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Robust support for tardigrade clades and their ages from three protein-coding nuclear genes

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Abstract. Coding sequences (5,334 nt total) from elongation factor-1α, elongation factor-2, and the largest subunit of RNA polymerase II were determined for 6 species of Tardigrada, 2 of Arthropoda, and 2 of Onychophora. Parsimony and likelihood analyses of nucleotides and amino acids yielded strong support for Tardigrada and all internal nodes (i.e., 100% bootstrap support for Tardigrada, Eutardigrada, Parachela, Hypsibiidae, and Macrobiotidae). Results are in agreement with morphology and an earlier molecular study based on analysis of 18S ribosomal sequences. Divergence times have been estimated from amino acid sequence data using an empirical Bayesian statistical approach, which does not assume a strict molecular clock. Divergence time estimates are pre-Vendian for Tardigrada/Arthropoda, Vendian or earlier for Eutardigrada/Heterotardigrada, Silurian to Ordovician for Parachela/Apochela, Permian to Carboniferous for Hypsibiidae and Macrobiotidae, and Mesozoic for *Isohypsibius/Thulinia* (both within Hypsibiidae) and *Macrobiotus/Richtersius* (both within Macrobiotidae).

Additional key words: Arthropoda, elongation factor-1α, elongation factor-2, Phylogeny, RNA polymerase II

Decisive tests of higher-level phylogenetic hypotheses within Metazoa are few, including those for the position of the lobopodous micrometazoans called Tardigrada. Frequently considered, along with Arthropoda and Onychophora, to constitute Panarthropoda, tardigrades are actually missing a number of key morphological panarthropod characteristics, including a mixocoel, metanephridia, and haemal system (Nielsen 1997). Dewel & Dewel (1997) list five synapomorphies between tardigrades and arthropods, but then qualify them by stating "all are somewhat ambiguous." These characters are: 1) dorsal and ventral plates and flanges; 2) head with three segments, frontal appendage fully incorporated into cephalon; 3) arthropod-like sensilla; 4) unciliated epithelium, and 5) bismuth staining of Golgi apparatus. Most conspicuously, panarthropods are all segmented, with limbs on most segments and a pair of ganglia in all segments.

Molecular studies also support the proximity of tardigrades to arthropods and onychophorans, but not decisively. Part of the problem is taxon sampling, in which arthropods are invariably included, but either tardigrades or onychophorans are missing (Giribet et al. 1996; Giribet & Ribera 1998; Wheeler et al. 1993; Aguinaldo et al. 1997; Garey 1999). In all of these studies, tardigrades and onychophorans group closely with arthropods, although not necesssarily as sister group, and node support values are either low or not indicated. In some studies, tardigrades (Eernisse 1997) or onychophorans (Ballard et al. 1992) actually make the extant arthropods paraphyletic. However, this latter scenario is highly unlikely, based on our own recent molecular studies in which bootstrap values for Arthropoda are up to 96% when a tardigrade, an onychophoran, and a wide diversity of arthropods are sampled for three genes (Regier & Shultz 2001a). In summary, the evidence for Panarthropoda is reasonably well supported but will not be beyond dispute until studies incorporate broader taxon sampling and more decisive characters, both morphological and molecular.

Within Tardigrada, a higher-level classification exists based on morphological characters (e.g., see our Table 1, modified from Dewel & Dewel 1997), but a cladistic analysis has not been performed. However, a recent analysis of 18S ribosomal nucleotides from 6

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tardigrades plus outgroups has confirmed the broad outline of that morphological classification, with all but one node strongly supported (Garey et al. 1999). In light of the difficulty of resolving deep-level relationships within arthropods, whose early Cambrian (or earlier) origin presumably approximates that of tardigrades, the Garey et al. result is encouraging for future phylogenetic studies. While there is no particular reason to doubt the results of Garey et al. given their agreement with morphology, in general, gene studies are considered more reliable when multiple genes are simultaneously analyzed (e.g., Baker & DeSalle 1997, Table 1 in Regier & Shultz 2001a). This is due both to the confidence that comes with increased data and to the effect of diluting single-gene biases that run counter to the true phylogenetic signal, e.g., non-homogeneous base compositions.

In the current report, we have taken another step towards constructing a robust higher-level phylogeny of tardigrades, namely, the identification of three additional genes that strongly resolve tardigrade relationships, both individually and particularly in combination. These genes will mitigate concern about possible bias of single-gene studies and will provide additional informative characters that will eventually be needed to resolve larger numbers of taxa. The present results are encouraging in that these three genes, when analyzed for a taxon set similar to that in Garey et al. (1999), very strongly confirm the previous results, including their only modestly supported order Parachela within class Eutardigrada. In addition, we have used an empirical Bayesian statistical approach to estimate the ages of well resolved tardigrade and other panarthropod clades. Perhaps it is not surprising that tardigrades, given their small size, are not well represented in the fossil record, and the earliest definitive tardigrade is only from the Late Cretaceous (Cooper 1964), although there is a Web-based description of a putative tardigrade from the Middle Cambrian of Siberia (http://biosys-serv.biologie.uni-ulm.de/sektion/dieter/noncrustacean/noncrustacean.html) with a statement that a formal description is in process.

Methods

Specimen preservation, taxon sampling, and data set

Live specimens of tardigrades were collected and transported either in water or dry in cryptobiosis for short periods at room temperature. Dried tardigrades were always hydrated and tested for movement prior to long-term storage. On occasion, live tardigrades were transferred to 30% or 100% ethanol for separation from debris. Long-term storage was at −85°C. Field-collected arthropod and onychophoran outgroup taxa were stored in 100% ethanol at room temperature for short periods before transfer to −85°C. Total nucleic acids were extracted in bulk from 10–100 tardigrade specimens or from correspondingly small pieces of outgroup taxa using the SV Total RNA Isolation kit (Promega) with the DNase step omitted. The extracts were dissolved in water to a final volume of 100 μl. Individual reverse transcription reactions used 0.1–1.0 μl of this solution as template. Specific mRNA sequences (5,334 nt total) for elongation factor-1α (EF-1α: 1,131 nt excluding terminal PCR primer sequences), the largest subunit of RNA polymerase II (Pol II: 2,025 nt excluding the terminal PCR primer sequences), and elongation factor-2 (EF-2: 2,178 nt excluding terminal PCR primer sequences) were reverse transcribed and amplified by the polymerase chain reaction using previously described conditions and oligonucleotide primers (Regier & Shultz 2001a and references therein). In all cases, nested PCR amplifications were performed. Identification of taxa that were sequenced, along with their higher classification and associated GenBank accession numbers, are shown in Table 1.

PCR fragments were sequenced directly from the M13 sequences present at the 5′ ends of all PCR primers, using fluorescent-labelled dye terminators and an automated DNA sequencer (model 3100, Applied Biosystems). The PREGAP and GAP4 programs within the Staden package (Staden et al. 1999) were used to edit and assemble contigs. The Genetic Data Environment software package (version 2.2, Smith et al. 1994) was used to manually align assembled sequences and to construct nucleotide data matrices for phylogenetic analysis. MacClade (Maddison & Maddison 1992) was used to create amino acid matrices. A few small indels were present in the multiple sequence alignments and were removed in assembling data sets for phylogenetic analysis. Within the sequence for *Echiniscus viridissimus,* there were four autapomorphic indels of 3–9 nt each, one in EF-1α, one in Pol II, and two in EF-2. Additionally, there were two informative indels in EF-2, a 3-nt insertion in Parachela (not sequenced for *Isohypsibius elegans*) and a 3-nt indel either for Parachela or for *Echiniscus viridissimus* + *Milnesium tardigradum*; the ambiguity comes from an uncertain ancestral state. The inclusion of additional arthropod taxa had no effect on relationships within tardigrades (unpubl. data).

Data analysis

Maximum-parsimony analyses of nucleotides and amino acid data sets were conducted with PAUP*4.0 (Swofford 1998) using equally-weighted character

Table 1. Tardigrade classificaion and taxon sampling. For each taxon, GenBank numbers are separated by gene—top row: EF-1α; middle row: Pol II; bottom row: EF-2.

Taxa	Abbreviation	Collection site	GenBank #
Tardigrada			
I. Heterotardigrada			
Echiniscus viridissimus Péterfi 1956	Evi	Tennessee, USA	AY305481 AY305632–AY305635 AY305527, AY305528
II. Eutardigrada			
A. Parachela			
1. Hypsibiidae			
Isohypsibius elegans (Binda & Pilato 1971)	Iso	Greenland	AY305482 AY305636, AY305637 AY305529
Thulinia stephaniae (Pilato 1974)	Thul	Wards, Inc. (in culture)	AY305486 AY305652–AY305655 AY305533
2. Macrobiotidae			
Macrobiotus islandicus Richters 1904	Mis	Greenland	AY305483 AY305638–AY305641 AY305530
Richtersius coronifer (Richters 1903)	Rco	Sweden	AY305485 AY305648–AY305651 AY305532
B. Apochela			
Milnesium tardigradum Doyére 1840	Hyp	Carolina Biological Supply (in culture)	AF063419 AF139016, AF240887, AF240888 AF240833
Arthropoda			
I. Chelicerata, Xiphosura			
Limulus polyphemus (Linneaus 1758)	Lpo	Western coast of North Atlantic	U90051 U90037 AF20821
II. Crustacea, Ostracoda			
Cypridopsus vidua (Müller 1776)	Ost	Maryland, USA	AF063414 AF138997–AF138999 AF240825
Onychophora			
I. Peripatopsidae			
Ooperipatellus nanus Ruhberg 1985	Ona	New Zealand	AY305484 AY305642–AY305644 AY305531
II. Peripatidae			
Peripatus sp. Guilding 1826	Per2_3	Ecuador	AF137395 AF139017, AY3055645–AY305647, AF240892 AF240835

transformations, both with and without third codon-position characters. Analysis consisted of a heuristic search using TBR branch swapping with random sequence addition (100 sequence-addition replicates). Bootstrap analysis (1000 bootstrap replications) was identical except for 10 sequence-addition replicates per bootstrap replication. Decay indices or Bremer support (Bremer 1994) partitioned by gene (Baker & DeSalle 1997) were calculated using TreeRot (Sorenson 1999).

Maximum-likelihood analysis and bootstrap analy-

sis (250 replications) of a nucleotide data set (third codon-position characters excluded) was performed with PAUP*4.0 under a general time-reversible model of sequence evolution (Rodriguez et al. 1990) with among-site rate-heterogeneity modeled by a gamma function approximated by 4 discrete rate categories (see Regier & Shultz 2001a for more details of the likelihood tree search strategy). The favored model of sequence evolution was selected from among 56, based on a likelihood-ratio test (Huelsenbeck & Rannala 1997) performed using Modeltest (version 3.06; Posada 1998).

Divergence-time estimations were carried out using Markov chain Monte Carlo procedures for an empirical Bayesian statistical analysis as implemented in the software programs of Thorne & Kishino (2002). Their programs combine evolutionary information separately inferred for multiple genes (in our case, 3) within a analytical framework that allows violation of the strict molecular clock assumption. Key features of the program are that rates at adjoining nodes are assumed to be autocorrelated, and that each gene has a separate autocorrelation parameter (Thorne et al. 1998; Kishino et al. 2001). The *estbranches* program estimates branch lengths given a topology, model (we selected the JTT model), and amino acid data set, as well as the variance-covariance structure of the branch length estimates. The *multidivtime* program then uses this output to estimate node divergence times for the ingroup, given upper- and lower-bound time constraints, various fixed parameters, and estimated priors (i.e., initial parameters whose values are sensitive to new data). For purposes of this study, we considered Tardigrada and Arthropoda to be sister taxa relative to Onychophora. With Onychophora as outgroup, we could then treat Arthropoda as part of the ingroup and apply constraints on that portion of the ingroup topology, since tardigrade fossils are relatively recent and would not represent useful constraints. In particular, we constrained the node separating the ostracod *Cypridopsis* and the xiphosuran *Limulus* to >601 mya but <1017 mya. Both lower and upper bounds are needed to effectively constrain the standard deviation of the estimated time. These constraints were derived from a separate study of 17 arthropods + 1 tardigrade + 1 onychophoran (Regier, Kambic, & Shultz unpubl. data; see also Discussion). Based on preliminary runs, we selected the following parameters: sampfreq =100; numsamps =40,000; burnin =2,000,000. The following priors were also chosen after exploring the effect of using a range of priors on the outcome: brownmean =0.250, brownsd =0.250, rttm =6.010 (intended to refer to 601 mya), rttmsd =2.080, bigtime =10.000. A fixed feature of *multidivtime* is that the posterior rate (and its prior, called *rtrate*) at the basal ingroup node and one of the two derived, adjoining, nodes (decided arbitrarily) must be identical. Preliminary studies demonstrated that the particular value chosen for *rtrate* had a significant effect on the time estimate for nodes near the base. Therefore, we used two distinct values of *rtrate*. In one case, the prior value was assumed to represent the average value across the entire ingroup based on the output from *estbranches* (rrate =2.000, rtratesd =1.000). For the other, we assumed the rate to be 3× faster (rrate =6.000, rtratesde =1.000), based on the idea that rapid changes may be confined to the early history of a lineage (Gould 1989, 1991). Given the two sets of priors, we have presented our time estimates as minimum and maximum values, along with their corresponding standard deviations.

Results

Parsimony analysis of the amino acid data set yields a single most-parsimonious tree in which Tardigrada and all of its internal nodes are strongly supported, i.e., bootstrap percentages are 99–100% and combined Bremer support values are 9–44 (Fig. 1). Examination of the partitioned Bremer support reveals no large conflict among the genes, although the three genes sampled are not equally informative. For example, Pol II strongly supports all five nodes of Tardigrada as well as Onychophora but not Arthropoda; EF-1α strongly supports Onychophora and Arthropoda but only one node within Tardigrada (i.e., Eutardigrada); EF-2 strongly supports Onychophora and 3 of 5 tardigrade nodes. From the differing combined branch lengths for terminal taxa (Fig. 1), it can also be inferred that rates of amino acid change have not been constant across lineages. For example, the various Eutardigrada have evolved 15–63% faster than Heterotardigrada.

Parsimony and likelihood analysis of nucleotides (first 2 codon positions only) yield the same topology as for amino acids, again with very strong node support. With codon position 3 included in a parsimony analysis, support for all nodes drops except for Onychophora; Arthropoda, but not Tardigrada or Onychophora, becomes paraphyletic.

Divergence time estimates for the 5 well supported groups within Tardigrada plus the phylum itself are displayed (Fig. 2). All groups are ancient, varying in ages from Proterozoic to Mesozoic.

Discussion

In the current study, nucleotide and amino acid characters from EF-1α, Pol II, and EF-2 in combination provide strong support for tardigrade relationships at all taxonomic levels (Fig. 1). Furthermore, the high

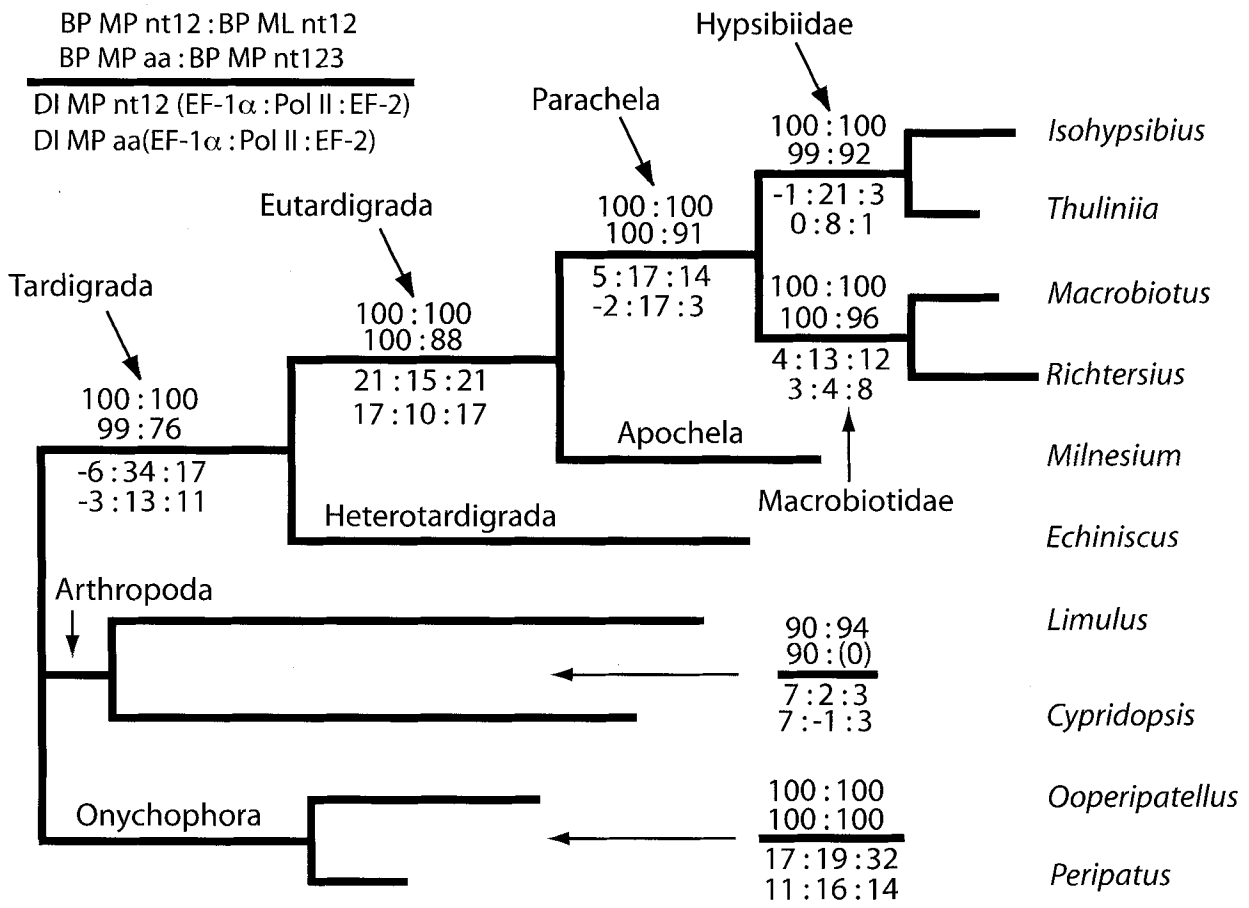
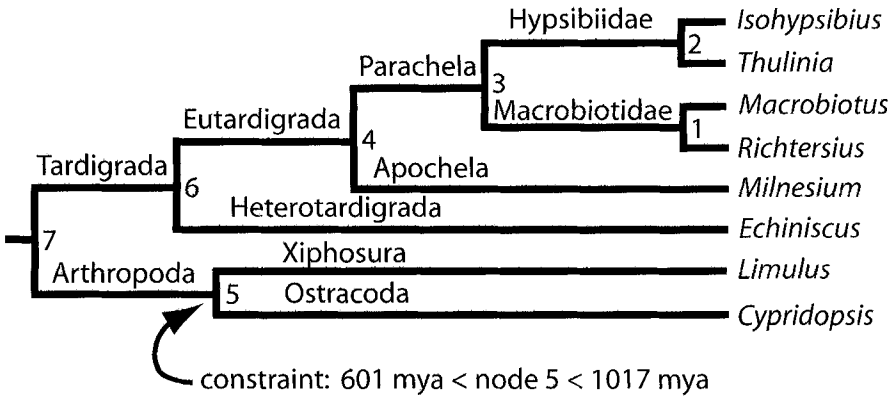


Fig. 1. Phylogenetic analysis of tardigrades. Combined EF-1α, Pol II, and EF-2 sequences were analyzed by maximum parsimony (MP) and maximum likelihood (ML) for nucleotides (nt12: first 2 codon positions only; nt123: all 3 codon positions) and for amino acids (aa). Bootstrap percentages (BP) are shown above branches (MP nt12, ML nt12, MP aa, MP nt123). All recovered the same topology except MP nt123 (not shown), for which Arthropoda became paraphyletic with respect to Onychophora. In that instance, the BP for Arthropoda was 0 and is shown in parentheses. Partitioned Bremer support by gene (DI) is shown below branches for MP analyses of nt12 and aa data sets. Branch lengths are from the MP aa analysis.

Bremer support values at all nodes suggest that the number of taxa could be increased considerably and still maintain satisfactory levels of branch support. Thus, these genes should be very useful for an expanded study of relationships within Tardigrada. Our results are also in complete agreement with Garey et al. (1999), who recovered the same groups from analysis of 18S ribosomal sequences, although in their case support for Parachela was modest. The finding of strong support contrasts with that in Arthropoda, where some taxonomic levels (e.g., relationships among Chelicerata/Myriapoda/Pancrustacea, relationships among classes of Pancrustacea, and relationships among orders of the myriapod classes Diplopoda and Chilopoda, and of Arachnida) are difficult to resolve with strong node support using the same genes, although others (e.g., Chelicerata and its classes and orders; Myriapoda and its classes and orders, Pancrustacea and its classes) are generally strongly resolved (Regier & Shultz 2001a,b; Regier, Wilson, & Shultz, unpubl. data). Generally speaking, levels of node support reflect rates and patterns of nucleotide substitution and of cladogenesis. These may be gene- and taxon-specific, so there is no single explanation for high node support. We note that the combined branch lengths from the root to the terminal taxa are longer for tardigrades than for arthropods and onychophorans (Fig. 1), implying higher rates of gene evolution, although this in itself doesn't necessarily lead to higher node support values, and indeed could result in decreased support through increased homoplasy. We also note that inter-nodal distances are rather uniform across tardigrades, which could reflect a fortuitous balance between rates of substitution and rates of cladogenesis,



node	min. age (s.d.) - max. age (s.d.)(mya)	geological time scale
1	120 (27) - 168 (38)	Cretaceous - Jurassic
2	166 (36) - 222 (40)	Jurassic - Triassic
3	265 (46) - 326 (39)	Permian - Carboniferous
4	433 (64) - 474 (33)	Silurian- Ordovician
5	623 (21) - 690 (77)	Vendian or earlier
6	627 (29) - 691 (85)	Vendian or earlier
7	700 (28) - 852 (97)	pre-Vendian

Fig. 2. Divergence time estimates for panarthropod clades. The topology (in cladogram format and not linearized with respect to time) is the same as that shown in Fig. 1, and the model, parameters, priors, and constraints are described and justified in Methods. Nodes are numbered. The time estimates in millions of years ago (mya) are displayed in the table (see below tree diagram) as ranges with standard deviations (s.d.) in parentheses. The time estimates that are underlined correspond to the results when the prior on the root node was set equal to the average rate across the entire tree (see Methods). The other estimate results when the prior is set equal to 3× the average rate.

but again this is only suggestive. Regardless of the particular explanation, our limited sampling of tardigrades has yielded robust resolution, and this augurs well for expanded studies using EF-1α, Pol II, and EF-2.

The one exception to the across-the-board strong support for tardigrade relationships occurs when nucleotides from the third codon position are not excluded from the nucleotide data set analyzed by maximum parsimony. In this case, support at all nodes except Onychophora decreases and Arthropoda becomes paraphyletic, with *Limulus* (Chelicerata) grouping strongly with Onychophora rather than with the other arthropod, i.e., *Cypridopsis* (Ostracoda). We have reported extensively on the difficulties that inclusion of third-codon-position characters introduce when analyzing deep-level relationships using a relatively small number of taxon exemplars (Regier & Shultz 2001a).

Lastly, our estimates of divergence times, while rather broad (especially when their standard deviations are included, as they should be), provide the most comprehensive proposal yet for the timing of cladogenic events within Tardigrada (Fig. 2). Our finding that the tardigrade and arthropod phyla diverged in the Proterozoic, likely >700 mya (and perhaps much greater), is consistent with other estimates for the divergence of basal metazoan groups inferred from analysis of multiple genes (Feng et al. 1997; Gu et al. 1998; Wang et al. 1999; Nei et al. 2001), but in conflict with one based on analysis of the 18S ribosomal DNA gene (Aris-Brosou & Yang 2002). In the 18S study, animals are estimated to have originated only 560 mya; that is, near the time of the Cambrian Explosion. However, this time may be a substantial underestimate as the fern/animal divergence (the next node basal on their tree) is estimated at only 633 mya, which is many hundreds of millions of years less than other estimates (e.g., see Wang et al. 1999, Nei et al. 2001). In addition, while Aris-Brosou and Yang provide no indicators of support of the 18S sequence data for their preferred topology, any such support is unlikely to be high given other 18S studies (e.g., see Peterson & Eernisse 2001). In the absence of strong support of a data set for a topology, time estimates should be treated with particular caution. By contrast, the EF-1α + Pol II + EF-2 data set in our study provides strong support for the topology used to estimate tardigrade divergence times.

Within Tardigrada, the basal split between Heterotardigrada and Eutardigrada is also ancient, i.e., Vendian or earlier. Even within the two families that were multiply sampled—Hypsibiidae and Macrobiotidae—divergence time estimates are from the Mesozoic.

Of course, these time estimates are no better than the method used for inference, and there is consider-

able controversy over the accuracy of molecular dating methods (summarized in Wray 2001). However, such methods are developing continually, and the method we explored (Thorne & Kishino 2002) has incorporated significant recent improvements. First, sequence evolution is not constrained to follow a strict molecular clock; instead, a relaxed-clock model is used, in which rates are autocorrelated over time. Another recent non-clock model (Huelsenbeck et al. 2000) allows rates to change in discrete jumps, and both approaches warrant exploration. Second, the Thorne & Kishino method combines evolutionary information separately for multiple genes rather than treating the multigene data as a single evolutionary unit. Third, character changes are assessed using a more realistic model than simply assuming equally weighted transformations. Fourth, the method (necessarily) incorporates minimum and maximum constraint times into the actual calculations of divergence time estimates.

Selecting meaningful constraint times within Tardigrada would have been highly desirable in implementing Thorne and Kishino's method, but it was not feasible given the paucity of the fossil record. To circumvent this limitation, we constrained the divergence time of our two sampled arthropods (an ostracod and a xiphosuran) to 601–1017 mya, and assumed Onychophora to be the outgroup. These upper and lower bounds were estimated in a separate molecular study using the same 3 genes from 17 arthropods + 1 tardigrade + 1 onychophoran and incorporating 9 fossil record constraints (Regier, Kambic, & Shultz, unpubl. data). Even on other grounds, a 601-mya minimum-age estimate is still reasonable. In particular, this minimum age would be on the recent end of the distribution of published molecular-based time estimates for the vertebrate/arthropod divergence (see Wang et al. 1999, Wray 2001), and it is within ~30 million years of recently discovered fossilized bilaterian embryos (Xiao et al. 1998, Chen et al. 2000). This is of particular note because fossils typically underestimate (and cannot overestimate) dates. Furthermore, origins of groups, as estimated from fossils, can be substantially more recent because of anagenesis than the divergence time of two extant taxa, as estimated from sequence data. Counterbalancing this is the recent finding that, under certain conditions, sequence data can overestimate divergence times (Rodríguez-Trelles et al. 2002). In summary, we present our results as a starting point for further discussion, but we feel they warrant serious consideration.

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References

Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, & Lake JA 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 387: 489–493.

Aris-Brosou S & Yang Z 2002. Effects of models of rate evolution on estimation of divergence dates with special reference to the metazoan 18S ribosomal RNA phylogeny. Syst. Biol. 51: 703–714.

Baker RH & DeSalle R 1997. Multiple sources of character information and the phylogeny of Hawaiian drosophilids. Syst. Biol. 46: 654–673.

Ballard JWO, Olsen GJ, Faith DP, Odgers WA, Rowell DM, & Atkinson PW 1992. Evidence from 12S ribosomal RNA sequences that onychophorans are modified arthropods. Science 20: 1345–1348.

Bremer K 1994. Branch support and tree stability. Cladistics 10: 295–304.

Chen J-Y, Oliveri P, Li C-W, Zhou G-Q, Gao F, Hagadorn JW, Peterson KJ, & Davidson EH 2000. Precambrian animal diversity: Putative phosphatized embryos from the Doustantuo Formation of China. Proc. Natl. Acad. Sci. USA 97: 4457–4462.

Cooper KW 1964. The first fossil tardigrade: *Beorn leggi* Cooper, from Cretaceous amber. Psyche 71(2): 41–48.

Dewel RA & Dewel WC 1997. The place of tardigrades in arthropod evolution. In: Arthropod Relationships. Fortey RA & Thomas RH, eds., pp. 109–123. Chapman & Hall, London.

Eernisse DJ 1997. Arthropod and annelid relationships reexamined. In: Arthropod Relationships. Fortey RA & Thomas RH, eds., pp. 43–56. Chapman & Hall, London.

Feng D-F, Cho G, & Doolittle RF 1997. Determining divergence times with a protein clock: Update and reevaluation. Proc. Natl. Acad. Sci. USA 94: 13028–13033.

Garey JR, Nelson DR, Mackey LY, & Li J 1999. Tardigrade phylogeny: Congruency of morphological and molecular evidence. Zool. Anz. 238: 205–210.

Giribet G & Ribera C 1998. The position of arthropods in the animal kingdom: A search for a reliable outgroup for internal arthropod phylogeny. Mol. Phylog. Evol. 9: 481–488.

Giribet G, Carranza S, Baguña J, Riutort M, & Ribera C 1996. First molecular evidence for the existence of a Tardigrada + Arthropoda clade. Mol Biol Evol 13(1): 76–84.

Gould SJ 1989. Wonderful Life. WW Norton & Co, New York. 347 pp.

——— 1991. The disparity of the Burgess Shale arthropod fauna and the limits of cladistic analysis: why we must strive to quantify morphospace. Paleobiology 17: 411–423.

Gu X 1998. Early metazoan divergence was about 830 million years ago. J. Mol. Evol. 47: 369–371.

Huelsenbeck JP & Rannala B 1997. Phylogenetic methods come of age: testing hypotheses in an evolutionary context. Science 276: 227–232.

Huelsenbeck JP, Larget B, & Swofford D 2000. A compound Poisson process for relaxing the molecular clock. Genetics 154: 1879–1892.

Kishino H, Thorne JL, & Bruno WJ 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol. Biol. Evol. 18(3): 352–361.

Maddison WP & Maddison DR 1992. MacClade: Analysis of phylogeny and character evolution, version 4.04 for Mac OS X. Sinauer, Sunderland, MA.

Nei M, Xu P, & Glazko G 2001. Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms. Proc. Natl. Acad. Sci. USA 98(5): 2497–2502.

Nielsen C 1997. The phylogenetic position of the Arthropoda. In: Arthropod Relationships. Fortey RA & Thomas RH, eds., pp. 11–22. Chapman & Hall, London.

Peterson KJ & Eernisse DJ 2001. Animal phylogeny and the ancestry of bilaterians: inferences from morphology and 18S rDAN gene sequences. Evol. Dev. 3: 170–205.

Posada D & Crandall KA 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics 14(9): 817–818.

Regier JC & Shultz JW 2001a. Elongation factor-2: A useful gene for arthropod phylogenetics. Mol. Phylog. Evol. 20(1): 136–148.

——— 2001b. A phylogenetic analysis of Myriapoda (Arthropoda) using two nuclear protein-encoding genes. Zool. J. Linn. Soc. 132: 469–486.

Rodriguez F, Oliver JL, Marin A, & Medina JR 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142: 485–501.

Rodríguez-Trelles F, Tarrío R, & Ayala F 2002. A methodological bias toward overestimation of molecular evolutionary time scales. Proc. Natl. Acad. Sci. USA 99: 8112–8115.

Smith SW, Overbeek R, Woese CR, Gilbert W, & Gillevet PM 1994. The genetic data environment and expandable GUI for multiple sequence analysis. Comput. App. Biosci. 10: 671–675.

Sorenson MD 1999. TreeRot, version 2b. Boston University, Boston, MA.

Staden R, Beal KR, & Bonfield JK 1999. The Staden package, 1998. In: Bioinformatics Methods and Protocols, Misener S & Krawetz S, eds., pp. 115–130. Humana Press, Totowa, NJ.

Swofford DL 1998. PAUP*4.0, version b10. Sinauer, Sunderland, MA.

Thorne JL & Kishino H 2002. Divergence time and evolutionary rate estimation with multilocus data. Syst. Biol. 51(5): 689–702.

Thorne JL, Kishino H, & Painter IS 1998. Estimating the rate of evolution of the rate of molecular evolution. Mol. Biol. Evol. 15(12): 1647–1657.

Wang DY-C, Kumar S, & Hedges SB 1999. Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. Proc. R. Soc. London B 266: 163–171.

Wheeler WC, Cartwright P, & Hayashi CY 1993. Arthropod phylogeny: a combined approach. Cladistics 9: 1–39.

Wray GA 2001. Dating branches on the tree of life using DNA. Genome Biology 3: reviews 1.1–1.7.

Xiao S, Zhang Y, & Knoll A 1998. Three-dimensional preservation of algae and animal embryos in a Neoproterozoic phosphorite. Nature 391: 553–558.