64 (1) 35–44

A Molecular Phylogeny of Hexapoda

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Received 27.ii.2006, accepted 23.viii.2006.
Available online at www.arthropod-systematics.de

> Abstract

We present a supermatrix approach to the phylogeny of Insecta that stemmed from a talk given at the 2nd Dresden Meeting on Insect Phylogeny (2005). The data included a fragment of the 28S (D1–D8) and complete sequences for the 18S, histone (H3), EF-1α, COI, COII, the 12S and 16S plus the intervening tRNA, and 170 morphological characters. Ribosomal RNA sequences were manually aligned to secondary structure. Two separate Bayesian likelihood analyses were performed, as well as a weighted parsimony analysis, on combined data. Partitioned datasets were also explored. Expected clades like Hexapoda, Insecta, Dicondylia, Pterygota, Neoptera, Dictyoptera, Paraneoptera, and Endopterygota were consistently recovered. However, conflicting hypotheses from independent datasets, as well as a lack of quantitative support from the combined supermatrix, suggest that the elucidation of relationships between non-holometabolous neopteran orders is far from resolved. Substitution rate heterogeneity among lineages, missing intermediate taxa, near simultaneous divergences, flawed phylogenetic models and nucleotide compositional bias are discussed as possible causes for unresolved interordinal relationships. The capacity of this dataset to convey information, its inherent limitations, and the role and responsibility of the systematist in interpreting data are explored.

> Key words

Insecta, Hexapoda, phylogeny, ribosomal RNA, secondary structure alignment, supermatrix, Bayesian analysis, weighted parsimony, among site rate variation, compositional bias.

1. Introduction

Our understanding of hexapod phylogeny has been dominated by two data sources; morphology, and nuclear small subunit rRNA (18S). Recently, a large fragment of the nuclear large subunit rRNA (28S) has been added by M.F. Whiting and others, along with the nuclear protein coding gene, histone H3 (OGDEN & WHITING 2003, 2005; WHITING et al. 2003; TERRY & WHITING 2005a). Several mitochondrial genes are also available for a large number of insect taxa, and recently, insect mitochondrial genomes have been explored (CAMERON et al. 2004, 2006a,b). Kjer was invited to present a combined data analysis of insect relationships, at the 2nd Dresden Meeting on Insect Phylogeny, which took place in September, 2005. In preparing for this presentation, we combined sequence data from GenBank with a new morphological matrix.

Analytical assumptions have a great influence on phylogenetic conclusions. The most influential and productive group of workers on insect molecular phylogenetics (WHITING et al. 1997, 2003; WHEELER et al. 2001; WHITING 2002a,b; SVENSON & WHITING 2004; TERRY & WHITING 2005a,b) favor unadjusted computer alignments, and most recently, a direct optimization approach using a program called POY (WHEELER 1996; GLADSTEIN & WHEELER 1997). While we credit these workers for their contribution to insect systematics, we feel that this approach suffers from some serious drawbacks (see KJER et al. in press). We therefore felt it important to provide alternative conclusions to those drawn from POY analyses of combined data. We show qualitative support through corroboration with partitioned analyses, followed by quantitative support with combined data. We also show the robustness of our conclusions to changes in analytical assumptions. These analyses are meant to provide the basis for a discussion of the issues from an alternative perspective, rather than to provide the definitive phylogeny of Insecta (which is not yet ready).

2. Methods

The data included complete 18S (1882 nucleotides = nts), a fragment of 28S (D1–D8: 2214 nts), Histone H3 (375 nts), the complete EF-1α (1243 nts, 414 amino acids = AAs), the complete COI (1551 nts, 518 AAs), the complete COII (684 nts, 228 AAs), complete 12S and 16S plus the intervening tRNA (1435 nts), and 170 morphological characters taken from the literature. We also examined the nuclear EF-2 gene (2188 nts, 729 AAs), but did not include it in the combined data because there were so few pterygote sequences available. In total, there were 14,209 characters in combinations of nucleotides, amino acids, and morphology. This represents 6 independent partitions: nuclear ribosomal genes (18S+28S), Histone H3, EF-1α, mitochondrial genes (COI, COII, 12S, 16S), and morphology. Data were put together with a supermatrix approach with 137 taxa from 18S providing a core (all taxa had 18S data). Other data were added to the 18S data, usually from the same genus. A few terminal taxa are chimeras combined from members of the same family. In a very few cases, when sampling within an order was limited to two taxa, we constructed a chimera of any two taxa within the order (permissible because monophyletic nodes with two taxa can freely spin without affecting the tree).

Ribosomal RNAs were aligned manually with reference to secondary structure, following KJER (2004). Two Bayesian likelihood analyses were performed using the program MrBayes (HUELSENBECK & RONQUIST 2002), one partitioned by gene, using a GTR+I+G model for the nucleotides (YANG 1994; YANG et al. 1994; GU et al. 1995), and an MK model for the morphology (LEWIS 2001), and the other using a site-specific rate model (SSR, described in KJER et al. 2001), with morphology excluded. Each analysis consisted of two separate runs of 750,000 iterations. After the "burnin" trees were discarded, trees from both analyses (all four runs) were pooled into a single treefile, from which a majority-rule consensus tree was constructed.

Weighted parsimony was also employed with a subset of the data we designated as "conservative". These data included the nuclear and mitochondrial rRNAs, and amino acids from protein coding genes (nucleotides excluded). We implemented pseudo-replicate reweighting, as in KJER et al. (2001) on the "conservative dataset".

In order to explore whether or not the morphology had a large effect on phylogenetic conclusions, a morphological matrix was constructed from the literature (KRISTENSEN 1975, 1981, 1991; BOUDREAUX 1979; HENNIG 1981). For the most part, orders were used as terminal taxa, except for *Timema* (Phasmatodea), *Cryptocercus* (Blattaria), *Liposcelis* (Psocodea) and *Boreus* (Mecoptera), which were coded separately. We focused on the characters these authors have used in coming to conflicting conclusions regarding the positions of the palaeopterous orders (Ephemeroptera and Odonata), Dictyoptera, and Endopterygota. One hundred and seventy binary characters were used. Other more recent work on morphology (KLASS 1997, 1998, 2003; WILLMANN 1997; GEREBEN-KRENN & PASS 1999; BITSCH & BITSCH 2000; KOCH 2000; STANICZEK 2000; BEUTEL & GORB 2001; HÖRNSCHEMEYER 2002 are only few examples), fossils (GRIMALDI 2001; ENGEL & GRIMALDI 2004; GRIMALDI & ENGEL 2005), sperm structure (DALLAI et al. 2002), embryology (MACHIDA et al. 2004) and many other systems discussed in this symposium were not included because we did not have the time to compile them for this largely molecular presentation. These are important works, which have greatly expanded morphological knowledge and massively revised previous interpretations, and should eventually be included.

3. Results and discussion

3.1. Combined data

Results from the Bayesian analyses are shown in Fig. 1. There is strong support for most relationships that would not surprise us; Pancrustacea, Hexapoda, Insecta, Dicondylia, Pterygota, Neoptera, Dictyoptera, Paraneoptera, and Endopterygota are supported, along with the monophyly of most orders. When we look for further resolution of the nodes that are in conflict among insect systematists, we find little information from this huge dataset. Interestingly, however, these analyses yield a clade Collembola + Protura + Diplura (i.e. the old "Entognatha"), and a clade Odonata + Ephemeroptera (i.e., the strongly disputed "Palaeoptera". A further interesting aspect is the group including Grylloblattodea, Mantophasmatodea, Phasmatodea, Embioptera, and Dictyoptera. This group was supported in 89% of the Bayesian trees, and also with the weighted parsimony analysis (Fig. 2). However, when we look for stability of analytical assumptions in other nodes by examining the weighted parsimony tree (Fig. 2), we see that what we might find most "interesting" in one analysis, are not found in the other. An exception to this is a strong resolution of a monophyletic Palaeoptera, from both Bayesian analyses, (Fig. 1: despite the inclusion of morphological characters that favor a clade containing Odonata and Neoptera), and from the weighted parsimony analysis

of the altered "conservative" dataset (Fig. 2; amino acids replacing nucleotide characters).

Bayesian analyses have been widely embraced in systematics, not because systematists are naturally Bayesians, but rather, because these analyses permit iterative refinement of multiple parameters, and also provide branch support that would be impossible under traditional likelihood analysis in a reasonable amount of time. (In other words, Bayesian analyses are fast and efficient.) However, we have been concerned about relatively high support, in the form of Bayesian posterior probabilities, for the same short internodes that have low bootstrap support when analyzed under parsimony and likelihood. Lewis et al. (2005) demonstrate this problem clearly, and we regard Bayesian branch support from short internodes with suspicion. P. Lewis and coworkers are working on software solutions for this problem, but none were available for these analyses. Bayesian posterior probabilities are interpreted as "the probability that a particular node is true…given the model and the data". It is important not to overemphasize the first part of the statement as evidence for truth, because we know that all models are simplifications, and the data may be biased. Since we are most interested in phylogeny, and since we believe that phylogenetic hypotheses without indications of branch support are nearly meaningless, we feel that the problems with posterior probabilities are serious. In order to interpret branch support (before there is a remedy the problem of high support for hard polytomies), we devised a temporary solution: alter both the model and the data, so that these less interesting parts of the definition play a less important role in the estimates of branch support. This is what we did here, by combining two Bayesian analyses; one with a partitioned GTR+I+G model, with morphology (under an MK model), and another with a site-specific rate model without morphology (Fig. 1). These are very different treatments of the data, but none-theless, recover remarkably similar phylogenies.

3.2. Partitioned data

While quantitative support should come from combined data (Figs. 1 and 2), qualitative support (influencing what we believe to be accurate, or at least corroborated) can come from partitioned data. Is there any corroboration for relationships among polyneopteran orders that we can deduce from independent datasets?

Morphology. A parsimony analysis of the morphology supported the clades Insecta, Dicondylia, Pterygota, Odonata + Neoptera, Neoptera, *Timema* + Euphasmida + Embioptera, Dictyoptera, Blattaria + Mantodea, Paraneoptera, Psocoptera + *Liposcelis* + Phthiraptera, Thysanoptera + Hemiptera, and Endopterygota. Within Endopterygota, relationships were (Coleoptera + (Neuroptera + (Hymenoptera + (Diptera + (Mecoptera + ((*Boreus* + Siphonaptera) + (Amphiesmenoptera))))))). Except for those noted above, orders were monophyletic (but this would be expected, since for the most part, orders were coded as terminal taxa, and this provides a strong constraint on combined analyses, both here, and in other studies that use this approach). There was no resolution among polyneopteran orders other than listed above. The point of our morphological analysis was not to provide anything new, but rather, to see how the traditional morphological characters would influence a combined analysis. Our morphological matrix was substandard in many ways. First, we omitted much of the recent work. Second, in our opinion, molecular systematists producing "groundplan" coded characters from the literature, without actually looking at specimens, is better than nothing…but just barely. What is desperately needed is a morphology matrix, put together by a morphologist, collaborating with a molecular systematist with coordinated taxa. In the future, we hope to collaborate with morphologists who wish to add a molecular component to their work.

Histone H3. This nuclear protein-coding gene with extreme conservation in amino acid structure, has been sequenced for a large number of insects. The only ordinal level node (and above) we recovered in a parsimony analysis of H3 was Mantodea. Every other taxon found in the combined analysis, at the level of order or above, was polyphyletic or paraphyletic. Model-based analyses may correct for homoplasy, so we ran a GTR+I+G Bayesian likelihood analysis of the H3 dataset. This analysis added Zygoptera and Crustacea to the clades found in the parsimony analysis, but clearly, the H3 by itself does not provide information in estimating relationships among insect orders. The reason for this is similar to problems encountered with the EF-1α, as discussed in Kjer et al. (2001). As is the case with proteins that are so tightly conserved, the signal is dominated by silent 3rd codon sites, and is highly homoplastic, even among closely related taxa. Conservative "looking" protein-coding genes (with invariant first and second codon positions) are inappropriate for deep-level phylogenetics unless there are sufficient amino acid changes to provide some nonhomoplastic signal. We examined amino acids in H3 as a potential source of conservative signal. There are 4 parsimony informative amino acid positions among arthropods (three of them within a span of four codons). All 4 shared the same pattern, grouping *Negha* (Rhaphidioptera), *Dolichopeza* (Diptera), and *Letothorax* (Hymenoptera) together, and apart

from *Anopheles* (Diptera), *Sialis* (Megaloptera), and *Hemerobius* (Neuroptera). There are apparently two permissible conformations; change one amino acid, and the other three amino acids change. This pattern must either represent convergence, or the polyphyly of both flies and neuropteroids. One may keep the H3 in, or throw it out, it doesn't matter. But the insect phylogenetics community should stop sequencing this uninformative marker for deep-level phylogenetics, and spend its resources on collecting appropriate data.

Elongation factor EF-2. Examining the EF-2, we conclude that there are not enough pterygotan sequences to be useful for estimating polyneopteran relationships. Among pterygotes, only Ephemeroptera, Dermaptera, Blattaria, Hemiptera, and Diptera were represented. However, a separate analysis of the EF-2 did recover Pancrustacea, adding to the number of molecular markers that consistently recover this group. The mitochondrial data, and the nuclear rRNA data also supported Pancrustacea.

Nuclear rRNAs. The combined results are still dominated by nuclear rRNAs. This is probably a good thing, as these markers possess meaningful characters for a wide range of divergence times. Characters in rRNA stems are also less prone to convergence, because the nucleotide state that is coded in the datamatrix is not under direct selection. Instead, the structure is selected, and any of the 4 nucleotide states may function structurally. Fig. 3 represents the single tree with the best likelihood score from a GTR+I+G Bayesian analysis of the nuclear rRNAs (remember, the "majority rule" consensus, from which posterior probabilities are calculated, is different from this tree). Interestingly, the nuclear rRNAs support Ephemeroptera + Neoptera. This is the same result as in the analysis of the 18S alone in KJER (2004). Thus the addition of the 28S data to the 18S data does not alter the positions of the two palaeopterous orders. A partitioned analysis of the EF-1α also recovered Ephemeroptera + Neoptera. However, Palaeoptera is monophyletic in the combined analyses (Figs. 1 and 2) as well as in a partitioned analysis of mitochondrial data (not shown). This result is coming from a few mitochondrial characters that conflict with other more conservative markers.

3.3 Interpretation of phylogenetic results

Support, accuracy, and error. We may believe that Palaeoptera is monophyletic, because of the combined analysis, or because of their wings. We may believe that Odonata is plesiotypic because it lacks direct sperm transfer, or because the two most conservative molecular markers put Ephemeroptera with Neoptera. Just as morphologists have argued for their beliefs in the face of conflicting morphological evidence, when the evidence is weak, molecular systematists may argue their beliefs in the face of conflicting molecular evidence. This conflict illustrates the problem we all face between the philosophy of science and the utility of phylogenetic hypotheses. We can never *prove* a phylogenetic hypothesis is true, and such an attempt may be counterscientific. However, as working systematists, we prefer "accurate" hypotheses, and the pursuit of accuracy should not be discouraged as meaningless, even under a "falsification" paradigm. Trees that are "wrong" are worse than worthless, because they actively promote confusion. Things like bootstrap values, or congruence do not demonstrate truth, but they can influence our beliefs. Here we present our results, but also provide our interpretation of those results.

Confounding factors in insect phylogenetics. Fig. 3 is presented to show the root of the problem with estimating relationships among the principal lineages of Neoptera. These lineages diverged from one-another hundreds of millions of years ago, and the fossil evidence indicates that this divergence was nearly simultaneous (LABANDEIRA & SEPKOSKI 1993; GRIMALDI & ENGEL 2005). The lengths of the internodes separating neopteran lineages in Fig. 3 are virtually zero. Adding additional data that is noisy or essentially randomized will never get us any closer to answering these difficult questions, even though it may provide "strong support" based on bias in the data due to branch length heterogeneity or nucleotide composition.

Another striking feature that can be seen in Fig. 3 is the excessive rate heterogeneity among orders. Diptera and Diplura evolve at rates that are orders of magnitude higher than the norm. Strepsiptera has similarly accelerated rates, as does Zoraptera (see phylogram in YOSHIZAWA & JOHNSON 2005), yet Odonata and Mantodea seem virtually frozen. While at least with Diptera, we can "believe" that it is endopterygotan, and not a crustacean, there is little to guide us with Zoraptera. YOSHIZAWA & JOHNSON (2005) place Zoraptera with the Dictyoptera. TERRY & WHITING (2005a) place Zoraptera with Dermaptera. We chose not to include Zoraptera in this analysis because of its excessive substitution rate acceleration, and associated alignment difficulties (similar to our decision to exclude Strepsiptera). By sequencing additional zorapterans, YOSHIZAWA & JOHNSON (2005) confirmed that the zorapteran sequence from WHEELER et al. (2001) was not a contaminant (as mistakenly suggested as a possibility by KJER 2004), and also confirmed the bizarre nature of zorapteran rRNA. One

could legitimately criticize us for omitting Zoraptera and Strepsiptera, but not for the concept that excessive substitution rate heterogeneity makes phylogenetics problematic, whether through long branch attraction in parsimony, or inadequate models in likelihood (YANG 1996; BUCKLEY et al. 2001). The diplurans always grouped with the proturans. The flies were never with Antliophora, but the fact that they always came out within Endopterygota gave us some degree of reassurance. No such reassurance would be possible with Strepsiptera or Zoraptera, regardless of their placement. Could our placement of Diptera and Diplura have been affected by their elevated substitution rates? Maybe. It would be good to examine this possibility in the future.

Effect of adding taxa. Another point of discussion that is very clear from Fig. 3 is whether or not taxon sampling will help solidify our beliefs about relationships among the major lineages of Neoptera. We think not. If one samples two widely separated taxa within an order (a cricket, and a grasshopper for example), then the branch leading to these taxa cannot be further subdivided unless some proto-orthopteran is included. The discovery of unexpected diversity is not impossible, as we have seen with the discovery of Mantophasmatodea (KLASS et al. 2002; PICKER et al. 2002), but at the level of orders, it would be very difficult to truncate the branches leading to any particular order shown in Fig. 3, except for the branches leading to Grylloblattodea, and Mantophasmatodea, which are lightly sampled in these analyses. Taxon sampling will certainly help with the resolution within orders, but when looking for relationships among orders, the sampling plan used herein is adequate. The situation is similar to morphology, where we find a wealth of characters that define, for example, Orthoptera or Coleoptera. The difficulty is finding synapomorphies that group the orders to one-another. Looking at the short internodes among the principal neopteran lineages, there was probably very little time in which to accumulate synapomorphies that link one order to another, as is indicated by the lack of space to accommodate additional taxa on the internodes of Fig. 3. What we need is more quality in the molecular data, care in its analysis, and a morphological data matrix to go with it. The addition of fossil data, even though extremely fragmentary, may help (WIENS 2005).

Missing data. These can cause problems, and these problems are difficult to spot if one has no phylogenetic expectations. However, if we expect the recovery of some minimal corroborated phylogeny, such as the monophyly of most insect orders, then we can assess how the supermatrix approach worked. For example, if one were to see Ephemeroptera come out in two places on the tree, and one of these "clades" included all the taxa for which there was mitochondrial data, and the other "clade" included all the taxa for which there was 18S data, we could say with some confidence that the supermatrix failed, because it was grouping taxa according to the presence or absence of data. If one could judge our analyses by the recovery of expected relationships, the supermatrix approach worked. Despite the fact that many of the data cells are missing in these analyses, orders grouped together, *without constraints*, with and without morphology. Once an order is grouped together, the missing data is simply filled in by the algorithm, such that it is assumed that other taxa in the order fit the "groundplan" estimated from the data cells that are filled.

3.4. Conclusions

Even with a data matrix in excess of 10,000 characters, we find little or no support for relationships among polyneopteran orders, except for Dictyoptera. We get phasmids with the Embioptera. The nuclear rRNAs place Mantophasmatodea with the Grylloblattodea, while the mitochondrial data place them with the Phasmatodea (see also results in TERRY & WHITING 2005 and CAMERON et al. 2006a,b).

Our approach was to address insect phylogenetics to a group of trained insect systematists by talking about the data and its properties. We look for corroboration among independent datasets (and find little), and we evaluate quantitative support from combined data (again, we find little). This approach is different from the direct optimization (POY) of combined data. This is not to say that our approach is the "correct" one. Rather, we think that looking at the data and discussing it as if we were interested in "truth", even if it can never be achieved, is useful in influencing what we believe to be supported by the data.

4. Acknowledgements

We thank Klaus-Dieter Klass for inviting us to participate in this forum and for his help in facilitating peer review for this work. We thank Kenneth (Tripp) MacDonald, Klaus-Dieter Klass and an anonymous reviewer for helpful comments on the manuscript. Financial support from NSF DEB 0423834, and the New Jersey Agricultural Experiment Station, is appreciated.

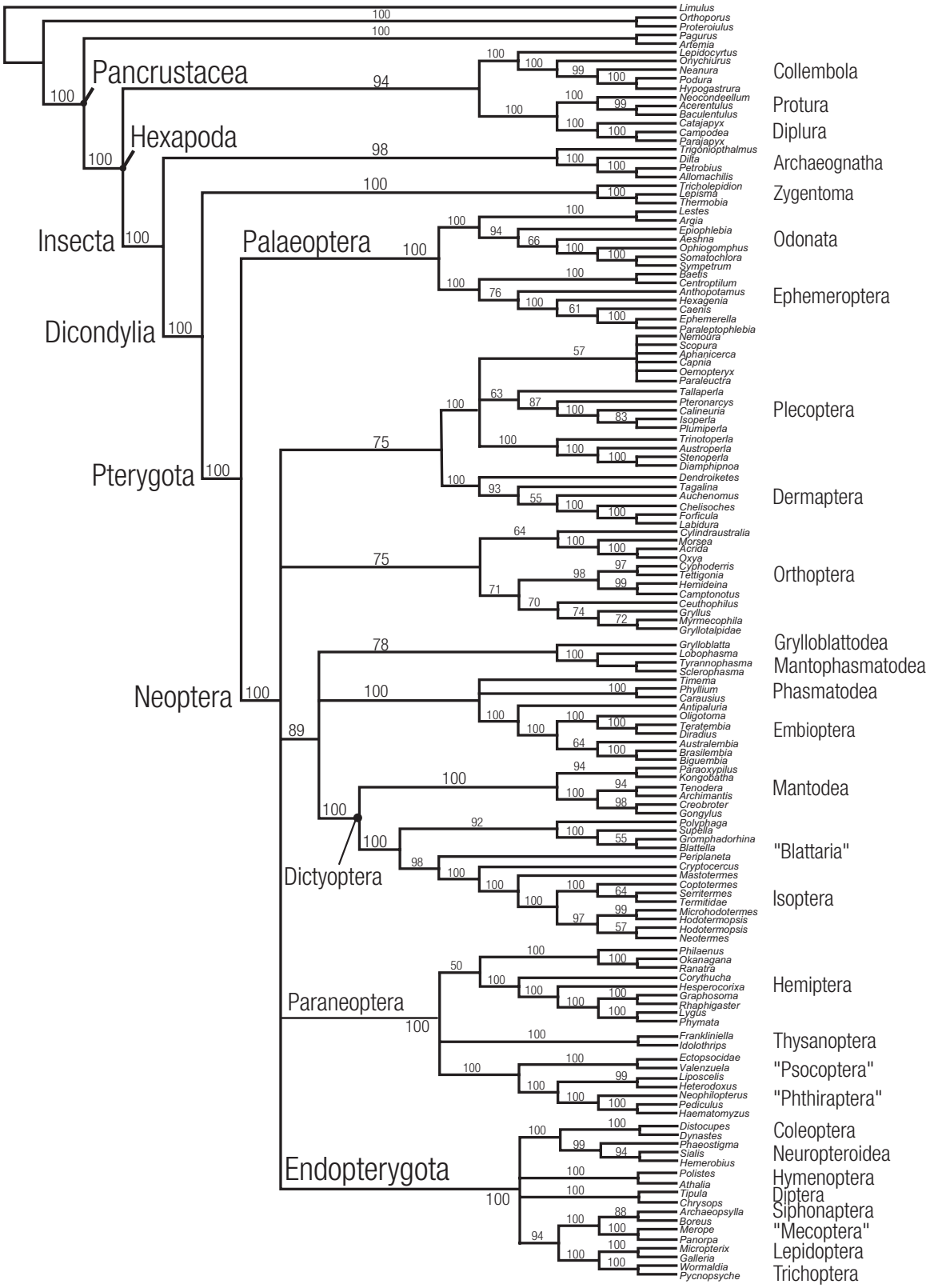

Fig. 1. Results from Bayesian analyses. This is a majority-rule consensus tree from two separate analyses; one using a GTR+I+G for the nucleotides, with an MK model for the morphology, the other with a SSR model, with morphology excluded. Numerals above the nodes represent the percentage that these nodes were present among the pool of most likely trees (from all analyses) after the likelihood scores had stabilized.

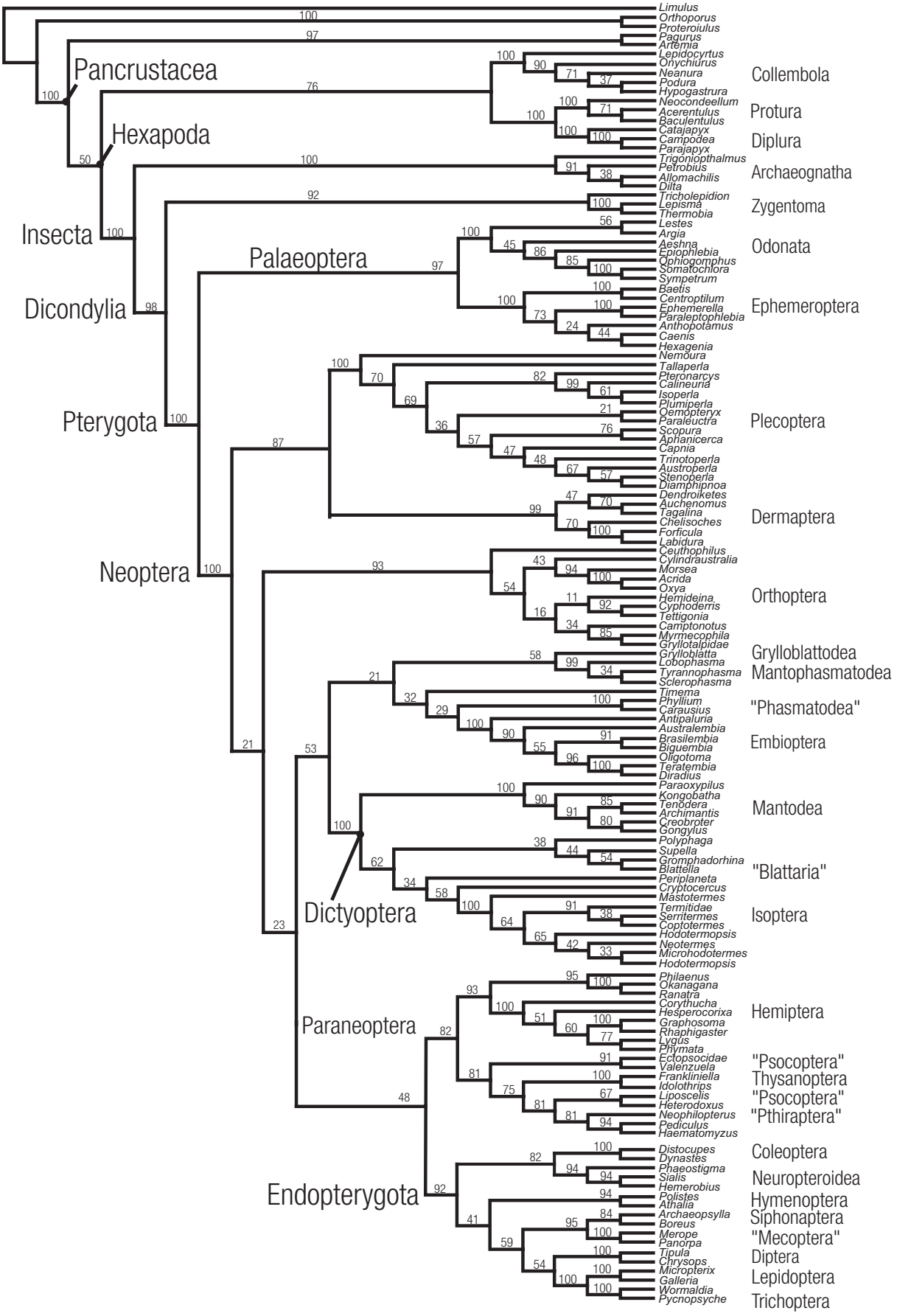

Fig. 2. Results from a weighted parsimony analysis of the combined "conservative" data. Numerals above the nodes are bootstrap values.

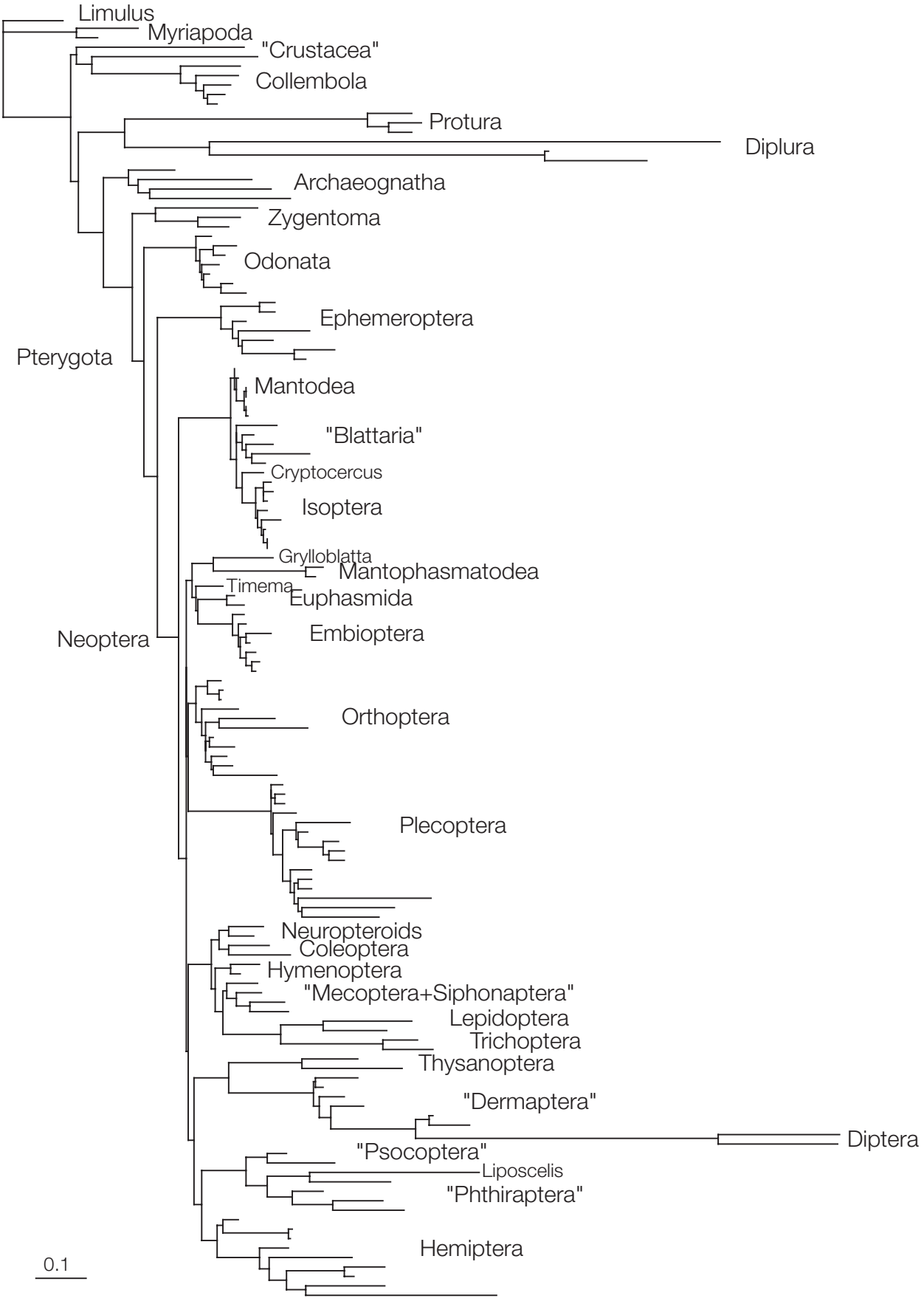

Fig. 3. Phylogram of the most likely tree from a Bayesian analysis of nuclear rRNA data. The lengths of branches are proportional to the number of nucleotide changes per site; scale: 0.1.

Tab. 1. Clades from the combined analysis that were supported by multiple independent datasets in partitioned analyses. MtB = Bayesian analysis of mitochondrial data. MtWP = weighted parsimony analysis of mitochondrial data. NrRNA = nuclear rRNA. Morph = morphology.

Pancrustacea	EF-2	MtWP	MtB	NrRNA	
Hexapoda		MtWP	MtB	NrRNA	Morph
Insecta	EF-1α		MtB	NrRNA	Morph
Dicondylia				NrRNA	Morph
Pterygota	EF-1α		MtB	NrRNA	Morph
Neoptera	EF-1α			NrRNA	Morph
Dictyoptera		MtWP	MtB	NrRNA	Morph
Cryptocercus + Isoptera			MtB	NrRNA	
Paraneoptera		MtWP	MtB	NrRNA	Morph
Endopterygota	EF-1α			NrRNA	Morph
Amphiesmenoptera				NrRNA	Morph

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