

Chapter 7

Molecular Timescale of Evolution in the Proterozoic

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1. INTRODUCTION

The late Precambrian (Neoproterozoic; 1000–543 Million years ago, Ma) was a transitional time in Earth history and the evolution of eukaryotes. Although atmospheric oxygen levels initially rose in the early Proterozoic

(~2300 Ma), perhaps to as much as 10% of present levels, a second major increase occurred at some point in the late Neoproterozoic (Canfield, 2005). By the early Phanerozoic, the atmospheric oxygen level was close to that of the present (Berner *et al.*, 2003). There has been speculation for years that the sudden appearance of many animal phyla in the early Phanerozoic (Cambrian Explosion) was causally tied to an increase in atmospheric oxygen, and that small and soft-bodied animals may have existed for a lengthy period before the Phanerozoic (e.g., Nursall, 1959). Nonetheless, the leading explanation for the Cambrian Explosion is that it represents a rapid evolutionary radiation of animals in the latest Precambrian or earliest Phanerozoic (Gould, 1989; Conway Morris, 2000).

Molecular clocks, which measure times of divergence between species from sequence data, have focused even greater attention on the Cambrian Explosion. Such studies in the last three decades have frequently found divergences among animal phyla to be hundreds of millions of years earlier than predicted by the fossil record (Brown *et al.*, 1972; Runnegar, 1982b; Wray *et al.*, 1996; Wang *et al.*, 1999; Hedges *et al.*, 2004; Pisani *et al.*, 2004; Blair *et al.*, 2005). Moreover, plants and fungi, two groups with a fossil record that, at least until recently, has been firmly rooted in the Phanerozoic, have also been suggested to have deep roots in the Neoproterozoic using molecular clocks (Berbee and Taylor, 2001; Heckman *et al.*, 2001; Hedges *et al.*, 2004; Padovan *et al.*, 2005). Not all molecular clock studies agree, and several recent studies (Aris-Brosou and Yang, 2002, 2003; Douzery *et al.*, 2004; Peterson *et al.*, 2004; Peterson and Butterfield, 2005) have obtained results more closely in line with the majority of fossil evidence (i.e., late Neoproterozoic).

Differing results among molecular clock studies are mirrored by differing opinions among palaeontologists as to the validity of the earliest fossils of multicellular life. This exemplifies the point that debates in this area are not necessarily between different fields (e.g., molecular evolution versus palaeontology) but are often within fields. For example, the earliest eukaryote fossils, assigned to the genus *Grypania* (Han and Runnegar, 1992) and now dated to ~1900 Ma are considered by some paleontologists to be prokaryotes (Samuelsson and Butterfield, 2001; Sergeev *et al.*, 2002), and none of the many trace fossils of animals from deep in the Precambrian (e.g., Seilacher *et al.*, 1998) are widely accepted (Jensen *et al.*, 2005). Nonetheless, the fossil record of fungi and land plants has been pushed back tens to hundreds of millions of years in recent studies (Yang *et al.*, 2004; Butterfield, 2005; Yuan *et al.*, 2005), and a greater diversity of animal fossils has come to light from Neoproterozoic Lagerstätten, especially phosphorites (Xiao *et al.*, 1998; Chen *et al.*, 2004). It is only to be expected that claims of the earliest fossil of any major group of organism will be controversial and subject to continued scrutiny.

Concurrent with developments in molecular dating and palaeontology have been discoveries in Earth history that have placed additional attention on the Neoproterozoic as a time period of great interest for the evolution of complex life. Chief among these has been the elucidation of a series of global glaciations (Snowball Earth events) which would have greatly restricted the habitable area for life on Earth and may have influenced patterns of speciation and macroevolution (Hoffman *et al.*, 1998; Hedges, 2003). However, as with Neoproterozoic molecular clocks and fossils, there has been disagreement among geologists as to mechanisms and extent of the glaciations (Hyde *et al.*, 2000; Young, 2002; Poulsen, 2003; Poulsen and Jacob, 2004).

Despite the uncertainties and debates within different fields, the interdisciplinary nature of this research in Neoproterozoic geobiology and astrobiology has been appealing to many and has invigorated the field. Here, we will review the current state of knowledge regarding evolutionary relationships and times of origin of organisms in the Proterozoic as they bear on these questions of how complex life evolved in the face of a changing planetary environment.

2. MOLECULAR CLOCK METHODS

Methods for estimating time from molecular sequence data have evolved over the four decades since the original proposal of a molecular clock (Zuckerandl and Pauling, 1962). At the basic level, molecular clocks provide a means of estimating the divergence time of species based on rates of sequence change in genes and genomes. These rates are usually established first by calibration with the fossil record.

The same mechanism driving radiometric clocks, stochasticity, is believed to be the engine of molecular clocks (Kimura, 1983). Of course, different genes and regions of the genome evolve at different rates, as a result of constraints imposed from natural selection, just as different isotopes decay at different rates. In both cases, it is this diversity of rates that allows use of these methods at different timescales. Nonetheless, molecular clocks are more variable than radiometric clocks, and this variation has been responsible for much of the debate over the use of clocks in recent years.

Fortunately, data sets have become larger and methods have become more sophisticated in parallel with developments in the field of molecular evolution (reviewed in Hedges and Kumar, 2003; Kumar, 2005). The importance of the size of a data set (number of sites and genes) cannot be stressed enough, because a large sample is needed for deriving any mean

estimate from a stochastic mechanism. Time estimates based on a single gene or small number of genes may have large associated errors or undetected biases, just as time estimates derived from a small number of radioisotope decays (in practice, the number of decays is not a limiting factor). Hence, it is preferable to estimate time from large numbers of genes.

While it is possible to restrict analysis to genes evolving at a constant rate among lineages or branches, a larger data set can be used by including those genes having rate variation among branches. This difference has led to the distinction of global clocks (rate constancy throughout tree) from local clocks (rate variation in localized parts of the tree) (Hedges and Kumar, 2003). The latter contains a great diversity of methods (Hasegawa *et al.*, 1989; Takezaki *et al.*, 1995; Sanderson, 1997; Schubart *et al.*, 1998; Thorne *et al.*, 1998), some of which are referred to as "relaxed clock" methods. Notwithstanding claims that time can be estimated "without a clock" (Sanderson, 2003; Bell *et al.*, 2005), the term "clock"—defined in most dictionaries as "a device used for measuring and indicating time"—applies appropriately to any method of estimating time.

The introduction of minimum and maximum constraints for calibrations in some local clock methods (Sanderson, 1997; Kishino *et al.*, 2001) has helped to focus greater attention on confidence intervals. However at the same time this has exposed weaknesses in these sources of data. In particular, the maximum time of divergence is almost never known with any certainty, although it has been widely used in molecular clock studies in recent years. As discussed elsewhere (Hedges and Kumar, 2004), this fact and the lack of knowledge of the probability distribution for most calibrations have resulted (mostly likely) in many underestimates and overestimates of time. Earlier molecular clock studies, in contrast, often used minimum calibration points, which are less subject to error, and interpreted their results with respect to that constraint (Hedges and Kumar, 2004). Nonetheless, it is preferable to obtain a mean estimate for the time of divergence rather than the minimum estimate, and therefore further improvements in these local clock methods seem likely.

Finally, a new method of incorporating statistical error of time estimates has been developed based on bootstrapping (Kumar *et al.*, 2005). This error estimation method can be used with any molecular clock method, and includes all types of variance, such as that resulting from differences in gene sampling, site sampling, error contributed by distance estimation procedures, variance in rates among lineages, and any error associated with calibrations. No previous molecular clock studies have incorporated all of these errors. With such methods, time estimates in future studies will likely have larger confidence intervals but should be more realistic.

3. MOLECULAR TIMESCALES

Some remarkable fossil discoveries have been made of Neoproterozoic organisms in recent years (Xiao *et al.*, 1998; Chen *et al.*, 2004; Yuan *et al.*, 2005) but the fossil record of this time period, and of earlier time periods, is much poorer than that of the Phanerozoic. For this reason, it is more difficult to estimate times of divergence between lineages from the fossil record alone, placing more importance on obtaining such estimates using molecular clocks. Below, we review those studies that have estimated divergence times of organisms that lived during the Precambrian, with emphasis on studies using relatively large data sets. Such information is critical to understanding how features of the planetary environment, including global glaciations, plate tectonics, and changes in atmospheric gases, influenced or were impacted by the evolution of life at that time.

3.1 Prokaryotes

There have been several molecular clock studies of prokaryotes that have estimated divergence times in the Precambrian. Without exception, all have found deep divergences, prior to the Neoproterozoic, of the major groups of archaeobacteria and eubacteria (Doolittle *et al.*, 1996; Feng *et al.*, 1997; Hedges *et al.*, 2001; Sheridan *et al.*, 2003; Battistuzzi *et al.*, 2004). One study (Sheridan *et al.*, 2003) used nucleotide variation in the gene for the small subunit ribosomal RNA whereas the other studies used amino acid sequences of multiple proteins. Taxon sampling was relatively limited in the earlier protein studies (Doolittle *et al.*, 1996; Feng *et al.*, 1997; Hedges *et al.*, 2001), with the recent study (Battistuzzi *et al.*, 2004) having the largest number of taxa. Also, it is now known that there are major rate differences among groups of prokaryotes and between prokaryotes and eukaryotes (Kollman and Doolittle, 2000; Hedges *et al.*, 2001). Some time estimates (Doolittle *et al.*, 1996; Feng *et al.*, 1997; Sheridan *et al.*, 2003) were made without accounting for those rate differences, and another (Hedges *et al.*, 2001) was made using a two rate model. The recent analysis (Battistuzzi *et al.*, 2004) was performed using a Bayesian local clock method that permitted rate variation among branches and the results of that study are reproduced here (Fig. 1). Genes that showed obvious evidence of lateral gene transfer were avoided. As can be seen, and not surprisingly, all of the major groups of prokaryotes were extant by the onset of the Neoproterozoic and therefore their major metabolic activities (e.g., anoxygenic photosynthesis, oxygenic photosynthesis, methanogenesis, aerobic methanotrophy, etc.) were present. In most cases, this only reinforces what has already been revealed with geologic and other evidence (Knoll, 2004).

high resistance to dehydration and have species that are currently terrestrial. These three groups were named, collectively, Terrabacteria (Battistuzzi *et al.*, 2004). This supports the paleontological and geological evidence that prokaryotes colonized the land surface in the Precambrian (Horodyski and Knauth, 1994; Watanabe *et al.*, 2000) and therefore their metabolic and erosional activities should be considered, as well as potential interactions with terrestrial eukaryotes.

3.2 Eukaryotes

The relationships of the major lineages of eukaryotes have become much better known during the last decade as more sequence data have been gathered and analyzed (reviewed in Baldauf *et al.*, 2000; Hedges, 2002; Keeling *et al.*, 2005). Because the relationships of single-celled eukaryotes (protists) are intimately tied to the relationships of multicellular eukaryotes (algae, plants, fungi, animals), it is usually more convenient to discuss this subject in terms of overall (higher-level) eukaryote phylogeny, as will be done here. The land plants, fungi, and animals will be discussed in separate sections.

The ease of sequencing ribosomal RNA (rRNA), and especially the small subunit, meant that an initial molecular view and framework of eukaryote phylogeny, from molecules, was based on that gene. For eukaryotes, those trees defined a crown consisting of plants, animals, fungi, and related protists, and a series of lineages along the stem or base of the tree, with the diplomonad *Giardia* as the earliest branch (Sogin *et al.*, 1989; Schlegel, 1994). Later analyses using complex models and different genes showed that some—but not all—basal branching lineages (e.g., microsporidia, *Dictyostelium*) actually belong higher in the tree, and their misplacement was the result of long-branch attraction or other biases (e.g., Philippe and Germot, 2000). Subsequently, much recent attention has been placed on building trees with as many genes and taxa as possible, and using different types of analyses, including complex substitution models. This has brought welcomed stability to some aspects of the tree, and remarkable volatility to others.

Some major questions that were once controversial have now been answered to the satisfaction of many in the field. For example, animals and fungi appear to be closest relatives (opisthokonts) to the exclusion of plants (although see Philip *et al.*, 2005), and red algae are on the “plant lineage” and not basal to the divergence of plants and opisthokonts as previously thought. There is growing support that amoebozoans are the closest relatives of opisthokonts (e.g., Amaral Zettler *et al.*, 2001; Baldauf, 2003), and that microsporidia are the closest relatives of fungi.

Recently, there has been an effort to summarize these and other aspects of the eukaryote tree in the form of a five-group arrangement: plants, unikonts, chromalveolates, rhizarians, and excavates (Keeling, 2004; Keeling *et al.*, 2005). Under this scheme, Plantae is defined by the presence of plastids acquired by primary endosymbiosis and includes the land plants, charophytes, chlorophytes, rhodophytes, and glaucophytes. The unikonts are defined by the presence of a single cilium-bearing centriole and include the opisthokonts (animals, fungi, choanoflagellates, ichthyosporeans, and nucleariids) and the amoebozoans. Rhizaria includes the cercozoans, foraminiferans, polycistines, and acanthareans. Chromalveolates include the alveolates (e.g., ciliates, apicomplexans, and dinoflagellates) and stramenopiles (e.g., brown algae, diatoms, haptophytes, and cryptomonads). The excavates include the discicristates (e.g., euglenids and kinetoplastids), oxymonads, and metamonads (e.g., diplomonads, parabasalids, and *Carpodimonas*); the content of the excavates and relationships among the included taxa are particularly controversial (see below). The above arrangement also agrees with a division of eukaryotes into unikonts and bikonts (Richards and Cavalier-Smith, 2005). The amount of evidence supporting inclusion of different taxa varies considerably, from hundreds of genes in some cases (e.g., animals joining with fungi) to relatively small amounts of morphological or molecular data in other cases (e.g., excavates).

As a point of discussion, this five-group arrangement serves a useful purpose. However, a major problem is that it avoids the question of the root. Technically, without a root there can be no evolutionary polarity or claim of monophyly (i.e., no “five groups”). In fact, analyses of the largest sequence data sets, using complex models of evolution, show with statistical significance that the root lies within one of these five groups, the excavates (Hedges *et al.*, 2001; Baptiste *et al.*, 2002; Hedges *et al.*, 2004), which breaks up the monophyly of the bikonts. This supports the earlier proposal of a basal position for *Giardia* (a diplomonad), based on rRNA sequences and cytological arguments (Sogin *et al.*, 1989). Because complex models of evolution have been used in these recent studies, there is no clear evidence yet that long-branch attraction or other substitutional biases are responsible for this root position.

One analysis demonstrated sensitivity of the topology to removal of fast-evolving sites, but those results were inconclusive because of a lack of statistical support for most nodes (Arisue *et al.*, 2005). With the great age of these lineages and the fact that long-branch attraction and other substitutional biases may lead to incorrect groupings (Philippe *et al.*, 2000), it is worth being cautious in interpreting any results, even if statistically significant. Future analyses of large numbers of taxa and genes, and testing of hypotheses concerning substitutional biases, should help better resolve the

tree of eukaryotes. However, the weight of the current sequence evidence argues, significantly, against the monophyly of at least one of the five groups, the “excavates,” and in favour of a root between the metamonads (or at least the diplomonads) and other eukaryotes (Fig. 2A). Thus, a six-group classification would divide the excavates into the discicristates and metamonads.

Another possible location of the eukaryote root, between opisthokonts and all other eukaryotes, has been proposed based on a gene fusion event, joining dihydrofolate reductase and thymidylate synthase (Philippe *et al.*, 2000; Stechmann and Cavalier-Smith, 2002) in many bikont eukaryotes. Evidence of a fusion of three genes in the pyrimidine biosynthetic pathway in unikonts led those same authors to revise their rooting scheme to include amoebozoans with opisthokonts (i.e., all unikonts) in the root (Stechmann and Cavalier-Smith, 2003). However, the subsequent finding of that triple gene fusion in a red alga (Matsuzaki *et al.*, 2004), which is clearly not related to opisthokonts or amoebozoans, undermined the usefulness of that gene fusion character.

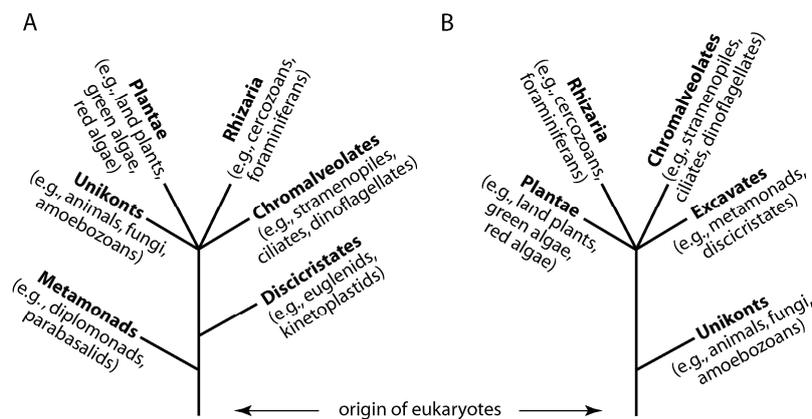


Figure 2. Two alternative hypotheses for the phylogenetic tree of eukaryotes. (A). The metamonad root, reflecting a six-group classification. This tree is favoured by phylogenetic analyses of DNA sequence data. (B). The unikont root, reflecting a five-group classification (metamonads and discicristates are combined into "excavates"). Under unikont rooting, the non-unikont eukaryotes (bikonts) are monophyletic.

Even more recently, evidence from the gene structure of myosin genes has been marshalled to further support a root between unikonts and bikonts (Richards and Cavalier-Smith, 2005) (Fig. 2B). In this case, gene and domain evolution is complex and there is homoplasy among the data. Moreover, most of the characters proposed as support for the root actually

support the largely uncontroversial grouping of animals, fungi, and amoebozoans (unikonts), which does not define the root position. Both of the two characters indicated as supporting the alternative branch (“bikonts”), which is critical for the claim of unikont rooting, are problematic. One character involves the two-gene fusion, but this turns out to be absent in species that are critical to defining the root (e.g., metamonads), and the clear case of homoplasy involving the triple-gene fusion shows that gene fusions in general are not necessarily reliable characters. The second character is an insertion of 60 amino acids in bikonts. However, only two of the 13 bikonts examined (*Trypanosoma* and *Phytophthora*) had this insertion and neither was a metamonad. Furthermore, a recent study (Hampl *et al.*, 2005) claimed to recover excavate monophyly but close scrutiny shows that those authors fixed the root to unikonts and therefore they did not actually test excavate monophyly with a prokaryote outgroup.

Time estimation of protist evolution has lagged behind that of other groups largely because of the complexity of relationships and slower accumulation of sequence data. Recently, a sequence analysis of the phylogeny and divergence times of eukaryotes, including the major groups of protists, was made using 22–188 proteins per node (Hedges *et al.*, 2004), (Fig. 3). Divergence times were estimated using both global and local (including Bayesian) clock methods, and the genes were analysed separately and as a single “supergene.” The diplomonad *Giardia* was found to be basal to the plant-animal-fungi clade, with significant bootstrap support, in Bayesian, likelihood, and distance analyses of 39 proteins (Hedges *et al.*, 2004). Two other protists lineages, the euglenozoans (105 proteins; 38,492 amino acids) and alveolates (73 proteins; 27,497 amino acids) were also found to be basal to the plant-animal-fungi clade with significant support.

In a separate phylogenetic analysis (Baptiste *et al.*, 2002) of a similar amount of sequence data (123 proteins, 25,023 amino acids; albeit with some missing sequences) and with a greater number of taxa, the same higher-level structure of the “protist tree” was found. In both cases, the results contradict the “five-group” classification of protists (Keeling, 2004; Keeling *et al.*, 2005) and opisthokont or unikont rooting of eukaryotes (Stechmann and Cavalier-Smith, 2002, 2003; Richards and Cavalier-Smith, 2005). A solution to this problem with the five-group classification is to separate the discicristates from a restricted excavate group (metamonads, and possibly oxymonads and malawimonads if future studies show them to be related). Therefore, if either the unikonts or the metamonads form the root of eukaryotes (Fig. 2), it does not contradict this six-group classification scheme. It is also possible that the root of eukaryotes is at yet another position, such as (for example) between the discicristates (e.g., euglenids) and all other eukaryotes.

The timetree (phylogeny scaled to evolutionary time) of eukaryotes shows that plants diverged from the animal-fungi clade approximately 1600 Ma and that animals diverged from fungi approximately 1500 Ma (Fig. 3), reflecting a relative consistency in these time estimates found in studies using large numbers of genes (Wang *et al.*, 1999; Hedges *et al.*, 2004; Blair *et al.*, 2005). In this timetree, the divergence of red algae (Rhodophyta) from the land plant lineage was approximately 1400 Ma, which is consistent with the date (1200 Ma) for the first fossils of red algae (Butterfield, 2000). Although plastids were obtained by some clades of protists through secondary endosymbiotic events, they arose initially on the plant lineage through primary endosymbiosis between a protist and a cyanobacterium. The date of that event is constrained to approximately 1500–1600 Ma (Fig. 3). The alveolates and euglenozoans branch more basally (~1900 Ma) in the timetree of eukaryotes, while the most basal branch (diplomonads) is dated to ~2300 Ma. A separate analysis of genes involved in the mitochondrial symbiotic event dated that event as 1840 ± 200 Ma (Hedges *et al.*, 2001), and together with these data (Hedges *et al.*, 2004) suggest a date of ~1800–2300 Ma for the origin of mitochondria.

The time estimates in this timetree (Fig. 3) compare closely with those in an analysis that focused on divergences among algae (Yoon *et al.*, 2004). In that study, DNA and protein sequences of several plastid genes were analyzed with a local clock method (rate smoothing) and the primary plastid endosymbiotic event was found to be “before 1558 Ma.” The split of red algae from green algae was found to be 1474 Ma, also comparing closely with that found in the other study, 1428 Ma (Hedges *et al.*, 2004), despite different genes and methods. In some earlier timing studies (Feng *et al.*, 1997; Nei *et al.*, 2001), sequences from different clades of protists were combined and therefore the results are not comparable, although time estimates for their hybrid protist lineages, ~1500–1700 Ma, are similar in general to those here (Fig. 3).

However, a recent time estimation study using a relatively large data set (129 proteins and 36 taxa) obtained younger dates, with the most basal branches among eukaryotes (in this case, between opisthokonts and all other eukaryotes) splitting only 950–1259 Ma (Douzery *et al.*, 2004). In particular, the split of red from green algae was dated as 928 (825–1061) Ma, which is only about 60% as old as the date obtained in those two other studies (Hedges *et al.*, 2004; Yoon *et al.*, 2004) and which directly conflicts with the oldest fossil of red algae at 1200 Ma (Butterfield, 2000). Their dates for the splits between green algae and land plants (729 Ma) and stramenopiles and alveolates (872 Ma) were also younger than the earliest fossils of those groups (e.g., green algae and stramenopiles), 1000 Ma (Woods *et al.*, 1998; Kumar, 2001). Douzery *et al.* (2004) explained the conflict by attributing

uncertainty (723–1267 Ma) to the geologic dating of the red algal fossil, and citing that same reference (Butterfield, 2000). However, this is incorrect because the paleontological reference (Butterfield, 2000) instead lists a date of 1198 ± 24 Ma for the fossil and claims that it is a refinement of an earlier interval spanning 723–1267 Ma.

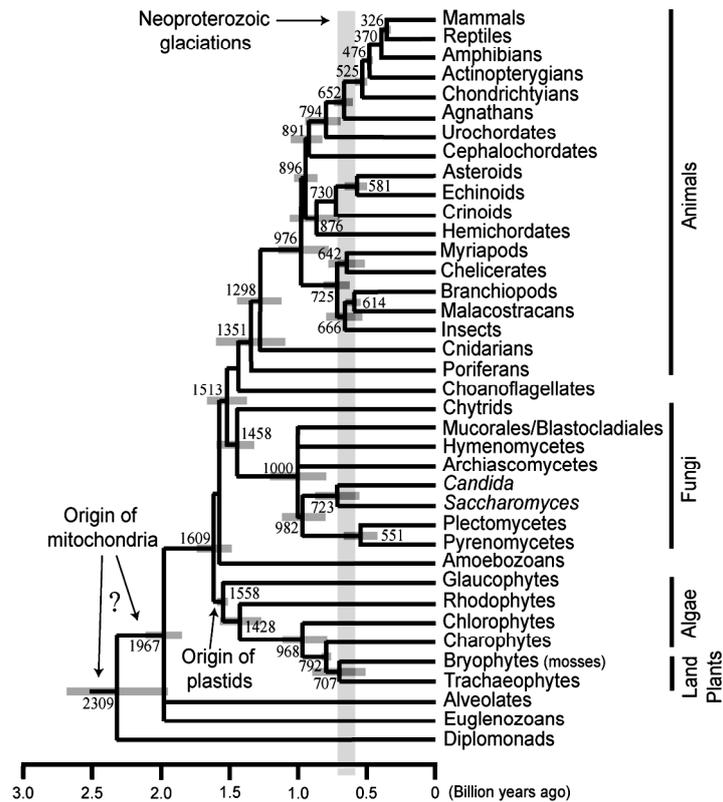


Figure 3. A timetree of eukaryotes based on several molecular studies. Divergence times of deuterostome animals are from Blair and Hedges (2005b), those of arthropods are from Pisani *et al.* (2004), the divergence time of chytrid fungi from higher fungi is from Heckman *et al.* (2001), that of glaucophyte algae from rhodophytes + chlorophytes (and their terrestrial descendants) is from Yoon *et al.* (2004), and the position of choanoflagellates and amoebozoans is constrained by phylogeny. Other divergence times, including those of algae, fungi, plants, other animals, and other protists, are from Hedges *et al.* (2004). The time of origin of the mitochondrion, and its debated position, is discussed elsewhere (Hedges *et al.*, 2001; Hedges *et al.*, 2004). The time of origin of the plastid is constrained at the base of the plastid-bearing clade (Hedges *et al.*, 2004). Gray horizontal bars are 95% confidence intervals.

One possible reason as to why the red algae time estimate of Douzery *et al.* (2004) conflicts with the fossil date is because they rooted their tree to a unikont (amoebozoan). Although they considered such a rooting to be correct, and a kinetoplastid rooting to be a "reconstruction artefact," they nonetheless calculated divergence times with the latter rooting for comparison. In doing so, they obtained an older date (899–1191 Ma, 95% credibility interval) for the chlorophyte-rhodophyte split. Nonetheless, even that estimate nearly conflicts with the fossil record (1200 Ma) and is 30–40% younger than the dates obtained by others (Hedges *et al.*, 2004; Yoon *et al.*, 2004) for this split.

An additional explanation for the young dates in that analysis (Douzery *et al.*, 2004) is that the calibrations used were applied incorrectly. For calibrations, they used minimum and maximum constraints based on the upper and lower time boundaries of the geologic periods containing the earliest fossils of a lineage. There are at least two problems with that approach. First, the geologic periods used were more inclusive than documented for the fossils. For example, they used the Devonian period (354–417 Ma) for the split of mammals and actinopterygian fishes. However, the fossil data are much better constrained than that, with the earliest fossils defining that split occurring in the very earliest Devonian, or more likely, late Silurian, 425 Ma (Donoghue *et al.*, 2003). 425 Ma is 20% older than 354 Ma, the minimum date used in the study (Douzery *et al.*, 2004). Even if the earliest fossils were in the Devonian, their date can usually be ascertained to a much finer level (e.g., age, stage, epoch) than major geologic period, and therefore this general approach is flawed and will result in an underestimate of divergence time.

The second problem is the assignment of a maximum date (constraint) for the calibration to the maximum age of the geologic period. For the time estimation analyses, assignment of a maximum calibration constraint means that the true divergence did not happen earlier than that time. But evolutionary biologists, including palaeontologists, usually never interpret the fossil record as a literal history of life, and most would agree that the true divergences occurred earlier (in many cases, even in earlier periods) than the first fossil occurrences. This approach of assigning a maximum close to the time of the first fossil occurrence would only be valid if the conclusions of the study were that the resulting times of divergence represented minimum estimates rather than mean estimates (for more discussion of this topic, see Hedges and Kumar, 2004). However, Douzery *et al.* (2004) interpreted their resulting time estimates as mean (true) times of divergence and drew attention to the conflict (difference) between their time estimates and other published dates that are older. If interpreted as minimum time estimates, their estimates would not be in conflict with older time estimates. It is likely

that these two problems with calibration methodology, combined with a forced unikont rooting, explain why those time estimates for protists and other eukaryotes (Douzery *et al.*, 2004) are much younger than other published analyses (e.g., Hedges *et al.*, 2004; Yoon *et al.*, 2004).

3.3 Land Plants

Normally land plants would not even be mentioned in a volume concerning the Precambrian, because evolutionary biologists have long considered that these organisms arose in the Phanerozoic. However, a molecular clock analysis of 54 proteins (5526 amino acids) found that the divergence of mosses (bryophytes) and vascular plants occurred 703 ± 45 Ma (Heckman *et al.*, 2001). Presumably the common ancestor of those two groups was a land plant, providing a minimum estimate for the colonization of land by plants. Subsequently, those data were analyzed further with local clock methods, including Bayesian and likelihood rate smoothing, and a similar date was obtained (707 ± 98 Ma; 95% confidence interval, 515–899 Ma) (Fig. 3) (Hedges *et al.*, 2004).

In contrast, a separate analysis of 27 plastid genes (Sanderson, 2003) resulted in a younger time estimate for the origin of land plants. The time estimate was presented as a range (425–490 Ma) although it was in reality two point estimates: 425 Ma using a global clock approach and 490 Ma using a local clock (likelihood rate smoothing). The errors or confidence intervals on those point estimates were not presented, but logically they must have extended older than 490 Ma and younger than 425 Ma. Nonetheless, it was concluded that “the nearness of these molecular age estimates to the first fossil evidence for land plants contrasts sharply with the results of Heckman *et al.* (2001).” However, a better agreement with the fossil record does not necessarily mean that those younger dates are closer to the true time of divergence.

One major difference in these two studies, not mentioned in the second (Sanderson, 2003), was that different lineages of basal land plants were compared: mosses in one case (Heckman *et al.*, 2001) and liverworts in the other (Sanderson, 2003). Because the relationships of the major clades of land plants (mosses, liverworts, hornworts, and tracheophytes) are not yet resolved (Hedges, 2002), it is not known whether the two studies addressed the same evolutionary divergence, except that in both cases the common ancestor was presumably a land plant. However, an even more significant difference in these two studies was in the calibrations used. The first study used an external calibration (1576 Ma for the plant-animal-fungi divergence) whereas the second study (Sanderson, 2003) used internal calibrations (330

Ma for the angiosperm-gymnosperm divergence and 125 Ma for "crown group eudicot angiosperms").

The external calibration of the first study was from an earlier molecular clock estimate (Wang *et al.*, 1999), derived in turn from an unusually robust vertebrate fossil calibration (Hedges *et al.*, 1996; Kumar and Hedges, 1998; Hedges and Kumar, 2004; van Tuinen and Hadly, 2004). As detailed in the previous section, a 1500–1600 Ma split of plants, animals, and fungi has been a relatively consistent result of several recent molecular clock studies using large numbers of genes, and is constrained by a 1200 Ma fossil (Butterfield, 2000). In contrast, the internal calibration used in the second study (Sanderson, 2003) is less robust for several reasons: (1) it is based primarily on the fossil record of only one of the two daughter lineages (stem group conifers), (2) the early evolution of the angiosperm clade (the other lineage) has been controversial from a phylogenetic standpoint and may have tens of millions of years of missing (or unidentified) fossil record (Stewart and Rothwell, 1993; Doyle, 1998; Crane *et al.*, 2004), and (3) new discoveries are extending the early fossil record of stem seed plants by tens of millions of years (Gerrienne *et al.*, 2004) indicating caution in interpreting the current fossil record of seed plants as being complete. For these reasons, molecular clock studies have often chosen the angiosperm-gymnosperm divergence as a time to estimate rather than calibrate (e.g., Savard *et al.*, 1994; Goremykin *et al.*, 1997; Soltis *et al.*, 2002). Although the title of the Sanderson (2003) study was “molecular data from 27 proteins do not support a Precambrian origin of land plants,” those data and analyses would result in a Precambrian time estimate if the calibration date were only 11% older (366 Ma instead of 330 Ma).

Finally, new fossil discoveries of the earliest land plants are bringing the group closer (within ~30 Ma) to a Precambrian origin without help from molecular clocks. Recently land plant megafossils have been discovered from the Ordovician (Wellman *et al.*, 2003) and early Middle Cambrian (Yang *et al.*, 2004), which predate the previously oldest megafossils of land plants by about 50–80 Ma. Fossil spores suggested to be of land plants have been known from the Cambrian (Strother and Beck, 2000) and Ordovician (Wellman *et al.*, 2003), but were controversial, and therefore these new megafossils provided support to the interpretation that these fossil spores are of land plants.

3.4 Fungi

Like plants, fungi are not often mentioned in discussions of life in the Precambrian. However, fungi have generally fewer morphological characters that are taxonomically useful and a poorer fossil record than animals or

plants, and therefore there is less of a tendency to interpret the fungal fossil record in a literal sense. Although an early molecular clock analysis of fungi using the small subunit rRNA gene (Berbee and Taylor, 1993) obtained relatively young times of divergence, close to fossil record times, an updated analysis using a refined calibration (Berbee and Taylor, 2001) showed deeper divergences, in the Proterozoic. Likewise, a time estimation analysis of up to 88 protein-coding genes (Heckman *et al.*, 2001; Hedges *et al.*, 2004) and another analysis of the rRNA gene (Padovan *et al.*, 2005) have also found Precambrian divergences among fungi (Fig. 3).

As is typical in comparing the results of molecular clock studies, variation in time estimates usually can be ascribed to the use of different calibrations. For example, differences between the early rRNA studies (Berbee and Taylor, 1993, 2001) and the study of multiple proteins (Heckman *et al.*, 2001) might at first be thought to relate to the different data sets, but the calibrations used were quite different. When rRNA data (Padovan *et al.*, 2005) were analyzed using the same calibration as in the protein study, 1576 Ma for the split of animals and fungi, the time estimates of the two studies were in relatively close agreement.

One particularly useful fossil constraint for fungi is a Devonian (~400 Ma) sordariomycete ("pyrenomycete"), an ascomycotan (Taylor *et al.*, 1999). Because the next youngest sordariomycete is considerably younger, and because the Rhynie Chert is an example of exceptional fossil preservation, there is no reason to assume that the sordariomycete lineage diverged from other fungi immediately prior to 400 Ma. Instead, it is best to interpret that calibration point (or constraint) as a minimum time of separation. However, in either case (minimum or as a fixed calibration), this fossil calibration still results in time estimates of fungal divergences deep in the Proterozoic (Heckman *et al.*, 2001; Padovan *et al.*, 2005).

The higher-level relationships of fungi have not yet been fully resolved (Hedges, 2002; Lutzoni *et al.*, 2004), and therefore it is not surprising that the various time estimation studies also differ somewhat in their estimates of relationships. Nonetheless, chytrids (Chytridiales) appear to be the most basal group of living fungi, diverging from other fungi ~1400 Ma (Fig. 3). The other major lineages of fungi, including the two largest groups—Ascomycota and Basidiomycota—are estimated to have split ~900–1000 Ma (Hedges *et al.*, 2004; Blair *et al.*, 2005). However, the relationships of those lineages, and of several that may have arisen even earlier (Blastocladales, Glomeromycota, and "Zygomycota") remain unresolved (Heckman *et al.*, 2001; Hedges, 2002; Lutzoni *et al.*, 2004; Padovan *et al.*, 2005).

With so many major lineages of fungi appearing hundreds of millions of years prior to the Phanerozoic, the virtual absence of Precambrian fungal fossils has been surprising. It has been suggested that such fossils do exist in

collections, but have been misidentified. For example, much of the late Precambrian Ediacara “fauna” has been interpreted alternatively as representing marine lichens or fungi (Retallack, 1994; Peterson *et al.*, 2003). More recently, Precambrian fungal fossils have been described by two other groups. In one case, fossils dated to ~850 Ma and 1450 Ma have been identified as being “probable fungi” (Butterfield, 2005), and in the other, fossils from phosphorite and dated to 551–635 Ma were interpreted as lichenized fungi (Yuan *et al.*, 2005). Such fossils fall short of documenting a diversity of Precambrian fungi implied by the time estimation studies, but they support the contention that fungi existed in the Precambrian and lay the ground for future studies of Precambrian fungi.

3.5 Animals

Estimating divergence times among animal phyla has been confounded by an incomplete understanding of phylogenetic relationships within the kingdom. In recent years, a new phylogeny of animals has been proposed, based predominantly on small subunit ribosomal RNA sequence analyses, that divides the bilaterally-symmetric animals (bilaterians) into three main groups: deuterostomes, edysozoans, and lophotrochozoans (Aguinaldo *et al.*, 1997; de Rosa *et al.*, 1999; Mallatt *et al.*, 2004). Despite a lack of strong statistical support for Ecdysozoa, this new animal phylogeny has had a large influence on studies of metazoan evolution and development, and has led some researchers to suggest that the last common ancestor of the bilaterians may have been a complex organism (Balavoine and Adoutte, 2003). Other studies using larger numbers of genes have supported and refuted certain aspects of this new phylogeny (Blair *et al.*, 2002; Wolf *et al.*, 2004; Philip *et al.*, 2005; Philippe *et al.*, 2005). Perhaps most controversial has been the position of nematodes (round worms) and platyhelminths (flatworms). These two phyla lack true body cavities and are traditionally placed basal to most other bilaterian phyla. Molecular evidence has mostly supported the elevation of platyhelminths (excluding Acoela) into protostomes (specifically within Lophotrochozoa), but there is currently no consensus as to the position of nematodes.

A number of studies over the past four decades have used molecular clocks to time divergences among animal phyla (e.g., Brown *et al.*, 1972; Runnegar, 1982a; Wray *et al.*, 1996; Hedges *et al.*, 2004; Blair *et al.*, 2005). Such analyses have consistently indicated deep origins for animal phyla (~800–1200 Ma), much earlier than predicted by the fossil record (i.e., Cambrian Explosion, ~520 Ma). Recently, some studies have proposed substantially younger molecular time estimates (Aris-Brosou and Yang, 2002, 2003; Douzery *et al.*, 2004; Peterson *et al.*, 2004; Peterson and

Butterfield, 2005). These studies claimed that through careful consideration of potential biases in both rate modelling and calibration, they produced molecular divergence times that were consistent (or more so) with the fossil record. However, upon closer inspection, these studies suffer from methodological biases that cast doubt on their results.

Most recent molecular clock analyses (e.g., Douzery *et al.*, 2004; Hedges *et al.*, 2004; Blair and Hedges, 2005a; Blair *et al.*, 2005) have used sequences concatenated from multiple genes, thus avoiding potential statistical biases from averaging multiple single-gene estimates (the "mean of the ratios" problem). However, the criticism (Rodríguez-Trelles *et al.*, 2002) that previous studies (e.g., Wang *et al.*, 1999) were biased in that manner is incorrect, because those studies addressed asymmetry in distributions of time estimates by using medians and modes, and eliminating outliers. Even without such corrections, the simulations of Rodríguez-Trelles *et al.* (2002) showed that there was relatively little bias under most normal conditions (parameters). Also, the results from concatenated-gene studies (e.g., Hedges *et al.*, 2004) corroborated the results of those earlier studies (e.g., Wang *et al.*, 1999), indicating that such statistical biases are not responsible for old (~1 Ga) divergence time estimates among animal phyla.

Differences in how fossil calibrations are applied probably explain most of the variation in time estimates among studies. As discussed above (section 3.2), one study estimating animal divergence times (Douzery *et al.*, 2004) used fossil time constraints that were substantially younger than the fossils themselves, producing artificially younger time estimates. In some cases (Peterson *et al.*, 2004; Peterson and Butterfield, 2005), younger divergence times among animals were attributed to the use of calibrations from the invertebrate fossil record, rather than from vertebrates. However, other studies have also used invertebrate fossil calibrations and did not recover such young divergence times among animal phyla (Hedges *et al.*, 2004; Pisani *et al.*, 2004; Blair and Hedges, 2005a). Also, the young times found by Peterson and Butterfield (2005) are likely the result—in large part—of their decision to use molecular differences for timing without any statistical correction for hidden substitutions (multiple hits).

A related methodological issue involves the estimation of evolutionary rates among lineages. Two recent studies claimed that evolutionary rates were higher during the time of the Cambrian Explosion, which when accounted for in rate models allowed for younger divergence times to be recovered (Aris-Brosou and Yang, 2002, 2003). However, simulations (Ho *et al.*, 2005) have suggested that this higher rate was an artefact of the particular method used in those two studies. Other problems associated with rate modelling in those studies have been discussed elsewhere (Blair and Hedges, 2005a).

Finally, although we have noted (above) possible explanations for why these recent molecular studies (Aris-Brosou and Yang, 2002, 2003; Douzery *et al.*, 2004; Peterson *et al.*, 2004; Peterson and Butterfield, 2005) have erred in their analyses, we wish to draw attention to a simple criticism that applies generally. They fail a basic test of consistency because they yield time estimates that are contradicted by the fossil record. For example, the Douzery *et al.* (2004) study estimated that the origin of various groups of algae (e.g., red, green) was hundreds of millions of years after their first fossil occurrences (see Section 3.2). When additional taxa were added to the data set of Aris-Brosou and Yang (2003), the divergence of animals and plants was found to be 671 Ma, nearly a half-billion years younger than the fossil constraint for that divergence (1200 Ma). In the other studies (Peterson *et al.*, 2004; Peterson and Butterfield, 2005), the relevant data were not assembled by those authors to conduct such a consistency test. Considering this, the relatively small size of that data set, and especially the lack of statistical corrections for multiple substitutions, these results likewise are placed in question.

The consistency test demonstrates that those studies are biased to produce young dates and therefore those time estimates are unreliable. Thus if any of these young time estimates for animal evolution are to be seriously considered, it is incumbent upon those authors to explain why their results are not consistent with other aspects of the fossil record. Not considering these aberrant results, molecular clocks continue to support a long history of animal evolution in the Proterozoic (Fig. 3).

4. ASTROBIOLOGICAL IMPLICATIONS

4.1 Complexity

It is logical to assume and expect that life begins in a simple state of organization and, through natural selection, develops greater complexity. For several reasons it is of interest to astrobiologists to know if there is any general and predictable pattern to this rise in complexity, because it would bear on our expectations of the existence of complex life (e.g., animal life) elsewhere in the Universe. For example, if the rise in complexity occurs quickly and easily, the probability that complex life occurs elsewhere is much higher than if it takes billions of years to develop complex life. Ward and Brownlee (2000), using this logic (in part), concluded that complex life is rare in the universe even though simple (prokaryote-like) life may be common.

The conclusion that complex life takes a long time to develop was based on a literal reading of the fossil record, which shows that most animal phyla first appeared in the earliest Phanerozoic, the Cambrian Explosion, nearly four billion years after the Earth was formed. However, most molecular phylogenies and timescales in the last four decades have indicated a deeper (Proterozoic) origin for the major groups of animals, as discussed above (Section 3.5). Also, an earlier origin of plants, fungi, and the major lineages of protists has been estimated (Fig. 3). But how does this new information bear on the rise in complexity? Biological complexity can be defined in many ways, including shape, size, number of cells, and number of genes, among many possibilities. However, the most common measure used to compare complexity across all of life is the number of cell types (Bonner, 1988; Bell and Mooers, 1997; McShea, 2001). Using this measure, and by estimating the number of cell types of common ancestors in the timetree of life (Figs. 1–3), it is possible to construct a contour for the rise in complexity of life on Earth (Hedges *et al.*, 2004) (Fig. 4). This shows that complexity began to rise much earlier in time, roughly 2–2.5 billion years after the Earth was formed. The animal grade of complexity then rose more rapidly (>10 cell types) between 1000–1500 Ma, not 500–600 Ma as predicted by a literal reading of the fossil record.

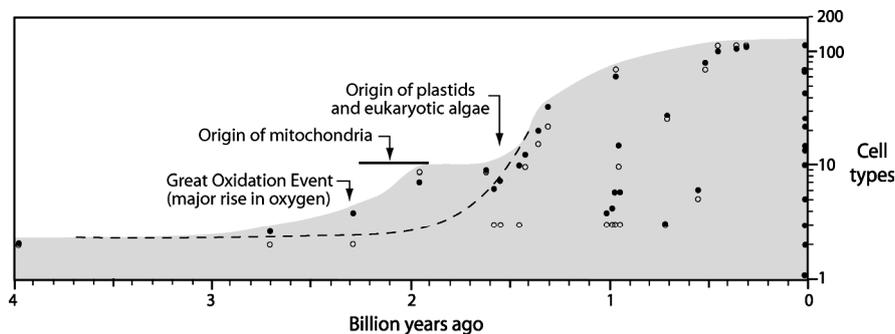


Figure 4. Increase in the maximum number of cell types throughout the history of life (after Hedges *et al.*, 2004). Data points are from living taxa (time zero) and common ancestors (earlier points) estimated with squared-change parsimony (solid circles) and linear parsimony (hollow circles) using a molecular timetree (Hedges *et al.*, 2004). The dashed line shows an alternative interpretation based on uncertainty as to the level of complexity of ancestors of early branching eukaryotes.

Perhaps a key factor in this rise of complexity was the major rise in oxygen in the early Proterozoic (~2300 Ma) (Holland, 2002). The mitochondrion appeared soon thereafter (Hedges *et al.*, 2001; Hedges *et al.*, 2004), allowing eukaryotes to gain much more energy in cellular respiration compared with glycolysis. This symbiotic event (Great Respiration Event) is estimated to have occurred 2300–1800 Ma (Fig. 4).

That additional energy source may have provided the fuel for the rise in complexity, feeding the associated energy requirements (e.g., cell-signalling, mobility, etc.). The addition of the plastid at 1500–1600 Ma, through a symbiotic event with a cyanobacterium, then gave eukaryotes the ability to produce oxygen. This was the beginning of eukaryotic algae and almost certainly led to an increase in eukaryotic diversity and biomass. The parallel diversification of animals and fungi after 1500 Ma are likely related to this Great Algification Event.

Returning to the original question, these new insights from molecular clocks increase the probability that complex life exists elsewhere in the universe because the time required for complexity—in our single example on Earth—is less than previously thought. If the rise in complexity is tied to an energy source such as oxygen, as suggested, then a further consideration must be the time required to evolve the biological machinery for producing that energy (oxygenic photosynthesis or some other process).

4.2 Global glaciations

The defining aspect of the Neoproterozoic, in terms of the planetary environment, appears to have been the multiple episodes of global glaciations (Hoffman *et al.*, 1998). Certainly, the freezing of the entire Earth, or most of it, is an event that must have had a major impact on life at that time. As this review has shown, all of the major groups and subgroups of prokaryotes living today must have experienced these glaciations (Fig. 1), as well as did a great diversity of eukaryotes (Fig. 3). At least three global glaciations have been identified, at ~713 Ma, ~636 Ma, and ~580 Ma (Hoffmann *et al.*, 2004), with each presumably lasting 10^5 – 10^7 years and triggered by either a geological or biological mechanism.

The geological trigger (Hoffman *et al.*, 1998) involves the long term carbon cycle and an unusual configuration of the continents. The long term carbon cycle normally provides a buffer for major shifts in temperature. Erosion of land exposes silicate minerals (e.g., CaSiO_3) and releases calcium, which combines with carbon dioxide in rainwater to form limestone. Return of the carbon to the atmosphere is delayed for millions of years until released by volcanism associated with subduction. If erosion is accelerated, then the carbon dioxide levels in the atmosphere are lowered, hence lowering the temperature. If erosion is slowed, as when land areas are covered with ice, atmospheric carbon dioxide increases and surface temperature increases. The presence of continents at high latitudes improves the buffering mechanism because they freeze over more quickly and provide an early brake on any tendency toward global cooling.

This geological trigger model proposes that it was the unusual configuration of the continents clustered at low latitudes (Rodinia) that led to the global glaciations (Hoffman *et al.*, 1998; Schrag *et al.*, 2002). An absence of high latitude continents removed the “early brakes” for global cooling and permitted ice sheets to extend further towards the equator than usual. Models show that if ice sheets (reflecting energy away from Earth) extend below $\sim 30^\circ$ north and south latitude they will quickly reach the equator (Snowball Earth) from a runaway albedo effect. Millions of years of volcanism, releasing carbon dioxide, may have been required to raise the temperature sufficiently to escape from a Snowball Earth (Hoffman *et al.*, 1998).

The biological trigger model (Heckman *et al.*, 2001; Hedges, 2003) also involves the long term carbon cycle but proposes that the critical excursions in rates of erosion came from biological sources, not the configuration of continents. Those biological sources probably were the fungi (including lichens) and land plants that evolved and colonized the land in the Proterozoic, although prokaryotes (e.g., cyanobacteria) also may have occupied land areas and may have contributed. The enhancement of weathering by organisms is well-established, and lichens can increase rates 10–100 times by themselves (Schwartzman and Volk, 1989; Schwartzman, 1999). Even prior to the elaboration of the Snowball Earth model (Hoffman *et al.*, 1998) and to the molecular evidence for an early origin of fungi and land plants (Heckman *et al.*, 2001), biological weathering has been considered in discussions of temperature changes in the Proterozoic (Carver and Vardavas, 1994; Retallack, 1994).

The exact configuration of the continents in the Neoproterozoic is far from certain (Meert and Powell, 2001) but the cyclic nature of the glaciations, over a long period (713–580 Ma) is not consistent with an unusual configuration of continents. On the other hand, a biological trigger may explain such cycles (Heckman *et al.*, 2001; Hedges, 2003). During each glaciation, most life on land would have been eliminated, followed by a post-Snowball recovery period that included expansion and diversification of life, and increased weathering leading to the next Snowball. If the carbon isotope excursion at the Precambrian–Cambrian boundary is included as a fourth event, even though it did not appear to be a full Snowball Earth, the time between each event appears to have decreased: 78 my (713–635 Ma), 55 my (635–580 Ma), and 37 my (580–543 Ma). The significance of such a decrease is unknown, but could be interpreted as an increasing development of the land biota, reducing the recovery time between successive glaciations.

4.3 Oxygen and the Cambrian explosion

A simple explanation for the Cambrian Explosion is that it records, in a literal sense, the evolutionary diversification (phylogenetic branching) of animal phyla in the latest Proterozoic and early Cambrian (Gould, 1989; Conway Morris, 2000). Alternatively, if the molecular time estimates showing a deep origin of animal phyla in the Proterozoic (e.g., Fig. 3) are correct, then an explanation is required as to why we see an explosion of fossils in the Cambrian, and almost nothing before that time. A leading theory is that the Cambrian Explosion reflects a major rise in oxygen levels (e.g., Nursall, 1959; Cloud, 1976; Runnegar, 1982a; Canfield and Teske, 1996). Oxygen would have been a limiting factor for body size and the production of hard parts (Rhoads and Morse, 1971; Bengtson and Farmer, 1992; Bengtson, 1994), although Ca^{2+} levels may also have been important (Brennan *et al.*, 2004). A corollary of this theory is that the early representatives of animals in the Proterozoic were small and soft-bodied (although still complex, with many cell types; see above), explaining why they have been difficult to identify in the fossil record. Although the exact timing of the increase in oxygen is not yet established, most agree that it happened in the Neoproterozoic, and probably in the latter half of that time period (Knoll, 2003; Canfield, 2005).

Recently, it was proposed that this Neoproterozoic rise in oxygen, as a trigger of the Cambrian Explosion, was caused by the colonization of land by organisms, principally fungi and plants (Heckman *et al.*, 2001; Hedges, 2003). Specifically, enhancement of weathering and burial of terrestrial carbon were mentioned as potential mechanisms. This model has been elaborated further to show that selective weathering of phosphorus also can accomplish a rise in oxygen (Lenton and Watson, 2004). Further research is needed to constrain the timing of the rise in oxygen and to search for fossil evidence of a Neoproterozoic land flora predicted by molecular analyses.

5. CONCLUSIONS

Knowledge of the phylogeny and times of origin of major groups of organisms in the Neoproterozoic would help answer many questions about the rise in complex life and its interaction with the planetary environment. Although the fossil record will improve with time, and it is the only source of information for extinct groups, it is unlikely to ever provide that knowledge by itself because of the vagaries of preservation. In contrast, such information is currently being obtained from the genomes of organisms, and molecular timetrees will only improve in the future as more genomes are

sequenced and analytical methods are refined. Nonetheless, it remains to be seen whether the current large and controversial gaps in the Proterozoic fossil record, implied by molecular timescales, will be reduced. The best evidence at present indicates that those gaps are real. Therefore, while it is true that the fossil record gives us some brilliant windows into the past, we should also expect a few dark hallways.

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