TRENDS in
Genetics

Genomic clocks and evolutionary timescales

mRNA editing in mammals

Bacterial genes in vertebrates

Cytosine methylation and sexual conflict

Genomic clocks and evolutionary timescales

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For decades, molecular clocks have helped to illuminate the evolutionary timescale of life, but now genomic data pose a challenge for time estimation methods. It is unclear how to integrate data from many genes, each potentially evolving under a different model of substitution and at a different rate. Current methods can be grouped by the way the data are handled (genes considered separately or combined into a 'supergene') and the way gene-specific rate models are applied (global versus local clock). There are advantages and disadvantages to each of these approaches, and the optimal method has not yet emerged. Fortunately, time estimates inferred using many genes or proteins have greater precision and appear to be robust to different approaches.

Molecular clocks are based on the observation, first noted four decades ago [1], that protein and nucleotide sequence divergence between species often increases in an approximately linear fashion over time. An especially convincing example of this involves the fast-evolving influenza A virus, where nucleotide sequences have been compared between the living virus and samples frozen (halting mutation) over several decades [2] (Fig. 1a). The linear 'clock-like' pattern is evident in nonsynonymous (amino acid altering) as well as synonymous substitutions, even though the perceived SUBSTITUTION RATE (see Glossary) at nonsynonymous sites is lower because purifying selection has removed deleterious mutations.

Different genes evolve at different rates because of natural selection on gene function. Molecular clocks therefore have wide applicability across the tree of life, from recent divergences among populations (using fast evolving genes) to the deepest splits among prokaryotes and eukaryotes (using the slowest evolving genes). The ultimate utility of molecular clocks is in estimating times of divergence for species that have little or no fossil record. For this purpose, molecular clocks are typically calibrated using selected fossil dates (Box 1), but care must be exercised in this because fossils always underestimate divergence time [3]. Nonetheless, time estimates from molecular clock studies show broad agreement with the fossil record throughout the four billion year history of life (Fig. 1b).

The theory and application of molecular clocks have been controversial since they were first conceived. By far, the most debated topic concerns variation in the MUTATION RATE and

substitution rate between LINEAGES in a phylogeny [4–9]. If the rate with which a particular gene or protein evolves is not the same in all lineages, then use of a single (global) rate will result in biased time estimates. Classic examples are the continuing debates concerning nucleotide and amino acid substitution rates in mammals, where rodents (e.g. mouse and rat) are claimed to evolve faster, and hominoids (humans and apes) slower, than other mammals [4–7,9]. Other related debates concern the influence of life history (e.g. generation time, body size, metabolic rate) on the rate of substitution, and lineage-specific rate variation in the mitochondrial genome of animals [4,5,10].

The existence of rate variation among lineages does not prevent clocks from being applied because RELATIVE RATE TESTS are available to identify lineages that violate RATE CONSTANCY and diverse methods exist that can estimate time when such variability is present [11–14]. Certainly, deciphering the cause of evolutionary rate differences among lineages is of fundamental importance in molecular evolution. However, here we focus instead on new approaches and methods that have been developed to accommodate large numbers (≥ 10) of nuclear genes and proteins in molecular clock studies.
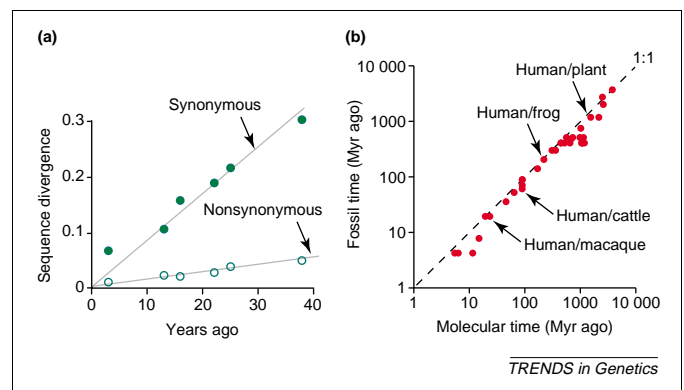
Fig. 1. Evidence of molecular clocks. (a) Demonstration of a constant rate of nucleotide substitution. Sequence data are from samples of influenza A virus frozen over several years to several decades, compared with the sequence of the living virus (modified from [2]). The linear relationship is seen in synonymous sites and nonsynonymous sites, with the latter showing a slower rate of change because of purifying selection against deleterious amino acid substitutions. (b) Agreement of molecular and fossil-record estimates of divergence time. Time estimates are from published studies using large numbers (≥ 10) of nuclear genes or proteins and robust calibrations [13,16,19,22,30,40–42] (see also legend for Fig. 6 for more details). Most data points fall below the diagonal line fixed at 1:1 because the fossil record provides minimum times of divergence, whereas molecular clocks estimate sequence change that has occurred from an earlier point when two lineages diverged.

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Glossary

Adaptive radiation: the rapid diversification of a group of species into various habitats over a relatively short period of geological time.

Lineage: a single branch, or series of connected branches, in an evolutionary tree usually leading to living species or group of species.

Mutation rate: the number of mutations occurring in germ-line cells per nucleotide site, per gene or genome, or per unit of time or cell division.

Outgroup: a species or group of species known to be outside of the group under analysis (e.g. plants or fungi would be outgroups when studying animals).

Rate constancy: the expectation of an equal number of substitutions per unit time, usually in comparisons of two or more lineages.

Relative rate tests: used in molecular clocks studies, they test rate constancy (the null hypothesis) by comparing the total amount of sequence change (number of substitutions) in two or more lineages since they shared a common ancestor. They are called "relative" because no prior knowledge of divergence time is needed.

Stringency of rate test: use of a smaller statistical cut-off value in the rate test resulting in a higher probability of Type I error (rejection of rate constancy when it is true).

Substitution rate: the rate at which a mutation is fixed in a population, either at the nucleotide or amino acid level. In almost all cases, substitution rate is lower than the rate of DNA mutation because purifying selection acts against deleterious mutations.

Type I error: rejection of the null hypothesis (in this case, rate constancy) when it is true.

Type II error: acceptance of the null hypothesis (in this case, rate constancy) when it is false.

Fig. 2. Branch length variation in a tree might not reflect underlying rate variation. Example of a tree produced from simulated data under a molecular clock model (300 amino acid residues, 5% sequence divergence per 100 million years). Letters A–H are the simulated sequences. Even under these conditions of rate constancy, where root-to-tip branch lengths are expected to be equal (a), branch length variation is observed (b) because of the stochastic nature of sequence change at the nucleotide and amino acid sequence level.

Tests of rate variation among lineages

One of the first steps in estimating time is to test for rate variation between lineages, keeping in mind that some variation is expected by chance [5,15] (Box 1; Fig. 2). Because the probability of rejecting the null hypothesis (rate constancy) is low for slow evolving and/or small genes and proteins, some rate variation could go undetected (TYPE II ERROR), possibly resulting in biased time estimates. But the STRINGENCY of the relative rate test can be increased by tightening the statistical cut-off value [16]. Although doing so will also necessarily cause some rate constant genes or lineages to be rejected (TYPE I ERROR), it
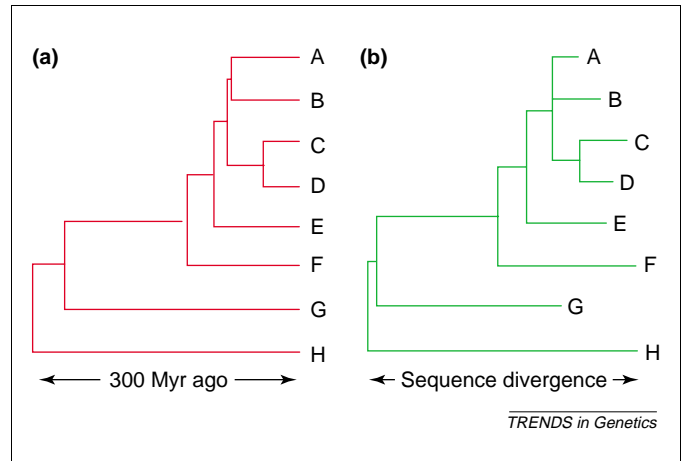
Box 1. Concepts and terms

Genomic clock methods

Current molecular clock methods fall into one or more of these four basic approaches for analysis of multiple genes or proteins: multigene global (MGG), multigene local (MGL), supergene global (SGL), and supergene local (SGL). They differ by whether genes are considered separately (multigene) or combined (supergene), and whether a single (global) or non-uniform (local) rate model is used for a given gene.

How time is estimated

With global clock methods, a rate is first calculated by estimating the amount of sequence change in two species with a particularly robust fossil record and dividing that amount by the time (calibration) of the earliest fossil of the two groups (fossils are usually dated by radiometric decay analysis of igneous rock above and below the fossil). Then, an unknown divergence time is estimated by dividing sequence change between two lineages in question by the calibrated rate. Local clock methods are similar in principle except that rates are not uniform between lineages. In practice, the various clock methods are quite sophisticated. They all require familiarity with theoretical aspects of molecular evolution (e.g. models of sequence change), the application of statistical methods (e.g. Maximum likelihood analysis, Bayesian analysis), and the biology and fossil record of the group (e.g. for rate calibration).

can be an effective way of removing rate heterogeneity. When this method was used with a dataset of vertebrates, it resulted in the rejection of a large number of proteins without much affect on the time estimates [16] (Fig. 3a). This indicates that the limited power of the rate test was not obviously causing a directional bias in average multigene time estimates (Fig. 3b).

It has been proposed that some lineages might increase in substitution rate concordantly during an ADAPTIVE RADIATION, resulting in biased time estimates [17]. If this occurred to the same magnitude in all lineages under analysis, relative rate tests would not be able to detect such correlated directional change. Although lineage-specific rate increases are seen commonly (e.g. eukaryotes compared with prokaryotes [13,18], Fig. 4), a simultaneous, genome-wide increase in nucleotide or protein substitution rate in many independent lineages is unlikely. Different genes and proteins (e.g. regulatory versus non-regulatory) would be expected to respond differently during an adaptive radiation, perhaps with no rate change expected for a large fraction of genes (e.g. glycolytic enzymes). Even if a genome-wide increase did occur independently in many lineages, relative rate tests should detect the increase when comparisons are made with distantly related species (OUTGROUPS). Moreover, a correlated rate increase in many lineages would cause a distortion in the timescale, leading to inconsistencies between molecular times and the earlier or later fossil record. In the case of avian and mammalian orders, where a coordinated rate increase has been suggested [17], time estimates before and after the period in question [late Cretaceous; 100–65 million years (Myr) ago] are mostly consistent with the fossil record, suggesting that the molecular timescale is not distorted [16,19,20].

Global clock methods

Global clock methods use a constant rate model of nucleotide or amino acid substitution in a given gene or genomic segment (not between genes). Although they are
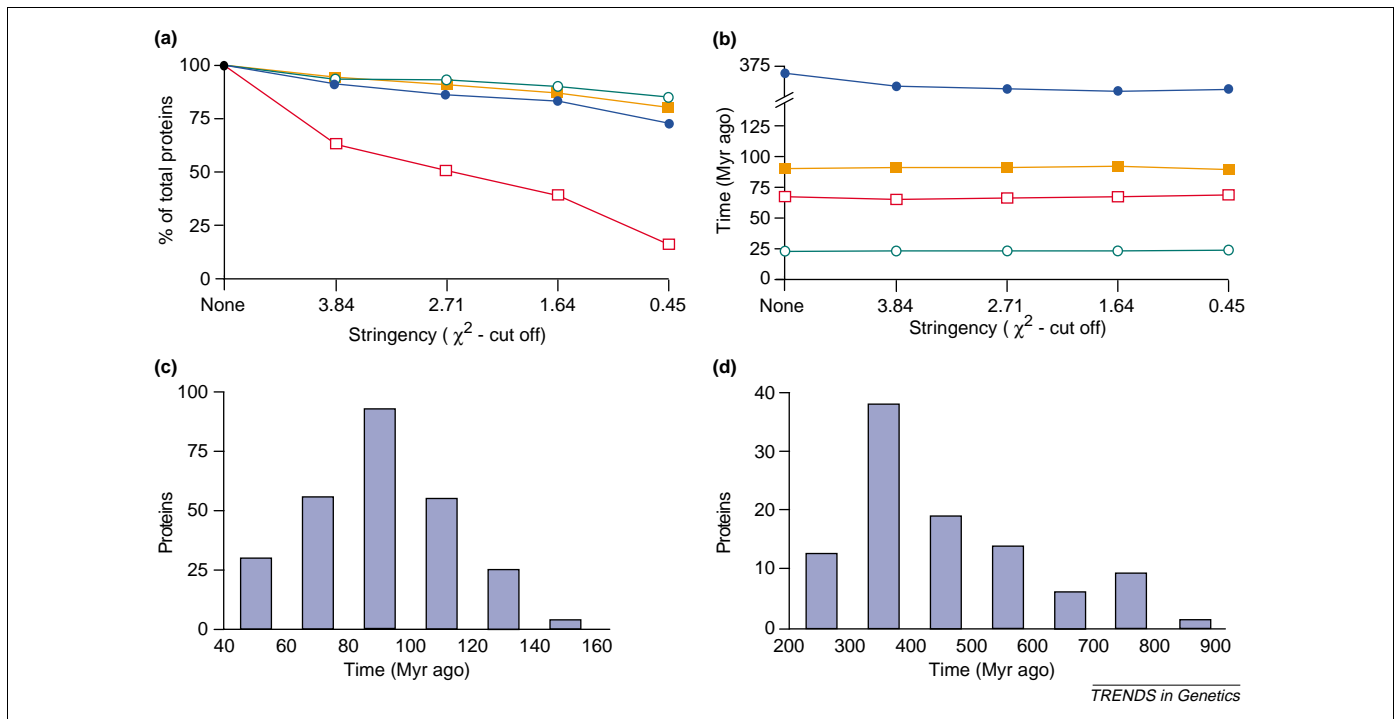
Fig. 3. Time estimates from many proteins show large variation, but possess a central tendency. Statistical characteristics of divergence time estimates from many proteins (modified from [16]). Myr, millions of years. (a) Increased stringency results in a decreasing number of proteins accepted as rate constant by the relative rate test, as expected. However, divergence time estimates are largely unaffected (b), indicating a lack of directional bias in proteins rejected by the relative rate test. Stringency is measured on the *x*-axis by the chi-square (χ^2) cutoff value indicating statistical significance, with 3.84 (5%) being the typical cutoff value and lower values (to the right) representing increased stringency. The four divergences examined are amniotes versus amphibians (blue filled circles, e.g. human versus frog; 125 total proteins), primates versus lagomorphs (orange filled squares, e.g. human versus rabbit; 124 proteins), ruminant versus suid artiodactyls (red open squares, e.g. cattle versus pig; 75 proteins), and hominoids versus Old World monkeys (green open circles, e.g. human versus macaque; 60 proteins). (c) Distribution of time estimates for the divergence of primates (e.g. human) and artiodactyls (e.g. cattle), illustrating a normal distribution (mean and mode = 92 Myr ago, coefficient of variation = 25%, 358 total and 333 rate constant proteins) and (d) the divergence of amphibians and amniotes, illustrating a right-skewed distribution resulting from outliers (mean = 441 Myr ago, mode = 360 Myr ago, coefficient of variation = 43%, 125 total and 107 constant rate proteins).

often characterized as assuming (*a priori*) rate constancy, relative rate tests are used in almost all global clock studies. Genes and lineages that are rejected in the rate tests are usually removed from later analyses if they cause an overall bias [16,19,21,22]. Each gene that is not rejected in relative rate tests can be considered to be evolving under a constant rate of substitution (the null hypothesis). The rates will, of course, differ between genes and possibly between species not examined.
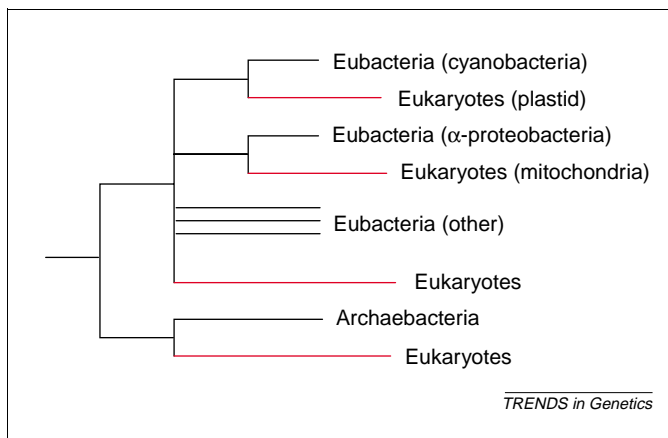
Fig. 4. Eukaryotes consistently evolve faster than prokaryotes. The tree indicates the four general locations where eukaryotic protein sequences typically cluster in the evolutionary tree of prokaryotes (modified from reference [13]). The rate of evolution on each lineage (branch) is indicated diagrammatically by relative branch length (long branch = fast; not proportional to time or actual rate).

There are two basic approaches to estimate divergence time when data from multiple constant-rate genes are available for a given set of species (Box 1). In the multigene method [16,19,23], divergence times are estimated for each gene separately, and the average or modal divergence time and error is estimated from pooled time estimates. Multigene time distributions are usually symmetric and have a strong central tendency [16] (Fig. 3). The wide range of gene-specific times reflects the stochastic nature of sequence change and the estimation (statistical) variance in correcting for multiple hits and converting distance to time using a clock calibration. Because each time estimate from a gene is essentially a sequence divergence normalized by the calibration distance, this method can be used with genes having widely varying species samples and rates of change [16]. Furthermore, individual analysis of each gene obviates the need to devise methods to combine multigene sequence data. Outliers can be seen in multigene distributions and trimmed from the dataset, or the median [23] or mode [16] can be used (Fig. 3). The presence of outliers can be an indication that the times are estimates of gene duplications, not speciation events [16]. They can also occur because of large extrapolation (or interpolations), small numbers of total substitutions, or short sequence lengths [3,24,25]. Avoiding such factors at the outset and using the mode (or trimming of outliers) can reduce or eliminate bias from contaminants, outliers and asymmetrical distributions [16].

The supergene approach involves concatenating

nucleotide or protein sequences from all relevant genes (or gene segments) of a species to form a single alignment for time estimation. Optimally, rate variation between genes and among sites within genes should be modeled [24,26]. Alternatively, evolutionary distance between species can be computed for each gene and then averaged over all genes for a given species pair [13,22–24,27,28]. In that case, divergence time is estimated by dividing the average distance by the average calibration distance. Even in this case, time can be calculated in various ways corresponding to different models of evolution and different methods of weighting each gene [24,28]. For example, the parameter describing the rate variation among sites (the 'variation parameter', often referred to as the 'gamma parameter' when a gamma distribution is used [15,29]) can be calculated for each gene separately or for the entire dataset (concatenated alignments) and distances can be weighted by sequence length (higher weight to longer sequences) or by the inverse of variance. Other variations are possible, and time estimates are particularly sensitive to the sequences used to estimate the rate variation parameter and how that parameter is applied in the analysis [24].

Averaging distances from many genes can have other problems related to statistical distributions obtained with different distances. For example, the distribution of evolutionary distance is close to normal for synonymous substitutions [6]. In such cases, a weighted or simple average can be taken. By contrast, protein distances among genes for a given pair of species typically follow a distribution with no central tendency (Fig. 5). A weighted average using the inverse of the variance or sequence length does not yield a distribution with significant central tendency. These distributions are more skewed for closely related species, even when the same set of genes for both species are available (Fig. 5). Therefore, the estimation of average distance may or may not be suitable for comparison among species pairs. Additional difficulties can arise if the same set of genes is not available from all species.

As yet, it is unclear which of these global clock methods is to be preferred. Multigene methods are unique in that they can help detect potential contaminants and outliers so that they can be removed from consideration (subsequently, a supergene might be constructed from such uncontaminated genes). Also, the focus on pairwise comparisons permits all available species for a given gene to be used rather than a selected subset. Currently, the application of different evolutionary models with different genes is facilitated with multigene methods. However, the concatenation of data in the supergene and average distance methods reduces the likelihood of biases owing to small sample sizes, and the method of time calculation is theoretically less likely to generate a biased estimate. Fortunately, these different approaches perform well and empirical results show more similarities than differences (Table 1). This is partly because biologists using these methods have been aware of the associated biases and have accounted for them [16,22,24,25,30].

Local clock methods

Local clock methods use a model of nucleotide or amino acid substitution in which rate is not constant among all branches
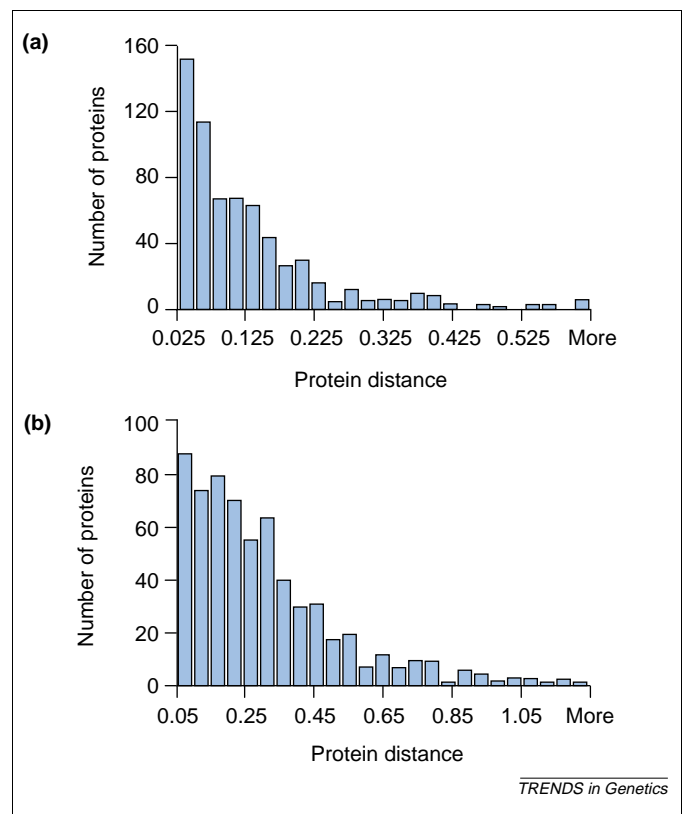

Fig. 5. Different proteins evolve at different rates because of natural selection for protein function. Multigene distributions of protein distances from (a) human-mouse and (b) human-chicken comparisons for the same 647 genes, showing variation in the rate of substitution among proteins. These distributions are derived from a dataset analyzed elsewhere [6].

of the tree, but can vary from one 'local' branch to another. Although these methods 'relax' one parameter (constant rate), they impose others, and therefore they are neither model-free nor assumption-free. An immediate advantage of local clock methods is that they can make use of genes discarded by rate tests in the global methods. However, smaller portions (e.g. branches) of the overall dataset are used for calculating rates (increasing stochastic error), and a greater number of parameters must be considered, thus increasing the variance of the time estimates. Also, the best way to distribute rate differences in a phylogeny, critical for determining times of divergence, is poorly understood. Nonetheless, there has been considerable interest in the development of local clock methods [11,12,21,31–36].

The two different approaches for handling sequence data under the global methods (multigene and supergene) can also be used with local methods. In one 'lineage-specific' method [21,37], individual branches rather than pairwise averages are used to estimate time. As normally applied, the method involves calibrating the molecular clock and estimating unknown times within a single evolutionary lineage. For example, it was used to estimate the times of early splits among prokaryotes and eukaryotes after discovering that eukaryotes have evolved consistently faster than prokaryotes across multiple genes [13] (Fig. 4). If the rate change occurred soon after the evolution of eukaryotes (as postulated in this case) instead of gradually ramping up over time, this method would be more appropriate than others that interpolate or smooth

Table 1. Divergence time estimates between selected eukaryotes involving more than ten nuclear genes or proteins[a]

Comparison (node)	Method[b]	Time in Myr ago (SE)	No. genes	No. sites	Ref.
Human versus chimpanzee	MGG	5.4 (0.6)	36	40 668 bp	[41]
	SGG	5.0[c]	53[d]	24 234 bp	[43]
	SGG	5.4[e] (0.9)	27	6586 aa	[44]
Human versus Old World monkeys	MGG	23.3 (1.2)	56	~22 000 aa[f]	[16]
	SGG	23.5 (4.1)	12	2305 aa	[44]
Artiodactyls versus perissodactyls	MGG	83 (4)	56	~22 000 aa[f]	[16]
	SGL	82 (2.1)	19	14 750 bp	[14]
Primates versus artiodactyls	MGG	92 (1.3)	333	~133 000 aa[f]	[16]
	SGG	98 (23)	27	6586 aa	[44]
	SGL	95 (3.4)	19	14 750 bp	[14]
Vertebrates versus arthropods	MGG	993 (46)	50	33 691 aa	[30]
	MGG	850	21	~8000 aa[f]	[23]
	MGG	830 (55)	17	5533 aa	[45]
	SGG	833–931 (153)	11	3310 aa	[24]
Animals versus plants	MGG	1547 (89)	49	28 582 aa	[30]
	MGG	1200	<37[g]	<15 000 aa[f]	[23]
	SGG	1392–1740 (252)	11	3310 aa	[24]

[a]Abbreviations: aa, amino acids; bp, base pairs; MGG, multigene global method; Myr, million years; SE, standard error; SGG, supergene global method; SGL, supergene local method.
[b]For definitions of methods see Box 1.
[c]Reported originally as 6.2 Myr ago using an orangutan calibration of 13 Myr ago; adjusted here for an orangutan divergence of 11.3 Myr ago [41] to permit comparison among the three studies.
[d]53 autosomal intergenic segments.
[e]Reported originally as 4.6–6.2 Myr ago using an orangutan calibration of 12–16 Myr ago; fixed here for an orangutan divergence of 11.3 Myr ago [41] to permit comparison among the three studies.
[f]Not reported, but estimated by assuming an average length of 400 amino acids per mammalian protein.
[g]Number of 'intergroup comparisons'; the number of enzyme sets and amino acids was not specified.

rate differences among nodes [11,12]. However, in most cases the actual pattern of rate change that took place between two nodes in a tree is unknown. Therefore, it might be useful to explore different models to determine their effects on divergence time. The resulting range of estimates might help in setting time constraints and drawing biological conclusions.

Another class of local clock methods uses Bayesian inference methodology to estimate divergence times [12,34–36,38]. Here, an attempt is made to fit a non-uniform model to describe the rate difference among lineages and model the correlation among lineage rates. As with the lineage-specific method, average rates of change are considered from different branches in this model. The time estimates ('posteriors') depend on a variety of initial variables ('priors') such as the rate of change and its variance (at the ingroup node) and selected divergence times and their variances.

Yet another local clock method uses maximum likelihood and a specified model of variation in evolutionary rate to distribute that variation among nodes and branches in a phylogeny [33]. In this case, the rate variation is permitted to evolve over time, even within a given branch in the tree. A variety of substitution models can be used to generate the topology and branch lengths before using the method. In a recent refinement, a 'roughness penalty' was added to buffer rate variation, because the estimated variance of local substitution rates was too high in the original method [11]. Time estimates depend on the amount of rate 'smoothing', and methods for determining the optimal level of smoothing have been proposed [11].

The Achilles' heel of all local clock methods is the model used to distribute rate variation among nodes and branches in the phylogeny. The number of different possible ways in which rate can vary throughout a phylogeny is nearly

limitless. Time estimates are dependent on the model, and yet no method currently exists that allows accurate prediction of the true model [39]. The use of fossil time constraints [11,12] helps to define a reasonable distribution of rates, but it is not yet known whether such constraints introduce any bias in the time estimates. Initial applications of these methods are producing promising results [14]. Extensive empirical, theoretical and simulation analyses are needed to compare these different local clock methods and reveal their strengths and weaknesses.

Evolutionary timescales
Global and local clock methods have been used with large numbers of genes and proteins to estimate divergence time in a diversity of organisms. The results have shown that fossil and molecular clock based estimates are in much better agreement than often appreciated. This is evident from a scatter plot (Fig. 1b) and a timeline of organismal evolution (Fig. 6) based on time estimates from large numbers of nuclear genes and corresponding dates from the fossil record. Fossil-based estimates of species origin and divergence times are necessarily minimum estimates, but they are usually in general agreement with molecular-clock-based dates when they are constrained by a robust fossil record and biogeography. Specific exceptions are for avian and mammalian orders in the Cretaceous, and for plants, animals and fungi in the late Precambrian.

It is possible to compare time estimates derived from different nuclear genes, methods and research groups, because of unusual attention given to certain evolutionary divergences (Table 1). Of course, different time estimates are expected under different assumptions and models of substitution. Also, different time estimates will be influenced by phylogeny, as in the current debate over the position of rodents among placental mammals [14]. Nonetheless, most
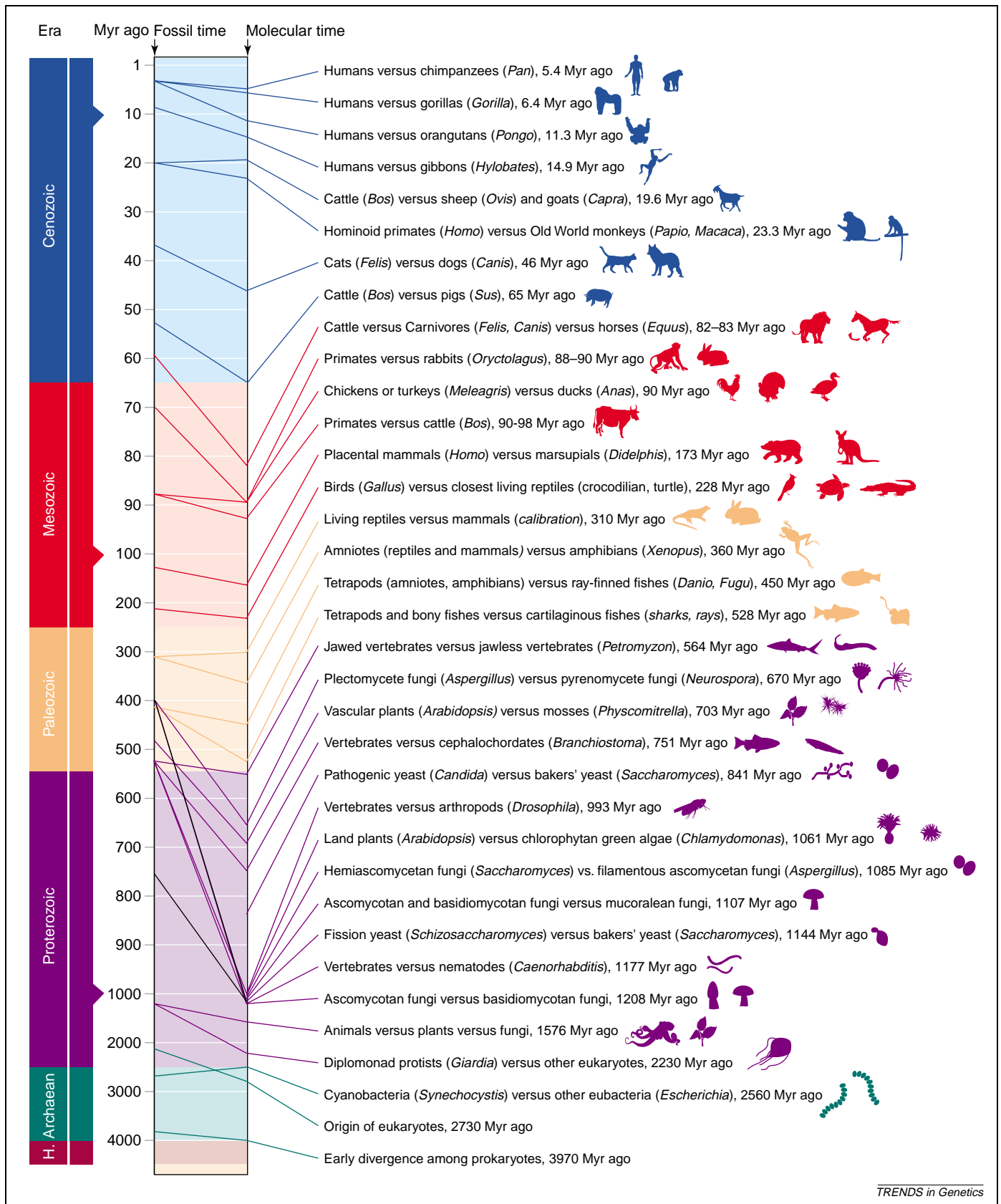
Fig. 6. Comparison of species divergence times from the fossil record and molecular clocks. Time estimates were based on published studies using large numbers (≥10) of nuclear genes or proteins and robust calibrations [13,14,16,19,22,30,40–42]. Note that the timescale is linear within 10–100, 100–1000, and 1000–5000 millions of years (Myr) ago ranges, but changes at the range boundaries. The two oldest fossil times are from fossil biomarker evidence. There is no fossil record for pathogenic versus baker's yeast. Divergence times involving rodents could not be placed on the timescale because of large differences in published molecular time estimates [14,16].

time estimates from diverse studies are remarkably similar and suggest that all such methods could be tracking the true signal of evolutionary time. Because the coefficient of variation in divergence time (among genes) is quite large (25–35%) [16], time estimates based on one or a few genes – which is typical in the literature – are less reliable. Therefore, the number of genes or proteins used should be considered as an important benchmark when comparing and evaluating time estimates from different studies.

Conclusions

The availability of genomic data from an increasing number of species, especially model organisms, has created a demand for improved methods of divergence time estimation to help understand the temporal component of the tree of life [3]. Here, we have focused on the development and comparison of new molecular clock methods that can be used with large numbers of genes. There is a surprising diversity of methods available and no clear evidence that any particular approach is superior. Additional empirical and simulation studies will guide the further development of these methods and help to reveal their strengths and weaknesses. Local clock methods are promising as they can use genes discarded by global clock methods, permitting a larger total number of genes for estimating time, a positive attribute in data-limited situations. Fortunately, these different methods have not so far yielded widely different results, showing promise for a bright future in molecular clock analysis.

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