Is something wrong with the tree of life?

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Summary

A recent study[1] of sequence data from many different proteins has suggested that contemporary prokaryotes and eukaryotes may have shared a common ancestor as recently as 2 billion years ago (the molecular clock). Strong evidence from the geological record, however, indicates that oxygen-producing microorganisms, perhaps similar to modern cyanobacteria, existed 3.5 billion years ago. The fossil evidence, therefore, suggests that any common ancestor of prokaryotes and eukaryotes must have existed at least 1.5 billion years earlier than suggested by the molecular clock evidence. The discrepancy between molecular and geological evidence for the age of modern cells is considered here, as are aspects of gene descent in the tree of life that might help to account for it.

Introduction

The last common ancestor of contemporary forms of life is generally believed to have existed about 3.5 billion years (Gyr) ago. That date, however, came under scrutiny recently with a report[1] in which the divergence times of eukaryotes, eubacteria and archaebacteria were estimated on the basis of amino acid sequence divergence for conservatively evolving proteins. A molecular clock for 57 enzymes was calibrated by plotting average sequence distance against the geological age of vertebrate groups whose divergence times are known from the fossil record. The reasonably constant rate of amino acid substitution found over the last 550 million years was then extrapolated into the depths of the Precambrian period, where cellular evolution has left relatively few traces in the fossil record. Divergence times for various unicellular organisms were inferred from the protein distance data. Very surprisingly, the analysis by Doolittle *et al.*[1] suggested that the last common ancestor (LCA) of all contemporary life existed only about two billion years ago. That finding, if true, would require thorough reevaluation of generally accepted ideas about early cellular evolution, which involve an assumption that the dates of divergence between these organisms were roughly twofold more ancient. The report activated considerable controversy[2,3] because it raised basic questions concerning the compatibility of what we know about early evolution from the geological record with what we infer from molecular data. The discrepancy needs to be addressed.

Geological and molecular evidence

Could it be that the first cells actually evolved only 2 Gyr ago? Why do we believe that life is about 3.5 billion years (Gyr) old in the first place? Three very strong lines of independent evidence from the geological record date the origins of life to within the first billion years of the earth's 4.5 Gyr history (Fig. 1a). The first of these is oxygen, contained as iron oxide in banded iron formations, which were deposited during the period from ~3.5 to ~2 Gyr ago. This is generally (but not universally, see ref. 4) accepted as evidence for the biological production of molecular oxygen in the oceans[5-7], because the atmosphere was oxygen-free during that time. About 2 Gyr ago, free oxygen started accumulating in the atmosphere, as indicated by the appearance of oxidized sediments of continental origin[7,8] and microfossils, which probably represent eukaryotic cells, appeared shortly thereafter[7,9]. Since the only known marine source of molecular oxygen is photosynthesis, with two photosystems as found in cyanobacteria (and plastids), a strong argument can be made that cyanobacteria were thriving 3.5 Gyr ago[10]. Secondly, the 3.8 Gyr δ-^{13}C record of organic carbon deposits provides evidence for a long, continuous history of biological CO_2 fixation[11]. Thirdly, the fossil record provides a continuum of cyanobacteria-like microfossils and stromatolites (fossilized bacterial mats) dating back into strata 3.5 Gyr of age[10,12]. We can thus be reasonably sure that CO_2-fixing, oxygen-evolving microorganisms with sizes and shapes strikingly similar to modern cyanobacteria arose and flourished within about 1 Gyr after the Earth's origin. But *were* they cyanobacteria?

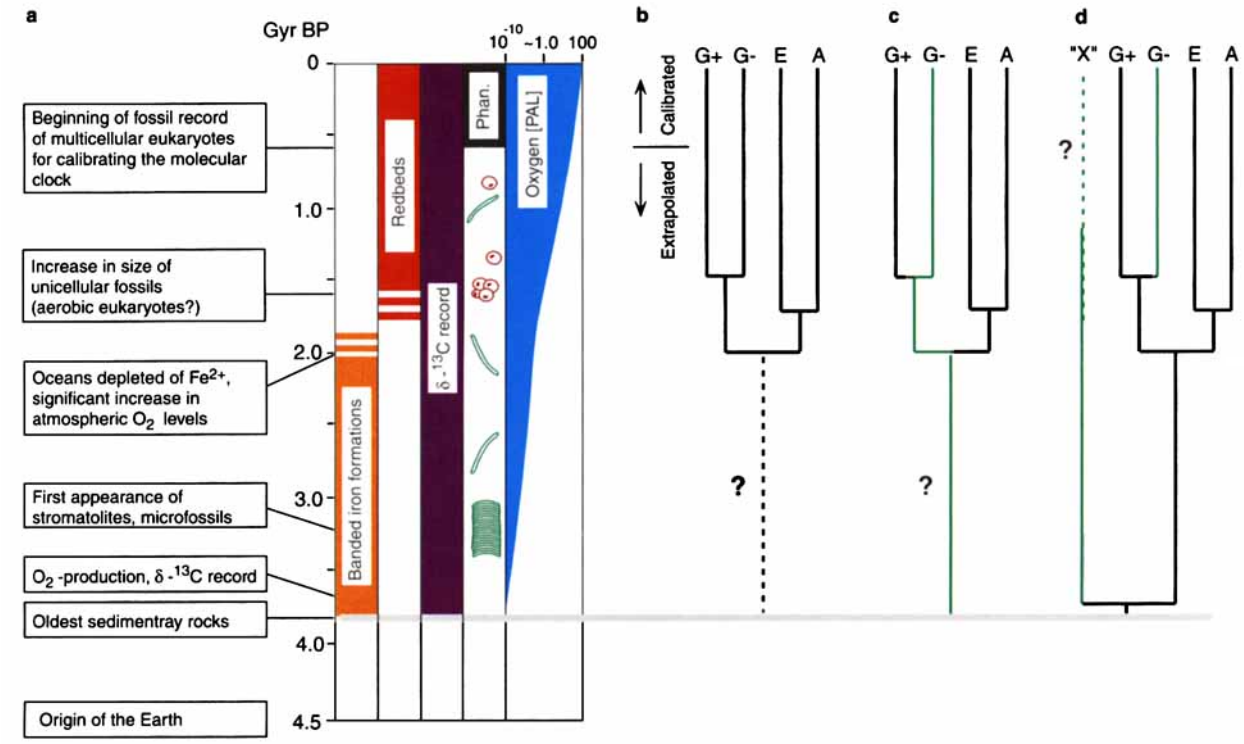

Fig. 1. Schematic comparison of the early history of life reflected in geological data with a recent study based on protein sequences. (a) Geological time scale with cardinal events in early evolution indicated. Coloured vertical panels reflect the continuity over time of indicators for O_2 production in the oceans (banded iron formations), O_2 appearance in the atmosphere (redbeds), biological CO_2 fixation (δ-^{13}C record), fossil life forms and the possible course of O_2 accumulation in the atmosphere as a percentage of present atmospheric levels [PAL]. Data are taken from refs 5-12, see also text. Phan, Phanerozioc. (b) Time scale for the evolution of contemporary life forms as suggested by the molecular clock in several proteins[1]. The segment of molecular evolution that can be directly correlated (calibrated) to the fossil record, and that which is inferred by extrapolation, are shown. The dotted line indicates the 2 Gyr (approx.) discrepancy between geological evidence for the age of life (3.8 Gyr) and the molecular estimate for the age of contemporary living things (2.0 Gyr). (c) Assuming that the molecular estimate is approximately correct, one possible explanation for the discrepancy is that the Last Common Ancestor was cyanobacteria-like, or at least had oxygenic metabolism, as indicated by the continuous green branch. This view turns much of what we believe about the evolution of metabolism upside down and for numerous reasons is unlikely to be true. Perhaps the most unacceptable problem with this view is that more 'primitive' (anoxygenic) forms of phyotosynthesis and strictly anaerobic forms of non-photosynthetic metabolism observed in extant cells would then have been derived from a much more complex and 'advanced' ancestral state, irreconcilable with traditional and modern views, both on the evolution of photosynthesis[13] and on the evolution of metabolism in general. G+, gram positive eubacteria; G-, gram negative eubacteria (includes cyanobacteria and α-proteobacteria, the probable ancestors of mitochondria); E, eukaryotes; A, archaebacteria. (d) Another possible explanation, assuming that the molecular estimate is approximately correct, is that the organisms that produced oxygen 3.5 Gyr ago ('X') were not members of the family of living organisms that we know today, and oxygenic photosynthesis evolved twice. This scenario in many ways represents the easiest and most convenient solution to the problem: we just 'invent' an imaginary form of life that oxidizes the planet and disappears, allowing us to hold on to comfortable views. Until clear evidence is presented that 'X' has ever existed, such conjecture is completely untestable and should be categorically rejected for that reason.

Unfortunately, ribosomal RNA phylogeny – the paradigm for early evolution – does not provide a direct answer to this question. This is because global rRNA trees have their own problem with cyanobacteria, in that the branches bearing these oxygen photosynthesizers are very short and occur very near the crown, rather than near the 'root' of the tree (see e.g. refs 14 and 15, but also ref. 16), suggesting at face value that the cyanobacterial lineage arose recently in evolution. If we accept (as most people do) that the miniscule length of cyanobacterial rRNA branches reflect approximately 3.5 Gyr of evolution, then in order to force the rRNA tree of life into compatibility with geological history, we have to drastically bend, stretch and compress its remaining branches, adding numerous corollary assumptions involving cataclysmic rate acceleration and deceleration along exactly those lineages where it is needed. Thus we can reconcile rRNA phylogeny with the geological record by assuming rate variation, but by itself the rRNA tree does not provide direct evidence for the existence of cyanobacteria 3.5 Gyr ago. We are then left with the problem that if all life can be traced to a common ancestor that is only about 2 Gyr old, as the protein study suggests, then it can hardly have been members of the cyanobacteria as we know them today that were photosynthesizing 3.5 Gyr ago, can it? That is the crux of the matter, and the starting point for some new questions.

Some will ask: 'Could it be that all contemporary forms of life descend *via* direct filiation from cyanobacteria-like forefathers', as sketched in Fig. 1c (and outlined in the legend)? Others will ask: 'Did some highly successful group(s) of

organisms – an outgroup to all contemporary life forms – exist for eons and produce the roughly 10^{15}-10^{16} tons of oxygen now found in sedimentary rocks, only to suffer subsequent extinction, forever obscuring molecular evidence of its existence?' (Fig. 1d and legend). Critics unwilling to entertain such possibilities will ask: 'Where is the molecular evidence that dismisses these alternatives and other similarly radical and revolutionary ideas about early evolution as untenable?' But for many the first question will be: 'Does the protein estimate withstand critical inspection?'

Stretching molecular evolution beyond fossil landmarks

Several factors inherent to molecular clock approaches that might yield lower divergence times for the domains[14] of life have already been briefly considered[2,3]. These include the possibility that the substitution rate for the proteins studied was conceivably lower during the Precambrian period in all lineages sampled, or that the divergence times used to calibrate the clock are slighter than the actual values. But there is no clear evidence to suggest that this is the case. So even with the assumption that the substitution rate was as constant 2 Gyr ago as it demonstrably was 0.2 Gyr ago, we can ask whether any rate-independent factors can be pinpointed that would produce a molecular underestimation of the age of the last common ancestor.

One of these has already been touched upon[3], namely the possibility that the approaches used to measure distances between proteins – and hence between organisms – systematically underestimate divergence towards the universal root. There are justifiable grounds for believing that this is indeed a problem. When ticking properly, molecular clocks (both in proteins and in DNA) do so in proportion to the number of substitutions (d) that have accumulated in two sequences since their separation[17]. Estimating divergence times thus requires reliable calculations of d; if that value is underestimated, so will be the divergence time inferred from it. But estimating d from the number of observable differences (p) between two sequences is much more

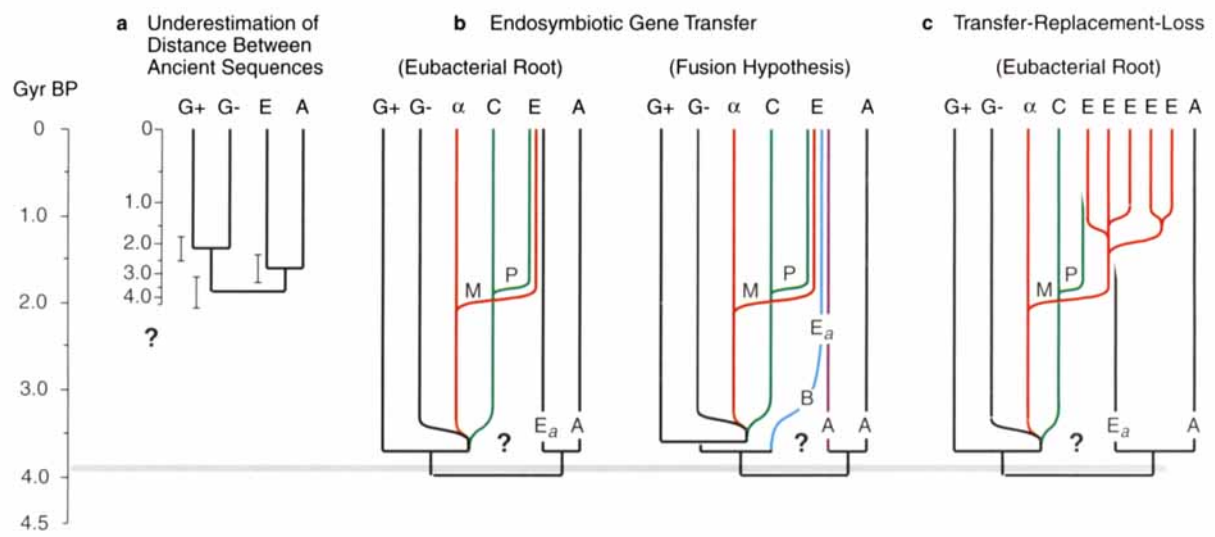

Fig. 2. Schematic depiction of two of numerous possible explanations for the discrepancy between molecular and geological estimates for the age of life. (a) Underestimation of evolutionary distance between sequences can potentially result from current substitution models. If so, underestimation is likely to become increasingly more severe with increasing divergence, 'compressing' the apparent time scale extrapolated from fossil calibration points. (b) Endosymbiotic gene transfer is known to introduce eubacterial genes into nuclear chromosomes. Several genes that we suspected had always resided in eukaryotic genomes have recently been shown, surprisingly, to derive from endosymbionts, as indicated by red and green branches in the eukaryotic lineage. Such genes reflect divergence between endosymbionts and their free-living eubacterial relatives. If not recognized, dates for eubacterial-eukaryotic divergence based upon data sets that include many such genes will be too recent. The endosymbiotic gene transfer scenario is sketched for two widely held theories for the origin of the amitochondriate eukaryotic lineage, which is assumed to have served as the recipient of organelles. Note however that the possibility that perhaps no eukaryotic lineage is primitively amitochondriate has recently been discussed (see refs 28 and 29). If that is true, then the lineages representing E_a in the figure, though conceptually satisfying for current endosymbiotic theory, are erroneous. Left: endosymbiotic gene transfer under a eubacterial root hypothesis[30]; right: under the fusion hypothesis[31]. Also note that neither hypothesis for the origin of the host can fully account for available protein data[16]. Paralogy for ancient genes is excluded here for convenience, but it is a widespread problem in real data. The schematic eubacterial phylogenies in (b) accept the branching order of rRNA trees as correct, but also embrace the plausible views that oxygenic photosynthesis only evolved once, that this occurred in the lineage to which living cyanobacteria belong, and that oxygen produced 3.5 Gyr ago came from photosynthesis. In order to integrate these views through projection of rRNA evolution onto the geological record, the cyanobacterial and all other terminal eubacterial branches of classical rRNA trees (cf. ref. 14) need to be conceptually 'stretched' to several times their apparent length, and all internal eubacterial branches 'compressed' by a similar factor. (c) An idealized example of endosymbiotic gene transfer from mitochondria followed by replacement and loss of the presumably preexisting 'endogenous' homologue. Subsequent to radiation of mitochondriate eukaryotes, plants are 'offered' a second copy from cyanobacteria; this copy, however, does not replace the mitochondrial copy, but rather is lost. α, α-protoebacteria; C, cyanobacteria, M, mitochondria; P, plastids of primary symbiotic origin[21]; E_a, amitochondriate eukaryotes, presumed to have served as the recipients of organelles; B, eubacterium; other designations as in Fig. 1, except that G– here indicates unspecified gram-negative eubacteria.

difficult than might appear at first sight, and many current approaches are thought to underestimate *d*. This is due in part to the fact that different positions of an alignment can evolve at dramatically different rates[18-22]. Underestimation of *d* is not considered to be a serious problem when divergence is low, but it becomes severe with increasing sequence dissimilarity[19]. The phylogenetic result of underestimation would be 'compression' of the apparent divergence of highly dissimilar sequences towards the base of the tree, because many substitutions are unobservable, even when the rate in different lineages is constant over time. As it relates to molecular dating of the tree of life, underestimation of *d* will compress the time scale with increasing divergence, as sketched in Fig. 2a. It is not known whether current substitution models underestimate as severely as suggested by the hypothetical compression in Fig. 2a, but nor is there any evidence to suggest that they do not. Furthermore, an uncertainty is attached to values of *d* in all substitution estimation models, and this standard error also increases steadily with increasing divergence[18,19], expanding the gray areas of molecular phylogeny as we go further back in time.

Endosymbiotic gene transfer – a twist in the trunk

Even if the molecular clock has ticked perfectly over the entire course of evolution, other factors can still influence estimates of divergence time for the kingdoms of life. One of the more commonly overlooked of these is endosymbiotic gene transfer: many genes in the nucleus are intruders, having been acquired from mitochondrial and chloroplast genomes. 'Endosymbiotic' gene transfer is a special case of simple 'horizontal' (lateral) gene transfer, in that it is explicitly unidirectional (eubacterium → nucleus) and occurs in the well-founded biological context of organelle origins. Horizontal gene transfer primarily explains confusing gene phylogenies[23], whereas endosymbiotic gene transfer primarily explains why organelles have retained so much of their biochemically eubacterial heritage despite having relinquished the majority of genes necessary to have done so. These were successfully transferred to the nucleus early on in evolution and the encoded proteins have either been reimported into the organelle of their genetic origin on a daily basis since, or have been functionally replaced through import of a duplicated nuclear homologue[24].

As shown in Fig. 2b, endosymbiotic gene transfer throws a monkey wrench into the phylogenetics of early evolution. This is because proteins encoded in eukaryotic chromosomes suddenly pop up on branches that belong to the eubacterial world. The resulting phylogenetic confusion is not too severe if one *expects* a given nuclear gene to stem from its donor organelle, for example in the case of nuclear-encoded proteins of the electron transport chains in chloroplasts and mitochondria. But what if we *don't* expect it? For example, have we not always confidently assumed that conservatively evolving enzymes of the eukaryotic cytosol reflect the evolution of the host nucleus? But that seemingly valid and harmless assumption may in many cases be wrong and very misleading in our attempts to understand where eukaryotes come from or how old they are. Recent studies suggest that several genes for cytosolic, glycolytic enzymes, thought for decades to represent the ancestral eukaryotic lineage, have been acquired by nuclei during the course of evolution, probably through endosymbiosis. Examples are glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), phosphoglycerate kinase (PGK, EC 2.7.2.3) and now triosephosphate isomerase (TPI, EC 5.3.1.1). The nuclear genes for cytosolic GAPDH[25,26] and TPI (P. Keeling and W.F. Doolittle, personal communication) in higher eukaryotes appear to have been donated by the ancestors of mitochondria. In the case of GAPDH, the tree is very complicated because an ancient but poorly resolved gene family exists in eubacteria, three members of which have persisted in the genomes of the cyanobacterium *Anabaena variabilis* and the proteobacterium *E. coli*. Confusingly, eukaryotic GAPDH trees sprout up on branches belonging to different members of that ancient gene family[26], and to further complicate matters, class I and class II GAPDH enzymes with virtually no sequence similarity exist in archaebacteria[27]. The nuclear gene for cytosolic PGK in plants appears to have been donated by the antecedants of plastids[27], replacing its preexisting counterpart specifically in the plant lineage. These examples from the glycolytic pathway should open our eyes to the view that for enzymes common to endosymbionts and host, each endosymbiotic event has a high likelihood of successful endosymbiotic gene transfer. And surprisingly, in the ensuing competition for survival between endogenous and intruding genes, the endogenous nuclear copy does not appear to enjoy a 'home field advantage'.

Data sets for GAPDH, PGK and TPI have several things in common: the enzymes belong to what is presumed to be one of the oldest biochemical pathways[32], they tend to occur in the same operon in several eubacteria[27], and sequences are known from each of the domains. For all three enzymes, only *after* sufficient reference sequences from eubacteria and archaebacteria were compared in detail did the eubacterial origin of the eukaryotic nuclear homologues become apparent. This underscores the need to have a broad prokaryotic sample for comparison before embracing the traditional *ad hoc* view that a given nuclear gene for an ancient protein is likely to provide evidence for the origin of the host. That view is reinforced by the surprising findings that even eukaryotes that lack mitochondria possess nuclear genes of eubacterial origin[26,33,34]; such data depict nuclear genomes as repositories of many acquired and apparently useful genes, but also blur the genetic identity of eukaryotes as having an independent evolutionary lineage.

Are eukaryotic genes of eubacterial origin like those

encoding GAPDH, PGK and TPI just rare and curious exceptions in the evolutionary history of nucleate cells, or are they the tip of an iceberg of unexpected eubacterial genes in the nucleus? That can only be answered on a gene-by-gene basis with the help of large prokaryotic sequence samples for each gene. Of the 57 data sets recently analyzed[1], only 12 contained four or more prokaryotic reference sequences and only nine contained archaebacterial homologs at all. So although those sequences were screened and purged of suspected lateral transfers prior to analysis, endosymbiotic gene transfer is easily overlooked, and the genes for GAPDH, PGK and TPI are among those that were studied[1]. Thus there certainly is some, and there might be quite a bit, of endosymbiotic gene transfer in the data that suggest a 2-Gyr age of life. For a number of reasons, it is still too early to tell where the majority of eukaryotic sequences came from[16], and one of these is the scanty eubacterial and archaebacterial lineage-sampling for individual genes.

Conclusion

So is something wrong with the tree of life, or is molecular data telling us something about early evolution in a language that we don't yet fully understand? Two rate-independent factors have been pointed out that can lead to underestimates for the age of living cells. A brief case was argued that since the geological record provides several independent lines of coherent evidence for the age of life and oxygenic photosynthesis, comparative protein data is probably easier to explain in the context of the geological record than *vice versa.* After all, the staggering speed of the evolutionary sequence from organic soup to genes to primitive anaerobic metabolism to water-splitting photosynthesis is a 'rate' problem of much more dramatic dimensions than anything that molecular sequences will ever have to offer.

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