MicroRNAs and phylogenomics resolve the relationships of Tardigrada and suggest that velvet worms are the sister group of Arthropoda

Lahcen I. Campbell[a,1], Omar Rota-Stabelli[a,1,2], Gregory D. Edgecombe[b], Trevor Marchioro[c], Stuart J. Longhorn[a], Maximilian J. Telford[d], Hervé Philippe[e], Lorena Rebecchi[c], Kevin J. Peterson[f,3], and Davide Pisani[a,3]

[a]Department of Biology, The National University of Ireland, Maynooth, Kildare, Ireland; [b]Department of Palaeontology, The Natural History Museum, London SW7 5BD, United Kingdom; [c]Dipartimento di Biologia, Università di Modena e Reggio Emilia, 41125 Modena, Italy; [d]Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, United Kingdom; [e]Centre Robert-Cedergren, Département de Biochimie, Université de Montréal, Montréal, QC, Canada H3C3J7; and [f]Department of Biology, Dartmouth College, Hanover, NH 03755

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Morphological data traditionally group Tardigrada (water bears), Onychophora (velvet worms), and Arthropoda (e.g., spiders, insects, and their allies) into a monophyletic group of invertebrates with walking appendages known as the Panarthropoda. However, molecular data generally do not support the inclusion of tardigrades within the Panarthropoda, but instead place them closer to Nematoda (roundworms). Here we present results from the analyses of two independent genomic datasets, expressed sequence tags (ESTs) and microRNAs (miRNAs), which congruently resolve the phylogenetic relationships of Tardigrada. Our EST analyses, based on 49,023 amino acid sites from 255 proteins, significantly support a monophyletic Panarthropoda including Tardigrada and suggest a sister group relationship between Arthropoda and Onychophora. Using careful experimental manipulations—comparisons of model fit, signal dissection, and taxonomic pruning—we show that support for a Tardigrada + Nematoda group derives from the phylogenetic artifact of long-branch attraction. Our small RNA libraries fully support our EST results; no miRNAs were found to link Tardigrada and Nematoda, whereas all panarthropods were found to share one unique miRNA (miR-276). In addition, Onychophora and Arthropoda were found to share a second miRNA (miR-305). Our study confirms the monophyly of the legged ecdysozoans, shows that past support for a Tardigrada + Nematoda group was due to long-branch attraction, and suggests that the velvet worms are the sister group to the arthropods.

Ecdysozoa | cycloneuralia | Lobopodia | Tactopoda

Ecdysozoa (1) is the clade of molting invertebrates that includes two of the ecologically most important and evolutionarily most successful animal phyla—the arthropods and the nematodes—as well as several other, less diversified taxa, including the tardigrades (water bears), the onychophorans (velvet worms), and the priapulids (penis worms). Although the monophyly of Ecdysozoa is now well established (2, 3), the phylogenetic relationships within this group have proven difficult to resolve (4–7). Morphological and embryological evidence suggests a close affinity among Arthropoda, Onychophora, and Tardigrada (the Panarthropoda) (8, 9), although the interrelationships among these three taxa are uncertain. Despite the concordance between these morphological studies and a few molecular analyses (10–14), most molecular studies instead support a close relationship between the water bears and the cycloneuralian ecdysozoans (nematodes, priapulids, and their close relatives), particularly the nematodes (2, 15–22). These alternative hypotheses of tardigrade relationships have important consequences for our understanding of morphological evolution within Ecdysozoa. For example, if tardigrades are cycloneuralians, then the telescopic mouth cone and plated pharynx shared by tardigrades and cycloneuralians should be considered cycloneuralian apomorphies, whereas the important characteristics of segmentation and the possession of paired limbs must be homoplastic—they either evolved convergently in arthropods and tardigrades or were lost in nematodes (23). Obviously, the opposite would be true if the tardigrades are panarthropods. Thus, accurately placing the tardigrades with respect to nematodes and arthropods is central to solving the interrelationships among the ecdysozoans and clarifying homologies within this group.

Although the rapidly growing influx of molecular data has radically altered our understanding of the animal tree of life, no dataset is homoplasy-free. Phylogenies derived from large, genomic-scale datasets of expressed sequence tags (ESTs) from many proteins minimize stochastic errors; however, they can exacerbate systematic errors (24), such as the well-known long-branch attraction (LBA) artifact (25). This is because systematic errors, unlike stochastic ones, are positively misleading; the error increases with an increase in the amount of data in the analysis (24). Although genomic-scale datasets are important for resolving difficult phylogenetic problems, suboptimal approaches to tree reconstruction, such as those using poorly fitting substitution models, can generate phylogenetic artifacts when applied to such datasets. Tools have been developed to ameliorate these problems, including comparing trees derived using differently fitting models (13, 14, 26), site-stripping (e.g., "slow-fast" analyses; ref. 27), signal dissection (28), and targeted taxon pruning (3, 26, 29). These tools have recently been applied to address, for example, the position of the Myriapoda (centipedes and their relatives) within Arthropoda (12, 14, 20, 30) and the position of the Ctenophora (comb jellies) among the non-bilaterian animals (12, 26, 31, 32).

Given the inherent difficulties and potential biases associated with the analyses of genome-scale datasets, the use of a single type of data might not be sufficient to solve a particularly difficult phylogenetic problem (33). We have contended that consilience (34)—the congruence of multiple lines of evidence—is a particularly cogent indicator of phylogenetic accuracy (14, 35, 36). A

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[1]L.I.C. and O.R.-S. contributed equally to this work.

[2]Present address: Research and Innovation Center, Fondazione Edmund Mach, San Michele all'Adige, Italy.

[3]To whom correspondence may be addressed. E-mail: kevin.j.peterson@dartmouth.edu or davide.pisani@nuim.ie.

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class of molecules whose utility for phylogenetic reconstruction has recently been recognized is the microRNAs (miRNAs), genomically encoded nonprotein coding RNAs of approximately 22 nucleotides in length that are found in many eukaryotes, including the metazoans (37, 38). MiRNAs are important post-transcriptional regulators (39), but it is their use as phylogenetic markers that is of interest here. MiRNAs have four properties that make them reliable indicators of phylogenetic relationships: (*i*) New miRNA families are continually added through time to evolving metazoan genomes; (*ii*) once a new miRNA is acquired, its mature sequence accumulates mutations only very slowly; (*iii*) the rate of miRNA acquisition outweighs the rate of miRNA losses in most metazoan taxa; and (*iv*) there is a low probability of convergent evolution of an miRNA gene (38, 40). Indeed, the use of miRNAs has already provided important insights into the interrelationships among annelids (41), sponges (42), arthropods (14) vertebrates (43), and brachiopods (44), and has helped place enigmatic taxa, such as acoel flatworms, into the animal tree of life (36).

In the present study, we investigated the phylogenetic relationships of the Tardigrada within Ecdysozoa by studying the consilience of two independent genomic datasets, ESTs and miRNAs. We first present our EST results and use these to ask whether alternative hypotheses of tardigrade relationships (arthropod vs. nematode affinity), as found in previous phylogenomic analyses, could be tree-reconstruction artifacts. We then assembled the miRNAs complements of a tardigrade and an onychophoran, and compare these with the miRNA complements of all other known metazoans. Finally, we compare the results of our EST and miRNA analyses to evaluate the extent to which these genomic markers corroborate or, alternatively, disagree with each other. These lines of evidence support the monophyly of Panarthropoda including Tardigrada. We show that support from previous studies for a nematode+tardigrade group is the result of an LBA artifact, and provide evidence that Onychophora is the sister group of Arthropoda. These results imply that panarthropod limbs and segmentation are homologous, and that characters shared by tardigrades, nematodes, and other cycloneuralians are ecdysozoan plesiomorphies.

Results

EST-Based Phylogenomic Analyses Support Panarthropoda and Lobopodia. To address the phylogenetic position of tardigrades, we assembled a dataset of 255 genes (49,023 reliably aligned amino acid positions) from all of the ecdysozoan phyla except the Loricifera. Because the use of poor-fitting models can cause the recovery of artifactual phylogenies, we first used Bayesian cross-validation (45) to rank substitution models according to their fit to our alignment. Results of our cross-validation analysis (Fig. S1) show a regular increase in the fit of the model to the data when moving from simple to more complex models, with the site-heterogeneous mixture model CAT-GTR+Γ having the best fit to our dataset. (All models tested used a gamma distribution to account for rate variation among sites.) Results of the Bayesian analyses performed using the CAT-GTR+Γ model are shown in Fig. 1*A*. The majority of internal nodes have a posterior probability (PP) = 1. Tardigrada is recovered within Panarthropoda as the sister group of Onychophora + Arthropoda, together called the Lobopodia (46), with PP = 1. Within Arthropoda, our analyses confirm the chelicerate affinity of the sea spiders and are consistent with the monophyly of Mandibulata (Myriapoda + Pancrustacea) (14, 30).

Our results do not support the monophyly of the Cycloneuralia, given that Nematoida (Nematoda + Nematomorpha) is recovered as the sister group of Panarthropoda, albeit with a low posterior probability (PP = 0.76), whereas Scalidophora

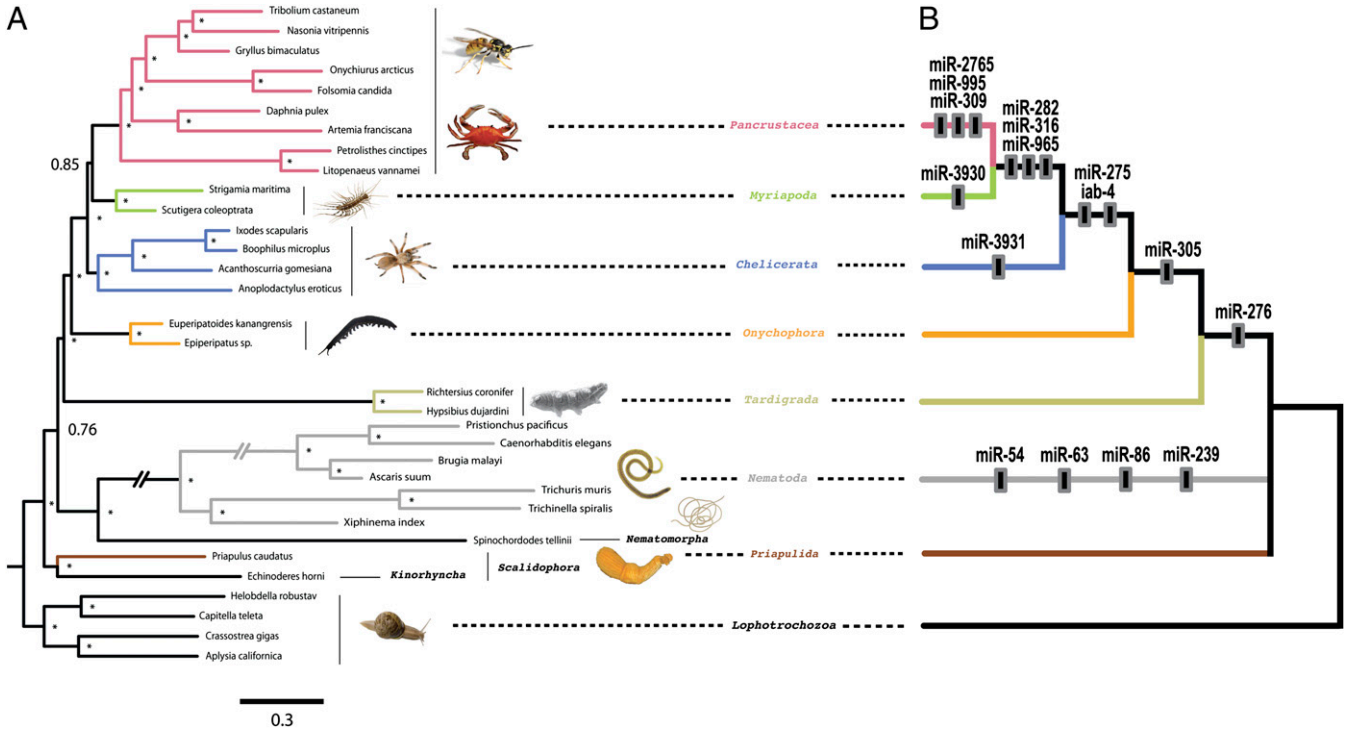

Fig. 1. Phylogenomics and miRNAs suggest velvets worm are the sister group to the arthropods within a monophyletic Panarthropoda. (*A*) Phylogenetic tree derived using Bayesian analysis of the EST data under the best-fitting CAT-GTR+Γ model supports tardigrades as the sister group of Lobopodia (Onychophora + Arthropoda). Support values represent posterior probabilities. Asterisks indicate a PP value of 1.0. Note that for Nematoda alone, the branch lengths are not shown to scale. (*B*) MiRNA distribution is consistent with the results obtained from the phylogenomic analysis. Single gray/black rectangles represent a miRNA gain. Clades are color-coded to highlight congruence between ESTs and miRNAs (see text for more details).

(Priapulida + Kinorhyncha) is recovered as the sister group of all other ecdysozoans. Nematoida was recovered with PP = 1. Because Nematomorpha is the taxon with the greatest amount of missing data in our EST dataset (Table S1), the strong support found for Nematoida (an otherwise well-accepted clade) suggests that missing data for Nematomorpha do not have a negative impact on our results.

Model Selection, Signal Dissection, and Targeted Taxonomic Pruning Highlight the Artifactual Nature of Tardigrada + Nematoda. To better understand the nature of the signal in our EST dataset, we performed three experiments to test whether the Tardigrada + Nematoda group recovered in previous analyses (2, 15–22) could result from a systematic error. First, Bayesian analyses were performed under a series of alternative models (Figs. S1 and S2). When the data were analyzed under poor-fitting site-homogenous models (i.e., WAG+Γ and GTR+Γ) (Fig. 2*A* and Figs. S1 *A* and *B* and S2 *A* and *B*), Panarthropoda was not recovered, and instead Tardigrada was found as the sister group of Nematoida (PP = 1 with both models). In contrast, analyses using the better-fitting site-heterogeneous CAT+Γ and CAT-GTR+Γ invariably identified Tardigrada as a member of Panarthropoda (Fig. 1*A* and Figs. S1 *C* and *D* and S2 *C* and *D*).

We next performed a signal-dissection analysis (13, 28), based on the slow-fast technique (27). We partitioned sites into subsets according to their rate of evolution, and independently analyzed these partitions. We hypothesized that if Tardigrada + Nematoda were an LBA artifact, then support for this group would be favored by the partitions of fast-evolving sites, whereas it would be minimized in partitions that exclude these sites (*Methods*). Consistent with our hypothesis, analyses of the fast-evolving sites show Nematoda + Tardigrada with PP = 0.88, whereas analyses of the slow-evolving sites show Tardigrada + Lobopodia with PP = 0.84 (Fig. 2 *B* and *C*, Fig. S3, and Table S2).

To further test whether Tardigrada + Nematoda is an LBA artifact, we performed a series of taxon pruning experiments. We selectively removed taxa to generate uninterrupted long-branches for Tardigrada, Onychophora, and Nematoda (*Methods*). As expected if Tardigrada + Nematoda is an LBA artifact, the results systematically support this group (Fig. 2*D* and Fig. S4).

In summary, three different experiments designed to uncover potential sources of systematic bias in our EST alignment suggest that a nematode (or cycloneuralian) affinity for Tardigrada is most likely an LBA artifact.

MiRNAs Corroborate the EST-Based Phylogenomic Analyses, and Confirm the Monophyly of Panarthropoda and Lobopodia. Our second dataset derives from the newly sequenced small RNA complements of the tardigrade *Paramacrobiotus* cf. *richtersi* and the onychophoran *Peripatoides novaezelandiae*, and characterization of their respective miRNA complements. Rota-Stabelli et al. (14) identified four miRNAs that characterize arthropods and had not yet been found in other ecdysozoans: miR-275, -276, -305, and -iab-4. There are also four miRNAs that are conserved between the nematode genera *Caenorhabditis* and *Pristionchus* (47): miR-54, -63, -86, and -239 (Fig. 1*B*). Consistent with our EST results, we did not find any nematode miRNAs in our tardigrade small-RNA library. Similarly, we did not find any potential miRNAs shared exclusively between the tardigrade and the onychophoran. Instead, in both the tardigrade and onychophoran libraries we found a single miRNA, miR-276, that formerly had been identified only in arthropods (14). In addition, in the onychophoran library, but not in the tardigrade library, we found a second miRNA, miR-305, which is also considered arthropod-specific (Fig. 1*B*). Based on these discoveries, we hypothesize that miR-276 is an apomorphy of Panarthropoda (Tardigrada + Lobopodia) and miR-305 is an apomorphy of Lobopodia (Onychophora + Arthropoda). Finally, our results suggest that miR-275 and miR-iab-4 are apomorphies of Arthropoda (Fig. 1*B*).

Discussion

Given the pervasiveness of systematic artifacts, care must be taken when evaluating topologies derived from large alignments, especially when well-supported competing hypotheses have been proposed. In the case of the tardigrades, molecular homoplasy certainly exists, as demonstrated by the fact some molecular studies support a nematode affinity of tardigrades, whereas others support an arthropod affinity. With respect to morphology, tardigrades have a melange of arthropod and cycloneuralian characters, suggesting that either the arthropod-like characters were lost in cycloneuralians or cycloneuralian-like characters

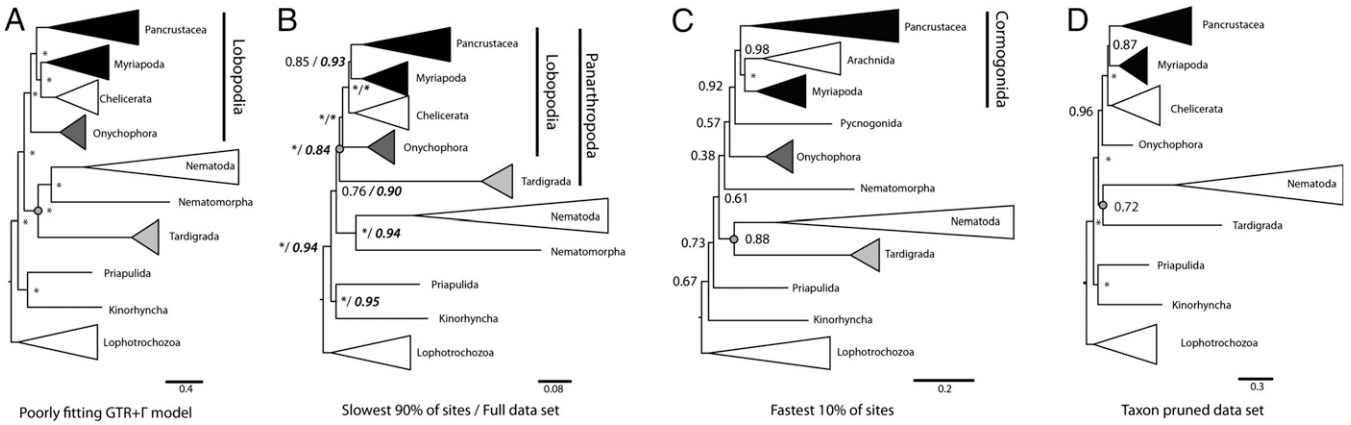

Fig. 2. Model selection, signal dissection and taxon pruning experiments show LBA explains previous support for a tardigrade/nematode clade. As in Fig. 1, these are trees from the EST data; node values represent posterior probabilities, and asterisks indicate a PP of 1.0. The node where the Tardigrada join the tree is identified by a circle. Clades have been collapsed for clarity. (*A*) Tardigrades are recovered as the sister group of Nematoida under the poorly fitting GTR+Γ model of sequence evolution (for Δ-likelihoods and SDs; Fig. S1 and *Methods*). (*B*) Tree recovered from the analysis of the slowest-evolving 90% of the sites in our dataset (Fig. S3*A*). The PP values are reported in italics, whereas support values obtained from the analysis of the complete dataset are in roman type (Fig. 1*A*). (*C*) Topology recovered from the 10% fastest evolving sites in our dataset, under CAT-GTR+Γ. The fast-evolving sites support Tardigrada as the sister group of Nematoda. (*D*) Phylogeny generated under a reduced-taxon set (one onychophoran, one tardigrade, and no nematomorph) designed to exacerbate LBA artifact.

were lost in arthropods (assuming that cycloneuralian and tardigrade characters are homologous). Consilience between our EST and miRNA analyses, as well as the experiments performed to identify LBA artifacts, congruently suggest that the closest affinity of tardigrades is with the Arthropoda and the Onychophora (i.e., Panarthropoda), not with the cycloneuralian ecdysozoans (nematodes). These results supersede our previous mitogenomic analyses (13), which could not reject a nematode affinity of Tardigrada because of the extremely high evolutionary rate of nematode mitochondrial genomes. The arthropod-like features of tardigrades, such as the paired ventrolateral appendages with segmental leg nerves and *Engrailed* expression in the posterior ectoderm of each segment (23, 48), appear to be panarthropod apomorphies that are not present in Cycloneuralia.

The position of tardigrades within the panarthropods is less certain. Overall, our results favor a sister group relationship between the Tardigrada and the Lobopodia. This relationship is favored because our EST and miRNA data both suggest a sister group relationship between onychophorans and arthropods and account for the uniquely shared features of onychophorans and arthropods (e.g., an open, hemocoelic circulatory system, a dorsal heart with segmental ostia, nephridia forming from segmented mesoderm), without the need to force their secondary loss in tardigrades as the result of miniaturization. Nonetheless, arthropods and tardigrades do share segmental ganglia in the nerve cord, in contrast to the unganglionated nerve cord in onychophorans (49), in which the commissures are not in segmental register. Our best tree, however, implies either convergent gain of segmental ganglia in tardigrades and arthropods or a secondarily unsegmented nerve cord in onychophorans, given that tardigrades share no miRNAs with arthropods to the exclusion of onychophorans and were not recovered as sister taxa in any of our EST analyses (Figs. 1 and 2 and Figs. S1 and S2). Analyses performed using the CAT+Γ model, similar to previous mitogenomic analyses (13), still pointed toward a Tardigrada + Onychophora group within Panarthropoda (Fig. S2*C*). CAT+Γ is not the overall best-fitting model for our dataset, however. When the overall best-fitting model (CAT-GTR+Γ) is used, our dataset support Lobopodia (Fig. 1), whereas mitogenomic data are known to be not very reliable markers for resolving deep divergences. In addition, no morphological evidence has been shown to support such a grouping, and no miRNA has been found to be shared exclusively between these two taxa. We conclude that by fully rejecting "Arthropoda + Tardigrada" (i.e., Tactopoda: ref. 50), which was never recovered in our analyses, and by favoring Lobopodia over Onychophora + Tardigrada, our results significantly reduce uncertainty regarding the placement of Tardigrada within Panarthropoda.

Our findings suggest that characters shared by tardigrades and cycloneuralians, such as a terminal mouth, protrusible mouth cone, triradiate pharynx, and a circumesophageal brain (9, 23, 51), are most likely ecdysozoan plesiomorphies. This is consistent with the fact that in our proposed phylogeny (Fig. 1*A*), even if the Tardigrada are excluded, the remaining cycloneuralian taxa do not form a monophyletic group (14). Instead, they are arranged as a paraphyletic grade at the base of Ecdysozoa (Fig. 1*A*). This hypothesis is also consistent with the fossil record of arthropods, in that taxa in the arthropod stem group, such as armoured lobopodians and anomalocaridids, show a melange of arthropod-like and cycloneuralian-like features, the latter (e.g., radially arranged mouthparts) then lost in the arthropod crown group (23, 50). Our phylogeny suggests that paired limbs and a shared mode of segment patterning (48) are apomorphic for Panarthropoda. Thus Tardigrades, as a living taxon with a mixture of cycloneuralian and arthropod characters, are placed center stage in our pursuit of understanding of the mechanisms underlying the construction of the most successful of all animal body plans, that of the arthropods.

Methods

EST Dataset Assembly. We assembled a 255-gene phylogenomic dataset of 49,023 amino acid positions from 33 ecdysozoan species by merging genes from two previous EST datasets (12, 14) (available on request). By merging these two datasets, we were able to improve taxonomic sampling with reference to (14) and particularly to (12). In addition, we were able to investigate the effect of including genes unique to (12) to the initial gene sets that we analyzed in (14) to address the problem of the relationships within Arthropoda. Improving taxonomic sampling is a key to alleviating LBA, and by merging the two datasets we were able to add data for one nematomorph, a second onychophoran, and an additional, relatively slowly evolving nematode. More details on dataset assembly, taxonomic sampling, and ortholog identification are provided in *SI Methods*. The average amount of missing data in our superalignment is ~36% (Table S1).

MiRNA Library Generation. Specimens of a velvet worm *Peripatoides novaezealandiae* were obtained commercially and identified by S.J.L.. A small-RNA library was constructed according to established protocols (38) and sequenced at 454 Life Sciences. The total RNA preparation of the tardigrade *Paramacrobiotus* cf. *richtersi* (~4,400 pooled individuals) was sequenced using Illumina technology at the Yale Center for Genome Analysis. Tardigrades were cultured by L.R. and T.M. and stored in RNAlater. MiRNA data for the arthropod subclasses Myriapoda and Chelicerata were obtained from previously described miRNA complements (14), and those for *Drosophila melanogaster*, *Daphnia pulex*, *Priapulus caudatus*, and *Caenorhabditis elegans* were obtained from miRBase (52). Sequences from the tardigrade and onychophoran small-RNA libraries were processed using PERL scripts written by L.I.C. and D.P. (available on request) and analyzed using miRMiner as described previously (14, 38).

Phylogenetic Analyses. All phylogenetic analyses were conducted under a Bayesian framework using PhyloBayes 3.2e (53). We first compared the fit of alternative models of evolution to our EST dataset. We used Bayesian cross-validation (45), as described in the PhyloBayes manual (53), to rank the fit of alternative substitution models to the data. The models compared were WAG+Γ, GTR+Γ, CAT+Γ, and CAT-GTR+Γ.

Phylogenetic analyses of the EST dataset were performed under each model, and results were compared to evaluate whether different phylogenies were obtained when different-fitting models were used. For every PhyloBayes analysis, two independent runs were executed. Convergence was tested using "bpcomp" in the PhyloBayes package. Analyses were considered to have converged when the maximum difference across bipartitions was <0.2 (see the PhyloBayes manual). For each analysis, the burn-in period was estimated independently, and trees sampled before convergence were not considered when summarizing the results of the two runs.

Site Stripping and Signal Dissection Analyses. These analyses used the slow-fast method (27) to estimate the rate of substitution of the sites in our alignment. First, the parsimony score of each site in our alignment was calculated for each of four groups with constrained monophyly (Pancrustacea, Chelicerata, Nematoda, and Lophotrochozoa). The rate of each site in our alignment was then estimated as the sum of its parsimony scores across all considered monophyletic groups. All parsimony analyses were performed using PAUP4b10 (54). Sites in our alignment were then ranked according to their substitution rates and partitioned into classes. Alignments were generated, according to the distribution of site rates, by systematically removing (*i*) approximately the fastest 10% of the sites, that is, all characters with a slow-fast–estimated rate of six or more steps (total number of remaining sites, 45,292); (*ii*) the fastest ~20% of the sites, that is, all characters with a slow-fast estimated rate of five or more steps (total number of remaining sites, 43,316); and (*iii*) the fastest ~30% of the sites, that is, all characters with a slow-fast–estimated rate of three or more steps (total number of remaining sites, 37,150). However, the number of substitutions in the sites that remained after exclusion of the first 10% of characters at just five or fewer steps is already low. This implies that the proportion of fast-evolving sites in our alignment is quite small. Accordingly, we did not create datasets excluding more than 30% of the fastest sites.

We also performed a signal-dissection analysis (14, 28) to compare the signal in the slow- and fast-evolving sites. Accordingly, two datasets were generated, containing approximately 10% (3,731 sites) and 30% (11,873 sites) of the fastest sites in our alignment. The five aligned datasets that resulted, namely the three sets composed of slow-evolving sites (approximately the slowest 70%, 80%, and 90%) and the two sets of fast-evolving sites (approximately the fastest 10% and 30%), were analyzed independ-

ently using PhyloBayes 3.2e to construct trees under the best-fitting model (i.e., the site-heterogeneous mixture model CAT-GTR+Γ).

Taxonomic Pruning Experiment. It is well known that the number and nature of the taxa used can affect phylogenetic inference and, in particular, can exacerbate or reduce LBA (2, 3). Thus, we carried out three taxon pruning experiments to evaluate the robustness of our EST results. We generated datasets that excluded (*i*) the tardigrade *Richtersius coronifer* and the onychophoran *Epiperipatus* sp., which resulted in uninterrupted branches for the tardigrades and the onychophorans; (*ii*) the nematomorph *Spinochordodes tellinii* and the tardigrade *R. coronifer*, which resulted in uninterrupted branches leading to the nematodes and the tardigrades; and (*iii*) the onychophoran *Epiperipatus* sp., the tardigrade *R. coronifer*, and the nematomorph *S. tellinii*, which resulted in uninterrupted branches leading to the onychophorans, tardigrades, and nematodes. In these experiments, the retained tardigrade was always *Hypsibius dujardini* because of its greater gene coverage. All of these datasets were analyzed under CAT-GTR+Γ.

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