early members of Plecoptera	Permian, 299–251	Grimaldi and Engel (2005) and Zwick (2000)	Gamma, shape=2.0, scale=13.5, offset=251.0
6	Hemiptera	<i>Paraknightia magnifica</i> (Heteroptera) from Newcastle Coal Measure, Australia	Late Permian, 260–251	Percival (1985)	Gamma, shape=2.0, scale=13.5, offset=251.0
7	Hymenoptera	<i>Cretotrigona prisca</i> (Hymenoptera: Apidae) from Maastrichtian amber from New Jersey	Late Cretaceous, 80–65	Michener and Grimaldi (1988a, 1988b) and Engel (2000)	Gamma, shape=2.0, scale=35, offset=65.0
8	Ephemeroptera	<i>Fuyous gregarius</i> and <i>Shantous lacustris</i> (Euplectoptera, extant mayflies) from the Daohugou formation of China	Late Jurassic, 176–145	Zhang and Kluge (2007)	Gamma, shape=2.0, scale=32.5, offset=145.0
9	Anisozygoptera	Liassogomphidae, for example <i>Liassogomphus brodiei</i> , from Liassic outcrops in Europe, including Switzerland, Germany, England, and Luxembourg	Early Jurassic, 201.6–197	Grimaldi and Engel (2005) and Nel et al. (1993)	Gamma, shape=2.0, scale=23.2, offset=197.0
10	Aeshnidae/Petaluridae/Gomphidae	Aeshnidae/Petaluridae/Gomphidae taxa	Middle to Late Jurassic, 161–145	Fleck et al. (2008)	Gamma, shape=2.0, scale=32.5, offset=145.0
11	Zygoptera	Triasolestidae taxa from Triassic deposits of South America, Australia and Central Asia	Triassic, 251–201.6	Carpenter (1992)	Gamma, shape=2.0, scale=22.4, offset=201.6
12	Archaeognatha	<i>Triassomachilis uralensis</i> , Triassic deposits in Russia	Triassic, 251–201.6	Sharov (1948) in Grimaldi and Engel (2005)	Gamma, shape=2.0, scale=22.4, offset=201.6

Note: All geological ages are in accordance with Walker and Geissman (2009).

to mitigate the loss of phylogenetic signal over time (Phillips and Penny 2003). Third, in addition to the standard phylogenetic methods employed in previous studies, this study uses the relaxed-clock method implemented in BEAST (Drummond et al. 2006), along with temporal information about arthropod divergences derived from the fossil record (Table 1). By exploiting temporal information to inform topological inference, this method removes the need to include outgroup taxa in the analysis; this allows us to assess the influence of outgroup choice on the results obtained.

METHODS

DNA Sequence Data

For our core data sets, sequence data from species of Odonata, Ephemeroptera, Neoptera, and outgroup taxa were collected from Genbank (www.ncbi.nlm.nih.gov) (see Supplementary Table S1 for details, <http://datadryad.org>, doi: 10.5061/dryad.7d2g2). We aimed to target a representative species sample from each order for genes with wide taxonomic coverage. A supermatrix approach to gene alignment was used; there

is evidence that missing data are not a serious problem for topological reconstruction, if most taxa have data for most bases in the sequence (Wiens and Morrill 2011, but see also Lemmon et al. 2009). Nevertheless, we avoided genes for which there were only a few representative species. In some cases, when not all of the sequence data were available for a single species, chimerical taxonomic units were assembled from 2 members of the same genus, or in a few cases from 2 members of the same family.

For our primary data set, “data set 1,” we compiled sequence data from 113 species; 35 from each of the Odonata, Ephemeroptera, and Neoptera, plus 8 outgroup taxa from Thysanura, Archaeognatha, Collembola, and *Daphnia* (Crustacea). This sampling scheme represents near-complete family-level coverage for the Odonata (26/32 families recognized in the World Odonata List; Schorr et al. 2010), a large proportion of Ephemeroptera families (28/42 families recognized by Barber-James et al. 2008), and near-complete ordinal-level coverage for the more diverse Neoptera (25/28 recognized orders) (see Supplementary Table S1 for more information). The sequences for data set 1 comprised four ribosomal genes: mitochondrial 12S and 16S (788 bp), nuclear 18S and 28S (3595 bp), as well as one nuclear protein-coding gene: histone 3 (H3) (342 bp of coding region); total sequence length was 4725 bp.

We also compiled a second data set, “data set 2,” with a greatly reduced taxon sample of 10 species per order, together with outgroups (38 species in total). For these taxa, 2 additional gene sequences were available: mitochondrial COII (684 bp), and nuclear EF1a (1077 bp of coding region) (total sequence length = 6365 bp).

Ribosomal sequences were aligned manually in Se-AL (Rambaut 1996). Where rRNA alignment was unclear, RNA secondary structures were referred to from the Gutell Lab database (Cannone et al. 2002). Any highly variable regions that could not be aligned confidently were excluded from phylogenetic analysis and the remaining sequences were concatenated. For further analyses, data were partitioned by gene because our markers differed in genomic location, base composition, and, perhaps, in levels of selective constraint. In addition, for protein-coding genes, an additional partition applied to the third codon positions, which are primarily synonymous and so subject to very different levels of constraint (see also Shapiro et al. (2006) who argue for this partition choice on empirical grounds).

Base Composition Bias

Base composition heterogeneity across taxa can add systematic error to analyses (Conant and Lewis 2001). Accordingly, all partitions in all data sets were assessed with the *basefreq* command in PAUP*4.0b10 (Swofford 2003), which uses a chi-squared test for base frequency differences among taxa. When significant heterogeneity was found in a partition, we compiled new versions of our alignment, using RY recoding (Phillips and Penny 2003). In practice, this involved coding all purines as

G and all pyrimidines as C for further analysis. Data were then retested for heterogeneity. In cases where RY recoding did not remove composition bias, biased partitions were excluded from the alignment. Details of all sequences recoded and excluded can be found in Supplementary Table S2.

Additional problems can arise with the use of mitochondrial markers which are rapidly evolving, subject to strand inversion bias (Hassanin et al. 2005) and have given anomalous results elsewhere in the arthropod tree (Delsuc et al. 2003; Nardi et al. 2003; Cameron et al. 2004; Hassanin 2006; Carapelli et al. 2007; Rota-Stabelli and Telford 2008; Timmermans et al. 2008; Masta et al. 2009; Rota-Stabelli et al. 2010a). Accordingly, we also repeated all analyses with these markers excluded.

Phylogenetic Analyses

Maximum-likelihood (ML) analyses were conducted in RAxML, v.7.0.4 (Stamatakis 2006; Stamatakis et al. 2008), applying the general time-reversible (GTR) model of sequence evolution with across site rate variation modeled to a Gamma distribution, (GTR+ Γ —corresponding to the GTRGAMMA model in RAxML) for each partition (Rodríguez et al. 1990). Twenty separate ML analyses were performed (using the “-f d” command in RAxML), and the tree with the highest likelihood was chosen from this set. A separate thorough bootstrap analysis with 100 replicates was also executed to examine the relative support for each clade.

For Bayesian analyses, topology and divergence dates were estimated simultaneously in BEAST v1.7 (Drummond and Rambaut 2007). Data were partitioned and for the four-nucleotide-encoded regions, a GTR+ Γ model was applied, for comparison with the ML results. For the RY-recoded regions only 2 substitution types were possible, and so we used the F81+ Γ model (Felsenstein 1981) (implemented in BEAST by fixing the transition/transversion ratio, to 0.5 in an HKY+ Γ model). The birth–death model of speciation and an uncorrelated lognormal-relaxed molecular clock model were employed, with all parameters estimated from the data. Clock and tree parameters were linked across partitions, but a separate substitution rate parameter “mu” was estimated for each. Prior distributions on the root and 11 other nodes were applied based on an interpretation of the arthropod fossil record. BEAST requires that these dated nodes be monophyletically constrained. We consequently placed date distributions and constraints only on nodes that were highly supported in the ML analysis. Full details of all prior distributions for divergence times are found in Table 1. All other priors were left as the default values in BEAUti (Drummond and Rambaut 2007). Supplementary Table S3 summarizes the distributions for the marginal priors specified for each calibrated node and compares these to the values obtained from running the complete prior (see below for details). This suggests

that, in this case, interaction between the different elements of our prior did not substantially distort our actual prior knowledge (Heled and Drummond 2012).

All BEAST analyses in this study were run for a total of 200 million generations and sampled every 20 000 generations. Convergence and the consequent proportion of burn-in were assessed using the diagnostic plots in the programs “Are We There Yet” (AWTY; Wilgenbusch et al. 2004) and Tracer v1.5 (available from <http://beast.bio.ed.ac.uk/>). The effective sample sizes (ESSs) of parameters of interest (species tree, relaxed-clock parameters) were greater than 1000, and all ESS values were above 100. Most analyses had a burn-in of 50 million generations resulting in 15 000 trees in the posterior distribution, and all had at least 10 000 sampled trees.

To test the strength of support for the 3 hypotheses using Bayesian methods, Bayes factors were calculated. To establish the prior probabilities of the 3 possible groupings, analyses were rerun with nucleotide bases recoded as N for 10 000 000 generations with 1 000 000 discarded as burn-in. Because both “models” (taxon branching orders) appear in the same Markov chain Monte Carlo (MCMC), the Bayes factor (i.e., the ratio of marginal likelihoods) can be calculated simply as: $B(\text{data}) = (\pi(M_1|\text{data})/p(M_1))/(\pi(M_2|\text{data})/p(M_2))$, where $\pi(M_i|\text{data})$ is the posterior probability of hypothesis M_i , and $p(M_i)$, its prior probability.

Reanalyses of Published Data Sets

For the reanalysis of Simon et al. (2009), amino acid sequence alignments of their “maxgene” data set were obtained from treebase (Morell 1996). We analyzed this data set as specified by Simon et al. (2009). For the ML analysis in RAxML, this corresponded to an approximate algorithm (PROTMIXWAG) to optimize tree topology before estimating parameters using the WAG+ Γ model (Whelan and Goldman 2001). For our BEAST analysis, we also used the WAG+ Γ model for comparison and placed a prior for the origin of the Pterygota on the root height (Table 1, Node 2), with all other prior parameters left as default.

For the reanalyses of Zhang et al. (2008) and Lin et al. (2010), nucleotide sequence data were obtained from Genbank or kindly provided by the authors. Two versions of this data set were analyzed using both ML in RAxML and Bayesian methods in BEAST. First, we analyzed the original data set as described by Lin et al. (2010). This data set included only the first and second coding positions of each mitochondrial protein sequence. For the RAxML analysis, following Lin et al. (2010), 2 partitions were allocated per gene and the GTR+I+ Γ model of sequence evolution was applied to each partition. For comparison, a GTR+I+ Γ model was also estimated for each partition in the BEAST analysis. Prior distributions were placed on the root age (the split between the Collembola and the Insecta: corresponding to Node 1 in Table 1) and on 5 other nodes: Pterygota,

Isoptera, Ephemeroptera and Odonata (corresponding, respectively, to Nodes 2, 6, 8 and 10 in Table 1) and an additional prior on the Archaeognatha (Node 12 in Table 1). All other BEAST priors were left as default (Drummond and Rambaut 2007).

In addition to the original data set of Lin et al., we analyzed an edited data set in which base composition bias was also addressed. For this edited data set, mitochondrial protein-coding genes were first obtained from Genbank, aligned by eye, partitioned and then tested for base composition heterogeneity, as described above. The third codon positions of all genes except ATP8 showed heterogeneity even after recoding and so were excluded from further analysis. The first and second codon positions of 3 genes (ND2, ND4, and ND5) were also RY-recoded. For this data set, each full-coding partition was assigned the GTR+ Γ model, and in BEAST, each RY-coded partition was assigned the F81+ Γ model.

RESULTS

Analysis of 113-Taxon Data Set 1

Figure 2 shows the maximum clade credibility tree for our primary data set 1, and Table 2 shows the corresponding results from both ML and Bayesian approaches. All analyses of this data set show support for a Palaeopteran clade. Tests for heterogeneity in base composition showed significant heterogeneity in 3 regions of our alignment (third codon positions of histone H3, and the mitochondrial rRNAs 12S and 16S—see Supplementary Table S2), but recoding- or excluding-affected sequences had little effect on the results (Table 2); neither did repeating analyses with substitution models chosen by jModelTest (Posada 2008) (not shown) or excluding mitochondrial markers altogether (Table 2). Finally, results were consistent when the outgroup taxa were removed, and the tree rooted using temporal information alone in BEAST (Table 2).

Although results for data set 1 are consistent, the Bayesian results are far more statistically precise: ML analyses show moderate differences in the bootstrap support for Palaeoptera and Chiasmomyaria, whereas BEAST showed very strong posterior support for the Palaeoptera. One key reason for this must be that our ML analysis was a free-rate method (which indirectly allows substitution rates to be unique for each branch in the tree), whereas our BEAST analysis drew each rate from a lognormal distribution, whose mean and variance were estimated concurrently with the tree (Drummond et al. 2006). This procedure reduces the number of rate parameters estimated from $2n-3$ for a tree of n tips, to $n+1$ (the dates of the $n-1$ nodes plus a mean and variance of the lognormal), and this reduction in the number of parameters will increase the precision of the results. However, it will also tend to favor branching patterns that lead to more clock-like patterns

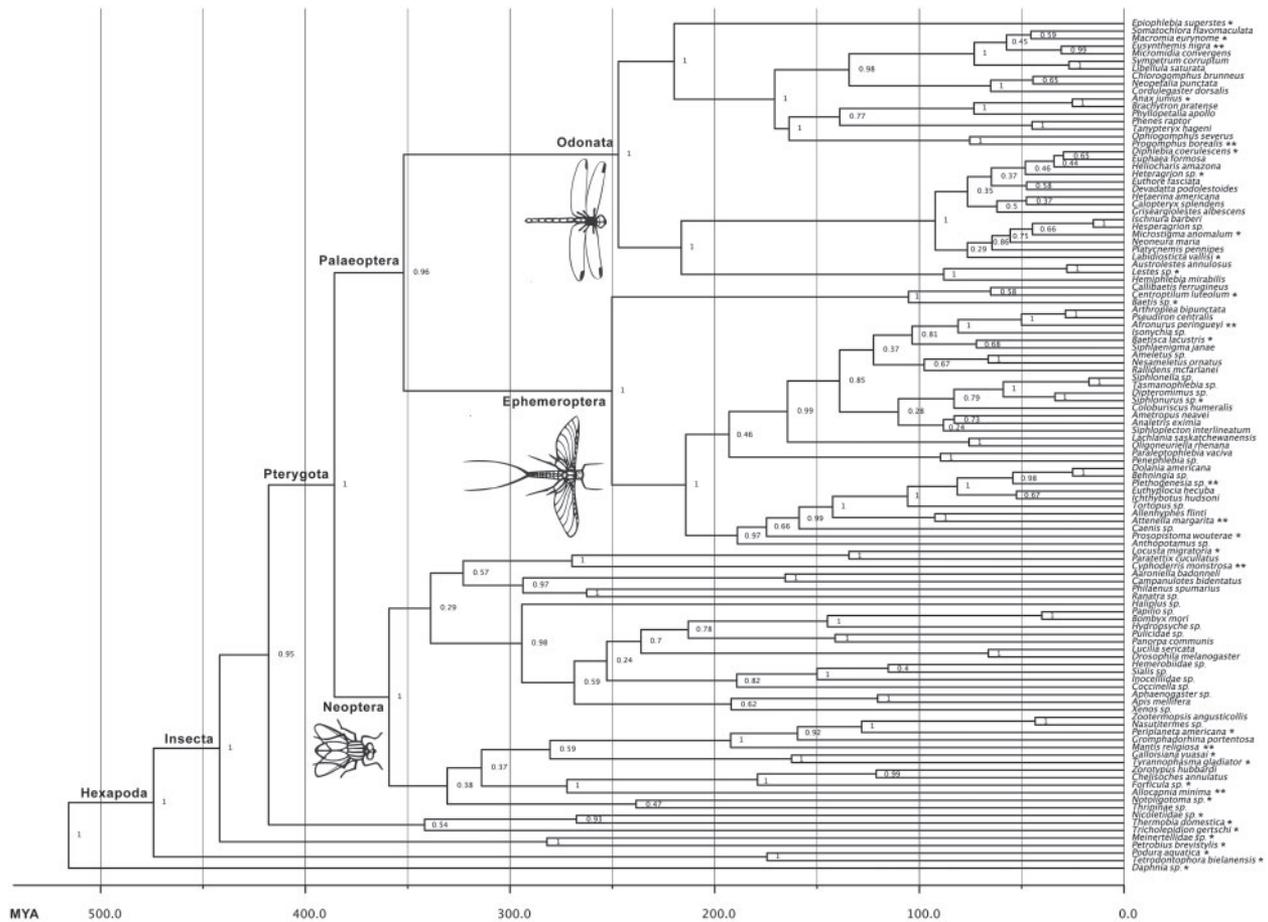


FIGURE 2. The Maximum Clade Credibility phylogeny for data set 1 as obtained from BEAST. Branch lengths are measured in millions of years (lines indicate 100 myr intervals). Stem branches leading to Pterygota and Palaeoptera are shown. *Indicates taxa that were also in data set 2; **Indicates taxa with members of the same family (Odonata and Ephemeroptera) or order (Neoptera) in data set 2.

of molecular evolution insofar as they are compatible with the temporal calibrations placed on the tree.

Additional analyses showed that our BEAST results were not sensitive to the particular form of the relaxed-clock model nor to the particular form of the prior placed on the uncalibrated node ages (results using an exponential model of rate change or a Yule process model of cladogenesis were qualitatively unchanged; see Supplementary Table S4). In contrast, removal of the fossil information on the internal nodes (Table 1) decreased support for the Palaeoptera—although it remained the favored hypothesis (see Supplementary Table S4). Investigation of our prior (i.e., without the molecular data) showed that Palaeoptera was indeed favored *a priori* when the fossil data were included (prior probabilities: Palaeoptera 51%, Chiasmomyaria 22%, and Metapterygota 27%). However, Bayes factors, which factor out the priors, indicate that there is strong evidence for the Palaeoptera over either of the alternative hypotheses, Chiasmomyaria, or Metapterygota (Table 2).

Because the use of a relaxed-clock model increases support for the Palaeoptera hypothesis over the Chiasmomyaria hypothesis, we next asked whether the

pattern of rate change implied by the Chiasmomyaria hypothesis was particularly extreme (Drummond et al. 2006; Smith et al. 2006). To investigate this possibility, we repeated our BEAST analysis, but with a constrained chiasmomyarian topology. Figure 3 shows the estimated rates for the basal branches of the tree under the unconstrained Palaeoptera and the constrained chiasmomyarian phylogenies. This plot indicates that the rates are very similar in the 2 cases. The branch representing the stem Neoptera is fast under both groupings, while that leading to the Palaeoptera is particularly slow (Figs. 2 and 3), but neither are outlying compared with the lognormal distribution of rates estimated for the whole tree (i.e., the point estimates for these branches fall well within the best-fit lognormal distribution for the tree as a whole; Fig. 3), and this distribution differs little in the 2 cases.

Finally, our time tree (Fig. 2) provides no evidence that the radiation of the basal pterygotes was particularly rapid. We estimate that around 30 million years (myr) separated the divergence of the Palaeoptera from the Odonata–Ephemeroptera split (Fig. 2; posterior median 31.7 myr [95% CI 2.–78.2]; prior median 30.1

TABLE 2. Results of phylogenetic analyses

Data set (no. taxa)	# Genes	Length (bp)	Grouping	ML	BEAST		BEAST (no OG)	
				Bootstrap	Posterior	Bayes factor	Posterior	Bayes factor
1. (113 sp)	Full	4732	Palaeoptera	0.61	0.99	—	0.95	—
			Chiaistomyaria	0.36	0.01	29.881	0.02	13.291
			Metapterygota	0.00	0.00	≥ 1000	0.03	11.176
	Composition bias corrected	4372	Palaeoptera	0.54	0.96	—	0.93	—
			Chiaistomyaria	0.44	0.04	8.342	0.03	13.044
			Metapterygota	0.00	< 0.01	247.898	0.03	13.842
Nuclear only; composition bias corrected	3935	Palaeoptera	0.61	0.98	—	0.95	—	
		Chiaistomyaria	0.34	0.02	15.931	0.03	13.917	
		Metapterygota	0.02	< 0.01	265.702	0.03	11.600	
2. (38 sp)	Full	6381	Palaeoptera	0.15	0.30	3.255	0.62	—
			Chiaistomyaria	0.38	0.28	1.558	0.20	1.348
			Metapterygota	0.47	0.42	—	0.18	1.571
	Composition bias corrected	6145	Palaeoptera	0.19	0.22	7.902	0.76	—
			Chiaistomyaria	0.66	0.76	—	0.18	1.711
			Metapterygota	0.15	0.02	41.088	0.16	1.885
Nuclear only; composition bias corrected	4890	Palaeoptera	0.09	0.30	4.971	0.75	—	
		Chiaistomyaria	0.68	0.61	—	0.13	2.807	
		Metapterygota	0.23	0.09	6.654	0.12	2.694	

Notes: Values indicate bootstrap support (ML) or posterior probability (BEAST) for the specified clade. ML or maximum posterior groupings are shown in bold. Bayes factors in bold indicate substantial support for the preferred model.

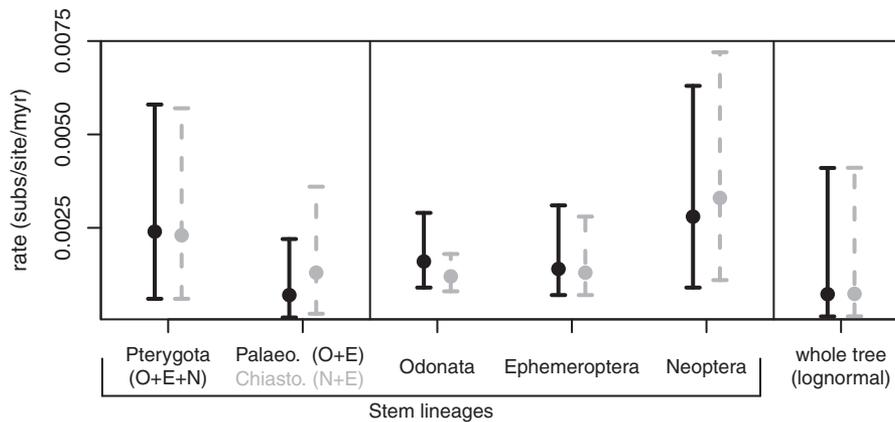


FIGURE 3. Rate measurements for the unconstrained Palaeoptera (black) and constrained Chiaistomyaria (dashed gray) BEAST trees for data set 1. Median posterior rate estimates (in substitutions per site per million years) are shown with 95% Credibility Intervals. Estimates are shown for stem lineages including the Pterygota (branch in Fig. 2), and Palaeoptera (branch in Fig. 2). Similar statistics for the whole tree were calculated using the maximum posterior estimates of the parameters of the lognormal distribution (which modeled rate variation across the tree).

myr [95% CI 0.0–89.1]). If we conservatively assume that the ancestral Palaeoptera had annual generations (as is typical of many extant Palaeoptera), and an effective population size of order 10^6 (typical of cosmopolitan insect species for which we have good estimates: Lehmann et al. 1998; Michel et al. 2006; Shapiro et al. 2007), then this implies a mean neutral coalescence time of 4 myr ($=4N_e$ generations), which is rapid compared with the ~ 30 myr of the branch. Furthermore, we place the origin of the crown-group Pterygota well before the near-simultaneous appearance of the Odonata, Ephemeroptera, and Neoptera in the lowermost Upper Carboniferous (Carpenter 1992; Rasnitsyn and Quicke 2002; Grimaldi and Engel 2005);

estimated branching of the Pterygota: median 385.3 myr [95% CI 353.3–418.0] and Palaeoptera: median 351.4 myr [95% CI 298.2–396.4]. Of course, these dates are for coalescence times rather than the appearance of morphological synapomorphies, but the estimates are nonetheless consistent with a substantial period of hidden divergence (see “Discussion” section).

Analysis of 38-Taxon Data Set 2

Although our primary data set 1 gave consistent results, results for our data set 2 (which contain many fewer taxa, but 2 additional genes) are highly

inconsistent. For the full data set, ML and Bayesian approaches both return a metapterygote clade (albeit with low support), which was decisively rejected for data set 1. Furthermore, results changed qualitatively with relatively minor changes in the data: correction for base composition heterogeneity, with or without the removal of mitochondrial markers, led to support for Chiasmomyaria from both ML and Bayesian methods (Table 2 and Supplementary Table S4). To test whether this inconsistency was due to the taxon-poor nature of the data set, we reanalyzed a subset of our taxon-rich data set 1, which contained the same taxa as data set 1. Results were again inconsistent and sensitive to small changes in data, with ML analyses now favoring either Chiasmomyaria or Palaeoptera (Supplementary Table S5). Despite this general pattern, however, the removal of the outgroup taxa, and the rooting of the tree with temporal information, again favored Palaeoptera under all conditions (as with data set 1), albeit without strong Bayes factor support in all cases (Table 2 and Supplementary Table S5).

Reanalyses of Published Data Sets

Analysis of our own data sets showed support for the Palaeoptera, but these results were consistent only when (i) taxon sampling was extensive (as for data set 1) or (ii) temporal information was used to root the tree. This suggests that inconsistency in the literature concerning the basal Pterygote node might reflect systematic error stemming from sparse taxon sampling and distant outgroups. Indeed, most published studies have used even fewer taxa than our data set 2 (although often using many more genes).

Some of these previous studies also found support for Palaeoptera as true clade. For example, Regier et al. (2010), used 60 genes from 10 pterygote taxa (2 Odonata, 2 Ephemeroptera, and 6 nonendopterygote Neoptera) and several closely related hexapod outgroups (including 2 Zygentoma, 2 Archaeognatha, 3 Collembola, and 1 Diplura), while Meusemann et al. (2010) used 129 genes (filtered from over 700) from 19 pterygotes (1 Odonata, 1 Ephemeroptera, and 17 nonendopterygote Neoptera) together with 3 Archaeognatha and 3 Collembola outgroups.

Although results of these 2 studies were consistent with each other, other multigene data sets have shown strong support for the alternative hypotheses (Zhang et al. 2008; Simon et al. 2009; Lin et al. 2010). For example, Simon et al. (2009) used a data set containing 125 genes from 8 taxa (1 Odonata, 1 Ephemeroptera, 4 Neoptera, and 1 Collembola [*Onychiurus arcticus*] as outgroup) and obtained strong support for Chiasmomyaria. A difference between this study and the similar studies of Meusemann et al. (2010) and Regier et al. (2010)—which supported Palaeoptera—is the choice of outgroup. The latter studies used wingless insect groups (Archaeognatha and Zygentoma) while Simon et al. (2009) use the more distantly related Collembola. To assess the impact of outgroup

choice, we reanalyzed the data set of Simon et al. (2009) in BEAST, with and without the outgroup taxa. When the outgroup was included, these data produced decisive evidence for Chiasmomyaria with both ML and Bayesian methods (100% bootstrap support and posterior probability). However, when the outgroup was excluded from the BEAST analyses, the supported topology was Palaeoptera, although posterior support (59%) was weak.

Results even more discordant with our own were obtained by Zhang et al. (2008). These authors obtained strong support for the Metapterygota using the complete mitochondrial genomes of 11 taxa (1 Odonata, 1 Ephemeroptera, 7 Neoptera, with 1 Thysanura, and 1 Archaeognatha as outgroup). The data set of Zhang et al. (2008) was extended by Lin et al. (2010), to include an additional 3 Neoptera, 2 Odonata, 1 Ephemeroptera, and 10 outgroup taxa. These authors found support for a modified Chiasmomyaria, but with the Plecoptera (stoneflies) grouping with Ephemeroptera; thus, this data set supported a paraphyletic Neoptera, which is at odds with most insect phylogenies (Zwick 2000; Kjer et al. 2006; von Reumont et al. 2009; Ishiwata et al. 2011).

To investigate the discrepancies between these results and our own, we reanalyzed the data set of Lin et al. (2010); the results of this reanalysis are in Table 3. ML analysis, as expected, favored the modified Chiasmomyaria found by Lin et al. (2010). However, the BEAST analysis of the same data obtained 99% support for Metapterygota, consistent with the original findings of Zhang et al. (2008). Finally, a BEAST analysis without outgroups found most support for a nonstandard Palaeoptera, with Odonata as sister group to an Ephemeroptera + Plecoptera clade. The grouping of the Plecoptera outside of the Neoptera (Lin et al. 2010) is contradicted by many other studies, using both morphological and molecular characters (Zwick 2000; Kjer et al. 2006; von Reumont et al. 2009; Ishiwata et al. 2011), a situation that may be analogous to other anomalous results in the arthropods using mtDNA (Delsuc et al. 2003; Nardi et al. 2003; Cameron et al. 2004; Hassanin et al. 2005; Hassanin 2006; Carapelli et al. 2007; Rota-Stabelli and Telford 2008; Timmermans et al. 2008; Masta et al. 2009; Rota-Stabelli et al. 2010b). Because of the nonstandard placement of the Plecoptera, we repeated all analyses with this sequence removed. In this case, each of the analyses with outgroups included, favored Metapterygota (Zhang et al. 2008)—that is a different basal branching to that supported by Lin et al. (2010)—while the BEAST analysis without outgroup again favored Palaeoptera.

DISCUSSION

The branching of the basal pterygotan lineages has proved a problem for biologists since it was first investigated in the early 20th century. Morphologists have argued for all 3 possible groupings, and so, later, have researchers using DNA sequence data. Even the

TABLE 3. Reanalyses of Lin et al. (2010) data set

Data set	# Taxa	# Genes	Grouping	Original data set			Composition bias corrected		
				ML	BEAST	BEAST (no OG)	ML	BEAST	BEAST (no OG)
Lin et al. (2010) All taxa	13 + 12 OG	13	Palaeoptera	0.00	0.00	0.00	0.02	< 0.001	0.00
			Chiaistomyaria	0.00	0.00	0.00	0.13	0.00	0.00
			Metapterygota	0.01	0.00	0.00	0.36	0.00	0.00
			Alternative topology	0.92^a	1.00^a	0.51^a	0.44^a	0.99^a	0.63^b
Lin et al. (2010), Plecoptera removed	12 + 12 OG	13	Palaeoptera	0.09	< 0.001	0.46	0.26	< 0.001	0.67
			Chiaistomyaria	0.18	0.01	0.41	0.02	< 0.001	0.24
			Metapterygota	0.67	0.99	0.13	0.72	1.00	0.09

Notes: Values indicate bootstrap support (ML) or posterior probability (BEAST) for the specified clade. ML or maximum posterior groupings are shown in bold. ^aA Chiaistomyaria grouping, with an Ephemeroptera + Plecoptera clade; ^bA Palaeoptera-like grouping, with an Ephemeroptera + Plecoptera clade.

recent application of large phylogenomic data sets has produced support for all 3 branching patterns. In some cases, inconsistency among molecular phylogenies may result from incomplete lineage sorting (i.e., between-locus variation in gene genealogies, which can arise when speciation events occur more rapidly than the coalescence of alleles [Pamilo and Nei 1988]). We have estimated that the basal radiation of the Pterygota was not particularly rapid (Fig. 2), but there is no way to convincingly reject incomplete lineage sorting with our data sets (containing, as they do, so few independent markers). However, we can show that other sources of systematic error are important. In particular, lineage sorting cannot explain the disagreements between studies using different mtDNA markers (e.g., Zhang et al. 2008; Lin et al. 2009; Tables 2 and 3), because all mitochondrial genes are inherited as a single unit (Moore 1995; Ballard and Whitlock 2004). Furthermore, with our data set 2, ML analyses of individual partitions gave quite inconsistent results, even when these are completely linked and so must have followed the same genealogy in reality; most notably, different groupings were supported by different codon positions of the EF1- α locus (Supplementary Table S6).

If incomplete lineage sorting is not the sole source of disagreement, then systematic error must also play a role (Baurain et al. 2007). Our results suggest that 2 factors are particularly important. First, we have shown that reduced taxon sampling can lead to results that are highly dependent on small changes in the data and assumptions of the analysis. Indeed, our 38-taxon data sets showed apparent support for all 3 grouping (Table 2 and Supplementary Table S5). This conclusion is consistent with other systematic explorations of taxon sampling (Zwickl and Hillis 2002). Second, we have shown that outgroup choice may be particularly difficult for the basal Pterygota. Outgroup choice has been a problem for morphological studies (because an outgroup is lacking for wing-based characters), but also for molecular studies, as is clear from Table 3. We have shown here that problems of outgroup choice may be mitigated by the use of temporal

information in estimating phylogenies (Drummond et al. 2006). Data sets that gave wildly inconsistent results when outgroups were used give consistent support for the Palaeoptera hypothesis when outgroups were removed and temporal information used to root the trees (Tables 2 and 3; Supplementary Table S5). In addition to exploiting additional data (Table 1 and Supplementary Tables S3 and S4), these results clearly rely on additional assumptions about the evolutionary process, preferring solutions that are relatively clock-like (Drummond et al. 2006), however, they do appear to be robust to minor variations in the data and model assumptions (Tables 2 and 3; Supplementary Tables S4 and S5). It is also notable that the support for Palaeoptera is in agreement with those previous studies that used large data sets of many nuclear loci and used the most closely related apterygote orders as outgroup taxa (Meusemann et al. 2010; Regier et al. 2010).

If future studies support the interpretation shown in Figure 2, what can we conclude about pterygotan evolution? First, if we accept the inferred timescale, then the near-simultaneous appearance of the Odonata, Ephemeroptera, and Neoptera in the lowermost Upper Carboniferous (Carpenter 1992; Rasnitsyn and Quicke 2002; Grimaldi and Engel 2005) would represent a preservational artifact, rather than a genuine rapid radiation. Other aspects of the record, in fact, support this interpretation. Most important is the speciose nature of the early records of Odonata-like, Ephemeroptera-like, Neoptera-like, and early palaeodictyopteroid insects, and the absence of fossils for the extant apterygote outgroups, Thysanura, Archaeognatha, and Diplura (Carpenter 1992; Rasnitsyn and Quicke 2002; Grimaldi and Engel 2005), although the topology implies long branches connecting these taxa to the pterygote insects.

Regardless of the timescale, other conclusions follow if we accept the Palaeoptera as a true clade (Meusemann et al. 2010; Regier et al. 2010; Fig. 2). In particular, all 3 hypotheses shown in Figure 1 are consistent with the palaeopterous condition (the inability to fold the wings) having a single origin, but only

the Palaeoptera hypothesis (Figs. 1a and 2) suggests that this state might be truly synapomorphic, rather than symplesiomorphic (a shared primitive state), as originally conjectured by Martynov (1924). As such, this grouping makes it far more probable that neopteran wing folding is the primitive state or at least evolved from a nonpalaeopterous ancestor—and this would have important implications for the origins of insect flight (Kristensen 1991; Kingsolver and Koehl 1994; Wootton and Kukalová-Peck 2000; Kukalová-Peck 2008; Edwards 1997). However, drawing strong conclusions is complicated by the fact that different sets of wing vein fusions are likely to be responsible for the palaeopteran trait in Odonata and Ephemeroptera, and indeed in members of the extinct Palaeodictyopteroidea—although some parts of the complex of wing vein fusions that separately prevent wing folding may be common to all of these groups. Furthermore, we currently lack agreement among morphologists about which veins in the very highly derived odonate wing correspond to which veins in other insects (Tillyard 1926; Hamilton 1972; Carle 1982b; Riek and Kukalová-Peck 1984; Watson and O'Farrell 1991), and about where the Palaeodictyopteroidea should be placed on the phylogeny. These issues will have to be decided by further morphological examination of extant and Palaeozoic insects.

SUPPLEMENTARY MATERIAL

Supplementary material, including data files and/or online-only appendices, can be found at <http://datadryad.org>, doi: 10.5061/dryad.7d2g2.

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