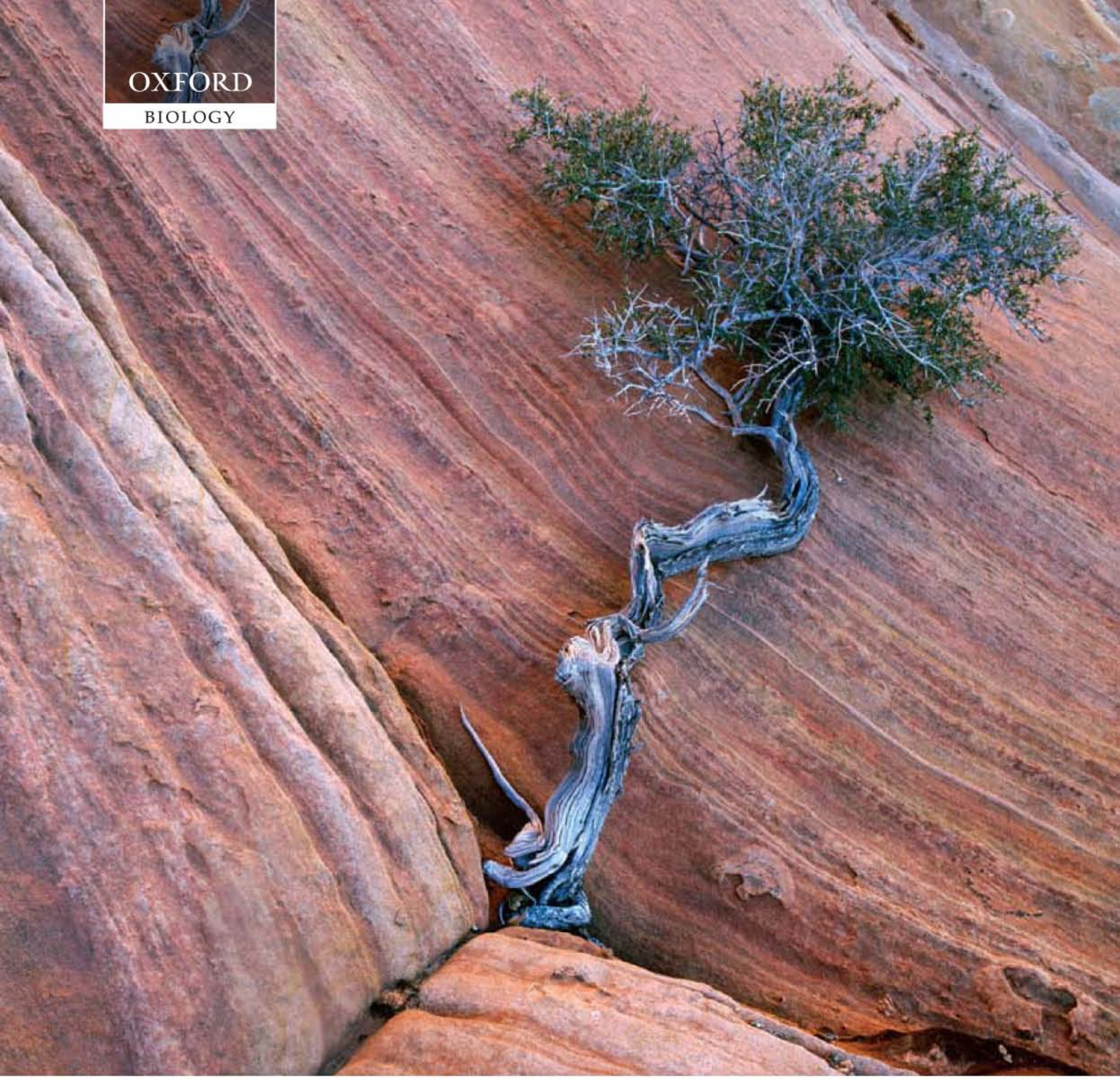

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the TIMETREE *of* LIFE

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Animals (Metazoa)

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Abstract

The relationships and molecular divergence times among metazoan (animal) phyla have been the subject of debate for decades. The current consensus suggests that most traditional hypotheses of metazoan phylogeny based on morphology are not supported by molecular phylogenetic analyses. In addition, the steady accumulation of sequence data and the increased sophistication of molecular clock methods have led to an expanded number of studies estimating divergence times among metazoan lineages. Most molecular clock studies, or their reanalyses, have found that the earliest divergences among living metazoans occurred deep in the Precambrian, hundreds of millions of years before the first animal fossils.

The evolution of large, heterotrophic metazoans (Fig. 1) has undoubtedly had a significant impact on the history of life by increasing the complexity of trophic interactions both in marine and terrestrial ecosystems. Metazoans are most closely related to Fungi as part of Opisthokonta, a eukaryotic supergroup which also includes unicellular choanoflagellates, icthyosporeans, and nucleariids (*1, 2*). The closest relatives of metazoans are the choanoflagellates; morphological similarities between the collar cells of sponges and the colonial habits of choanoflagellates were noted over 150 years ago (*3*). In addition, important molecular characteristics traditionally thought to be unique to metazoans, such as cell signaling and adhesion protein families, have also been found in choanoflagellates (*4, 5*). Here I review the relationships and divergence times among the metazoan phyla.

Relationships among the metazoan phyla have undergone major revisions over the past two decades (*3, 6*). The traditional view of simpler forms giving rise to more complex lineages has been challenged by the accumulation of developmental and molecular data. One important discovery has been the paraphyly of phylum Porifera (the sedentary filter-feeding sponges) which has altered interpretations of the last common ancestor of metazoans (Fig. 2). The Calcarea, which possess calcareous skeletons, may be more closely related to the Eumetazoa (all metazoans other than sponges) than the siliceous Hexactinellida or Demospongiae (*7–9*). The exact relationship between the siliceous sponges has not yet been determined, although some data have suggested they may form a monophyletic group, the Silicea (*3*).

The monophyly of Eumetazoa has been supported by molecular data as well as a number of morphological characteristics, such as the presence of body symmetry, a nervous system, and a mouth and gut. Although traditionally grouped together as the Coelenterata, molecular data have not supported a close relationship between Cnidaria and Ctenophora, and have instead placed Cnidaria as the closest relative of the Bilateria (*7, 9–11*). The relationships of Ctenophora and Placozoa to other metazoans have yet to be firmly established. In addition, the absence of bilateral symmetry and mesodermal tissues in the basal Eumetazoan lineages has been challenged by developmental and gene expression studies in jellyfish and complete genome analysis of the sea anemone *Nematostella* (*12–14*). These new data suggest a need to reevaluate the characteristics typically used to distinguish the diploblastic "Radiata" from the triploblastic Bilateria (*15*).

Within the monophyletic Bilateria, substantial changes have been made to the traditional scheme of

Fig. 1 An onycophoran (*Peripatus juliformis*) from St. John, United States Virgin Islands. Credit: A. Sanchez.

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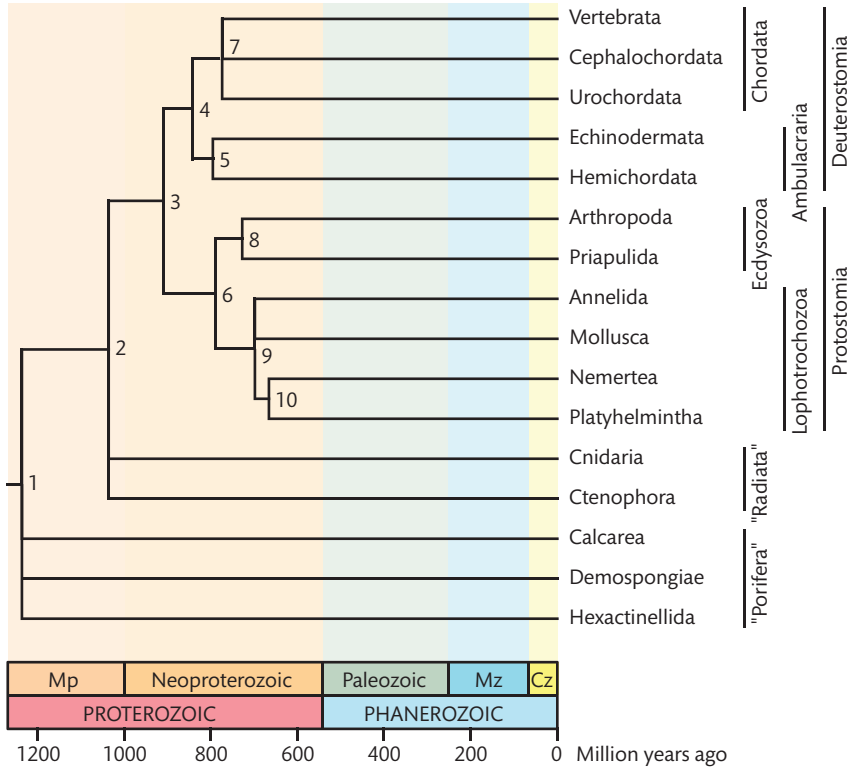

Fig. 2 A timetree of metazoan phyla. Divergence times are shown in Table 1. *Abbreviations*: Cz (Cenozoic), Mp (Mesoproterozoic), and Mz (Mesozoic).

increasing grades of complexity from basal, acoelomate flatworms up to complex, segmented protostomes and deuterostomes (*16*). The accumulation of molecular sequence data, primarily from the small subunit ribosomal RNA, suggests instead a division of the Bilateria into three major clades: Lophotrochozoa (*17*), Ecydsozoa (*18*), and Deuterostomia. Lophotrochozoa and Ecdysozoa together form the Protostomia, whose monophyly is often assumed but has been supported only recently with large, multigene studies (*19–22*). Initially, these new divisions, along with the apparent lack of any basal lineages, suggested that the last common bilaterian ancestor was a large, possibly segmented organism likely to have left traces in the fossil record (*23, 24*). However, two lineages of bilaterians, the Acoelomorpha flatworms (*25*) and the enigmatic Myxozoa parasites (*26*, but see *27*), have since been shown to be basal to the rest of Bilateria, suggesting the last common ancestor may have been less complex (*28, 29*).

The Lophotrochozoa clade was first suggested based on the affinity of lophophorates (Brachiopoda, Bryozoa, and Phoronida) with protostomes, specifically with mollusks and annelids (*17*). Further studies have supported the placement of additional groups within Lophotrochozoa, such as Rotifera, Acanthocephala, Gnathostomulida, and Gastrotricha, among others (*6*). The Platyhelmintha (excluding the basal Acoelomorpha) are also included in Lophotrochozoa, an unusual relationship first suggested by small subunit ribosomal RNA and *Hox* data (*18, 30*), and later supported by multigene studies (*20, 31*, but see *32*). Two main subclades have been suggested within Lophotrochozoa; the Platyzoa, which contains acoelomate Platyhelmintha, Rotifera, Acanthocephala, Gastrotricha, and Gnathostomulida; and the Trochozoa, which includes lophophorates, annelids, mollusks, and Nemertea (*33*). Overall, the relationships within Lophotrochozoa have not yet been firmly established, as most studies are based on one or two genes (ribosomal RNAs) with limited taxon sampling.

The second major protostome clade, Ecdysozoa, has received considerably more attention. The process of moulting, or ecdysis, has been suggested as the shared-derived character uniting this group of morphologically diverse phyla. Within Ecdysozoa, the monophyly of

Table 1. Divergence times (Ma) their confidence/credibility intervals (CI) among animals.

Timetree		Estimates							
Node	Time	Refs. (*50*, *60*)		Ref. (*61*)(a)		Ref. (*61*)(b)		Ref. (*62*)	
		Time	CI	Time	CI	Time	CI	Time	CI
1	1237	1351(*60*)	1586–1116	766	803–731	1122	1360–932	–	–
2	1036	1298(*60*)	1443–1153	676	709–645	907	1070–775	902	959–845
3	910	976(*60*)	1166–786	643	669–615	845	993–731	627	727–527
4	842	896	1022–932	601	625–579	788	930–675	–	–
5	795	876	1074–725	548	554–534	713	847–608	–	–
6	790	–	–	619	648–594	790	921–685	–	–
7	774	843	1067–685	547	584–518	744	891–620	–	–
8	728	–	–	584	616–552	728	857–621	–	–
9	698	–	–	586	612–563	698	815–610	–	–
10	666	–	–	565	595–534	666	784–574	–	–

Node	Estimates							
	Ref. (*67*)		Ref. (*68*)		Ref. (*69*)		Ref. (*70*)	
	Time	CI	Time	CI	Time	CI	Time	CI
1	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–
3	813	975–651	931	1237–625	993	1084–902	581	610–557
4	–	–	–	–	–	–	536	544–524
5	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–

Node	Estimates							
	Ref. (*73*)(a)		Ref. (*73*)(b)		Ref. (*79*)		Ref. (*80*)	
	Time	CI	Time	CI	Time	CI	Time	CI
1	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–
3	695	761–642	1141	1389–934	955	1135–775	–	–
4	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–
7	–	–	–	–	756	870–642	751	814–689
8	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–

Note: Node times in the timetree represent the mean of time estimates from different studies, except for original times (a) in refs. (*61*, *73*), where the times obtained after corrections (b) were used (see text for details).

the Panarthropoda subclade (Arthropoda, Tardigrada, and Onychophora) has been supported by a number of studies, while the relationships among the remaining Introverta phyla (Priapulida, Kinorhynca, Loricifera, Nematomorpha, Nematoda) have not been resolved (*34*). The inclusion of the pseudocoelomate Nematoda within Ecdysozoa has created the most controversy. The Ecdysozoa clade was first proposed based on an analysis of small subunit ribosomal RNA; the authors claimed that the use of a slower evolving nematode sequence overcame the typical long-branch attraction artifacts that place nematodes basal to other bilaterians (*18*). Additional studies of molecular sequence data (*20, 35, 36*), *Hox* gene homology (*37*), intron positions (*38*), gene expression patterns (*39, 40*), and other lines of evidence, have supported Ecydysozoa. On the other hand, genome-level studies utilizing the complete sequences of vertebrates and the two model organisms, *Drosophila* and *Caenorhabditis*, have supported a closer relationship between vertebrates and arthropods, which corresponds to a more traditional Coelomata hypothesis (*32, 41–44*). The position of Nematoda remains an active area of research and debate (e.g., *45–49*), along with the affinity of Chaetognatha, or arrow worms.

Phylogenetic rearrangements have also occurred within the Deuterostomia. Among the chordates, recent molecular studies have challenged the traditional position of Urochordata as the closest relative of the group containing Vertebrata and Cephalochordata, instead suggesting a closer relationship between Vertebrata and Urochordata (*20, 21, 50–52*). However, further analysis may be needed as this unusual relationship significantly alters traditional interpretations of chordate evolution (but see *53*). Recent multigene studies have also solidified support for a close relationship between Echinodermata and Hemichordata as Ambulacraria (*50, 51*). This arrangement has important implications for the ancestral condition of deuterostomes, suggesting that "chordate" features such as gill slits and notochords may have been present in the last common ancestor (*6, 54, 55*). In addition, molecular studies have shown that the marine worm *Xenoturbella* is a deuterostome closely related to Ambulacraria (*51, 56*).

Molecular divergence times among metazoan phyla have received considerable attention, with two main hypotheses emerging. The first hypothesis posits that metazoans originated only shortly before their appearance in the fossil record during the so-called "Cambrian Explosion." A minority of molecular investigations have supported this view (e.g., *57*), and have suggested that the evolution of *Hox* genes and other developmental pathways in bilaterians led to a dramatic restructuring of trophic interactions in the late Precambrian oceans, culminating in the appearance of large complex fauna in the Cambrian (*58*). The second hypothesis, that metazoans arose hundreds of millions of years before the Cambrian, implies a long history of cryptic evolution not present in the fossil record. A majority of molecular dating studies support this second scenario (Table 1); however, this remains a very active area of investigation. Estimates for the earliest divergences within Metazoa, such as those among members of the paraphyletic Porifera and the Eumetazoans, range from 1350 to 660 Ma with an average of 1240 Ma (*57, 59–61*). Estimates for the divergence of Bilateria and Cnidaria (perhaps with Ctenophora and Placozoa) have ranged between 1300 and 600 Ma, with an average of 1035 Ma (*57, 60–63*).

The divergence between protostomes and deuterostomes has dominated molecular clock studies. Most analyses have yielded divergence times that predate the Cambrian Explosion of animal phyla. Estimates range from 1200 to 580 Ma, with an average of 910 Ma (Table 1). An early study based on seven genes proposed a controversial estimate of ~1200 Ma for the divergence between protostomes and deuterostomes (*64*); similar studies using small numbers of genes have produced times between 900 and 600 Ma (*57, 62, 65–68*). The first large multigene study, incorporating rate variation among sites and rate-tested genes, estimated a divergence time of 993 Ma with 50 genes (*69*). Using a different approach, Otsuka and Sugaya (*63*) used theoretical rates of base-pair changes in mitochondrial rRNA to estimate the divergence between protostomes and deuterostomes at 920 Ma.

Other studies have used likelihood and Bayesian "relaxed clock" methods to estimate the divergence between protostomes and deuterostomes, and other splits in the tree of metazoan phyla. An analysis of 22 nuclear and mitochondrial genes using Bayesian methods to correct for temporal rate variation suggested a divergence time of 581 Ma (*70*); the results from this study, however, were affected by significant methodological biases. Specifically, a rate model was used as a Bayesian prior that was biased toward decreasing rates, causing divergences earlier than the Cambrian to be underestimated and divergences later than the Cambrian to be overestimated (*71, 72*). For example, divergences among living mammals were found to be in the Paleozoic and among living birds in the Jurassic, much older than has been found in other molecular clock studies.

A large multigene study by Douzery *et al.* (*73*) using 129 proteins and a different Bayesian method calculated the divergence between protostomes and deuterostomes to be 695 Ma (741–642 Ma). Roger and Hug (*74*) and Hug and Roger (*75*) conducted reanalyses of this large data set, questioning many aspects of the study and its results. However, these reanalyses overlooked a significant issue with both the minimum and maximum constraints used in the original study, as was noted earlier (*76*). In the study of Douzery *et al.* (*73*), each minimum calibration constraint was fixed as the younger boundary of the major geologic period containing the pertinent fossil rather than to the actual (older) geologic time constraints of the fossil itself, causing the resulting time estimates to be underestimates. Douzery *et al.* also fixed maximum calibration constraints, arbitrarily, to the older boundary of the major geologic period containing the fossil rather than to an evolutionary event that might bear on the constraint. For example, the maximum calibration for the split of actinopterygian fish from mammals, 417 Ma, was essentially the same time as the oldest fossil on either branch, 416 Ma (*77*). However, there is little fossil information from this time period (Silurian) to establish that the divergence occurred precisely when the fossils appeared; more than likely it was much earlier, which would result in older Bayesian posterior time estimates. Also, one of the maximum calibrations, the split between chelicerates and other arthropods (543 Ma), was fixed within the Cambrian, which can lead to circular reasoning when the results are then used to support a reconciling of the Cambrian Explosion in the fossil record with molecular clock times, as was proposed in that study (*73*).

A separate reanalysis of the Douzery *et al.* data set (S. B. Hedges, personal communication) was conducted using the same methods as the original authors, but with corrected minimum calibrations, based on the fossil record. This led to a protostome–deuterostome divergence time of 742 Ma (817–692 Ma), 200 million years before the Cambrian boundary and 47 million years older (31% of time to Cambrian) than the date reported in the original study (*73*). Therefore, a simple and necessary correction of calibrations yielded a divergence time that overturns the primary conclusion of Douzery *et al.*, the reconciliation of molecular clock times and fossil times. Furthermore, after the removal of the maximum calibration in the Cambrian (543 Ma) and keeping all corrected minimum calibrations, the time became 797 Ma (898–719 Ma), 255 million years before the Cambrian boundary and 102 million years older (67% of time to Cambrian) than the date reported in the original study. Moreover, correcting the actinopterygian-mammal maximum calibration to 495 Ma (from 417 Ma), removing other unjustified maximum calibrations, and keeping all corrected minimum calibrations resulted in a time of 1141 Ma (1389–934), 599 million years before the Cambrian boundary and 446 million years older (392% of time to Cambrian) than the date reported in the original study (*73*). These reanalyses provide corrected divergence time estimates of the protostome–deuterostome divergence for this large data set (Table 1), and their trends agree with those of Hug and Roger (*75*) in showing the sensitivity of these data to maximum calibrations. However, Hug and Roger (*75*) estimates are not included in Table 1 because those authors did not recommend any estimates based on their reanalysis.

A seven-gene data set of mostly protostomes has been analyzed in three separate studies by Peterson *et al.* (*78*), Peterson and Butterfield (*57*), and Peterson *et al.* (*61*). The first two analyses resulted in young time estimates largely agreeing with a direct reading of the fossil record (Cambrian Explosion). For example, the protostome–deuterostome divergence was estimated to be 592–556 Ma (*78*) and 579 Ma (*57*). However, three reanalyses (*71, 74, 75*) identified methodological problems in the original studies which were responsible for the underestimation of divergence times, such as the use of uncorrected distances and fixed calibrations (maximum = minimum). The original studies (*57, 78*) also used a constant rate method rather than a relaxed clock method.

The most recent study by Peterson *et al.* (*61*) involved a Bayesian analysis of the seven-gene data set. The use of relaxed clock methods resulted in older divergence time estimates, although the dates were still much younger than other molecular studies. For example, the protostome–deuterostome divergence was estimated as 643 Ma (669–615 Ma); this date increased to 733 Ma when probability distributions on fossil calibrations were used (*61*). However, a potential problem with this study was that five maximum calibrations were used, four of which were placed in the latest Precambrian and Cambrian (the Cambrian Explosion). The resulting time estimates for animal phyla were therefore prohibited from being much older than those constraints, thus tightly linking the posteriors (time estimates) to the priors (calibrations). The conclusions drawn by Peterson *et al.* (*61*), of young time estimates consistent with the fossil record, were thus an example of circular reasoning. Although they claimed that the results were robust to the use (or not) of maximum calibrations, this was based on the

experimental removal of only one of the five maximum calibrations.

The seven-gene data set of Peterson *et al.* (*61*) was reanalyzed (S. B. Hedges, personal communication) using the same method of analysis, all original minimum calibrations, and the mid-Phanerozoic maximum calibration (insects), but with the four remaining maximum calibrations that created the circularity removed. The resulting protostome–deuterostome time estimate (845 Ma) was 202 million years older than reported by Peterson *et al.* (643 Ma) (Table 1), demonstrating that their young time estimates were a direct result of the use of the suspect maximum calibrations. Other studies using both constant rate and relaxed clock methods have estimated the divergence between protostomes and deuterostomes in a narrow range: 976 Ma (*60*) to 955 Ma (*79*). The importance of establishing a robust time estimate for the divergence of protostomes and deuterostomes will undoubtedly lead to the development of additional molecular clock methods and large, genome-scale data sets for future analyses.

The divergence of Chordata and Ambulacraria within Deuterostomia has been estimated between 1001 and 590 Ma (*63–66*). A recent multigene study estimated a time of 896 Ma for the divergence of vertebrates and echinoderms using 71 nuclear proteins (*50*). Divergence time estimates for the earliest divergence within Chordata range from 890 to 547 Ma (*50, 59, 61, 73, 79, 80*). However, this divergence may need to be reevaluated in light of the recent phylogenetic evidence for a closer relationship between Vertebrata and Urochordata. Estimates for the divergence between Echinodermata and Hemichordata range from 875 to 535 Ma (*50, 57, 61*). Within the Protostomia, the divergence between Lophotrochozoa and Ecdysozoa has been dated at approximately 800 Ma (*57, 61, 63, 73*) with the deepest divergences within Ecdysozoa and Lophotrochozoa dated at ~700 Ma (*57, 61*). All of these divergences predate the first fossil evidence for animal phyla in the Cambrian.

Establishing a robust timetree for Metazoa has important implications for understanding evolution in the Neoproterozoic. Essentially all molecular clock studies suggest that bilaterians had already radiated 100 million years or more before the Precambrian–Cambrian boundary. Studies proposing younger divergences consistent with a literal interpretation of the Cambrian Explosion have been shown to suffer from methodological biases, such as incorrect assumptions about rate models or the use of inappropriate fossil constraints. Geological evidence has suggested that the interval between approximately 800 and 550 Ma was one of planetary unrest, with multiple rounds of global glaciation (Snowball Earth, *81*), changes in sea water chemistry (*82–84*), and increases in atmospheric oxygen levels (*85, 86*). Changing environmental pressures, along with the evolution of complex genetic pathways for skeletogenesis (*87*) and organ formation (*88*), had an evident effect on the evolutionary trajectory of metazoans during this important period in Earth's history**.**

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