The Molecular Clock*

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2013

Abstract

The molecular clock uses evolutionary changes in proteins and DNA to measure the passage of time. Yet molecular evolution is clocklike only to a first approximation. Uncertainties arise because of variation in rates of molecular evolution, because of difficulty in calibrating clocks, and because we measure molecular changes only indirectly. Statistical methods now cope with all of these uncertainties. As these methods have matured and molecular data sets have increased in size, the molecular clock has grown increasingly reliable.

1 Introduction

The molecular clock plays a central role in dating events in evolutionary history. These range from branch points throughout the tree of life to changes in population sizes during the past few thousand years. Yet it is surprising that the molecular clock works at all, for it is based on a hypothesis that (in simplest form) is certainly false. No modern geneticist would claim that molecules evolve at a constant rate within all parts of any large phylogeny.

To understand this apparent paradox, we will need to consider how the clock works and also how it can fail. We will then survey methods used to overcome the various problems. I will conclude that, difficulties notwithstanding, the molecular clock is more reliable now than ever.

2 Historical overview

Although the first methods for sequencing proteins came into use in the 1940s and 1950s, it was not until the early 1960s that we began to see broad studies that compared the proteins of different species. These showed that rates of evolution vary a great deal from one protein to another but vary much less among species or across time. Each protein seemed to evolve at a constant rate, as shown in figure 1.

That rate, moreover, was surprisingly high. The prevailing view saw natural selection as the primary agent of change. But selection operates by removing individuals from the population, and this is costly. If too many individuals are removed, the population cannot survive. For this reason, there is a limit to the number of loci that can be simultaneously under strong selec-

*Published 2013 by Elsevier in *Treatise on Geochemistry*, 2nd edition (Heinrich D. Holland and Karl K. Turekian, eds.), volume 14, pp. 55–61.

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tion. Kimura (1968) calculated that if molecular evolution were driven mainly by natural selection, the observed rate would entail a cost that was simply too great to bear.

Several authors saw the molecular pattern in terms of functional constraint (Doolittle and Blombäck, 1964; Kimura, 1968; Margoliash, 1963). Under this view, the regions of proteins that evolve quickly are those that are free to vary, because they have only minor effects on protein function. This implied that most amino-acid changes were caused not by selection but by the neutral evolutionary forces of mutation and genetic drift (Doolittle and Blombäck, 1964; Kimura, 1968, 1983; Margoliash, 1963). This view became increasingly plausible during the 1960s and 1970s, as molecular data accumulated. As it did, evolutionists gravitated toward the idea that molecules evolve at a relatively constant rate—the idea of the *molecular clock*.

This idea was first proposed by Zuckerkandl and Pauling (1962). At that time it was highly controversial, for it seemed to contradict what was known about the pace of evolutionary change. It had long been clear from fossils that some species evolved faster than others and that individual species evolved at varying rates (Simpson, 1953). Thus, evolutionists were predisposed against the molecular clock. Those with an intimate knowledge of the fossil record found it easy to agree with Darwin that natural selection was the principle agent of evolutionary change. On the other hand, those who studied molecules were more impressed by the neutral forces of mutation and genetic drift.

During the 1960s and 1970s, the clock hypothesis received support from several kinds of evidence. Judging from their morphology and from fossils, it appeared that frogs and opossums have evolved slowly. Indeed, the opossum is sometimes called a living fossil. If the rate of molecular evolution were closely tied to the rate of evolution in morphology, then the proteins of such species should evolve slowly. This however is not the case. The proteins of these creatures evolve as fast as those of other animals (Maxson et al., 1975; Wallace et al., 1971).

To estimate a rate of evolution, one must compare species that have been separated a known length of time. Unfortunately, we are often ignorant about separation times and therefore cannot estimate rates. Yet we can still use these problematic comparisons to test the clock hypothesis via what is now called the *relative rate test* (Sarich and Wilson, 1967; Wilson and Sarich, 1969). The simplest case involves three species: two close relatives (say mouse and rat) and one less close (say squirrel). The separation time of mouse and squirrel equals that of rat and squirrel. According to the clock hypothesis, equal separation time implies equal genetic difference. Thus, the mouse–squirrel difference should equal the rat–squirrel difference, at least approximately. Because this test does not require geological estimates of separation times, it has been applied widely since the late 1960s.

By the 1980s, opinion had swung strongly in favor both of the molecular clock and of the theory of neutral evolution (Kimura, 1983). Yet things were about to change. By the end of the decade, skepticism toward the molecular clock was gathering (Gillespie, 1991). Today, few geneticists would argue that the rate of molecular evolution is constant.

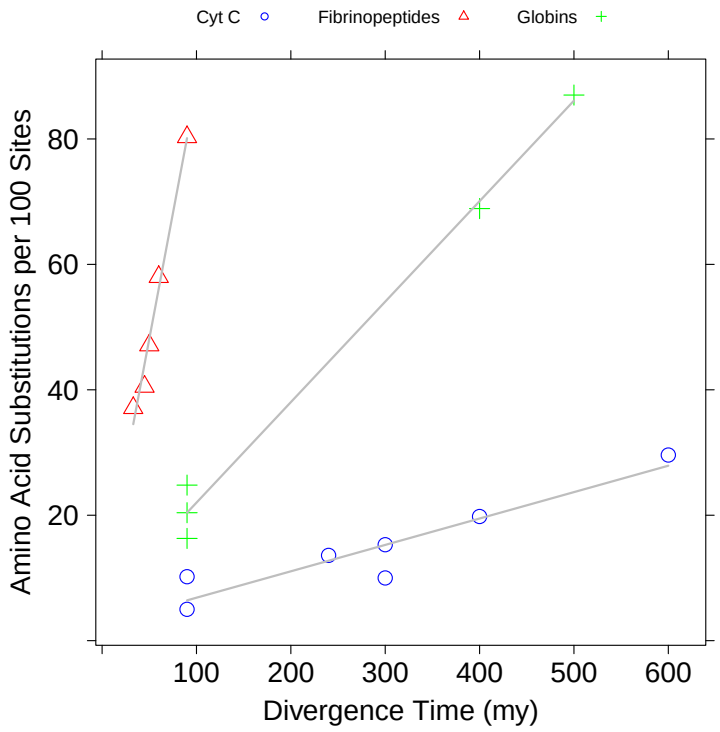

Figure 1: Early data showing a roughly constant rate of evolution in cytochrome *c* (Dickerson, 1971).

3 A numerical example: the chimp-human common ancestor

Before discussing the difficulties of molecular dating, let us consider a concrete example: the date of the last common ancestor of chimpanzee and human. To estimate this date, we'll use genetic data from human, chimpanzee, and orangutan. We'll also need an externally-derived date in order to "calibrate the clock"—to estimate the rate of evolutionary change.

Molecular clocks are ordinarily calibrated using fossils, and this process introduces a considerable undertainty into molecular dates, as discussed below. Here, I will simply assume that the orangutan–human common ancestor lived 14 My ago.

The genetic data in this example consists of 929 bases of mitochondrial DNA, sequenced in a human, a chimpanzee, and an orangutan (Hasegawa et al., 1985, Table 1). In these data, human differs from chimpanzee and orangutan at 79 and 149 nucleotide sites, respectively. Re-expressed as fractions of the total sites, these numbers become $p_{ch} = 79/929 = 0.085$, and $p_{oh} = 149/929 = 0.1539$.

Some of these nucleotide sites have probably changed more than once, so p_{oh} and p_{ch} underestimate the number of evolutionary changes per site. This is the problem of *saturation*, which is

discussed further below. Various methods have been introduced to correct this bias, the simplest of which was introduced by Jukes and Cantor (1969) in 1969. This method assumes that each nucleotide mutates to each other nucleotide at the same rate. Although this is surely an oversimplification, it is good enough for this numerical example.

Under the Jukes-Cantor model, there is a simple relationship between the fraction p of sites that differ and the average number d of evolutionary changes per site:

$$d = -(3/4)\ln(1 - (4/3)p)$$

By plugging p_{oh} and p_{ch} into the right side of this equation, we obtain estimates of the corresponding numbers, d_{oh} and d_{ch}, of evolutionary changes per site. This gives $d_{oh} = 0.1723$ and $d_{ch} = 0.0903$. These numbers are only slightly larger than p_{oh} and p_{ch}, suggesting that saturation had only a modest effect.

We estimate the rate of evolution by dividing d_{oh} by the combined lengths of the two branches leading from orangutan and human back to their common ancestor. As this ancestor (by assumption) lived 14 My ago, the combined branch length is 28 My, and the rate of evolution is $r = 0.0062$ changes per site per million years. Given this rate, we estimate the age of the chimpanzee–human common ancestor as $d_{ch}/2r = 7.33$ My.

This analysis is not adequate by modern standards. In the first place, it ignores the facts that different parts of this DNA sequence evolve at different rates and that, at any given nucleotide site, some changes are more likely than others. Furthermore, it assumes that the rate of change has been constant and takes no account of uncertainties. Nonetheless, the answer is consistent with current knowledge about the age of the chimpanzee–human common ancestor.

4 Difficulties with the molecular clock

4.1 Uncertainties

Evolutionary rates are estimated from the number of nucleotide substitutions that accumulate between DNA sequences separated for a known length of time. Unfortunately, neither the number of substitutions nor the separation time is often known exactly, and this can add both uncertainty and bias to the molecular clock.

For example, in the chimp-human-orangutan example above, we did not count evolutionary changes directly. Instead, we counted nucleotide differences. As discussed above, these counts underestimate the number of evolutionary changes because of the problem of saturation. This effect is insignificant in very recent comparisons but increases with age. It is exacerbated when nucleotide sites vary in rate, because rates at rapidly-evolving sites may be underestimated. These problems are addressed by fitting models of the substitution process such as the Jukes-Cantor model used above. In modern work, more elaborate models are often used (Nei and Kumar, 2000, sec. 3.2–3.4). If the model is appropriate, saturation adds noise but not bias to the molecular clock. Unless saturation is extreme, estimates of dates are relatively insensitive to this component of the model: we get approximately the same answer from many different models (Yang, 2006, p. 143).

There are also problems involving "calibration points"—the time values used to calibrate the clock. In the numerical example above, a value

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was simply assumed. Real analyses are based on intervals anchored either by well-dated fossils or by the dates at which one population separated from another. There are difficulties either way.

Suppose, for example, that we identify one fossil as the earliest known ape and another as the oldest known Old World monkey. These fossils must be younger than the ape–monkey common ancestor. Nonetheless, many studies take the date of the fossils as a proxy for the date of this ancestor. Two sorts of error are possible here. First, we may be wrong in identifying these fossils as an ape and an Old World monkey. Second, we have ignored the interval that separated the fossils from their common ancestor. Yet that interval must have been long enough for the ape and the monkey to evolve the distinctive features that allow us to recognize them as different.

Many calibrations are based not on fossils but on the date at which a population split in two. For example, Knowlton et al. (1993) studied the DNA of 7 pairs of sibling species of snapping shrimp *(Alpheus)*, which were separated when the Isthmus of Panama rose above the sea roughly 3–3.5 my ago. We need not worry here about the phylogenetic placement of fossils or about the relationship between fossil dates and dates of separation. Yet this system turns out to be more complex than it seems at first. Genetic divergence varied by over 3-fold among the 7 pairs of species, and the greatest divergence was found between pairs that live in deeper water or which avoid heavy sedimentation. As Knowlton et al suggest, these species may have been isolated earlier during the uplift. Clearly, we should not think of the uplift as a discrete event that happened at a precise point in time. It was a gradual process that took place over millions of years. It is an open question when during this process any particular pair of species was separated. When the uplift is used to calibrate clocks, those clocks will inherit this uncertainty.

Another bias arises when calibrations are based on the time that two populations have been separate. Two genes, one from each population, have clearly been separate at least as long as the populations have, provided that there is no interbreeding between the populations. And it would be a remarkable coincidence if the common ancestor of the two genes lived in the very generation when the populations split. It is far more likely that the common ancestor lived much earlier. The difference between the separation time of the populations and that of the genes is typically thousands of generations (Tajima, 1983).

To calibrate the clock, we need to divide the genetic difference between two homologous genes by the separation time of those genes. If we rely instead on the (smaller) separation time of populations, our rate estimates will be too large, for they will interpret a given genetic difference as the product of a smaller amount of time. This problem is most pronounced with recent calibrations, because in such cases the bias forms a larger fraction of the whole.

Phylogeneticists have recently begun using probability distributions to model the uncertainty about separation times. These uncertainties appear as priors in Bayesian statistical methods (Yang and Rannala, 2006). This reduces the bias caused by poor calibrations, allows us to incorporate several different calibration points, and surely improves estimated dates. Yet there is still uncertainty, and no molecular clock can yield dates with less uncertainty than that of its calibration point (Yang and Rannala, 2006, p. 224).

Some recent calibrations are based on ancient DNA, such as that obtained from Neanderthal

skeletons. In such cases, calibration contributes only a small uncertainty to estimated dates (Ho et al., 2011).

4.2 Purifying selection

Deleterious mutations are ordinarily removed by natural selection. This process, however, can be slow, and mildly deleterious mutants may persist for thousands of generations (Pereira et al., 2011). While they do, they contribute to genetic variation. If we knew the number introduced each generation and how strongly these are opposed by selection, we could predict the rate at which they are removed from the population. But these facts are not known, so deleterious alleles make an obscure contribution to observed genetic differences.

This is especially true for DNA that does not recombine, such as that in mitochondria (Rice, 1994). In such systems, some chromosomes may carry a deleterious allele at one locus and an advantageous one at another. The advantageous allele interferes with selection's efforts to remove the deleterious one. Furthermore, mutations in the mitochondrion are more likely to be deleterious in the first place, simply because the functional fraction of the genome is larger there than in the nucleus. In mitochondrial DNA, it appears that some deleterious alleles persist for tens of thousands of years and add appreciably to genetic differences measured over such intervals (O'Fallon, 2010).

Although we may not know the precise level of this deleterious contribution, we do know that nearly all deleterious alleles are eventually removed by selection. Consequently, they contribute only a small fraction to the genetic difference between individuals who have been separated for millions of years. The problem is important only at smaller time scales, where deleterious alleles make a larger but unknown contribution. This may explain why mitochondrial rates of evolution appear much higher when estimated over (say) 50,000 y rather than 5,000,000 y (see Ho et al., 2011, and references therein). This problem has made it difficult to use molecular mitochondrial clocks to date events within the past quarter million years. Fortunately, the problem does not seem to affect human nuclear DNA, where there is no detectable difference between the rate estimated from parent–offspring comparisons and that estimated from chimpanzee–human comparisons (Roach et al., 2010). The difference between the two presumably reflects both the absence of recombination in mitochondria and the larger deleterious fraction among mitochondrial mutations.

4.3 Variation in rates

The numerical example above was based on the strict form of the molecular clock hypothesis. In other words, it assumed a constant rate of molecular evolution. However, evidence against this hypothesis began to accumulate early in the history of the molecular clock. Studying protein sequences, several authors showed in the early 1970s that variation in rates was larger than expected under the clock hypothesis (Langley and Fitch, 1973; Ohta and Kimura, 1971). These early tests left some ambiguity, but by the early 1980s it was possible to show decisively that protein sequencies did not conform to the clock (Gillespie, 1984; Hudson, 1983).

At about the same time, it became possible to study variation in the DNA sequences themselves (Kreitman, 1983). Motivated by these new data, theoreticians introduced new and more powerful tests of the clock hypothesis (Felsen-

stein, 1981; Hudson et al., 1987; McDonald and Kreitman, 1991; Tajima, 1993). These innovations soon made it clear that the molecular clock does not hold as a general principle applying to all forms of DNA.

These variations occur for a variety of reasons. In coding sequences, the rate of neutral evolution depends on the fraction of the protein that is free of functional constraint. That in turn depends on the three-dimensional shape into which the protein folds. If a substitution alters this shape, it may increase or decrease the rate at which the protein evolves (Bromham and Penny, 2003).

Selection can also alter the enzymes responsible for DNA replication and repair. If such a change reduces the fidelity with which DNA is replicated or the efficiency with which it is repaired, then the rate of neutral evolution will increase (Bromham and Penny, 2003).

Rates of evolution may also respond to population size. In a small population, selection is ineffective, so a larger fraction of nucleotide sites is effectively neutral, and molecular evolution is faster (Ohta, 1992).

Many mutations are thought to occur during cell division. If the number of germ-cell divisions varies less across species than does the generation time, then short-lived species will evolve faster than those with long lifespans. There is evidence for this phenomenon both in animals and in plants (Hasegawa et al., 1989; Lanfear et al., 2007; Smith and Donoghue, 2008; Thomas et al., 2010; Wu and Li, 1985). This hypothesis may also explain the difference in rates of evolution between the X and Y chromosomes. Among mammals, there are more germ cell divisions per generation within males than within females. Consequently, the hypothesis predicts a larger rate for the Y chromosome (which spends all its time in males) than for the X chromosome (which spends most of its time in females). This is indeed the case (Yi, 2007, pp. 146–147).

The rate of evolution may also be affected by metabolic rate. Metabolism produces highly oxidative byproducts, which may cause mutations. If so, then species with high metabolic rates would also have high rates of evolution. The evidence on this hypothesis is mixed. The hypothesis seems to hold among vertebrates (Gillooly et al., 2005, 2007; Martin and Palumbi, 1993), but not among other metazoans (Lanfear et al., 2007). Studies involving temperature also provide ambiguous evidence. Among exotherms, metabolic rate is correlated with temperature. Thus, we would expect a higher rate of evolution among tropical plants than temperate ones, and this seems to be so (Wright et al., 2006). We would not expect the same pattern among endothermic species such as mammals and birds, yet the pattern seems to exist there too (Gillman et al., 2009). This issue is still unsettled (Gillman et al., 2011; Weir and Schluter, 2011), and the evidence is hard to evaluate. It seems likely that metabolic rate does affect the rate of evolution along with other sources of variation that are not controlled in these studies.

Natural selection surely underlies many of the causes of variation in rates of evolution. This suggests that we might avoid much of that variation by focusing on parts of the genome with no apparent function. If a DNA sequence has no function, then selection cannot produce effects that vary over time or in different species. Such regions of the DNA should behave in a more clocklike fashion.

Yet several of the factors discussed above—generation time, metabolic rate, and precision of DNA replication and repair—should affect the entire genome, not just the functional portion. Consequently, it should come as no surprise

that variation in rates of evolution is also found in neutral DNA (Thomas et al., 2003, p. 790). Nonetheless, it appears that some portions of the genome do evolve in a clock-like fashion. For example, Kim et al. (2006) find no generation-time effect in portions of the primate genome that are rich in CpG dinucleotides. Instead, substitutions accumulate there at a constant, clock-like rate. Hwang and Green (2004) made a similar point earlier, arguing that CpG sites were clocklike and exploiting this fact to infer phylogenies. There is also evidence that the generation-time effect is absent in nonsynonymous sites for mammals (Nikolaev et al., 2007). If these findings hold up, it should be possible to avoid rate variation by focusing on clock-like portions of the genome.

5 Coping with an imperfect clock

Is the clock still useful, if it ticks at an erratic rate? In contemplating this question, it is useful to recall that no one has ever claimed that the rate of molecular evolution was *precisely* constant. Most early advocates of the clock would probably have agreed with Dickerson (1971, p. 43), who argued that it was constant "to a good first approximation." Kimura (1983, p. 79) put it like this a decade later:

> These results suggest that although the strict constancy may not hold, yet a rough constancy of the evolutionary rate for each molecule among various lineages is a rule rather than an exception.

The idea of a molecular clock came initially from plots such as the one in figure 1, which show the steady increase with time in protein differences.

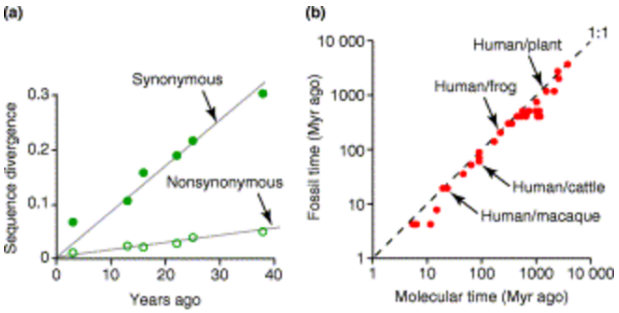

Figure 2: Evidence of a molecular clock. (a) Sequence from influenza virus A compared with samples frozen in the past. (b) Molecular estimates of divergence time compared with estimates from fossils. Reprinted from Hedges and Kumar (2003).

Figure 2 shows that these plots have not gone away in the age of DNA sequences. In spite of all the variation in rates, it remains true that many genes show a "rough constancy of the evolutionary rate." The question is, how can we use that rough constancy to infer dates without being misled by the variation?

5.1 Excluding variant lineages

Some authors exclude lineages that show evidence of rate variation, based on some statistical test (Bromham et al., 1998; Hedges et al., 2004; Kumar and Hedges, 1998; Takezaki et al., 1995). This method has been criticized, however, because the tests involved are low in power unless sequences are quite long—several thousand nucleotide sites (Bromham et al., 2000). If we are unable to exclude lineages with modestly elevated rates, then our rate estimates may be biased. In the future, the force of this objection is likely to wane as the cost of sequencing DNA declines. When most analyses are based on entire genomes, there will be less reason for concern

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about the need for a few thousand nucleotides. Furthermore, there are other ways to ameliorate the problem.

One of these was introduced by Kumar and Subramanian (2002). They exclude loci that show evidence of variation, not in the overall rate of substitution, but in the "pattern" of substitutions—the ratios of rates between pairs of nucleotide states. (For example, the ratio of $A \rightarrow T$ changes to $A \rightarrow G$ changes.) Kumar and Subramanian study only the loci that show no evidence of variation in this pattern. In this subset, evolutionary rates were approximately constant per year throughout mammalia. This is remarkable, as exclusions were based not on rate but on pattern. This approach is promising, because pattern varies in several dimensions whereas rate varies in only one. Thus, a test based on pattern may be more powerful than one based on rate.

To ameliorate the problem of low power, one can also increase the stringency of the relative rate test by tightening the statistical cutoff value. This makes the test more sensitive to lineages whose rate differs only modestly. In the study of Hedges and Kumar (2003), the more stringent test excluded many lineages but did not affect time estimates much.

The success of any of these measures will depend on how variation is distributed across the phylogeny. If most lineages shared the same rate and a few had markedly different rates, then statistical power would not matter much. Any sensible test would identify the variant lineages. On the other hand, if there were a continuum of variation, a weak test would identify only the most divergent lineages, and the less divergent ones would cause bias. In the latter case, estimated dates should change as the stringency of the test is increased. But this is not what happened in the study of Hedges and Kumar (2003), who found essentially no change as stringency increased. This suggests that variant lineages are grossly variant, at least for this data set. Such lineages should be easy to detect and exclude.

5.2 Relaxed clocks

A variety of "relaxed clock" methods now exist for estimating dates in the absence of a global clock. These methods all allow the rate to differ in different parts of the phylogenetic tree. Without imposing some sort of constraint, it is not possible to obtain consistent estimates of separation times (Britton, 2005). All methods therefore make restrictive assumptions about the way rates vary across the tree. Some require the user to divide the tree into several partitions, which are then allowed to evolve independently. Others assume that rates are autocorrelated along branches or that each rate is drawn from some given probability distribution (reviewed by Bromham and Penny, 2003; Kumar, 2005; Rutschmann, 2006; Welch and Bromham, 2005). More recently, these ideas have been implemented in a Bayesian context, which provides a natural means to incorporate uncertainty about input parameters such as dates of calibration (Aris-Brosou and Yang, 2002; Drummond and Rambaut, 2007; Rannala and Yang, 2007; Ronquist and Huelsenbeck, 2003; Yang and Rannala, 2006).

These approaches avoid the bias caused by excluding anamalous sequences. Yet all rely on restrictive assumptions about how variation is distributed across the phylogeny, and these assumptions are difficult to test. We should therefore prefer methods that are insensitive to violations of their assumptions—that give approximately correct answers even with data that were not

generated as assumed. The various relaxed-clock methods differ in this respect. Some yield biased results if their assumptions are violated (Aris-Brosou and Yang, 2002; Ho et al., 2005; Lepage et al., 2007), but others seem less sensitive (Drummond et al., 2006; Ho et al., 2005; Kumar, 2005).

6 Using multiple genes

In recent studies, there is a trend toward larger samples of genes. This should improve the clock in several ways. In the first place, there should be less uncertainty in multi-gene estimates, simply because they are based on more data. But this is not the whole story. All clock estimates struggle with a fundamendal confound between branch lengths and rates: we can double the rate and halve the branch length without affecting the expected number of substitutions. Under a strict clock, there are enough constraints to break this confound, and dates are easy to estimate. Estimation is not so easy under a relaxed clock, and this is why all such methods impose some sort of constraint. Things improve, however, when there are multiple genes. In that case, different genes may vary in different ways, yet all share the same branch lengths. This makes it easier for multigene methods to break the confound between rates and branch lengths (Yang and Yoder, 2003, p. 706).

Even if there were no such confound, clocks would still benefit from multiple loci. Unless the variations at different loci are perfectly correlated, we can reduce the error variance by averaging over loci (Rannala and Yang, 2007). This will not help much however, if rate variation is caused primarily by factors such as metabolic rate and life span, which affect all loci equally. This issue is seldom studied, but one study found no evidence of correlation between rates at different loci (Thorne and Kishino, 2002, p. 700). Perhaps for this reason, data sets involving multiple multiple genes often produce well-behaved dates.

7 Conclusions

Few geneticists would now support the hypothesis that each genomic region evolves at a rate that is constant across species. There is abundant evidence not only that rates vary but also that these variations can be large. Yet most geneticists would also maintain that the molecular clock is still useful. This continued optimism results in part from the evidence that variations at different loci tend to cancel one another out, so that multilocus data are more clock-like than any single locus. It also reflects the apparent success of various statistical methods for dealing with an imperfect clock. Geneticists disagree about which method is best—about whether it is best to exclude anamolous loci or to correct the variation statistically, using a relaxed clock. Nonetheless, it is clear that molecular evolution is sufficiently clock-like to be useful in estimating dates. In the future, the accuracy of molecular clock estimates will improve as we rely increasingly on samples involving many genetic loci.

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