Neutralism and selectionism: the molecular clock

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Abstract

The neutrality theory predicts that the rate of molecular evolution will be constant over time, and thus that there is a molecular clock for timing evolutionary events. It has been observed that the variance of the rate of evolution is generally larger than expected according to the neutrality theory. Several modifications of the theory have been proposed to account for the ‘overdispersion’ of the molecular clock, by postulating effects attributed to generation-time, population size, slightly deleterious mutations, repair mechanisms, and the like. An extensive investigation of two proteins, glycerol-3-phosphate dehydrogenase (GPDH) and superoxide dismutase (SOD), manifests that none of these modifications can simultaneously account for the disparate patterns observed in both proteins. GPDH evolves very slowly in *Drosophila* species, but several times faster in mammals, other animals, plants, and fungi. SOD evolves very fast in *Drosophila* species and also in mammals, but much more slowly in other animals and still slower when plants and fungi are compared to one another, or to animals. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The hypothesis of the molecular clock of evolution emerged from early observations that the number of amino acid differences in a given protein appeared to be proportional to the time elapsed since the divergence of the organisms compared (Zuckerkandl and Pauling, 1962; 1965; Margoliash, 1963). This observation was later stated by Kimura (1983, p. 98) as an ‘empirical principle’: ‘For each protein, the rate of evolution in terms of amino acid substitutions is approximately constant per year per site for various lines, as long as the function and tertiary structure of the molecule remain essentially unaltered’. The time constancy was accounted for with the hypothesis that many amino acid (and nucleotide) substitutions may be of little or no functional consequence and that, therefore, most substitutions that occur in evolution will be of this kind rather than involve amino acid replacements strongly

constrained by natural selection (Zuckerkandl and Pauling, 1965).

Kimura’s neutrality theory of molecular evolution provided a mathematical formulation for the hypothesis of the molecular clock (Kimura, 1968; see also Kimura, 1969, 1983). New alleles arise in a species by mutation. If alternative alleles are neutral with respect to natural selection (i.e. do not modify the Darwinian fitness of their carriers), their frequencies will only change by accidental sampling errors from generation to generation, that is, by genetic drift. Rates of allelic substitution will be stochastically constant – they will occur with a constant probability for a given protein. Kimura showed that the rate of substitution of adaptively equivalent (‘neutral’) alleles, k , is precisely the rate of mutation; u , of neutral alleles, $k = u$.

According to the neutrality theory, evolution at the molecular level consists for the most part of the gradual, random replacement of one allele by another that is functionally equivalent to the first. The theory assumes that favorable mutations occur, but are sufficiently rare that they have little effect on the overall evolutionary rate of nucleotide and amino acid substitutions. A large proportion of all possible mutants are deleterious, but these are eliminated or kept at very low frequencies by natural selection, with little or no consequence in the rates of molecular evolution.

Abbreviations: CR, Central repeat region; CSP, Circumsporozoite protein; MSP-1, Merozoite surface antigen protein-1; MSP-2, Merozoite surface antigen protein-2; R1a, R1b, R2a; Nucleotide repeat 1a, 1b, 2a in *Msp-1*; RAT, Repeat allototype; RHR1, RHR2, RHR3, Repeat homology region 1,2,3

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In order to account for the apparent observation that the rate of amino acid substitution was constant over time, the neutrality theory postulated that the neutral mutation rate of amino acids was constant over time (Kimura, 1968, 1969, 1983). Ohta and Kimura (1971) statistically tested the neutrality hypothesis under the assumption that the number of substitutions has a Poisson distribution, with mean kt (where t is the age of the lineage in years). The Poisson process predicts that the variance, V , will be equal to the mean, M , so that their ratio, $R = V/M = 1$. Ohta and Kimura's (1971; see Kimura, 1983; Gillespie, 1991) investigation of three proteins (α globin, β globin and cytochrome c) concluded that $R \sim 2$ and suggested reasons why the clock might be somewhat 'overdispersed,' that is, $R > 1$. Langley and Fitch (1974; Fitch, 1976) conducted an investigation of the same three proteins plus fibrinopeptide A in 18 vertebrate species and statistically rejected the neutrality hypothesis for overall rates (comparisons among branches), for relative rates (comparisons among proteins within branches), and for the total process. The matter was considered settled by many after an extensive study by Gillespie (1989; see 1991). The value of R for 20 proteins spread from 0.32 to 43.82; 12 values were significantly greater than 1, as was the average, $R = 6.95$ (corrected value by using weights from replacement substitutions; uncorrected $R = 8.26$).

A number of hypotheses have been proposed to account for the overdispersion of R and these will be briefly examined below. I want first to summarize our observations concerning the evolution of two proteins, part of an extensive investigation precisely designed as a test of the molecular clock hypothesis.

2. Evolution of glycerol-3-phosphate dehydrogenase

The nicotinamide adenine dinucleotide (NAD)-dependent cytoplasmic GPDH (glycerol-3-phosphate dehydrogenase) plays a crucial role in metabolism because of its keystone position in the glycerophosphate cycle, which in *Drosophila* and other dipterans provides energy for flight in

the thoracic muscles (O'Brien and MacIntyre, 1978; Bewley, 1983). In *Drosophila melanogaster*, the *Gpdh* gene consists of eight exons and produces three isozymes by differential splicing of the last three exons (Cook et al., 1988; Bewley et al., 1989; von Kalm et al., 1989). We have analyzed in my laboratory 768 nucleotides from exons 3–6, coding for 256 amino acids, in 27 dipteran species (Kwiatowski et al., 1997; Barrio and Ayala, 1997). The species studied are from two families: *Tephritidae*, represented by the medfly *Ceratitis capitata*; and *Drosophilidae* represented by three genera: *Scaptodrosophila* (one species), *Chymomyza* (two species), and *Drosophila* (23 species, classified within five subgenera: *Dorsilopha*, *Drosophila*, *Hirtodrosophila*, *Sophophora*, and *Zaprionus*). We have investigated the amino acid and nucleotide composition of the homologous GPDH region in nine other species, encompassing three mammalian orders (human, mouse, and rabbit), three metazoan phyla (the nematode *Coenorhabditis elegans*, plus arthropods and chordates, represented by the mammal and fly species already listed), and three multicellular kingdoms (the angiosperm *Cuphea lanceolata*, the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, and the animals already listed).

The rate of amino acid replacements is not constant, but rather seems to increase with the time elapsed. The average number of amino acid replacements per 100 residues (Table 1) is 0.8–1.2 between species from different *Drosophila* groups or subgenera, which diverged 45–55 My ago, but 3.0 between species from different genera, which diverged only somewhat earlier (~60 My ago). When comparisons are made between the medfly *Ceratitis capitata* and the *Drosophilids*, the number of amino acid replacements becomes 9.4 ± 0.1 , for a time elapsed only twice as long as between the *Drosophila* genera or subgenera. The corresponding rates of amino acid replacement are shown in Fig. 1 (left). The rate of amino acid replacements observed in *Drosophila* lineages is $0.9\text{--}1.1 \times 10^{-10}$ /site/year, a very slow rate (comparable to the rates of 0.25–1.7 observed for histone proteins; Wilson et al., 1977). But when *Ceratitis* and *Drosophila* species are compared, the lineage rate

Table 1
Evolution of GPDH: amino acid replacements between increasingly divergent species^a

Comparison	My	Mean	Rate
Within <i>Drosophila</i> groups	5–25	0.0–0.8	0–2.1
Between <i>Drosophila</i> groups	45 $\pm$ 10	0.8 $\pm$ 0.0	0.9 $\pm$ 0.0
Between <i>Drosophila</i> subgenera	55 $\pm$ 10	1.2 $\pm$ 0.0	1.1 $\pm$ 0.0
Between dipteran genera	60 $\pm$ 10	3.0 $\pm$ 0.1	2.7 $\pm$ 0.1
Between mammals	70 $\pm$ 15	7.4 $\pm$ 1.6	5.3 $\pm$ 1.1
Between dipteran families	100 $\pm$ 20	9.4 $\pm$ 0.1	4.7 $\pm$ 0.1
Between animal phyla	650 $\pm$ 100	54.7 $\pm$ 0.3	4.2 $\pm$ 0.0
Between kingdoms	1100 $\pm$ 200	87.0 $\pm$ 0.8	4.0 $\pm$ 0.1

^a The species compared and taxonomic position are given in Ayala et al. (1996). The plus/minus values are crude estimates of error for My, but are standard deviations for mean and rate. Mean values are per 100 residues for differences between species; the rates are lineage values, in units of 10^{-10} amino acid replacements/site/year. Replacements are corrected according to Dayhoff (1978). The matrices of amino acid and nucleotide differences are given in Barrio and Ayala (1997); Kwiatowski et al. (1997).

Fig. 1. Rate of molecular evolution in the protein GPDH. The rate numbers on the right refer to lineage changes in units of 10^{-10} amino acid replacements/site/year. Left. The three lines correspond to the best-fitting rates for three sets of species considered: within the genus *Drosophila* (white circles, rate 1.1), between the genera *Drosophila* and *Chymomyza* (gray circles, rate 2.7), between the fruitfly families *Drosophila* and *Ceratitis* (black circle, rate 4.7). Right. The three lines correspond to the best-fitting rates for three sets of species: from different *Drosophila* subgenera (rate 1.1), animal phyla (flies vs. mammals vs. nematodes, rate 4.2), and kingdoms (animals vs. plants vs. fungi, rate 4.0). The black circle represents the rate of evolution for mammals (rate 5.3).

becomes five times greater, 4.7×10^{-10} /site/year (slightly slower than the rates observed in some intracellular enzymes, such as 5.3×10^{-10} for triosephosphate isomerase, or 6.7×10^{-10} for cytochrome *c*).

The different rates of evolution displayed in Fig. 1 become even more disparate when we take into account that these rates apply to largely overlapping lineages. Consider, for example (Fig. 2), the evolution from node 3 to a *Chymomyza* species and a *Drosophila* species (say, *D. melanogaster*). The average rate of amino acid evolution is 2.7×10^{-10} /site/year (Table 1, line 4) over the 120 My of evolution separating these two species (60 My from node 3 to the *Chymomyza* species and 60 My from node 3 to the

Drosophila species). But during 100 of the 120 My (55 My from node 4 to the *Drosophila* species and 45 My from node 5 to the *Chymomyza* species), the rate of evolution is $0.9\text{--}1.1 \times 10^{-10}$ (Table 1, lines 2 and 3). Thus, the acceleration in rate of evolution could only have occurred over the 20 My of evolution indicated by thick lines in Fig. 2 (between nodes 3 and 4 and between nodes 3 and 5). The rate of GPDH evolution during those 20 My must have been 11×10^{-10} /site/year, or more than 10 times faster than for the remainder 100 My, in order to give an average of 2.7×10^{-10} over the 120 My. (The number of amino acid replacements between the *Drosophila* subgenera, or between the two *Chymomyza* species, is mostly 2, whereas between *Chymomyza* and *Drosophila* is 8 or 9.)

Similarly, the rate of GPDH divergence between the medfly *Ceratitis* and *Drosophila* is 4.7 times greater (Table 1, line 6, and Fig. 1, left) than between *Drosophila* species. But the accelerated rate of evolution could only have occurred for a fraction of the 200 My elapsed (between nodes 1 and 2 and between node 1 and *Ceratitis*), during which the rate of GPDH evolution must have been much more than five times faster than between *Drosophila* species.

Fig. 1 (right) displays the number of amino acid replacements against time for the dipteran lineages (highly compressed), as well as for three mammals (filled circle), three animal phyla, and three kingdoms. If we exclude the *Drosophila* lineages, the relationship is fairly linear, corresponding to a rate of amino acid replacement evolution of $4.0\text{--}5.3 \times 10^{-10}$ /site/year (Table 1, lines 5, 7, 8), fairly similar to the rate observed between *Ceratitis* and the *Drosophila* (4.7 $\times 10^{-10}$ /site/year, line 6). At this scale, the rate of evolution of GPDH appears fairly clock-like. Nevertheless,

Fig. 2. Phylogeny of nine fruitfly species. The thicker branches between nodes 3 and 4 and between 3 and 5 indicate times with greatly accelerated evolution of GPDH. The time scale is based on data from Kwiatowski et al., (1994, 1997); Ayala et al., (1996).

this ‘global’ rate would yield very erroneous conclusions if it were used for timing events or for deciding branching topologies in the evolution of dipterans. It can be seen in Table 1 that the apparent rate of amino acid replacements is fairly constant for comparisons between species diverged >70 My ago (i.e. $4.0\text{--}5.3 \times 10^{-10}$ /site/year), but this is 2–5 times as large as between species diverged <60 My ago.

3. Evolution of superoxide dismutases

The superoxide dismutases (SOD) are abundant enzymes in aerobic organisms, protecting the cell against the harmfulness of free oxygen radicals (Fridovich, 1986). These enzymes have active centers that contain either iron or manganese, or both copper and zinc (Fridovich, 1986). The Cu,Zn SOD is a well-studied protein, found in eukaryotes but also in some bacteria. The amino acid sequence is known in many organisms, plant, animals, fungi, and bacteria (Kwiatowski et al., 1991; Smith and Doolittle, 1992; Fitch and Ayala, 1994; Ayala et al., 1996).

The Cu–Zn SOD of *D. melanogaster* is a dimer molecule consisting of two identical polypeptide subunits associated with two Cu^{2+} and two Zn^{2+} per molecule. Each subunit has a molecular weight of 15,750 daltons and consists of 151 amino acids, the same as in other fruitfly species. The *Drosophila Sod* gene consists of two exons, separated by an intron 300–700 bp in length, located between codons 22 and 23. In *Chymomyza*, *ScaptoDrosophila*, and *Ceratitis*, there is an additional short intron (<100 bp) between codons 95 and 96 (Kwiatowski et al., 1992a,b, 1994). We have sequenced the *Sod* gene in 27 fruitfly species, the same set (with two inconsequential differences) sequenced for

Table 2

Evolution of SOD: amino acid replacements between increasingly divergent species^a

Comparison	My	Mean	Rate
Within <i>Drosophila</i> groups	5–25	0.0–11.4	0–22.7
Between <i>Drosophila</i> groups	45 ± 10	15.0 ± 0.2	16.6 ± 0.2
Between <i>Drosophila</i> subgenera	55 ± 10	17.8 ± 0.3	16.2 ± 0.3
Between dipteran genera	60 ± 10	21.4 ± 0.2	17.8 ± 0.2
Between mammals	70 ± 15	24.0 ± 0.4	17.2 ± 0.3
Between dipteran families	100 ± 20	31.8 ± 0.6	15.9 ± 0.3
Between animal phyla	650 ± 100	68.3 ± 0.9	5.3 ± 0.1
Between kingdoms	1100 ± 200	72.5 ± 1.2	3.3 ± 0.1

^a Species compared and other conventions are the same as in Table 1.

Gpdh (Kwiatowski et al., 1992a,b, 1994; Barrio and Ayala, 1997).

The data are summarized in Table 2. Fig. 3 (left) gives the *Sod* rate of amino acid evolution for the same set of dipteran species compared in Fig. 1. The rate of amino replacement is fairly linear over time, about $16\text{--}18 \times 10^{-10}$ amino acid replacements/site/year, although there are some conspicuously divergent points (see Fig. 3, left). The linearity of SOD evolution in these dipteran lineages contrasts with the sharp increase observed earlier in GPDH evolution when *Chymomyza* or *Ceratitis* are compared with the *Drosophila*, rather than *Drosophila* species with each other.

Fig. 3 (right) displays the SOD amino acid replacements for the same set of species investigated for GPDH in Fig. 1 (right) (except that the plant is now *Ipomoea batatas* rather than *Cuphea lanceolata*). As noted earlier (Ayala, 1986; but see also Lee et al., 1985), the rate of evolution of SOD seems to slow down as the lineages compared become

Fig. 3. Rate of molecular evolution of the protein Cu,Zn SOD. The rate numbers on the right refer to lineage changes in units of 10^{-10} amino acid replacements/site/year. Left. The three lines correspond to the best-fitting rates for the same three sets of species as in Fig. 1. The three rates (15.9, 16.2, and 17.8) are very similar to one another, in contradistinction to the disparate rates of GPDH for the same sets of species. Right. The three lines correspond to the best-fitting rates for three sets of species: from different *Drosophila* subgenera (rate 16.2), animal phyla (rate 5.3), and kingdoms (rate 3.3). These three rates are disparate and are much higher for species recently diverged (16.2, *Drosophila*) than for those that diverged long ago (3.3, for kingdoms), just the opposite to the shift occurring in GPDH.

increasingly remote. This state of affairs is precisely the reverse of the pattern observed in GPDH, where the rate of amino acid replacement is slower when the species compared are closely related.

4. Discussion

The contrasting patterns of SOD and GPDH evolution cannot be attributed to distinctive characteristics of particular taxa, since the same set of species are compared for each enzyme (with the inconsequential exception that the plant species is *Cuphea lanceolata* for GPDH, but a different angiosperm, *Ipomoea batatas* for SOD). For the same reason, the rate fluctuations cannot be attributed to telescoping consequences of the different time intervals. The SOD rate is much faster for recently diverged taxa than for those that diverged long ago (16.2×10^{-10} for *Drosophila* versus 3.3×10^{-10} between kingdoms), but for GPDH it is exactly the other way around (1.1×10^{-10} for *Drosophila* versus 4.0×10^{-10} between kingdoms). Moreover, the mammal rate is similar to the *Drosophila* rate in SOD, but much faster in GPDH. The generation-time hypothesis (Wu and Li, 1985; Gaut et al., 1992; Li et al., 1996) cannot account for the erratic patterns of GPDH and SOD. This hypothesis assumes that rates of evolution will be increased in organisms with shorter generation times. But the set of species analyzed is, as noted, the same for GPDH and for SOD. It should also be noted that we have used a variety of algorithms for correcting for overlapping and back substitutions. These various algorithms all yield the same results, except for trivial numerical differences (Kwiatowski et al., 1992a, b, 1994, 1997; Barrio and Ayala, 1997).

Several modifications of the neutral theory have been proposed seeking to account for the excess variance of the molecular clock. Five biological variables have been invoked:

1. Generation time. Shorter generation time will accelerate the clock because it shortens the time to fix new mutations. It has been noted, for example, that the rate of molecular evolution is faster in rodents than in cows or primates (Gu and Li, 1992; Ohta, 1993).
2. Population size. A larger population size will slow the clock, because it increases the time required to fix new mutations. It has been suggested that population size and generation time are often inversely related (there are more mice than elephants), which thus might cancel out, and yield approximate constant rates over time (Ohta, 1972; Kimura, 1983). But this inverse relationship is far from general. Several species of *Proechimys* rats in Venezuela and of *Spalax* mole rats in Israel have narrow ecological niches and small population sizes, while common rats and mice are cosmopolitan and several orders of magnitude more numerous. *Drosophila willistoni* is exceedingly numerous throughout the Amer-

ican topics, while its sibling species, *D. insularis*, is restricted to two small islands in the Caribbean, where it is much rarer than *D. willistoni*; surely the population size of the two species differs by many orders of magnitude (and there is no reason to think that matters were different throughout the history of these two species). Yet, rates of molecular evolution among rodent species or among *Drosophila* species do not differ in patterns consistent with their differences in population size.

3. Slightly deleterious mutations. This hypothesis was proposed by Ohta (1973, 1974) and is called 'the model of effectively neutral mutations' by Kimura (1983, p. 240). Such mutations behave as neutral in small populations, but will be selected against in large populations. If selection coefficients against mutations follow an exponential distribution, the rate of substitution is inversely proportional to the effective population size of the species (Ohta, 1977).
4. Species-characteristic differences in polymerases or other biological properties that affect the fidelity of DNA replication, and hence the incidence of mutations, neutral or not (Britten, 1986). Without any specific knowledge about each particular species, this hypothesis simply says that molecular evolutionary rates will differ among species. But this hypothesis predicts that differences between species will be consistent across different genes, a prediction that does not hold for the combined cases of GPDH and SOD.
5. Changes in the function of a protein as evolutionary time proceeds. This might particularly be expected in the case of gene duplication, when one or both duplicates evolve new functions and hence an accelerated rate of evolution might be expected. Accelerated evolution following duplication has been observed, for example, in the globin genes (Goodman, 1976; Goodman et al., 1988). Except when postulated in association with gene duplication, this hypothesis is a special case of the general claim for a predominant role of natural selection in molecular evolution.

The covarion hypothesis (Fitch and Markowitz, 1970) becomes helpful to account for the reduction in rate of evolution that obtains in some proteins when the species compared become increasingly remote, as in the case of SOD (Fitch and Ayala, 1994). But the covarion model cannot be extended to GPDH, whose rate of evolution increases as the organisms compared become more remote (Ayala, 1997). Similarly, the combined consideration of GPDH and SOD evolution in the same set of species excludes any of the other proposed modifications of the neutrality theory listed above. The hypothesis of the generation-time effect cannot account for the divergent patterns of evolution of both GPDH and SOD, since the same set of species are compared in both cases, and thus identical generation times have been involved at all times in the evolution of these species. Similarly, the postulates of vari-

Table 3

Rates of evolution of GPDH and SOD and estimates of divergence time derived from the *Drosophila* rate (Ayala, 1997)^a

Taxa compared	Rate of evolution		Normalized rate		Clock estimates, My	
	GPDH	SOD	GPDH	SOD	GPDH	SOD
<i>Drosophila</i> subgenera	1.1	16.2	1.0	1.0	55	55
Mammal orders	5.3	17.2	4.8	1.1	340	74
Dipteran families	4.7	15.9	4.3	1.0	470	98
Animal phyla	4.2	5.3	3.8	0.33	2,500	211
Kingdoms	4.0	3.3	3.6	0.20	3,990	224

^a The rate of evolution is in units of 10^{-10} amino acid replacements/site/year. The normalized rate is relative to the rate between the *Drosophila* subgenera. The clock estimates of time divergence use the average amino acid replacements of the particular organisms and assumes that the replacement rate is constant, so that the protein evolves as a molecular clock that ticks at the *Drosophila* rate.

able population size, slightly deleterious mutations or differences in the efficiency of repair mechanisms may be adjusted to account for the evolution of one or the other protein, GPDH and SOD, but not for both, without stretching ad hoc their elasticity to make the molecular clock hypothesis universally applicable to any possible empirical state of affairs and, therefore, without any predictive power and untestable (Popper, 1959; Ayala, 1994).

I have noted (Ayala, 1997) that constraints occur in the evolution of GPDH in *Drosophila*, which considerably restrict the number of sites that can accept amino acid replacements and the particular replacements that can occur at each site. It remains obscure why greater constraints would occur in *Drosophila* than in the *Chymomyza* or *Ceratitidis* lineages (or, indeed, in other animals, plants, and fungi). But, in any case, the issue is not whether biologically ascertainable processes are at work, which of course they are, in GPDH, SOD, or any other enzymes. The issue rather is whether the processes are of such regularity that some sort of molecular clock may be assumed. The stark contrast between the pattern of evolution of GPDH and SOD may be an aberration rather than representative of prevailing modes of protein evolution, since protein evolution seems so often to behave in a clock-like manner. But the congruence between observations and the clock predictions are often obtained due to the fact that the data collected do not have sufficient resolution to manifest likely discrepancies.

The operational risks of assuming that protein clocks are fairly reliable are made evident in Table 3. The rate of GPDH evolution is nearly four times faster between animals and plants than between *Drosophila* species, whereas the rate of SOD evolution is five times slower. If we were to use the observed rate of *Drosophila* evolution to estimate the time of divergence between plants and animals, GPDH would yield an estimate of 3,990 My, SOD an estimate of 224 My, both grossly erroneous. The practical conclusions to be drawn are that (i) protein clocks should be used cautiously and weighed against any other available evidence, rather than considered decisive; (ii) several protein clocks should be used whenever feasible, particu-

larly when important evolutionary events need to be determined (Wray et al., 1996; but see Ayala et al., 1998).

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