

Further use of nearly complete 28S and 18S rRNA genes to classify Ecdysozoa: 37 more arthropods and a kinorhynch

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Abstract

This work expands on a study from 2004 by Mallatt, Garey, and Shultz [Mallatt, J.M., Garey, J.R., Shultz, J.W., 2004. Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin. *Mol. Phylogenet. Evol.* 31, 178–191] that evaluated the phylogenetic relationships in Ecdysozoa (molting animals), especially arthropods. Here, the number of rRNA gene-sequences was effectively doubled for each major group of arthropods, and sequences from the phylum Kinorhyncha (mud dragons) were also included, bringing the number of ecdysozoan taxa to over 80. The methods emphasized maximum likelihood, Bayesian inference and statistical testing with parametric bootstrapping, but also included parsimony and minimum evolution. Prominent findings from our combined analysis of both genes are as follows. The fundamental subdivisions of Hexapoda (insects and relatives) are Insecta and Entognatha, with the latter consisting of collembolans (springtails) and a clade of proturans plus diplurans. Our rRNA-gene data provide the strongest evidence to date that the sister group of Hexapoda is Branchiopoda (fairy shrimps, tadpole shrimps, etc.), not Malacostraca. The large, Pancrustacea clade (hexapods within a paraphyletic Crustacea) divided into a few basic subclades: hexapods plus branchiopods; cirripedes (barnacles) plus malacostracans (lobsters, crabs, true shrimps, isopods, etc.); and the basally located clades of (a) ostracods (seed shrimps) and (b) branchiurans (fish lice) plus the bizarre pentastomids (tongue worms). These findings about Pancrustacea agree with a recent study by Regier, Shultz, and Kambic that used entirely different genes [Regier, J.C., Shultz, J.W., Kambic, R.E., 2005a. Pancrustacean phylogeny: hexapods are terrestrial crustaceans and maxillopods are not monophyletic. *Proc. R. Soc. B* 272, 395–401]. In Malacostraca, the stomatopod (mantis shrimp) was not at the base of the eumalacostracans, as is widely claimed, but grouped instead with an euphausiacean (krill). Within centipedes, *Craterostigma* was the sister to all other pleurostigmophorans, contrary to the consensus view. Our trees also united myriapods (millipedes and centipedes) with chelicerates (horseshoe crabs, spiders, scorpions, and relatives) and united pycnogonids (sea spiders) with chelicerates, but with much less support than in the previous rRNA-gene study. Finally, kinorhynchs joined priapulans (penis worms) at the base of Ecdysozoa.

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1. Introduction

Ecdysozoa (Aguinaldo et al., 1997) is a large clade of animals that grow by molting a cuticle at least once during their life cycles (Schmidt-Rhaesa et al., 1998). This clade

contains two of the most speciose animal phyla, arthropods and nematodes, together with tardigrades (the tiny water bears), onychophorans (velvet worms), nematomorphs (horsehair worms), priapulans (penis worms), kinorhynchs (mud dragons), and loriciferans. Although still somewhat controversial, the Ecdysozoa hypothesis has received increasing support over the past few years: See Giribet (2003a), Mallatt et al. (2004), Philip et al. (2005), and Philippe et al. (2005) for a history of the controversy, along

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with references and the best new evidence for and against Ecdysozoa.

Arthropods are abundant, morphologically diverse, speciose, and important economically, so their systematics has long been of interest to zoologists. This attention increased in the past decade as it became evident that the phylogenetic relationships derived from gene-sequences, although mostly agreeing with traditional morphology-derived relationships, sometimes show startling differences (Fortey and Thomas, 1998; Friedrich and Tautz, 1995; Giribet et al., 2001, 2005; Regier et al., 2005a,b; Wheeler et al., 1993). Constructing the phylogeny and interrelationships of arthropods is challenging because they appeared before ~520 million years ago, and the major subgroups of Chelicerata, Crustacea, Myriapoda, and Hexapoda were all present by ~325 million years ago, so their morphological and genetic characteristics experienced early diversification followed by long, subsequent, evolutionary changes (Chen et al., 2001; Dunlop, 2002; Edgecombe, 2004; Kukulová-Peck, 1991; Spears and Abele, 1998; Waloszek et al., 2005).

This paper is a sequel to a recent study (Mallatt et al., 2004) on the relationships within arthropods and other ecdysozoans. That study used nearly complete sequences of 28S and 18S rRNA genes from about 40 ecdysozoan taxa, and its main findings were: (1) priapulans are the sister group of the other represented Ecdysozoa; (2) Panarthropoda (tardigrades, onychophorans, and arthropods) and Arthropoda are each monophyletic; (3) within Arthropoda, hexapods are nested in a paraphyletic Crustacea, the entire group being called Pancrustacea (or Tetraconata: Dohle, 2001); (4) Myriapods join with chelicerates and pycnogonids as Paradoxopoda (or Myriochelata: Pisani et al., 2004); this Paradoxopoda, however, contrasts with the dominant view that myriapods join with hexapods and crustaceans as Mandibulata (e.g., Edgecombe et al., 2003; Giribet et al., 2001, 2005; Scholtz and Edgecombe, 2005); and (5) Collembola (springtails) are in a monophyletic Hexapoda.

Here, we further tested these findings and sought additional relationships, by sequencing nuclear rRNA genes from 37 additional arthropods from a broad range of subclades within the phylum. We also added sequences from eight other arthropods, obtained from GenBank. This effectively doubles the number of complete, arthropod rRNA-gene-sequences to over 80. Finally, we sequenced a non-arthropod ecdysozoan, a kinorhynch, to test the common view that kinorhynchs are related to priapulans (and loriciferans) in the larger clade Scalidophora (Garey, 2001; Lemburg, 1995; Nielsen, 2001; Neuhaus and Higgins, 2002).

Although we use a single gene family, with about 3850 informative nucleotides, these rRNA genes have been shown to contain good signal for resolving divergences in metazoan phylogenies that span the Cambrian to the present (e.g., Giribet, 2002; Mallatt and Winchell, 2002). Additionally, our rRNA-gene data can be combined with the growing number of sequences from many different genes (Brown et al., 2001; Philippe et al., 2005; Regier et al.,

2005a,b), and with morphological characters, in future “total evidence” analyses (Edgecombe, 2004; Giribet et al., 2001, 2005).

2. Materials and methods

2.1. Specimens, sequences, and alignments

In total, we analyzed nearly complete sequences of the two largest subunits of the nuclear ribosomal RNA genes for 81 ingroup terminals plus 8 outgroups. The taxa used are listed in Table 1, whereas more detailed information on the 38 newly sequenced taxa, including sources and voucher-specimen data, is presented in Supplementary material, S1.

Several aspects of these sequences should be noted. First, we used the same non-ecdysozoan outgroups as in the previous study: four spiralian and four deuterostomes. Second, the *Meganycitiphanes/Nycitiphanes* (krill), *Oxyethira* (caddisfly), and *Merope* (scorpionfly) sequences became available to us only after the intensive calculations for our main analyses (in Fig. 1) were under way, so these three species were used in analyses containing smaller subsets of taxa. Third, in a few cases it was necessary to assemble composite sequences from two genera in a family (see the *Meganycitiphanes/Nycitiphanes*, *Poppeal/Spissistilus*, *Myrmecial/Leptothorax*, and *Florometral/Antedon* entries in Table 1). Fourth, all our 28S + 18S rRNA-gene-sequences were over 90% complete, except for two taken from GenBank: That of krill *Meganycitiphanes/Nycitiphanes* was about 87% complete, and that of the treehopper *Poppeal/Spissistilus* was about 83% complete. Dozens more arthropod sequences, available but not used here, are two-thirds complete, consisting of 18S and the 5' half of the 28S rRNA gene (Giribet et al., 2005; Ogden and Whiting, 2003; Svenson and Whiting, 2004; Terry and Whiting, 2005; Whiting, 2002a; Whiting et al., 2003). Fifth, although the rRNA genes we sequenced contained parts of the 5.8S rRNA gene, only the 28S and 18S genes were used in this study (as in the previous study).

Extraction of DNA, primers used, PCR amplification conditions, and sequencing and fragment assembly, were as in previous studies (Luan et al., 2005; Mallatt et al., 2004). The genes of all taxa were aligned manually in GCG SeqLab (Wisconsin Package Version 10.3: Accelrys Inc., San Diego, CA) against a reference set of rRNA-gene-sequences from other metazoans, and with the aid of the secondary-structure models of the frog *Xenopus laevis* and the sea urchin *Strongylocentrotus purpuratus* rRNA (Gutell, 1994; Schnare et al., 1996). To rule out the possibility that these structure models apply only to deuterostomes, and not to ecdysozoans nor other eukaryotes, they were compared to models of yeast, *Saccharomyces cerevisiae*, rRNA, and were found to be essentially the same, indicating strongly conserved secondary structures across eukaryotes (see Gutell et al., 1993, for the yeast 28S rRNA, and http://www.psb.ugent.be/rRNA/secmodel/Scer_SSU.html for yeast 18S rRNA).

Table 1
Information on species used in this study

Classification	Species	GenBank Nos.	
		28S rRNA genes	18S rRNA genes
Arthropoda			
Chelicerata			
Xiphosura	<i>Limulus polyphemus</i>	AF212167	U91490
Arachnida			
Acari	<i>Dermacentor</i> sp.*	AY859582	Z74480
Pseudoscorpiones	<i>Chaloeiridius</i> cf. <i>termitophilus</i> *	AY859558	AY859559
Opiliones	<i>Siro rubens</i> *	AY859602	U36998
Solifugae	<i>Eremobates</i> sp.*	AY859572	AY859573
Scorpiones	<i>Pandinus imperator</i>	AY210830	AY210831
Uropygi	<i>Mastigoproctus giganteus</i> *	AY859587	AF005446
Amblypygi	<i>Paraphrynus</i> sp.*	AY859594	AF005445
Araneae	<i>Misumenops asperatus</i>	AY210461	AY210445
	<i>Aphonopelma hentzi</i>	AY210803	X13457
Pycnogonida			
	<i>Anoplodactylus</i> sp.*	AY859550	AY859551
	<i>Callipallene</i> sp.	AY210807	AY210808
	<i>Colossendeis</i> sp.	AY210809	AF005440
Myriapoda			
Diplopoda			
Polyxenida	Polyxenidae sp.*	AY859595	AY859596
Sphaerotheriida	Sphaerotheriidae sp.*	AY859605–07	AY859608
Spirostreptida	<i>Orthoporus</i> sp.	AY210827–28	AY210829
Polydesmida	<i>Cherokia georgiana</i> *	AY859562	AY859563
Chilopoda			
Notostigmophora			
Scutigermorpha	<i>Scutigera coleoptrata</i> *	AY859601	AF173238
Pleurostigmophora			
Craterostigmomorpha	<i>Craterostigma tasmanianus</i> *	AY859568–69	AF000774
Lithobiomorpha	<i>Lithobius</i> sp.	AY210824–25	AF000773
Scolopendromorpha	<i>Cormocephalus hartmeyeri</i>	AY210812	AF173249
Geophilomorpha	<i>Zelation antipodus</i> *	AY859616–19	AY859620
Symphyla	<i>Hanseniella</i> sp.	AY210821–22	AY210823 (also see AF173237)
Crustacea			
Branchiura	<i>Argulus</i> sp.	AY210804	M27187
Pentastomida	<i>Raillietiella</i> cf. <i>hemidactyli</i> *	AY744894, DQ013856–57	AY744887
Ostracoda	Cyprididae sp.	AY210815	AY210816
Copepoda	Cyclopidae sp.	AY210813	AY210814 and L81940
Thecostraca			
Cirripedia	<i>Megabalanus</i> sp.*	AY859588	AY520632
	Sacculinidae sp.*	AY859599	AY859600
Malacostraca			
Leptostraca	<i>Nebalia</i> sp.*	AY859589–90	L81945
Eumalacostraca			
Anaspidacea	<i>Anaspides tasmaniae</i> *	AY859548–49	L81948
Stomatopoda	<i>Squilla empusa</i>	AY210841–42	L81946
Euphausiacea	<i>Meganctiphanes norvegica</i> , <i>Nyctiphanes australis</i>	AY744900, AF169701, AF169735	AY744892
Mysidacea	<i>Heteromysis</i> sp.*	AY859578–79	AY859580
Isopoda	<i>Armadillidium</i> sp.*	AY859552–53	AJ287061
Decapoda	<i>Crangon franciscorum</i> *	AY859564–66	AF859567
	<i>Gaetice depressus</i> *	AY859575–76	AY859577
	<i>Panulirus argus</i>	AY210832–35	U19182
	<i>Homarus americanus</i> *	AY859581	AF235971
Branchiopoda			
Anostraca	<i>Artemia</i> sp.	AY210805	X01723
Spinicaudata	<i>Eulimnadia texana</i> *	AY859574	AF144211
Notostraca	<i>Triops</i> sp.	AY210844	AF144219
Cladocera	<i>Daphnia pulex</i> and <i>D. pulex</i>	AF346514	AF014011
Hexapoda			
Entognatha			
Collembola	<i>Podura aquatica</i>	AY210838–39	AF005452
	<i>Sminthurus viridus</i> *	AY859603	AY859604
	<i>Triacanthella</i> sp.*	AY859609	AY859610
Protura	<i>Baculentulus tianmushanensis</i> *	AY859554–56	AY037169

Table 1 (continued)

Classification	Species	GenBank Nos.	
		28S rRNA genes	18S rRNA genes
Diplura			
Japygidae	<i>Parajapyx emeryanus</i> *	AY859591–93	AY037168
Campodeidae	Campodeidae sp.*	AY859560	AY859561
Insecta			
Archeognatha	<i>Dilta littoralis</i> *	AY859570–71	AF005457
Dicondylia			
Thysanura	<i>Ctenolepisma longicauda</i>	AY210810	AY210811, AF005458
Odonata	<i>Leucorrhinia frigida</i> (or <i>L. intacta</i> or <i>L. proxima</i>)*	AY859583	AY859584
Ephemeroptera	<i>Callibaetis ferrugineus</i> *	AY859557	AF370791
Neoptera			
Mantodea	<i>Mantis religiosa</i> *	AY859585	AY859586
Isoptera	<i>Zootermopsis angusticollis</i> *	AY859614	AY859615
Blatteria	<i>Gromphadorhina laevigata</i>	AY210818–19	AY210820
Orthoptera	<i>Acheta domesticus</i> *	AY859544	X95741
	Gomphocerinae sp.*	AY859546	AY859547
Hemiptera	<i>Poppea evelyna</i> , <i>Spissistilus festinus</i>	AF304634	U06477
Hymenoptera	<i>Myrmecia croslandi</i> , <i>Leptothorax acervorum</i>	AB052895	X89492
	<i>Vespa pensylvanica</i> *	AY859611–12	AY859613
Coleoptera	<i>Tenebrio</i> sp.	AY210843	X07801
Lepidoptera	<i>Attacus ricini</i>	AF463459	AF463459
Diptera	<i>Aedes albopictus</i>	L2260	X57172
	<i>Chironomus tentans</i>	X99212	X99212
	<i>Drosophila melanogaster</i>	M21017	M21017
Trichoptera	<i>Oxyethira rossi</i>	DQ202352 (Karl M. Kjer)	AF423801
Mecoptera	<i>Merope tuber</i>	DQ202351 (Karl M. Kjer)	AF286287
Onychophora	<i>Peripatus</i> sp.	AY210836	AY210837
	<i>Peripatoides novaezealandiae</i>	AF342791–93	AF342794
Tardigrada	<i>Milnesium</i> sp.	AY210826	U49909
Nematoda	<i>Ascaris lumbricoides</i>	AY210806	U94366
	<i>Caenorhabditis elegans</i>	X03680	X03680
	<i>Meloidogyne artiellia</i>	AF248477	AF248477
	<i>Trichinella spiralis</i>	AF342803	U60231
	<i>Xiphinema rivesi</i>	AY210845	AF036610
Nematomorpha	<i>Chordodes morgani</i>	AF342787	AF036639
	<i>Gordius aquaticus</i>	AY210817	U51005
Priapulida	<i>Priapulid caudatus</i>	AY210840	Z38009
	<i>Halicryptus spinulosus</i>	AF342788–89	AF342790
Kinorhyncha	<i>Pycnophyes</i> sp.*	AY859597	AY859598, AY428820
Outgroups			
Lophotrochozoa (Spiralians)			
Nemertea	<i>Amphiporus</i> sp.	AF342785–86	AF119077
Mollusca	<i>Placopecten magellanicus</i>	AF342798	X53899
Platyhelminthes	<i>Stylochus zebra</i>	AF342800	AF342801
Echiura	<i>Urechis caupo</i>	AF342804	AF342805
Deuterostomes			
Echinodermata	<i>Florometra serratissima</i> and <i>Antedon serrata</i>	AF212168	D14357
Hemichordata	<i>Ptychodera flava</i>	AF212176	AF278681
Chordata	<i>Ciona intestinalis</i>	AF212177	AB013017
	<i>Hydrolagus colliei</i>	AF061799	Stock (1992)

Note. The 38 species indicated by an asterisk (*) were newly sequenced for 28S genes (and for 18S genes when needed) in the present study.

Our alignment method should be clarified further. First, the primary structure of each rRNA sequence is divided into segments—loop, half-stem, loop, half-stem, and so on—by referring to the secondary-structure models. Then, the individual segments are aligned across taxa. We do not account for paired bases on stems (Wheeler and Honeycutt, 1988) or treat stems differently from loops, although alignment methods that do this are starting to become available for 18S rRNA genes (Jow et al., 2002; Kjer, 2004; Telford et al., 2005). Such new methods, though labor-intensive,

could be used on our 28S and 18S rRNA genes in the future.

For our main phylogenetic analyses (in Fig. 1), we used the same parts of the 28S and 18S genes as in the previous study (Mallatt et al., 2004; also see Mallatt and Sullivan, 1998; Mallatt and Winchell, 2002). The 28S gene has a well-defined, conserved core (present in all eukaryotes and prokaryotes), and 12 divergent domains (variable regions that only exist in eukaryotes and whose nucleotides are difficult to align across taxa: see

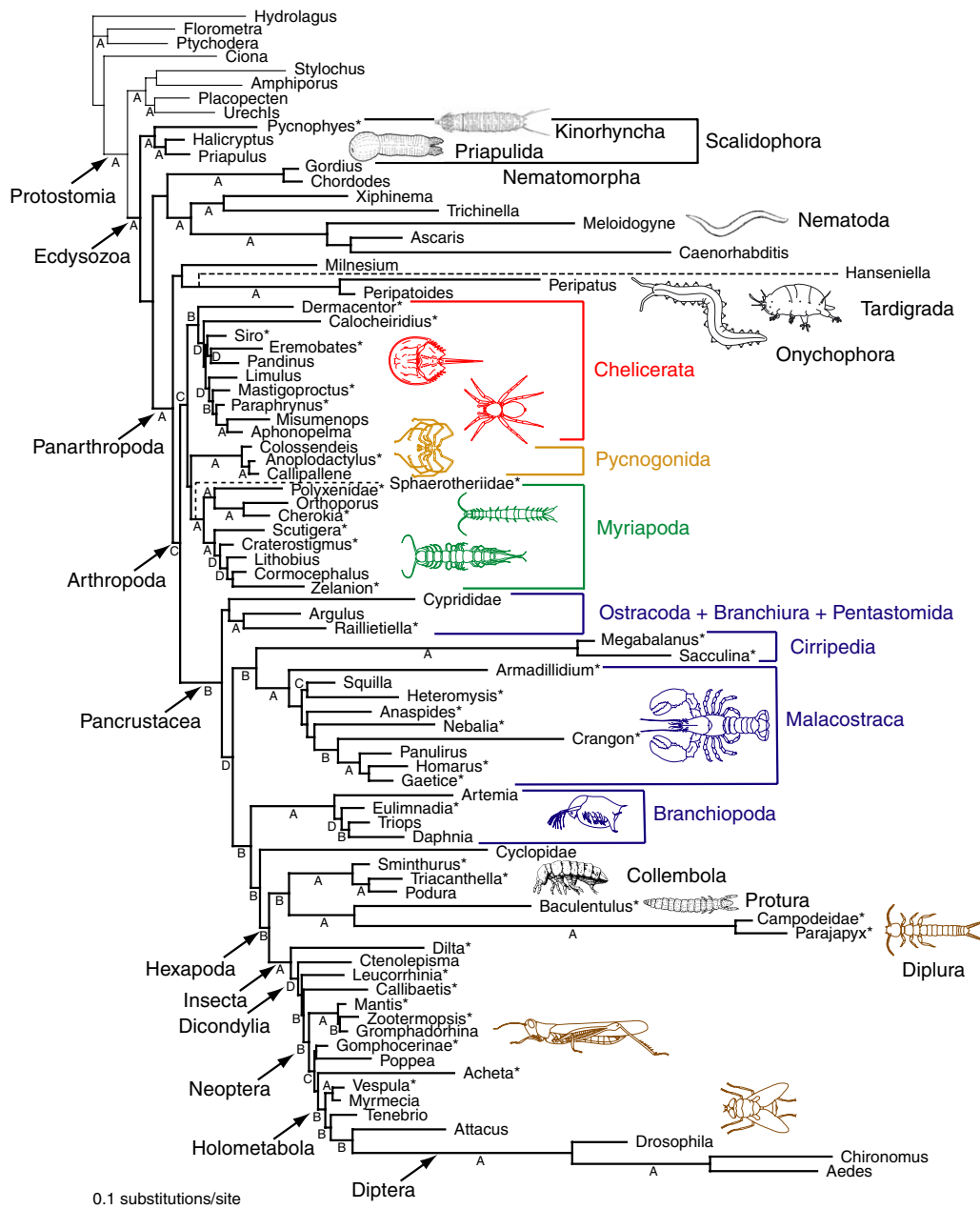


Fig. 1. Maximum likelihood tree calculated from our main data set: combined 28S + 18S rRNA genes, 84 taxa, and the standard alignment of 3852 nt. Asterisks denote the 38 species whose 28S genes were newly sequenced for this study. The LnL value of this tree is $-70,907.9$. Precise support-percentages for the nodes are listed in column 1 of Table 2, but significance is indicated here by classifying the supported nodes in four categories of decreasing strength, labeled (A–D): category A, nodes supported by all tree-building methods (Bayesian $\geq 95\%$; ML, MP, and LD $\geq 60\%$); category B, nodes supported by ML (but not necessarily by MP or LD); category C, nodes supported by Bayesian inference only, by both the Bayesian–covarion and Bayesian–ASRV methods. Category D: nodes supported by just one of the two Bayesian methods. Nodes without any letter are unsupported. Branches for two questionable sequences, from the myriapods *Hanseniella* sp. and Sphaerotheriidae sp., are sketched into this tree as dashed lines, showing their (unsupported) locations from a separate analysis that included these two taxa (our 86-taxon analysis). Their branch lengths, relative to those of nearby clades, are also indicated; note that the branch to *Hanseniella* is extremely long.

Hassouna et al., 1984; Hillis and Dixon, 1991; Mallatt et al., 2001). As before, we here omitted the divergent domains and used only the core of the 28S gene. The 18S gene also has a core, and intervening *variable regions* (the red helices in Figure 1 of Wuyts et al., 2002). However, unlike in the 28S gene, the variable regions of the 18S gene contain many stretches of slowly evolving sites that are

readily alignable across all eukaryotes, so we do not exclude them entirely. Instead, we exclude only those variable sites of 18S for which more than $\sim 30\%$ of the taxa did not share the same nucleotide (after Hillis and Dixon, 1991). Overall, 2307 sites were used from the 28S gene, and 1545 from the 18S gene, for a total of 3852 sites in the main analyses of concatenated 28S + 18S genes. All our

alignments, including one that indicates the features of secondary structure, can be accessed at <http://www.wsu.edu/~jmallatt/alignments.html> and in Supplementary material, S3.

Manual alignments have been criticized for often being subjective and non-repeatable (e.g., Giribet et al., 2002b; Ogden et al., 2005). It can be argued that our using a conserved secondary structure that was defined before aligning the data, and our eliminating the well-defined hypervariable regions a priori, help to ameliorate this criticism. Also to this end, we generated a second, more-conserved alignment, by trimming the 18S gene down to its core only, so both rRNA genes were limited to their well-defined core sites. More specifically, we removed about 250 more sites from the 18S rRNA gene. Results from this “conserved alignment” (with 3588 nt.) could then be compared to those from the longer, standard alignment (3852 nt.).

2.2. Phylogenetic analyses

Gene trees relating the taxa were constructed by different tree-building algorithms, all using PAUP* 4.0 beta 10 (Swofford, 2002; Swofford et al., 1996). These algorithms were: equally weighted parsimony (MP), minimum evolution using LogDet-Paralinear distances (LD; with $P_{inv} = 0.35$), and maximum likelihood (ML). For ML, the GTR + I + Γ model best fit our concatenated 28S + 18S genes and our 28S genes alone, but the simpler GTR + Γ model fit the 18S genes best—all as judged by the AIC approach to model selection in Modeltest (Posada and Buckley, 2004; Posada and Crandall, 1998). For the ML analyses we used eight discrete gamma categories, as opposed to four categories in the past studies. Increasing the categories to eight was the best compromise between achieving higher likelihood scores and avoiding excessive computation time.

For the LD and MP methods, support for clades was estimated with 500 replicates of non-parametric bootstrapping. For ML, with its higher computational demands, only the single best tree could be calculated in the large analyses with 78–86 taxa. However, ML-support values were obtained by splitting these taxa into two subgroups, of (1) 37 pancrustaceans plus 4 outgroups, and (2) 21 chelicerates and myriapods plus 5 outgroups (see Note in Table 2); and 100 ML-bootstrap replicates were then performed on each subgroup.

Likelihood-based Bayesian inference (Huelsenbeck et al., 2001) was also performed on the data sets, mostly as described by Mallatt et al. (2004) and Winchell et al. (2004), using MrBayes 3.01 (Huelsenbeck and Ronquist, 2003a,b; Huelsenbeck et al., 2001). Here, however, partitioned analyses were run, after we used AIC in Modeltest to determine the best models for the 28S and 18S-gene partitions. These models included six transition probabilities with invariant sites and a discrete gamma correction (command `nst=6 invgamma`) for the 28S-gene partition, and six transition

probabilities with a discrete gamma correction but without invariant sites (command `nst=6 gamma`) for the 18S-gene partition. As with ML, the number of gamma categories was set at eight. Four Markov chains were run for 1,500,000 generations. Stabilization of model parameters usually occurred before 200,000 generations and never after 750,000. From the trees left after stabilization (burn-in), we sampled every 100th tree, and these were used to calculate the majority-rule consensus tree. The values on this tree are the posterior probabilities of the nodes.

These Bayesian analyses were run both with and without the covarion model (Ané et al., 2005; Galtier, 2001; Huelsenbeck, 2002; Inagaki et al., 2004; Penny et al., 2001). Whereas ordinary, non-covarion ML and Bayesian methods can model evolutionary differences in substitution rates across the sites in a gene—the so-called among-site rate variation (ASRV)—these methods do not allow the rate of substitution at a site to vary between lineages across a tree (that is, to vary over time). Covarion models, by contrast, do model such site-specific rate variation (as well as ASRV), and have been shown to construct better-supported trees from rRNA genes than do the ordinary, ASRV-only methods (Galtier, 2001). However, all current versions of the covarion method assume stochasticity; i.e., that the evolutionary-rate changes occurred uniformly throughout the tree; but in reality these rate changes concentrate in fast-evolving lineages (e.g., in the highly divergent rRNA genes of dipteran insects, rhabditid nematodes, and symphylian myriapods: Friedrich and Tautz, 1997; Giribet and Ribera, 2000; Mushegian et al., 1998). Because this gross violation of a key assumption is so likely to characterize our rRNA data, the covarion method might merely yield better likelihood support without any real improvement in accuracy. This is why we do not rely solely on the covarion-based findings, but also report the ordinary ASRV-Bayesian findings.

Two of our sequences—from the myriapods Sphaerotheriidae sp. and *Hanseniella*—were highly derived, so they were given special treatment. The peculiarities of *Hanseniella* rRNA genes were discussed previously (Giribet and Wheeler, 2001; Mallatt et al., 2004), and our Sphaerotheriidae 28S gene was suspiciously long (>8800 nt) and difficult to amplify and sequence, so it may be a pseudogene (Spears and Abele, 1998, p. 176) or an odd secondary allele among heterogeneous rRNA genes (Rooney, 2004). Thus, we excluded both these sequences from our main phylogenetic analyses with 84 taxa but included them in a separate analysis with 86 taxa.

Non-stationarity of nucleotide frequencies across taxa is a feature of many 28S and 18S rRNA data sets (e.g., Mallatt and Winchell, 2002; Mallatt et al., 2004; Winchell et al., 2002), including the present one (χ^2 test of homogeneity of base frequencies for 84 taxa: $P < 0.000000005$; see Supplementary material, S2). The accuracy of likelihood-based (and parsimony) methods can be lowered by such non-stationarity (Jermin et al., 2004; Omilian and Taylor, 2001).

Table 2
Summary of results of different tree-building methods: the numbers are Bayesian posterior probabilities and non-parametric-bootstrap percentages, and are listed in the order: Bayesian–covarion/Bayesian–ASRV /ML/MP/LD

Clade	1 84 taxa, standard alignment, 28S + 18S (main data set: Figs. 1 and 4)	2 84 taxa, conserved alignment, 28S + 18S	3 78 taxa (stationary), standard alignment, 28S + 18S	4 86 taxa, standard alignment, 28S + 18S	5 28S only, 84 taxa, standard alignment (see Fig. 2)	6 18S only, 84 taxa, standard alignment (see Fig. 3)
1. Scalidophora (Kinorhyncha + Priapul)	100/100/ —/97/100	100/100/ —/93/100	100/100/ —/97/100	100/100/ —/96/100	100/100/ —/82/89	100/100/ —/ <50/73
2. Panarthropoda	100/100/ —/75/94	100/100/ —/87/95	100/100/ —/77/95	100/100/ —/87/92	100/100/ —/82/85	<50/ <50/ —/ <50/ <50
3. Arthropoda	97/100/ —/ <50/ <50	51/83/ —/ <50/ <50	100/99/ —/50/54	<50/ <50/ —/ <50/ <50	<50/51/ —/ <50/ <50	100/100/ —/ <50/ <50
4. Paradoxopoda (myriapods + chelicerates + pycnogonids)	100/100/ <50/ <50/98	98/100/ —/ <50/96	99/99/ —/ <50/93	<50/ <50/ —/ <50/ <50	100/94/ <50/ <50/71	<50/ <50/ 58/ <50/90
5. Myriapoda	100/100/ 76/83/88	100/100/ —/57/55	100/100/ —/79/91	<50/ <50/ —/ <50/ <50	<50/ <50/ <50/ <50/ <50	100/100/ 83/68/95
6. Diplopoda	100/100/ 72/87/92	100/100/ —/71/66	100/100/ —/83/93	<50/ <50/ —/ <50/ <50	100/100/ <50/71/74	51/77/ 53/52/65
7. Helminthomorpha (<i>Orthoporus</i> + <i>Cherokia</i>)	100/100/ 100/100/100	100/100/ —/100/100	100/100/ —/100/100	100/100/ —/100/100	100/100/ 100/100/100	100/100/ 98/99/100
8. Chilopoda	100/100/84/76/100	98/98/ —/ <50/95	100/100/ —/76/ 100	99/99/ —/65/ 100	100/100/ <50/ 53/80	100/100/ 97/75/98
9. Pleurostigmophora (<i>Craterostigma</i> + <i>Lithobius</i> + <i>Zelanion</i> + <i>Cormocephalus</i>)	88/97/ <50/ <50/61	96/99/ —/ <50/68	75/97/ —/ <50/61	<50/62/ —/ <50/59	77/86/ <50/ <50/78	<50/ <50/ <50/ <50/ <50
10. <i>Craterostigma</i> sister to other Pleurostigmophora	92/100/ <50/ <50/ <50	96/100/ —/ <50/ <50	82/100/ —/ <50/ <50	<50/63/ —/ <50/ <50	98/100/ 59/ <50/ <50	<50/ <50/ <50/ <50/ <50
11. Chelicerata	100/100/ 75/59/ <50	100/100/ —/ <50/ <50	100/100/ —/59/69	100/100/ —/66/63	100/94/ <50/ <50/ <50	100/100/ 84/61/70
12. <i>Dermacentor</i> sister to other Chelicerata	94/62/ <50/ <50/ <50	96/100/ —/ <50/ <50	93/69/ —/ <50/69	98/59/ —/ <50/ <50	<50/ <50/ <50/ <50/ <50	70/90/ 50/50/50
13. <i>Eremobates</i> (solifug) + <i>Pandinus</i> (scorpion)	93/99/ <50/ <50/ <50	79/83/ —/ <50/ <50	94/97/ —/ <50/ <50	96/97/ —/ <50/ <50	<50/ <50/ <50/ <50/ <50	<50/ <50/ <50/ <50/ <50
14. Tetrapulmonata (<i>Mastigoproctus</i> + <i>Paraphrynus</i> + <i>Misumenops</i> + <i>Aphonopelma</i>)	100/100/ 71/97/ <50	100/100/ —/95/ <50	100/100/ —/98/ <50	100/100/ —/98/ <50	100/100/ 52/96/70	<50/ <50/ <50/ <50/ <50
15. <i>Limulus</i> sister to Tetrapulmonata	75/97/ <50/ <50/ <50	77/92/ —/ <50/ <50	71/91/ —/ <50/ <50	78/95/ —/ <50/ <50	<50/ <50/ <50/ <50/ <50	78/88/ <50/ <50/ <50
16. Pycnogonida	100/100/ 100/100/100	100/100/ —/100/100	100/100/ —/100/100	100/100/ —/100/100	100/100/ 100/100/100	100/100/ 100/100/100
17. <i>Callipallene</i> + <i>Anoplodactylus</i>	100/100/ 88/92/98	100/100/ —/98/100	100/100/ —/91/97	100/100/ —/88/98	100/100/ 99/97/99	75/86/ <50/ <50/ <50
18. Pancrustacea	100/100/ 99/73/ <50	100/100/ —/78/ <50	100/100/ —/83/75	100/100/ —/75/ <50	100/100/ 100/81/ <50	100/100/ <50/ <50/ <50
19. <i>Argulus</i> , <i>Raillietiella</i> , and ostracod (cypridid) as basal Pancrustacea	95/71/ <50/ <50/ <50	87/ <50/ —/ <50/ <50	98/82/ —/64/ <50	95/70/ —/ <50/ <50	87/94/ 53/ <50/ <50	<50/ <50/ <50/ <50/ <50
20. <i>Raillietiella</i> (pentastomid) + <i>Argulus</i> (branchiuran)	100/100/ 99/92/100	100/100/ —/94/100	100/100/ —/94/99	100/100/ —/93/100	100/100/ 99/88/100	54/95/ 51/51/69
21. Branchiopoda + Copepoda (cyclopoid) + Hexapoda	100/100/ 94/ <50/ <50	100/100/ —/ <50/ <50	100/100/ —/ <50/ <50	100/100/ —/ <50/ <50	100/100/ 88/ <50/ <50	<50/ <50/ <50/ <50/ <50
22. Copepoda (cyclopoid) + Hexapoda	100/100/ 81/ <50/ <50	100/99/ / —/ <50/ <50	100/100/ —/ <50/ <50	100/100/ —/ <50/ <50	100/94/80/ <50/ <50	<50/ <50/ <50/ <50/ <50
23. Cirripedia + Malacostraca	100/100/ 94/ <50/ <50	100/100/ —/ <50/50	100/100/ —/50/67	100/100/ —/ <50/ <50	95/83/ 68/55/ <50	<50/ <50/ <50/ <50/ <50
24. Branchiopoda	100/100/ 100/100/100	100/100/ —/100/100	100/100/ —/100/100	100/100/ —/100/100	100/100/ 100/100/100	100/100/ 100/100/100
25. Phyllopora (<i>Eulimnadia</i> + <i>Triops</i> + <i>Daphnia</i>)	87/97/ <50/98/100	92/97/ —/99/100	95/99/ —/97/100	93/99/ —/98/100	85/93/ <50/98/100	<50/ <50/ 55/ <50/ <50
26. <i>Triops</i> + <i>Daphnia</i>	100/100/ 77/70/ <50	100/100/ —/73/ <50	100/100/ —/72/ <50	100/100/ —/71/ <50	85/93/ 55/55/ <50	<50/ <50/ <50/ <50/ <50
27. Hexapoda	90/99/ 77/ <50/ <50	93/99/ —/ <50/ <50	84/97/ —/50/50	90/99/ —/ <50/ <50	97/100/ 83/ <50/ <50	<50/ <50/ <50/ <50/ <50

Table 2 (continued)

Clade	1	2	3	4	5	6
	84 taxa, standard alignment, 28S + 18S (main data set: Figs. 1 and 4)	84 taxa, conserved alignment, 28S + 18S	78 taxa (stationary), standard alignment, 28S + 18S	86 taxa, standard alignment, 28S + 18S	28S only, 84 taxa, standard alignment (see Fig. 2)	18S only, 84 taxa, standard alignment (see Fig. 3)
28. Entognatha (Collembola + Protura + Diplura)	<u>100/100/</u> <u>93/58/<50</u>	<u>100/100/</u> <u>—/61/50</u>	—	<u>100/100/</u> <u>—/59/<50</u>	<u>98/100/</u> <u>74/65/<50</u>	<u>99/99/</u> <u>68/<50/<50</u>
29. Protura + Diplura (Nonoculata)	<u>100/100/</u> <u>100/96/100</u>	<u>100/100/</u> <u>—/97/100</u>	—	<u>100/100/</u> <u>—/93/90</u>	<u>100/100/</u> <u>99/93/91</u>	<u>99/98/</u> <u><50/87/98</u>
30. Insecta	<u>100/100/</u> <u>100/70/91</u>	<u>100/100/</u> <u>—/75/90</u>	<u>100/100/</u> <u>—/99/97</u>	<u>100/100/</u> <u>—/68/91</u>	<u>100/100/</u> <u>99/66/<50</u>	<u>96/100/</u> <u>89/<50/<50</u>
31. Dicondylia	<u>57/95/</u> <u>56/<50/<50</u>	<u><50/78/</u> <u>—/<50/<50</u>	<u>60/86/</u> <u>—/<50/<50</u>	<u>50/80/</u> <u>—/<50/<50</u>	<u><50/56/</u> <u><50/<50/<50</u>	<u><50/<50/</u> <u><50/<50/<50</u>
32. Callibaetis (ephemeropteran) + Neoptera	<u>83/99/</u> <u>60/<50/<50</u>	<u>60/95/</u> <u>—/<50/<50</u>	<u>95/98/</u> <u>—/<50/<50</u>	<u>69/83/</u> <u>—/<50/<50</u>	<u><50/83/</u> <u><50/<50/<50</u>	<u><50/<50/</u> <u><50/<50/<50</u>
33. Neoptera	<u>84/100/</u> <u>68/<50/<50</u>	<u>60/96/</u> <u>—/<50/<50</u>	<u>97/100/</u> <u>—/56/55</u>	<u>70/84/</u> <u>—/<50/<50</u>	<u><50/83/</u> <u><50/<50/<50</u>	<u>86/100/</u> <u><50/<50/<50</u>
34. Dictyoptera (<i>Mantis</i> + <i>Zootermopsis</i> + <i>Gromphadorhina</i>)	<u>100/100/</u> <u>100/100/100</u>	<u>100/100/</u> <u>—/100/100</u>	<u>100/100/</u> <u>—/100/100</u>	<u>100/100/</u> <u>—/100/100</u>	<u>100/100/</u> <u>99/100/100</u>	<u>100/100/</u> <u>99/100/100</u>
35. Dictyoptera sister to other neopterans	<u>99/95/</u> <u><50/<50/<50</u>	<u>96/99/</u> <u>—/<50/<50</u>	<u>98/93/</u> <u>—/<50/<50</u>	<u>97/98/</u> <u>—/<50/<50</u>	<u>99/100/</u> <u><50/<50/<50</u>	<u><50/<50/</u> <u><50/<50/<50</u>
36. <i>Zootermopsis</i> (isopteran) + <i>Gromphadorhina</i> (blattodean)	<u>93/98/</u> <u>72/75/58</u>	<u>92/98/</u> <u>—/80/73</u>	<u>92/99/</u> <u>—/71/58</u>	<u>89/98/</u> <u>—/75/59</u>	<u>95/99/</u> <u>84/79/87</u>	<u><50/<50/</u> <u><50/<50/<50</u>
37. Holometabola	<u>100/100/</u> <u>98/57/<50</u>	<u>97/100/</u> <u>—/60/<50</u>	<u>100/100/</u> <u>—/83/<50</u>	<u>100/100/</u> <u>—/56/<50</u>	<u>84/80/</u> <u>57/<50/<50</u>	<u>96/100/</u> <u>70/<50/<50</u>
38. <i>Tenebrio</i> + [Lepidoptera + Diptera] (Figs. 1–3)	<u>69/97/</u> <u>85/<50/<50</u>	<u>62/93/</u> <u>—/<50/<50</u>	—	<u>76/97/</u> <u>—/<50/<50</u>	<u><50/<50/</u> <u>56/<50/<50</u>	<u>62/88/</u> <u><50/<50/<50</u>
39. Lepidoptera (<i>Attacus</i>) + Diptera (Figs. 1–3)	<u>100/100/</u> <u>93/62/<50</u>	<u>97/100/</u> <u>—/67/<50</u>	—	<u>100/100/</u> <u>—/60/<50</u>	<u>100/100/</u> <u>79/<50/<50</u>	<u>93/100/</u> <u>65/<50/<50</u>
40. Mecopteroidea (Fig. 4A)	<u>—/—/—</u> <u>67/<50/<50</u>	—	—	—	—	—
41. Lepidoptera + Trichoptera (Fig. 4A)	<u>—/—/—</u> <u>100/99/100</u>	—	—	—	—	—
42. Malacostraca	<u>100/100/</u> <u>100/84/98</u>	<u>100/100/</u> <u>—/70/86</u>	<u>100/100/</u> <u>—/75/90</u>	<u>100/100/</u> <u>—/82/98</u>	<u>100/100/</u> <u>99/55/74</u>	<u>100/100/</u> <u>71/63/65</u>
43. Eumalacostraca vs. Leptostraca	<u>100/100/</u> <u><50/51/<50</u>	<u>98/100/</u> <u>—/<50/<50</u>	<u>100/100/</u> <u>—/<50/<50</u>	<u>98/100/</u> <u>—/<50/<50</u>	<u>79/51/</u> <u><50/<50/<50</u>	<u>59/<50/</u> <u><50/<50/<50</u>
44. <i>Squilla</i> (stomatopod) + <i>Meganyctiphanes</i> (euphausiacean) (Fig. 4B)	<u>—/—/—</u> <u>79/83/70</u>	—	—	—	—	—
45. Decapoda	<u>100/100/</u> <u>98/<50/75</u>	<u>100/100/</u> <u>—/<50/74</u>	<u>100/100/</u> <u>—/<50/73</u>	<u>100/100/</u> <u>—/<50/75</u>	<u>100/100/</u> <u>100/79/97</u>	<u><50/<50/</u> <u><50/<50/<50</u>
46. Reptantia (<i>Panulirus</i> + <i>Homarus</i> + <i>Gaetice</i>)	<u>100/99/</u> <u>91/100/100</u>	<u>79/<50/</u> <u>—/99/100</u>	<u>100/99/</u> <u>—/100/100</u>	<u>100/100/</u> <u>—/100/100</u>	<u><50/<50/</u> <u><50/80/100</u>	<u>100/100/</u> <u>95/98/100</u>
47. <i>Panulirus</i> + <i>Homarus</i> (lobsters)	<u>100/100/</u> <u><50/72/<50</u>	<u>99/90/</u> <u>—/65/50</u>	<u>100/100/</u> <u>—/72/<50</u>	<u>100/100/</u> <u>—/72/<50</u>	<u>95/<50/</u> <u><50/66/<50</u>	<u>99/100/</u> <u><50/65/<50</u>

Note. Underlined values are significant; i.e., Bayesian posterior probabilities $\geq 95\%$ and bootstrap percentages $\geq 60\%$. A dash means no data were calculated.

Note. Due to the high computational demands of ML, ML bootstrap values could not be calculated for the data sets with 78–86 taxa. Instead, all the ML-boot values in this table (except for the few values taken from Fig. 4) were calculated from two subsets of taxa: Subset (1) Forty one taxa containing Pancrustacea and outgroups; and Subset (2) Twenty six taxa containing myriapods, chelicerates, and outgroups.

ML Subset 1 consisted of: *Artemia*, *Eulimnadia*, *Triops*, *Daphnia*, Cyclopidae sp., *Ctenolepisma*, *Tenebrio*, *Attacus*, *Drosophila*, *Vespula*, *Myrmecia*, *Acheta*, Gomphocerinae sp., *Mantis*, *Zootermopsis*, *Gromphadorhina*, *Poppea*, *Callibaetis*, *Leucorrhinia*, *Dilta*, *Triacanthella*, *Sminthurus*, Campodeidae sp., *Baculentulus*, *Squilla*, *Panulirus*, *Homarus*, *Gaetice*, *Crangon*, *Anaspides*, *Heteromysis*, *Nebalia*, *Armadillidium*, *Megabalanus*, *Argulus*, *Raillietiella*, Cyprididae sp., *Scutigera*, *Limulus*, *Colossendeis*, and *Milnesium*.

ML Subset 2 consisted of: *Orthoporus*, *Cherokia*, Polyxenidae sp., *Cormocephalus*, *Craterostigmus*, *Zelanion*, *Scutigera*, *Lithobius*, *Mastigoproctus*, *Misumenops*, *Aphonopelma*, *Paraphrynus*, *Calocheiridius*, *Siro*, *Eremobates*, *Dermacentor*, *Pandinus*, *Limulus*, *Anoplodactylus*, *Callipallene*, *Colossendeis*, *Squilla*, *Argulus*, *Eulimnadia*, Gomphocerinae sp., and *Milnesium*.

To test if that did occur, we eliminated taxa with the most atypical nucleotide ratios until achieving a 78-taxon set that was stationary ($P = 0.264$: Supplementary material, S2).

The eliminated sequences were: the AT-rich flies of the clade Diptera (*Drosophila*, *Chironomus*, and *Aedes*), the GC-rich velvet worm *Peripatus*, and the especially GC-rich

dipluran hexapods (Campodeidae sp. and *Parajapyx*). Trees calculated from the stationary, 78-taxon set could then reveal if non-stationarity had disrupted the main, 84-taxon tree.

For later, we note that the rRNA genes of the proturan hexapod *Baculentulus* are moderately GC-rich, but not enough to disrupt stationarity, so *Baculentulus* was retained in the stationary set.

2.3. Parametric bootstrapping/hypothesis testing

Results of our tree-based analyses that seemed particularly interesting or controversial were tested statistically with parametric bootstrapping based on ML (Huelsenbeck et al., 1996). The results to be tested required only small groups of <35 taxa, so ML could be used without excessive computation time. As described by Mallatt et al. (2004), first ML was used to find the optimal constrained and unconstrained trees, and then the ML-bootstrap tests were performed on 100 simulated data sets. The necessary simulations were generated with the program Seq-Gen version 1.2.5 (Rambaut and Grassly, 2001).

2.4. Levels of significance

In the Bayesian trees, clades were accepted at $\geq 95\%$ posterior probability, whereas in the non-parametric-bootstrap trees, bootstrap values $\geq 60\%$ were accepted as significant. This is the same as in the previous study (Mallatt et al., 2004). For parametric bootstrapping, which may be susceptible to type I statistical error (rejecting hypotheses too readily: Antezana, 2003), we adopted the more-stringent rejection level of $P \leq 0.01$.

3. Results

Fig. 1 shows the ML tree that was calculated from the combined 28S+18S rRNA genes in the main, 84-taxon analysis with the standard alignment of 3852 nt. Support levels are categorized on the nodes of the tree, with the precise support-percentages given in Column 1 of Table 2.

Table 3 lists all significantly supported clades in the main tree of Fig. 1. These clades are grouped into four categories, A to D, ranked from most to least strongly supported:

- A. Supported by *all* the tree-building methods (Bayesian, ML, MP, and LD).
- B. Supported by ML bootstrapping (but not necessarily by MP or LD).
- C. Supported by Bayesian inference alone, by both the covarion and ASRV methods.
- D. Supported by just one Bayesian method, either covarion, or ASRV.

The reason why Categories C and D are less reliable is that Bayesian inference tends to be overconfident; that is,

Bayesian posterior probabilities can be inflated estimates of significance (Suzuki et al., 2002). By contrast, bootstrapping (and other resampling methods such as jackknifing) is a more conservative method.

Among the best-supported findings of this study are (Table 3, Fig. 1): In Category A: the kinorhynch *Pycnophyes* groups with the priapulans in Scalidophora (the third scalidophoran phylum, Loricifera, was not sampled). Among basal hexapods, the proturan and diplurans group as Nonoculata (after Luan et al., 2005). Within insects, the mantis, termite, and cockroach form a monophyletic Dictyoptera. The pentastomid *Raillietiella* groups with the branchiuran crustacean *Argulus*. In Malacostraca, the stomatopod (mantis shrimp) *Squilla* groups with the krill *Meganctiphanes* (Fig. 4B). In Category B: within chelicerates, the spiders *Misumenops* and *Aphonopelma* unite with the amblypygid (whip spider) *Paraphrynus* and with the uropygid (whip scorpion) *Mastigoproctus* as Tetrapulmonata. Pancrustacea is monophyletic. Hexapoda is recovered as the sister group to the cyclopid copepod, and this clade is sister to branchiopod crustaceans. Within branchiopods, the notostracan (tadpole shrimp) *Triops* is sister to the cladoceran (water flea) *Daphnia*. In hexapods, Entognatha consists of Collembola (springtails) and Nonoculata. In insects, Holometabola (the forms with complex metamorphosis) is supported. The cirriped barnacles group with the malacostracans. A monophyletic Decapoda includes the shrimp *Crangon* and the Reptantia (crab *Gaetice* and the lobsters).

The C and D groups, supported only by Bayesian inference, include: Arthropoda; Paradoxopoda; and within the chilopod myriapods (= centipedes), *Craterostigma* is sister to a group consisting of all the other pleurostigmophoran centipedes: namely, to *Lithobius*, the scolopendromorph *Cormocephalus* and the geophilomorph *Zelanion*. In chelicerates, the xiphosuran (horseshoe crab) *Limulus* groups with Tetrapulmonata to the exclusion of other arachnids. The branchiuran *Argulus*, the pentastomid *Raillietiella*, and the cypridid ostracod are basal to a group consisting of all other pancrustaceans.

Most of these results were furthermore obtained from the more conserved alignment with 3588 nt. (84 taxa) and from the 78-taxon set that was stationary for nucleotide frequency (Tables 2 and 3), and their trees were almost identical to those in Fig. 1 (not illustrated). Results from the 78-stationary set were especially similar to those of the main 84-taxon set (Column 3 in Table 2). The conserved alignment, on the other hand, shows weaker support for some clades (the six labeled “CN<” in Table 3), but the support for most such clades was already weak in the main data set with the standard alignment (e.g., *Limulus* as sister to the tetrapulmonate arachnids; the solifug *Eremobates* plus the scorpion *Pandinus*; *Argulus*, *Raillietiella*, and cypridid as basal pancrustaceans; Dicondylia; Arthropoda). This slight loss of support does not affect the main findings of this study: It may merely result from the reduced number of nucleotides, from 3852 nt. in the

Table 3

Summary of clades supported by the combined 28S + 18S rRNA genes (from Table 2: 84 taxa, standard alignment), grouped in categories from A (most support) to D (least support)

Category A: clades supported by all methods (Bayesian $\geq 95\%$; ML, MP, and LD $\geq 60\%$):

- a. Scalidophora
- b. Panarthropoda (mostly 28S)
- c. Myriapoda (86<) (mostly 18S)
- d. Diplopoda (86<) (mostly 28S)
- e. Helminthomorpha
- f. Chilopoda
- g. Pycnogonida
- h. *Callipallene* + *Anoplodactylus* (mostly 28S)
- i. Branchiopoda
- j. Nonoculata
- k. Insecta
- l. Dictyoptera
- m. *Raillietiella* (pentastomid) + *Argulus* (branchiuran)
- n. Lepidopteran + Trichopteran (in Fig. 4A)
- o. Malacostraca
- p. *Squilla* (stomatopod) + *Meganyctiphanes* (euphausiacean) (in Fig. 4B)
- q. Reptantia (CN<) (mostly 18S)

Category B: clades supported by ML, $\geq 60\%$ (but not necessarily by MP or LD):

- a. Chelicerata
- b. Tetrapulmonata (mostly 28S)
- c. Pancrustacea
- d. Branchiopoda + copepod (cyclopoid) + Hexapoda (mostly 28S)
- e. Copepod (cyclopoid) + Hexapoda (mostly 28S)
- f. *Triops* + *Daphnia* (mostly 28S)
- g. Hexapoda (mostly 28S)
- h. Entognatha
- i. *Callibaetis* (ephemeropteran) + Neoptera
- j. Neoptera (mostly 18S) (86<)
- k. *Zootermopsis* (isopteran) + *Gromphadorhina* (blattodean) (mostly 28S)
- l. Holometabola (in Fig. 4A)
- m. Mecopteroidea (in Fig. 4A)
- n. Cirripedia + Malacostraca (mostly 28S)
- o. Decapoda (mostly 28S)

Category C: clades supported only by Bayesian inference, $\geq 95\%$, by both covarion and ASRV methods:

- a. Arthropoda (CN<) (86<) (mostly 18S)
- b. Paradoxopoda (86<) (mostly 28S)
- c. Dictyoptera sister to other neopterans
- d. Eumalacostraca versus Leptostraca
- e. *Panulirus* + *Homarus* (lobsters)

Category D: clades supported only by Bayesian inference, $\geq 95\%$, by covarion or ASRV (not by both):

- a. Pleurostigmophora (86<) (mostly 28S)
- b. *Craterostigmus* sister to other Pleurostigmophora (mostly 28S)
- c. *Limulus* sister to Tetrapulmonata (CN<) (78<) (mostly 18S)
- d. *Eremobates* (solifug) + *Pandinus* (scorpion) (CN<)
- e. *Argulus*, *Raillietiella*, and cypridid ostracod as basal pancrustaceans (CN<)
- f. Phyllopora (mostly 28S)
- g. Dicondylia (CN<) (78<)

Note. Meaning of the annotations: (i) “(CN<)” means a clade was not significantly supported in the conserved alignment, which had ~250 fewer nucleotide sites than in the standard alignment. (ii) “(78<)” means a clade was not supported in the data set with 78 taxa, which showed stationarity of nucleotide frequency across taxa. (iii) “(86<)” means a clade was not supported in the data set that contained the two questionable sequences, from the myriapods *Sphaerotheriidae* sp. and *Hanseniella*. (iv) Explanation of “(mostly 28S)” and “(mostly 18S)”: Even though all clades in this table are derived from combined 28S + 18S genes, “(mostly 28S)” means that the clade also appeared in the 28S-gene tree (Fig. 2) but not in the 18S-gene tree (Fig. 3); and “(mostly 18S)” means the clade also was in the 18S tree but not in the 28S tree; such annotations were left off clades that appeared in both, or neither, of the 28S or 18S trees. Notice that more clades appeared in the 28S tree than in the 18S tree.

standard alignment down to 3588 nt. in the conserved alignment, and it need not imply the standard alignment was suboptimal.

The results of the 86-taxon analysis, which included the divergent *Hanseniella* and *Sphaerotheriidae* sp. sequences,

are also summarized in Table 2 (Column 4), and the positions of these two odd sequences are shown on the tree in Fig. 1. Neither’s position had significant support, but each had a destabilizing effect, lowering the support for nearby nodes—namely for Myriapoda, Diplopoda, Paradoxopoda,

and Arthropoda (clades 3–6 in Table 2). Thus, *Hanseniella* and Sphaerotheriidae are not reliably classifiable with our rRNA sequences, at least not with the tree-building methods employed.

Both the 28S-gene tree (Fig. 2) and the 18S-gene tree (Fig. 3) resemble the 28S+18S tree (Fig. 1), but with less resolution and lower support-values at some nodes (also see Table 2, columns 5–6). The 28S and 18S-gene trees share many supported nodes, such as Scalidophora, Chelicerata, Pancrustacea, Branchiopoda, ‘Protura and Diplura’ as Nonoculata, Insecta, and Malacostraca. As in the previous study (Mallatt et al., 2004), the 28S rRNA gene makes the stronger contribution, judging from the fact that three

times as many clades from the 28S tree appear in the combined-gene tree as do clades from the 18S tree. More specifically, the ratio is 15/5 clades, as documented by the number of “mostly 28S” versus “mostly 18S” annotations in Table 3.

4. Discussion

4.1. General

This study expands a previous investigation of ecdysozoan phylogeny (Mallatt et al., 2004) by more than doubling the number of nearly complete 28S+18S rRNA genes

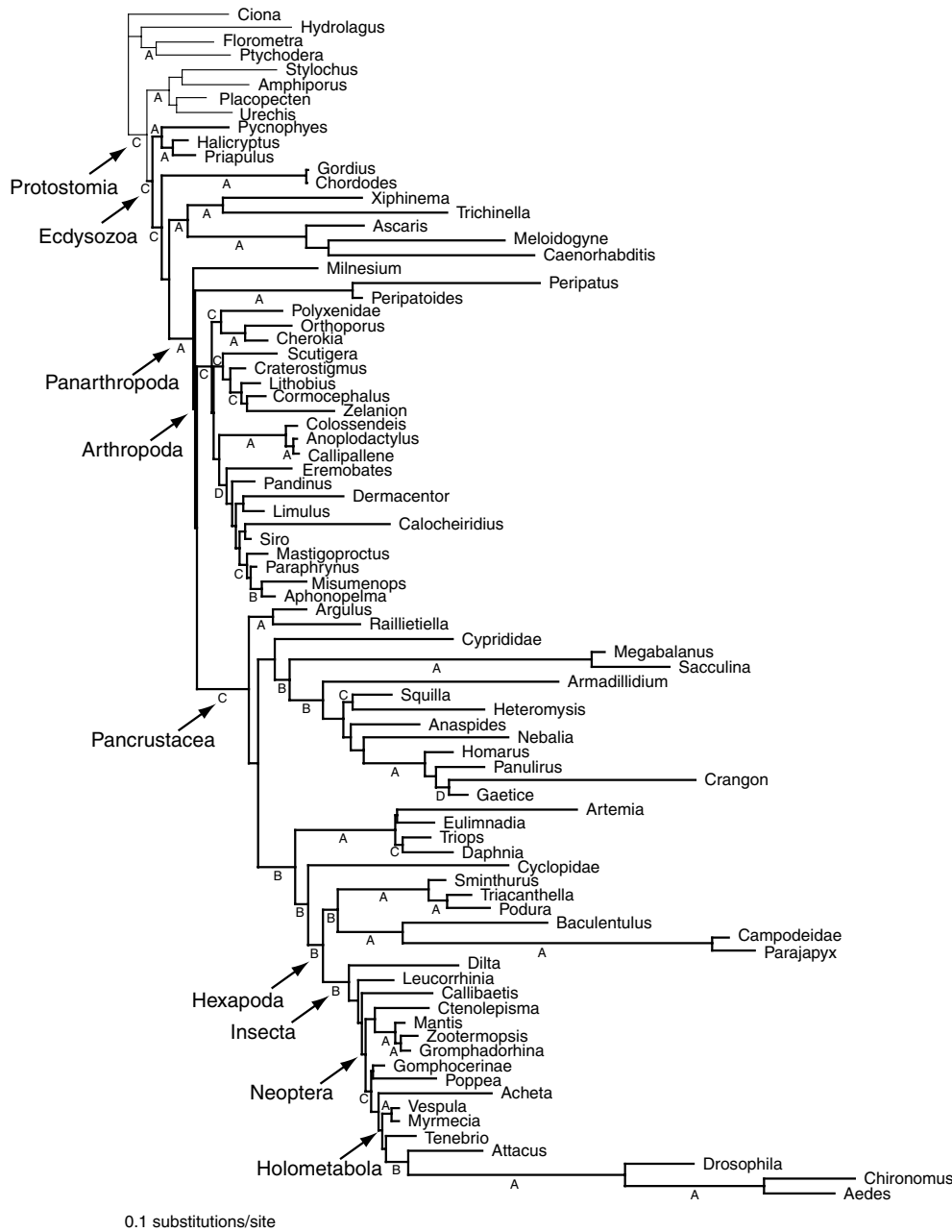


Fig. 2. Maximum likelihood tree from 28S rRNA genes alone (2307 nt). The LnL value of this tree is -41,858.1. Precise support-percentages for the nodes are listed in column 5 of Table 2, and the labeling of nodes is as in Fig. 1: from A (most supported) to D (least supported).

from each major group of arthropods. It recovered most widely accepted clades: Chelicerata, Chilopoda, Hexapoda, Pancrustacea, Scalidophora (Kinorhyncha + Priapulida), Tetrapulmonata in chelicerates, entognathan and holometabolan hexapods—plus such other clades as cirripedes with malacostracans, branchiopods near hexapods, and pentastomid with branchiuran (Fig. 1). These results were robust to analytical treatment (Tables 2 and 3), both to alignment variations and to the deletion of taxa to restrict analyses to stationary nucleotide frequencies. The

latter finding suggests that when taxon sampling is broad, non-stationary sequences may not be as disruptive as previously thought (Zwickl and Hillis, 2002). Further attesting to the robustness of the results, many of them were obtained from several different likelihood-based methods; i.e., both ML and Bayesian. There was, however, some incongruence between these likelihood findings and those from MP and LD, perhaps due to the different ways the methods treat ASRV (see Mallatt et al., 2004, for a full discussion of this).

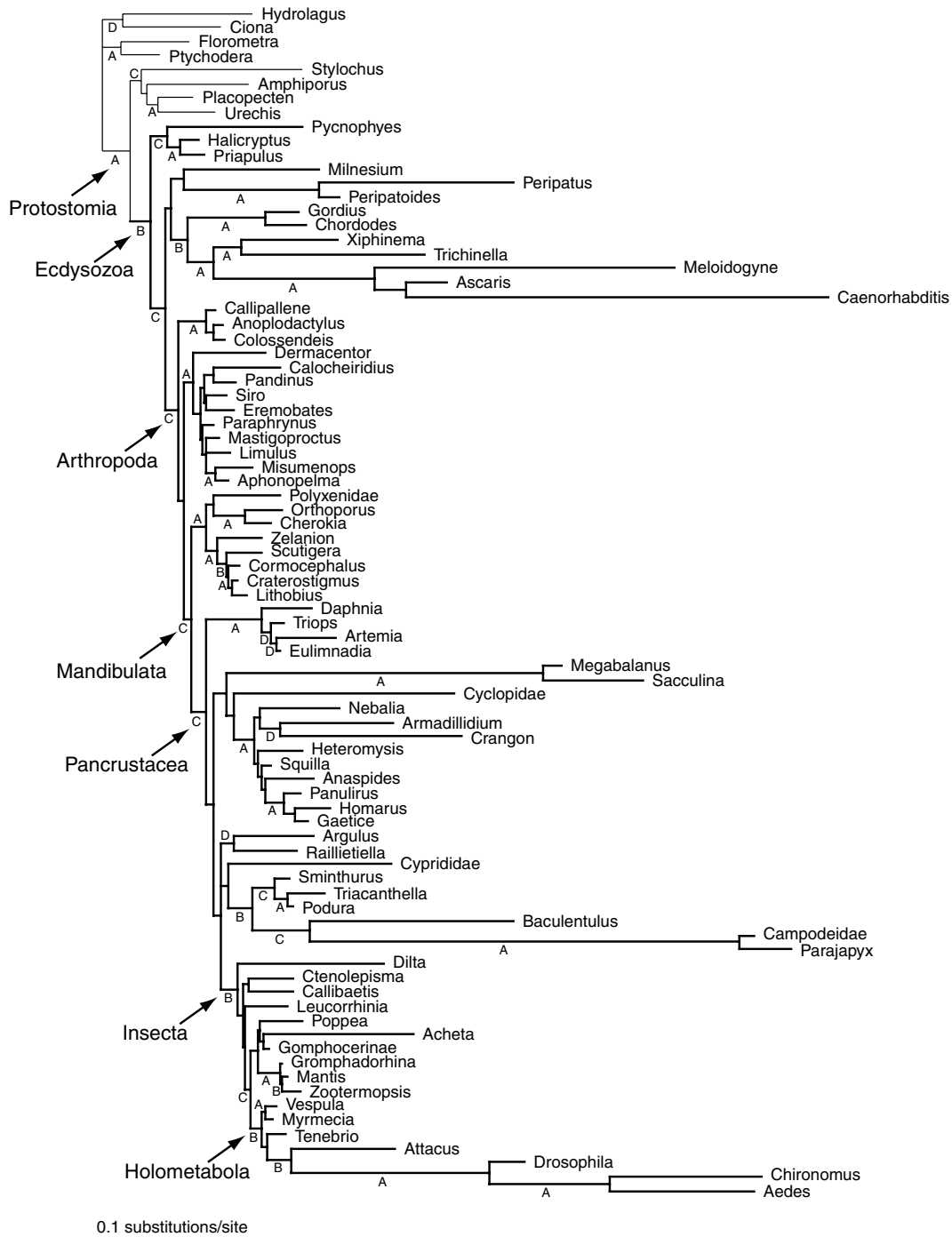


Fig. 3. Maximum likelihood tree from 18S rRNA genes alone (1545 nt). The LnL value of this tree is $-28,497.2$. Precise support-percentages for the nodes are listed in column 6 of Table 2, and the labeling of nodes is as in Fig. 1: from A (most supported) to D (least supported).

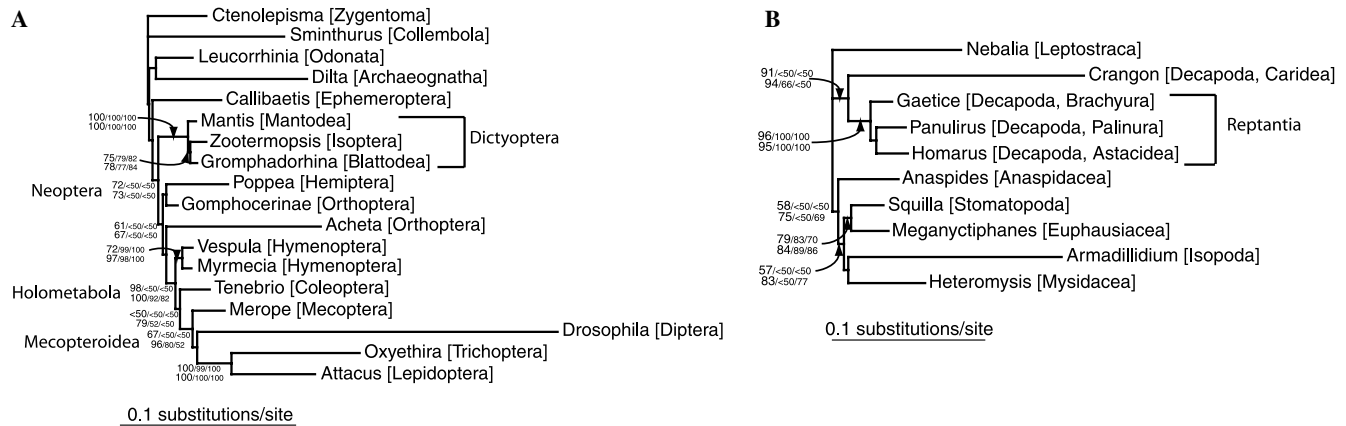


Fig. 4. Maximum likelihood trees from combined 28S + 18S genes, calculated from two subsets of taxa and including three species not present in the main tree of Fig. 1: (A) Insects, including scorpionfly *Merope* and caddisfly *Oxyethira* sequences (LnL = -16,866.6); (B) Malacostracans, including krill *Meganyctiphanes* (LnL = -13,334.9). Numbers at the nodes are ML, MP, and LD bootstrap percentages, respectively. ML values $\geq 60\%$ indicate significant support. (No Bayesian analyses were performed on these small data sets.) Two sets of bootstrap values are given for each node, the lower set being obtained when the most divergent and disruptive sequence is omitted; that is, after the omission of *Drosophila* in (A) and of *Armadillidium* in (B).

4.2. Clade-specific findings

4.2.1. Kinorhynchs

Morphologists unite kinorhynchs, priapulans, and loriciferans into Scalidophora (Lemburg, 1995; Neuhaus and Higgins, 2002; Schmidt-Rhaesa et al., 1998) or Cephalorhyncha (Nielsen, 2001), because these groups share a protrusible introvert (mouth cone) with scalids, among other characters. Molecular studies based on 18S rRNA genes (e.g., Garey, 2001; Giribet and Ribera, 2000; Giribet et al., 2000) or on a combination of multiple loci (Giribet et al., 2004b) support a clade of 'kinorhynch + priapulans' (no genes have yet been published from loriciferans). This kinorhynch + priapulans clade is upheld by the present findings, which unite these two taxa with strong support (Fig. 1, Table 2).¹

The other non-arthropod ecdysozoans—nematodes, nematomorphs, tardigrades, and onychophorans—need not be discussed because they showed exactly the same relations as in the previous study (Mallatt et al., 2004).

4.2.2. Main arthropod lineages

The Pancrustacea (= Tetraconata) concept, which joins hexapods and crustaceans in a clade, continues to find support in the present study. It is also upheld by other molecular and morphological evidence (Boore et al., 1998; Dohle, 2001; Giribet et al., 2001, 2005; Mittmann, 2002; Regier et al., 2005a,b; Richter, 2002; Turbeville et al., 1991; Zrzavý and Štys, 1997) but contradicted in analyses that include Paleozoic fossils (Wheeler et al., 2004).

By comparison, the Paradoxopoda (= Myriochelata) hypothesis, which places chelicerates with myriapods, has

not received as much support in the past. Paradoxopoda was previously obtained by certain studies that used nuclear rRNA (Friedrich and Tautz, 1995; Giribet et al., 1996), mitochondrial (Hwang et al., 2001), Hox (Cook et al., 2001), and nuclear protein-coding genes (Pisani et al., 2004), yet no morphological synapomorphies seem to unite chelicerates and myriapods. Neuroanatomical studies by Dove and Stollewerk (2003) and Kadner and Stollewerk (2004) did find similarities in the pattern of neurogenesis of one spider and one millipede that differed from the *Drosophila* pattern, but the limited taxonomic sampling could not distinguish whether the mode of neurogenesis in the spider and millipede is apomorphic (supporting Paradoxopoda) or plesiomorphic and thus not supporting a clade. Other recent neuroanatomical studies, by contrast, support Mandibulata, not Paradoxopoda (Harzsch et al., 2005; Müller et al., 2003).

Paradoxopoda was strongly supported by nearly complete rRNA sequence data in the previous study (Mallatt et al., 2004), but with the larger number of taxa in the present study, it now is more ambiguous. Despite the support obtained for Paradoxopoda from Bayesian tree-building methods (Fig. 1), the parametric-bootstrapping test shows the Paradoxopoda hypothesis does not fit our data any better than does the alternate, Mandibulata, hypothesis that places myriapods with Pancrustacea (see Table 4, Hypothesis 1); and Mandibulata was also recovered in our 18S-gene analysis (Fig. 3). Nor does another set of genes that is well-sampled across arthropods, elongation factor-1 α and 2 and RNA polymerase II, resolve the relationships among myriapods, chelicerates, and Pancrustacea (Regier and Shultz, 2001). Nor did the combined analysis of morphology and nine genes of Giribet et al. (2005), where only 2 of the 20 explored parameter sets supported Paradoxopoda. Thus, it is still uncertain whether Paradoxopoda, Mandibulata, or some other grouping, is correct.

¹ As this manuscript neared completion, a paper appeared (Petrov and Vladychenskaya, 2005) that also recovered a kinorhynch + priapulans clade from rRNA genes.

Table 4

Parametric bootstrapping: results of hypothesis testing, based on concatenated 28S + 18S rRNA-gene sequences, with rejection at $P \leq 0.01$

Alternate hypothesis that was tested	δ^a value	Range of 100 simulated-difference values	Probability	Accept or reject?
1. Mandibulata, consisting of Myriapoda and Pancrustacea	1.97	0–3.1	0.05	Accept
2. Pycnogonids as basal arthropods (= Pycnogonida vs. Cormogonida)	4.3	0–8.1 (99% = 0–3.2)	0.01	Reject (borderline)
3. Arachnida	4.3	0–6.1	0.02	Accept
4. Traditional centipedes: <i>Craterostigma</i> + (<i>Cormocephalus</i> + <i>Zelanion</i>)	16.5	0–5.9	$\ll 0.01$	Reject
5. Cirripedes in Maxillopoda (<i>Megabalanus</i> , Sacculinidae sp., Cyprididae sp., Cyclopidae sp., and <i>Argulus</i>)	16.4	0–5.1	$\ll 0.01$	Reject
6. Ostracod (Cyprididae) + Cirripedes	5.3	0–2.1	$\ll 0.01$	Reject
7. Hexapods + malacostracans	37.5	0–4.5	$\ll 0.01$	Reject
8. Copepod (Cyclopidae) not in the 'Hexapoda + Branchiopoda' group	0.96	0–3.5	0.04	Accept
9. <i>Daphnia</i> + <i>Eulimnadia</i> joined within Branchiopoda	9.5	0–4.8	$\ll 0.01$	Reject
10. Collembola as basal hexapods, or immediate sister of hexapods	14.0	0–3.5	$\ll 0.01$	Reject
11. <i>Acheta</i> + Gomphocerinae sp. (orthopteran insects)	3.0	0–3.4	0.02	Accept
12. <i>Squilla</i> as basal eumalacostracan	24.1	0–5.3	$\ll 0.01$	Reject

Note. The sets of taxa used in each of the 12 tests, plus the likelihood value of each optimal tree, are as follows:

Hypothesis 1. LnL = -31,523.9: *Halicryptus*, *Pycnophyes*, *Gordius*, *Milnesium*, *Trichinella*, *Peripatoides*, *Anoplodactylus*, *Callipallene*, *Colossendeis*, *Cormocephalus*, Cyclopidae sp., *Argulus*, Cyprididae sp., *Mastigoproctus*, *Leucorrhinia*, *Craterostigma*, *Homarus*, *Anaspides*, *Megabalanus*, *Calocheiridius*, *Eulimnadia*, *Sminthurus*, *Cherokia*, *Zelanion*, *Scutigera*, *Siro*, *Eromabates*, Polyxenidae sp., *Attacus*, *Dermacentor*, *Aphonopelma*, *Pandinus*, *Limulus*, and *Lithobius*.

Hypothesis 2. LnL = -31,523.9: same taxa used as for Hypothesis 1.

Hypothesis 3. LnL = -12,666.6: *Limulus*, *Aphonopelma*, *Pandinus*, *Eromabates*, *Calocheiridius*, *Paraphrynus*, *Siro*, *Dermacentor*, *Misumenops*, *Mastigoproctus*, *Anoplodactylus*, Polyxenidae sp., *Craterostigma*, and *Argulus*.

Hypothesis 4. LnL = -9,324.57. Polyxenidae sp., *Orthoporus*, *Cherokia*, *Scutigera*, *Lithobius*, *Craterostigma*, *Cormocephalus*, and *Zelanion*.

Hypothesis 5. LnL = -21,221.2. Gomphocerinae sp., *Eulimnadia*, *Megabalanus*, Sacculinidae sp., Cyprididae sp., Cyclopidae sp., *Argulus*, *Nebalia*, *Crangon*, *Panulirus*, *Gaetice*, *Squilla*, *Heteromysis*, *Anaspides* *Armadillidium*, and *Meganctiphanes*.

Hypothesis 6. LnL = -19,779.0. Same taxa as used for Hypothesis 5, except without Cyclopidae sp.

Hypothesis 7. LnL = -25,183.8. Cyclopidae sp., *Argulus*, Cyprididae sp., *Megabalanus*, *Armadillidium*, *Homarus*, *Anaspides*, *Nebalia*, *Artemia*, *Eulimnadia*, *Ctenolepisma*, *Baculentulus*, *Mantis*, *Sminthurus*, *Daphnia*, *Heteromysis*, *Crangon*, *Leucorrhinia*, and *Attacus*.

Hypothesis 8. LnL = -20,801.2. Cyclopidae sp., *Argulus*, Cyprididae sp., *Megabalanus*, *Armadillidium*, *Homarus*, *Anaspides*, *Nebalia*, *Artemia*, *Eulimnadia*, *Ctenolepisma*, *Baculentulus*, *Mantis*, and *Sminthurus*.

Hypothesis 9. LnL = -13,861.5. *Artemia*, Cyclopidae sp., *Squilla*, *Argulus*, *Ctenolepisma*, *Leucorrhinia*, *Eulimnadia*, *Sminthurus*, *Triops*, and *Daphnia*.

Hypothesis 10. LnL = -19,048.6. Cyclopidae sp., *Squilla*, *Argulus*, *Eulimnadia*, *Triacanthella*, *Sminthurus*, *Podura*, *Ctenolepisma*, *Dilta*, *Leucorrhinia*, *Callibaetis*, *Vespa*, Gomphocerinae sp., Campodeidae sp., *Baculentulus*, and *Mantis*.

Hypothesis 11. LnL = -14,977.0. *Acheta*, Gomphocerinae sp., *Leucorrhinia*, *Poppea*, *Ctenolepisma*, *Merope*, *Tenebrio*, *Vespa*, *Oxyethira*, *Mantis*, *Zootermopsis*, *Callibaetis*, *Gromphadorhina*, *Myrmecia*, *Attacus*, and *Drosophila*.

Hypothesis 12. LnL = -13,334.9. *Nebalia*, *Squilla*, *Crangon*, *Gaetice*, *Panulirus*, *Homarus*, *Anaspides*, *Meganctiphanes*, *Heteromysis*, and *Armadillidium*.

Note. All 12 of these subsets of taxa used the standard alignment with 3852 nt. All had stationary base frequencies, as judged by the χ^2 test of homogeneity in PAUP*, except for #10, 'Collembola as basal hexapods,' where the CG-rich dipluran sequences caused non-stationarity.

^a The test statistic, δ , is the difference between the likelihood value of the optimal tree and the tree constrained to fit the alternate hypothesis (see Mallatt et al., 2004).

4.2.3. Pycnogonids

Also debated is the position of pycnogonids; that is, whether sea spiders are the sister group to chelicerates (Waloszek and Dunlop, 2002), or are the sister group of all other extant arthropods, which have been termed Cormogonida (Giribet et al., 2001; Giribet et al., 2002a, p. 8; Zrzavý et al., 1998). Morphologically, pycnogonids are difficult to place because they have many autapomorphies, and most of the synapomorphies said to unite them with chelicerates seem uncertain: Dunlop and Arango (2005) concluded that the only valid synapomorphy of pycnogonids and chelicerates is a first pair of pincer-like appendages—the chelifores of pycnogonids and the cheliceres of chelicerates. However, new neuroanatomical evidence shows that pycnogonid chelifores are innervated by the protocerebrum (Maxmen et al., 2005), while

cheliceres are deutocerebral, so they might not be homologous appendages.

Molecular and total-evidence data are not consistent about the position of pycnogonids. The genes for elongation factor-1 α and 2, and RNA polymerase II place pycnogonids with Chelicerata with strong support (Regier and Shultz, 2001), but other analyses do not (Zrzavý et al., 1998). A recent sensitivity analysis using morphology and nine molecular loci favored pycnogonids-with-chelicerates over Cormogonida (Giribet et al., 2005, their Figure 6), as was found by morphological analysis alone (Giribet et al., 2005, their Figure 3). However, when character #122 referring to the presence of cheliceres/chelifores is re-coded according to the new neuroanatomical evidence of Maxmen et al. (2005), Cormogonida is favored. In the previous study based on nearly complete rRNA genes

(Mallatt et al., 2004), pycnogonids were decisively linked to the chelicerates (and to myriapods). In the present study with improved taxon sampling, our tree-based results also favored this hypothesis (Fig. 1), but parametric bootstrapping only marginally rejected the alternate, Cormogonida hypothesis ($P=0.01$: Table 4, Hypothesis 2). Thus, the present rRNA-gene evidence that joins pycnogonids with chelicerates and myriapods, while positive, is not conclusive.

4.2.4. Chelicerates

We present nearly complete rRNA-gene-sequences from nine of the 12 major chelicerate groups, lacking Schizomida, Ricinulei, and Palpigradi. Support for relationships within Chelicerata (Fig. 1, Table 2) was low for most clades. Low support was also noted by previous authors who used mostly 18S rRNA genes (Giribet and Ribera, 2000), and these authors related this lack of resolution to the very conserved rRNA sequences in this clade (note the short branch lengths of most chelicerate sequences in Fig. 1). Our results do support Tetrapulmonata, however, represented here by two spiders, the amblypygid *Paraphrynus* (whip spider), and the uropygid *Mastigoproctus* (whip scorpion, or vinegaroon). Tetrapulmonata is a widely accepted clade, supported by the presence of two pairs of book lungs (with many exceptions in spiders) and distinct, two-segmented cheliceres (also found in Ricinulei) (Dunlop, 2002; Giribet et al., 2002a; Shultz, 1990; Wheeler and Hayashi, 1998).

As seen in Fig. 1 and Tables 2 and 3, some of our methods weakly join the aquatic xiphosuran *Limulus* with tetrapulmonates (Bayesian-ASRV), or place the tick *Dermacentor* as the sister of all other chelicerates (Bayesian-covarian = 94%). Such groupings would invalidate the accepted clade Arachnida, which comprises the terrestrial chelicerates (Ax, 1999; Coddington et al., 2004; Dunlop, 2002; Shultz, 1990). Also, the positions obtained here for *Limulus* and *Dermacentor* imply so many losses and re-acquisitions of aquatic characters among chelicerates as to seem unlikely. For these reasons, we tested the results with parametric bootstrapping and found that they do not reject Arachnida after all (Table 4, Hypothesis 3).

Finally, our Bayesian analyses hint at a relationship between the solifug *Eremobates* and the scorpion *Pandinus*. Apparently, this precise pairing was never proposed before, although morphological analyses have suggested a clade of solifuges + pseudoscorpions + scorpions (see Giribet et al., 2002a, their Figure 3A).

4.2.5. Myriapods

Myriapoda and its two largest subclades, Diplopoda (millipedes) and Chilopoda (centipedes), are well-defined morphologically (Edgecombe, 2004; Edgecombe et al., 1999; Edgecombe and Giribet, 2002, 2004; Enghoff, 1984; Sierwald et al., 2003). Our rRNA-gene data recovered Myriapoda, Diplopoda, and Chilopoda with support from every tree-building method (Fig. 1, Tables 2 and 3). We achieved this only after omitting the odd sphaerotheriid and symphylian sequences, but previous analyses using

other molecular and morphological information supported these clades even with sphaerotheriid and symphylian sequences included (Giribet et al., 2001, 2005; Regier et al., 2005b).

Within chilopods (Fig. 1, Table 2), where we sequenced members of all five orders, some support was obtained for *Scutigera* as sister to the others (i.e., from Bayesian-ASRV and LD methods). This split matches the accepted division of centipedes into Notostigmophora (= Scutigeraomorpha) and Pleurostigmophora (the four remaining orders), which are distinguished by dorsal versus lateral tracheal openings (Edgecombe et al., 1999; Edgecombe and Giribet, 2002, 2004; Giribet et al., 1999; Giribet and Edgecombe, 2006; but see Ax, 1999). Within Pleurostigmophora, the Bayesian-ASRV method placed *Craterostigma* as the sister to the other three taxa, whereas most previous studies, based on morphology and other molecular data, instead placed Lithobiomorpha there (Borucki, 1996; Dohle, 1985; Edgecombe, 2004; Edgecombe et al., 1999; Edgecombe and Giribet, 2002, 2004; Giribet et al., 1999; Shear and Bonamo, 1988; but see Giribet and Edgecombe, 2006). Our parametric-bootstrapping test rejected this accepted view (Table 4, Hypothesis 4). Our finding lacks morphological support, however, because craterostigmomorphs share several traits with scolopendromorphs (e.g., *Cormocephalus*) and with geophilomorphs (e.g., *Zelation*), exclusive of *Lithobius*. Such traits include maternal brood care and anatomical characters in the maxillipede, anal legs, circulatory system, and testes (see Edgecombe and Giribet, 2004, for details).

Within Diplopoda, we had reliable sequences from just three of the sixteen recognized orders (Sierwald et al., 2003). However, we recovered Polyxenidae as sister to the two helminthomorphs *Orthoporus* and *Cherokia* (Fig. 1), which is consistent with morphological evidence (Edgecombe, 2004; Enghoff et al., 1993; Sierwald et al., 2003; Wilson and Anderson, 2004).

4.2.6. Pancrustacea—major splits

The relations within Pancrustacea, especially among the major groups of crustaceans, have prompted a century-and-a-half of debate among morphological taxonomists (for references, see Ax, 1999; Giribet et al., 2005; Richter, 2002; Schram and Koenemann, 2004; and Wills, 1998). Nor have the 18S rRNA-dominated gene-sequences used in previous molecular-taxonomic studies (Giribet and Ribera, 2000; Spears and Abele, 1998) or combined analyses of multiple loci and morphology (Giribet et al., 2001, 2005) provided as much resolution as is desired. Our study, based on more rRNA-gene characters, shows increased support for many pancrustacean clades (Fig. 1), plus overall agreement with a recent study that used genes for two elongation factors and RNA polymerase II (Regier et al., 2005a). The high-order clades found by both these studies were largely unanticipated by morphology-based taxonomy, although Schram and Koenemann (2004) recovered these clades in broad outline in a recent, preliminary morphological analysis.

Before we discuss and test the clades we found, we note that our rRNA-gene data strongly reject the existence of a “Maxillopoda” clade (parametric bootstrapping: Table 4, Hypothesis 5)—Maxillopoda being a debated grouping of cirripedes, ostracods, copepods, and branchiurans, which share some similarities of body segmentation and mouth parts (Ax, 1999; Walossek and Müller, 1998; Wills, 1998).

Our findings (see also Regier et al., 2005a) divide Pancrustacea into these major clades:

1. *Branchiurans (Argulus)*, *pentastomids (Raillietiella)*, and *ostracods (Cyprididae sp.)* as sisters to all other pancrustaceans. Although this was found in both the present topology and that of Regier et al. (2005a), it was not strongly supported in either study: in our rRNA-gene tree, the clade of ‘all other pancrustaceans’ received only Bayesian-covariation support (Fig. 1 and clade #19 in Table 2). Still, the fact that two independent molecular-phylogenetic studies based on different sets of genes place branchiurans and ostracods as the basal lines of pancrustaceans is noteworthy.

Overall, the placement of ostracods is the most uncertain. The elongation-factor/RNA-polymerase genes of Regier et al. (2005a) actually recovered Ostracoda as polyphyletic, and only one of their two ostracod groups went at the base of Pancrustacea. Morphological similarities hint instead at a relationship between ostracods and cirripedes (Ax, 1999; Ruppert et al., 2004). A recent total-evidence analysis, based on morphology and nine genes (Giribet et al., 2005), also grouped ostracods with cirripedes (stable to parameter-set variation) while otherwise matching our present finding of pentastomids and branchiurans as sister to all other pancrustaceans. Despite this, our rRNA-gene data reject the ostracod + cirripede clade, according to parametric bootstrapping (Table 4, Hypothesis 6).

2. *Hexapods plus branchiopods (and copepods)*. Much interest and debate center around which arthropod group is the nearest relative of hexapods, with recent discussion focusing on whether it is Malacostraca (Dohle, 1998; Giribet et al., 2005; Harzsch, 2002; Hwang et al., 2001; Sinakevitch et al., 2003; Wilson et al., 2000), Branchiopoda (Adoutte and Philippe, 1993; Friedrich and Tautz, 2001; Gaunt and Miles, 2002; Schram and Koenemann, 2004), or a monophyletic Crustacea (see Giribet et al., 2005). Both our rRNA genes and the genes for elongation factors and RNA polymerase II (Regier et al., 2005a) provide significant likelihood support for the branchiopods being closest to hexapods, and our parametric bootstrapping test strongly rejected the alternate, malacostracan-hexapod association (Table 4, Hypothesis 7).

3. *Cirripedes with malacostracans*. The genes encoding elongation factors and RNA polymerase II strongly supported this clade (Regier et al., 2005a), and our rRNA genes supported it in all the likelihood-based analyses (Fig. 1, Tables 2 and 3). Our parametric bootstrap tests also rejected two alternative hypotheses about cirripedes (Table 4, Hypotheses 5 and 6): that they are maxillopods or are the sister group of ostracods. The grouping of

cirripedes with malacostracans is startling because their key morphological-taxonomic characters differ so fundamentally: e.g., reduction versus importance of the second antennae (Ruppert et al., 2004). Also, both the rRNA and protein-encoding genes of cirripedes are divergent (see the long branches to the cirripedes in Fig. 1, and in Figure 1 of Regier et al., 2005a), and such long branches can be artifactually misplaced on gene trees. Even so, the independent recovery of a cirripede + malacostracan group from two different sets of genes is reasonably good evidence, and a potential developmental synapomorphy has been identified in the shared presence of eight mesodermal teloblasts (and probably, of ectodermal teleoblasts as well: Anderson, 1973; Dohle and Scholtz, 1988; Scholtz, 2002).

The position of copepods deserves discussion. The genes for elongation factors and RNA polymerase II firmly joined copepods with ‘cirripedes + malacostracans’ (Regier et al., 2005a), but our rRNA genes instead joined the single copepod (*Cyclopidae sp.*) to hexapods with ML and Bayesian support (Fig. 1; also see Mallatt et al., 2004). Our finding seems odd, however, because the copepod + hexapod clade is not supported by morphological characters (Giribet et al., 2005, their Figure 3) and this clade was shown to be unstable to parameter variation in a sensitivity analysis (Giribet et al., 2005). Nor can our parametric bootstrap test reject an alternative hypothesis that excludes copepods from the clade of Hexapoda + Branchiopoda (Table 4, Hypothesis 8). In fact, in that parametric test the best alternate tree grouped the copepod with cirripedes and malacostracans—exactly as indicated by the elongation-factor and RNA polymerase II genes (Regier et al., 2005a). This suggests our rRNA-based placement of the copepod with hexapods is an analytical artifact.

We could not obtain usable specimens or 28S rRNA-gene-sequences from two important clades of crustaceans, cephalocarids, and remipedes. However, previous molecular evidence indicates that these two clades group together (Spears and Abele, 1998) as the sister of the ‘branchiopod + hexapod’ clade (Regier et al., 2005a).

4.2.7. *Pentastomids*

Pentastomids are wormlike parasites in the respiratory tracts of vertebrates, mostly in reptile lungs (Ruppert et al., 2004). Their unusual morphology has confounded attempts to find their relations within the animal kingdom (Jenner, 2004), and cladistic analyses using morphology fail to place pentastomids within arthropods (Giribet et al., 2005, their Figure 3). However, their sperm structure relates them to branchiuran crustaceans (Jamieson and Storch, 1992; Riley et al., 1978; Wingstrand, 1972). This relationship also is supported by amino-acid sequences from mitochondrial genes (Lavrov et al., 2004), by 18S rRNA-gene-sequences (Abele et al., 1989; Spears and Abele, 1998; Zrzavý, 2001), and more recently by 18S plus half of the 28S rRNA gene (Giribet et al., 2005). The strong support attained here, from nearly complete 28S plus 18S rRNA genes, is further

confirmation of a pentastomid + branchiuran clade (*Raillietiella* plus *Argulus* in Fig. 1).

4.2.8. Branchiopods

The common view divides Branchiopoda into Anostraca (fairy shrimps without a carapace; e.g., *Artemia*) and Phyllopoda (with a carapace and internalized eyes, among other characters: Ax, 1999; Ruppert et al., 2004; <http://tolweb.org/tree/phylogeny.html>). Within Phyllopoda, the Notostraca (e.g., *Triops*) are said to be the sister group of a clade containing Cladocera (e.g., *Daphnia*) and Spinicaudata (e.g., *Eulimnadia*), the latter two sharing a large, compressed carapace with two valves that show growth rings, and other traits (Ax, 1999; Braband et al., 2002). Our rRNA-gene findings agree with this common view by supporting Phyllopoda (*Eulimnadia*, *Triops*, and *Daphnia*), but disagree by joining *Daphnia* with *Triops* instead of with *Eulimnadia* (Fig. 1; Tables 2 and 3) (see also Giribet et al., 2005). This ‘*Daphnia* plus *Triops*’ clade passed the test of parametric bootstrapping (Table 4, Hypothesis 9), indicating that similar, bivalved carapaces may have evolved independently in Cladocera and Spinicaudata. Alternatively, it could mean that notostracans lost such a carapace (Richter, 2004). Indeed, the posterior part of the carapace of larval *Triops* has bilateral humps that might be the *anlagen* of a bivalved carapace (Møller et al., 2003).

4.2.9. Hexapods—major splits

Hexapod monophyly was recovered with the Bayesian-ASRV and ML methods (Fig. 1), which also found a main division into Entognatha and Insecta (Ectognatha). The relations among the entognath groups—collembolans, proturans, and diplurans—are much debated. This problem was recently reviewed by several authors (Giribet et al., 2004a; Wheeler et al., 2001; Willmann, 2003). Luan et al. (2005) used the same techniques employed here on 18S and partial-28S rRNA sequences of many entognath taxa. We used fewer taxa than Luan et al. did and a much larger part of the 28S rRNA gene, but we likewise found the proturan *Baculentulus* to unite with a monophyletic Diplura (Campodeidae sp. and *Parajapyx*) in a clade that Luan et al. (2005) named Nonoculata (also found by Giribet et al., 2004a). By contrast, most morphological studies united proturans with collembolans (e.g., Hennig, 1953, 1981; Koch, 1997; Kraus, 1998, 2001; Kristensen, 1991, 1998; Wheeler et al., 2001) and some joined diplurans with insects (Koch, 1997; Kukulová-Peck, 1987; Wheeler et al., 2001).

Here, we also found significant support for uniting collembolans with the Protura + Diplura clade (Fig. 1: ML and Bayesian methods), in a new internal arrangement of Entognatha. Parametric bootstrapping shows that our rRNA genes reject an alternate hypothesis that Collembola are outside hexapods (Cook et al., 2005; Nardi et al., 2003) or are the most basal hexapods (Table 4, Hypothesis 10).

As a caveat to these conclusions, the rRNA genes of the entognaths showed non-stationarity of nucleotide frequency. This is because the genes of diplurans, and to a lesser extent, of the proturan, are GC-rich. Thus, the dipluran and proturan sequences might have attracted together artifactually. However, this result was stable to parameter variation in the stability analyses of Giribet et al. (2005), and stability analysis can detect putative long-branch attraction (Giribet, 2003b). Also, the Log-Det-Paralinear method that we used here is designed to solve this non-stationarity problem (Lake, 1994)—although the inability of this method to model ASRV in PAUP* hinders its reliability (see Luan et al., 2005, for more discussion).

4.2.10. Insects

In our tree (Fig. 1), the archeognathan insect *Dilta* was the sister to Dicondylia (all other represented insects), as is widely accepted based on mandibular articulations and other morphological traits (see Figure 1 in Giribet et al., 2004a; and Fürst von Lieven, 2000). However, this result was only supported by our Bayesian-ASRV method (Table 2; also see Kjer, 2004).

Our tree shows the mayfly *Callibaetis* as the sister to neopteran insects, with 95% Bayesian-ASRV and 60% ML bootstrap support. This relation was also found by Kjer (2004) based on 18S gene data, and by some morphological studies (Boudreaux, 1979; Matsuda, 1970). However, the problem of the relationship among Ephemeroptera (mayflies), Odonata (dragonflies), and Neoptera is a difficult one, and ‘Odonata + Neoptera’ has also received support (reviewed by Ogden and Whiting, 2003; Ogden et al., 2005; Willmann, 2003), especially from characters of the mandible (Fürst von Lieven, 2000; Staniczek, 2000).

Within Neoptera, the insects that can fold their wings, we recovered a well-supported Dictyoptera (mantodean *Mantis*, isopteran *Zootermopsis*, and blattodean *Gromphadorhina*), as in previous studies (Kjer, 2004; Terry and Whiting, 2005; Wheeler et al., 2001; Whiting et al., 2003). Within Dictyoptera, our data joined the isopteran and blattodean (Fig. 1) by the Bayesian, ML, and MP methods, and this is also the most widely recognized subgroup (Svenson and Whiting, 2004; Terry and Whiting, 2005; but see Wheeler et al., 2001).

Our Bayesian analyses (Fig. 1) indicate that Dictyoptera is the sister group to the remaining neopterans, whose basal subclades include the Orthoptera (Gomphocerinae sp. and *Acheta*) and the hemipteran *Poppea*. This is consistent with one of the trees from Wheeler et al. (2001, their Figure 17) and with Whiting et al. (2003), which were based on morphological and extensive molecular evidence. The failure of the cricket *Acheta* to group with the grasshopper Gomphocerinae sp. in our tree (Fig. 1) is probably an artifact, because our parametric bootstrapping accepts orthopteran monophyly (Table 4, Hypothesis 11).

The clade Holometabola (insects that undergo complex metamorphosis) is supported by much morphological and molecular evidence (see references in Wheeler et al., 2001; Kjer, 2004; Terry and Whiting, 2005; Whiting, 2002a,b; Whiting et al., 1997; and Willmann, 2003), and by the Bayesian and ML methods of the present study (Fig. 1, Table 2). Relations among the holometabolan orders are debated, but one group that seems firmly established is Trichoptera (caddisflies) with Lepidoptera (moths and butterflies) (Castro and Dowton, 2005). The rRNA genes uphold this group by strongly joining *Oxyethira* with *Attacus* (Fig. 4A). Another proposed clade in Holometabola is Mecopteroidea (Kristensen, 1998; Wheeler et al., 2001, p. 119), which contains Lepidoptera, Trichoptera, Diptera (true flies; e.g., *Drosophila*), and Mecoptera (scorpionflies; e.g., *Merope*). Our limited results uphold this clade (Fig. 4A), with 67% ML bootstrap support—and with the higher support-value of 96% after the divergent dipteran (*Drosophila*) sequence is excluded (but cf. Whiting, 2002b).²

In summary, none of the relationships we obtained for insects clashes with previous phylogenetic hypotheses, perhaps attesting to the accuracy of these results from rRNA genes.

4.2.11. Malacostraca

The relationships among the many subgroups of malacostracans are not well established (alternate hypotheses are summarized by Jarman et al., 2000; Richter and Scholtz, 2001; and Spears et al., 2005). We sampled only ten malacostracans, so our contribution to the topic is limited. Also, the isopod *Armadillidium* has divergent and unusually large 28S and 18S rRNA genes (Choe et al., 1999; Giribet and Wheeler, 2001), probably disrupting our malacostracan trees.

Despite these difficulties, our data yielded several significant results. First, Bayesian inference recovered the leptostracan *Nebalia* as sister to all other malacostracans (= Eumalacostraca: Clade 43 in Table 2). Eumalacostraca was also supported by previous morphological and molecular studies (Babbitt and Patel, 2005; Giribet et al., 2001, 2005; Richter and Scholtz, 2001; Spears and Abele, 1998).

² While this paper was in review, we pushed our computing capacity to its limit by adding the *Merope* (scorpionfly), *Oxyethira* (caddisfly), and *Meganctiphanes* (krill) sequences to the 41 pancrustacean sequences that had generated the ML bootstrap values in Fig. 1 (listed as ML Subset 1 in the Note of Table 2), and ran an ML-bootstrap analysis on this augmented, 44-taxon alignment (50 bootstrap replicates). Overall, the new ML percentages were very similar to those indicated in Figs. 1 and 4. On the new tree, *Merope* and *Oxyethira* were in the same places, with the same or better boot values as in Fig. 4A (*Oxyethira* + *Attacus*: still 100%; Mecopteroidea: 74% cf. 67%). *Meganctiphanes* still joined with *Squilla*, with 66% bootstrap support now, compared to 79% in Fig. 4B; this lowering of support probably occurred because the divergent cirripede sequences, absent from the analysis of Fig. 4B, were nearby in the new analysis.

Our data placed the stomatopod (mantis shrimp) *Squilla* with the euphausiacean (krill) *Meganctiphanes* with ML, MP, and LD support (Fig. 4B).² This is surprising because stomatopods are traditionally placed instead at the base of Eumalacostraca, based on characters of their carapace and rostrum, among others (Richter and Scholtz, 2001, their Figure 7; Schram and Koenemann, 2004; Spears et al., 2005, their Figure 1). However, parametric bootstrapping showed that our data reject this traditional view (Table 4, Hypothesis 12).

Our four decapod sequences—caridean (true shrimp) *Crangon*, crab *Gaetice*, spiny lobster *Panulirus*, and American lobster *Homarus*—were united in a well-supported Decapoda, with the shrimp as the sister of the three Reptantia (Figs. 1, 4B). This matches previous morphological and molecular studies (e.g., Ah Yong and O'Meally, 2004; Richter and Scholtz, 2001). Within Reptantia, our Bayesian and MP tests joined the lobsters *Panulirus* and *Homarus* (Table 2, Clade 47), although the morphology-plus-rRNA study of Ah Yong and O'Meally (2004) instead joined *Homarus* with crabs.

The relationships of our remaining malacostracan sequences are not clear from the main, 84-taxon tree in Fig. 1, but in the ML tree for malacostracans (with krill added: Fig. 4B), another potential group emerges. This group consists of the anaspidacean *Anaspides*, stomatopod *Squilla*, euphausiacean *Meganctiphanes*, isopod *Armadillidium*, and mysid *Heteromysis*. Except for the stomatopod, whose placement was discussed above, this same group was obtained from morphological characters by Richter and Scholtz (2001), who likewise identified Anaspidacea as its basal clade (also see Jarman et al., 2000). Our ML support for this group is weak (58%), but when the divergent *Armadillidium* sequence is removed, support for the group and some of its subclades rises to significant levels of $\geq 75\%$ (Fig. 4B).

5. Summary

In this study, with its expanded set of over 80 ecdysozoan taxa, nearly complete 28S and 18S rRNA-gene sequences proved valuable for discerning deep to recent divergences among the arthropods. As shown in Fig. 1 and Tables 3 and 4, here are the main clades recovered, based on ML-bootstraping ($\geq 60\%$), Bayesian posterior probabilities ($\geq 95\%$), or parametric bootstrapping ($P \leq 0.01$):

1. Kinorhyncha is the sister phylum of Priapula, at the base of Ecdysozoa.
2. Although Bayesian analyses support Paradoxopoda (myriapods + chelicerates + pycnogonids), parametric bootstrapping did not reject the alternate hypothesis of Mandibulata (myriapods + pancrustaceans), and it only barely rejected the hypothesis that pycnogonids are the sister group to all other arthropods ($P = 0.01$).
3. Within Chelicerata, Tetrapulmonata was recovered (spiders + uropygid + amblypygid). Resolution among

chelicerates was otherwise low, but did not reject the monophyly of Arachnida.

4. Myriapoda, Diplopoda, and Chilopoda were each monophyletic. Within Chilopoda, Craterostigmomorpha, and not Lithobiomorpha, was at the base of Pleurostigmophora.
5. Pancrustacea was monophyletic and contained: (1) branchiopods with hexapods (cephalocarids and remipedes not studied); (2) cirripedes with malacostracans; and, (3) ostracods, and then a clade of branchiurans + pentastomids, as the sister groups to all other pancrustaceans. Except for an uncertain placement of our copepod sequence, these findings agree with another large-scale study, which used genes for two elongation factors and RNA polymerase II (Regier et al., 2005a).
6. Hexapoda was monophyletic, and divided into Entognatha and Insecta (= Ectognatha). Entognatha consisted of Collembola and Nonoculata (Protura plus a monophyletic Diplura).
7. Relationships within Insecta were congruent with current phylogenetic hypotheses (for example, Neoptera, Holometabola, and Dictyoptera were recovered).
8. Within Malacostraca, the stomatopod was not sister to all other eumalacostracans, as is widely held, but instead joined with the euphausiacean (krill).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2006.04.021](https://doi.org/10.1016/j.ympev.2006.04.021).

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