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Perspectives

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Molecular Clock: An *Anti*-neo-Darwinian Legacy

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AS early as the time of the rediscovery of Mendel's laws, George H. F. Nuttal measured the amount of precipitate of normal sera from great apes, monkeys, and some other mammals. His crude method, using rabbit antiserum directed against whole human serum, indicated that except for flying lemurs, the amount of precipitate declined with the evolutionary distance from humans (Nuttal 1904; Klein 1995). The immunological method was later refined and played an important role in reconstructing primate phylogenies (Goodman 1962). In 1962, almost in parallel, Emile Zuckerkandl collaborated with Linus Pauling at Caltech on hemoglobin evolution and expressed the idea of "molecular anthropology" as a new discipline (Zuckerkandl 1963). The idea was optimistic and ahead of the times, but Morris Goodman shared it. In the same year, Zuckerkandl and Pauling (1962) calibrated the amino acid substitution rate in mammalian hemoglobins and estimated the divergence times of orthologous and paralogous hemoglobins. Clearly, the immunological and protein sequence data had already provided the germ for immunological and protein clocks (see also Margoliash 1963 for cytochrome c; Doolittle and Blombäck 1964 for fibrinopeptides).

The time was ripe for Zuckerkandl and Pauling (1965, p. 138) to advocate the concept of a molecular evolutionary clock: "Anyone who recognizes the value of the immunological approach for estimating phyletic distance with certain limits should find it impossible to deny that the comparison of amino acid sequences is potentially an even better tool. It is only potentially less equivocal, more accurate, suited for absolute instead of only relative evaluations, and able to extrapolate from the present to the past." The stochastic nature of the molecular clock was well recognized and described by a Poisson process for the first time. It was also pointed out that, for a molecular clock to exist, amino acid changes must be limited almost exclusively to functionally nearly neutral changes, although not only random genetic drift but also Darwinian selection was invoked for fixation of such changes. Thus, the discovery of a molecular clock supported the concept of near neutrality at the molecular level. From a historical point of view, it is of interest to ask who was responsible for the monumental proposition of a molecular clock, Zuckerkandl or Pauling. Thirty years later, Pauling recalled, "I think it was my idea, but I am not sure. We were just collaborating on these studies. Perhaps it was Emile's idea" (Morgan 1998, p. 166). This recollection might be a Freudian memory lapse (E. Zuckerkandl, personal communication). The idea must be Emile Zuckerkandl's, since it is clear in Zuckerkandl and Pauling (1962), an article almost entirely written by Zuckerkandl (see also Zuckerkandl 1987).

The high evolutionary rate estimated from hemoglobin and other proteins was a key to development of the neutral theory by Motoo Kimura, rather than, as sometimes asserted, the large extent of electrophoretically observed polymorphism (Dietrich 1994; Suárez and Barahona 1996). Indeed, Kimura's 1968 article begins with a discussion of the amino acid substitution rate obtained from Zuckerkandl and Pauling (1965). From this, Kimura estimated the nucleotide substitution rate on the basis of codon degeneracy and extrapolated this rate to the entire genome on the basis of the total number of base pairs estimated by Muller (1958). This rate was too high to be accounted for by natural selection, according to Haldane's (1957) cost of natural selection. Kimura often said that Muller's estimate (4×10^9 bp/human sperm) was critically important in advocating his thesis (see also Kimura 1983). Interestingly, James F. Crow, examining similar data, concluded that the rates were quite consistent with Haldane's cost of natural selection. Crow recalled that he was referring only to coding regions of DNA (Dietrich 1994). In accord with Crow (1968), King and Jukes (1969) pointed out that Kimura's estimate of per-genome substitution rates might

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be exaggerated for several reasons, especially the existence of massive amounts of noncoding DNA. In addition to its provocative title, "Non-Darwinian Evolution," KING and JUKES (1969) is filled with statements that are insightful even today: (1) natural selection was rightly epitomized as the editor, rather than the composer (SIMPSON 1964), of the genetic message; (2) similar at least in principle to the mutationism discussed later in this article, mutation was regarded as the major driving force in evolution; (3) it was convincingly argued that different proteins and different sites within specific proteins evolve at different rates; and (4) uniform rates of evolutionary change within a protein were thought to lend credence to the proposition of neutrality. Like King and Jukes, WILSON and SARICH (1969) also noted that the immunological clock is difficult to explain in terms of natural selection.

Although KIMURA (1969) was impressed by the remarkable rate constancy in protein clocks, he nevertheless regarded this only as additional support for his neutral theory. Two years later, however, considering the large amount of noncoding DNA in the vertebrate genome and redundancy in the genetic code, he changed his mind and agreed that the constant rate, rather than the high rate, in amino acid or nucleotide substitutions is the strongest evidence for neutrality: "Probably the strongest evidence for the theory is the remarkable uniformity for each protein molecule in the rate of mutant substitutions in the course of evolution" (KIMURA and OHTA 1971a, p. 467). By that time, another important finding—that the rate of molecular evolution differs greatly from protein to protein (*e.g.*, MARGOLIASH *et al.* 1968; DICKERSON 1971)—was explicitly incorporated into the theory. Selective constraint and the fraction of selectively neutral mutations are vital concepts in using the neutral theory to explain this prominent feature of molecular evolution. The concept was rephrased in various ways, such as that "the more stringent the functional requirement of the molecule [or part of the molecule], the slower is its evolutionary change" (KIMURA and OHTA 1973, p. 25). However, despite all this progress in molecular evolution, Kimura remained skeptical of his theory until the high rate of substitutions at synonymous sites (KIMURA 1977) and in pseudogenes (LI *et al.* 1981; MIYATA and YASUNAGA 1981) was actually observed, consistent with their having little or no apparent selective constraint.

The accuracy of the molecular clock, even with the early recognition of its stochastic nature, has long been a target of controversy. An early controversy also arose about the immunological clock. On the basis of assumed early divergence times of hominoids, GOODMAN (1962, 1963) claimed the rate slowdown in the lineage leading to humans. On the other hand, SARICH and WILSON (1967) and WILSON and SARICH (1969) contended that no such "hominoid slowdown" occurs, postulating a recent Pliocene ancestor between humans and apes. The rate slowdown in hominoids was attributed to the generation-time effect that assumes that species with shorter generation times evolve more rapidly than species with longer generation times. This correlation between the generation time and the substitution rate is thought to arise if DNA replication-dependent errors are the major source of mutations and organisms with shorter generation times undergo more replications per unit of time than those with longer generation times. Sarich and Wilson favored the immunological method; it allowed them to obtain a more reliable date for the ape–human divergence than the protein clock, since humans and apes are too closely related for a protein clock to tick often enough. For this obvious reason, it has become a common practice to select rapidly and slowly evolving molecules for studies of closely and distantly related species, respectively. At any rate, for the Pliocene common ancestor between humans and apes to be valid, the rate slowdown had to be rejected. To this end, SARICH and WILSON (1973) invented the so-called relative rate test that does not require debatable fossil dates.

Early work on protein clocks (*e.g.*, FITCH and MARGOLIASH 1967; KIMURA and OHTA 1971b; UZZELL and CORBIN 1971) revealed that the variance of the number of amino acid substitutions is generally larger than that expected from a Poisson clock. On the basis of this observation, some speculated that different amino acid sites in the same proteins do not evolve at the same rate. If the rate parameter in the Poisson process varies among sites according to the gamma distribution, a "negative binomial" clock results, as proposed by UZZELL and CORBIN (1971). Subsequently, LANGLEY and FITCH (1974) developed an elaborate method for testing Poisson clocks for amino acid substitutions in various proteins in a given phylogenetic tree. The test allowed separate examination of a Poisson clock among branches over proteins (later called "lineage effects") and among proteins within branches (later called "residual effects"). Although clock-based estimates of species divergence times correlated well with paleontological dates, the subtests rejected both Poisson models in light of more variations in the total number of substitutions on different branches and in the relative rates across branches than those predicted by the Poisson models. The idea of separating residual effects from lineage effects apparently influenced John H. Gillespie, who forcefully and consistently defended neo-Darwinism or the synthetic theory of evolution. Using the ratio (R-statistic) of the variance to the mean number of substitutions, he found that protein clocks are overdispersed ($R > 1$) and argued that protein evolution is even episodic, with short bursts of rapid evolution followed by long periods of slow evolution (GILLESPIE 1984). The overdispersion caused by strong residual effects demanded a mechanistic explanation. One suggestion was that amino acid sites per protein do not evolve independently. If so, it is necessary to model the entire sequence, rather than individual sites, on which

natural selection acts (Gillespie 1984, 1991; Takahata 1987, 1991; Cutler 2000). Another mechanistic interpretation for the rate heterogeneity was provided by fixation of nearly neutral, but slightly deleterious, mutations (Ohta 1987, 2002).

On the other hand, lineage effects are generally thought to be associated with systematically varying mutation rates among lineages and have been extensively studied with emerging DNA sequence data in the 1980s. It soon became clear that replication-dependent errors are more apparent at the DNA level than at the protein level, or lineage effects are strong for silent substitutions while residual effects are strong for amino acid substitutions. These contrasting patterns for silent and amino acid substitutions are consistent with the earlier finding obtained by DNA–DNA hybridization (Laird *et al.* 1969) and the immunological method. Lack of lineage effects on the rate of DNA sequence evolution is usually interpreted as a result of mutational processes other than errors in DNA replication (*e.g.*, DNA repair).

Wu and Li (1985) were the first to apply the relative rate test to DNA sequences. Comparing DNA sequences from rodents and humans mostly with cattle as an outgroup, they found that rodents evolve approximately twice as fast as humans and that the generation-time effect is stronger for silent substitutions than for amino acid substitutions. However, a heated debate again arose because the same test applied to mammalian DNA sequences did not show lineage effects for either type of substitution (Easteal 1988). One possibility for this discrepancy was incorrect branching orders of genes and species assumed in the previous test (Easteal *et al.* 1995; Kumar and Hedges 1998; Bromham *et al.* 1999). It is now clear that any kind of molecular clock ticks erratically, but it is nevertheless widely used for estimating species divergence times. A rationale behind this is the expectation that molecule-specific rate differences in different species average out if many molecules can be used (Kumar and Hedges 1998).

In the 21st century, research interest in the molecular clock has continued to grow. As demonstrated in Kumar (2005), the number of articles dealing with the molecular clock tends to exceed 100 annually. When writing this *Perspectives*, I could not survey all of these articles so that it is likely that I have overlooked important ones. Nevertheless, the following few articles will convey new trends in the field in the genomic era. An examination of a large number of fourfold degenerate sites between orthologous genes in various mammalian species showed a lack of significant lineage effects, although this holds true only if they evolve with homogeneous substitution patterns (Kumar and Subramanian 2002). By contrast, where orthologous gene pairs evolve with heterogeneous substitution patterns, these gene pairs tend to show large relative rate differences. The rate unit for the molecular clock has been intensively debated (*e.g.*, Nei 1975 for early debates). The observed rate constancy was apparently per year in contrast to the per-generation constancy of mutation rates revealed by classical genetics methods. Using DNA sequence data from several major taxonomic groups, which span 10 orders of magnitude in body size and the biological temperature, Gillooly *et al.* (2005) found that nucleotide substitution rates are strongly body size and temperature dependent. Combining principles of allometry and neutral evolutionary dynamics, they suggested a single molecular clock that ticks at a constant rate per unit of mass-specific energy rather than per year or per generation. So, the conclusion was that the mutation rate is intimately related to body size and temperature; the heavier the body weight and the colder the body temperature, the slower the mutation rate.

In relation to the heterogeneous mutation rate over the genome, Kim *et al.* (2006) paid special attention to CpG dinucleotides because the frequent C-to-T change by deamination appears to be replication independent. Indeed, hypervariable CpG sites exhibit relatively constant rates of substitutions over time while non-CpG sites exhibit generation-time-dependent rates of substitutions. Furthermore, in relation to the generation length as a life history trait, Elango *et al.* (2006) carried out a relative rate test for humans and chimpanzees using baboons or rhesus monkeys as an outgroup. Human DNA might have evolved at a significantly slower rate than chimpanzee DNA. However, the difference was so slight that if the rate slowdown in humans is attributed to the generation-time effect, this human-specific life history trait must have evolved very recently.

It is now well documented that the mutation rate *per se* varies greatly among broad taxonomic groups (Drake *et al.* 1998; Britten 1986 for early work). An important source for this rate heterogeneity is definitely differential fidelity of DNA replication and/or DNA repair among different taxonomic groups, as mentioned. Now, there are large-scale experiments for understanding the magnitude and type of spontaneous mutations. Recent advances in sequencing techniques permit direct knowledge about the mutation rate and spectrum in mutation-accumulation lines of *Escherichia coli* and *Salmonella enterica* (Ochman 2003), *Caenorhabditis elegans* (Denver *et al.* 2004), and Drosophila (Haag-Liautard *et al.* 2007). In this context, one can hardly forget the laborious work on viability polygenes in *Drosophila melanogaster* by Terumi Mukai and his colleagues (Mukai 1964; Mukai *et al.* 1972). Recently, there have been a number of reanalyses and new estimates, which throw doubt on Mukai's original values (Keightley and Eyre-Walker 1999; Haag-Liautard *et al.* 2007). The rate and spectrum of spontaneous mutations thus accumulated differs markedly from indirect estimates inferred from long-term evolution. This difference is expected because the organisms in these experiments were kept in conditions optimal for survival and reproduction. A great majority of spontaneous mutations are mildly deleterious: they

are retained in laboratories but eliminated in nature by purifying selection. These examples show some interesting and promising ways to connect the molecular clock to the study of species-specific life history traits and spontaneous mutation.

Focusing on mechanistic aspects of molecular clocks, I have tried to minimize a possible overlap with recent publications (*e.g.*, Bromham and Penny 2003; Hedges and Kumar 2003; Kumar 2005). These articles not only describe well the history of the study of the molecular clock, but also include the latest problems and excitement in the study of the tree of life, such as the emergence of eukaryotes and metazoan phyla, the Cambrian explosion of vertebrates, the emergence of tetrapods, the mammalian radiation, and the trichotomous relationship among humans, chimpanzees, and gorillas. It is now generally accepted that, although it is uncertain and rejected for a substantial proportion of proteins and genomic regions in comparisons of main taxonomic groups, the molecular clock can put a new timescale on the history of life, thereby allowing exploration of the mechanisms and processes of organismal evolution. Similarly, a molecular clock is an irreplaceable source of information in evolutionary biology and it would be foolish to abandon it altogether, as Bromham and Penny (2003) put it.

Despite inherent fluctuations and various interpretations, the molecular clock has become a most useful tool—perhaps *the* most useful—for studying molecular evolution. The simplest interpretation is to assume Kimura's neutral theory. Then the reasonable assumption that the mutation rate for a given DNA region is constant over time leads directly to a molecular clock. Exact rate constancy is not likely, for many reasons, but the assumption is good enough for practical use. Although neutrality is the conventional assumption, even Gillespie-like processes can average out to rough constancy over long periods. The clock need not be exact; an approximate molecular clock can still be very useful. The number of molecular changes can be calibrated by reference to events whose times are known (such as dated fossils) and converted to units of absolute time. A molecular clock is now a standard assumption in almost every study of molecular evolution.

It seems, however, that the most important implication of the molecular clock is concerned with the link between molecular and phenotypic evolution. This question has persisted since originally raised by Zuckerkandl and Pauling (1965). Published in the era of neo-Darwinism when the importance of natural selection in evolution was overvalued, the article raised the contrasting view: "Many phenotypic differences may be the result of changes in the patterns of timing and rate of activity of structural genes rather than of changes in functional properties of the polypeptides as a result of changes in amino acid sequence" (p. 100). Clearly, this view demanded some yet largely unknown changes in noncoding regions. It was reincarnated when King and Wilson (1975) proposed regulatory mutations for major biological differences among species (Wilson 1985; Zuckerkandl 1987).

In contrast, Kimura (1983, 1990) was primarily concerned with coding regions and demanded a bifurcation of molecular evolution from phenotypic evolution in terms of the mechanism. As a population geneticist, he asked why natural selection is prevalent at the phenotypic level and yet neutral drift prevails at the molecular level. He reasoned that this bifurcation is based on the effect of stabilizing selection for a quantitative character determined by a large number of segregating loci or sites. Under stabilizing selection, deleterious mutations are eliminated and a large fraction of genetic variation thus retained in a population is neutral or nearly so. However, if the environment changes, such variation may have a better chance of being advantageous, thereby generating the punctuated equilibrium-like pattern of evolution—long periods of status quo interspersed with rare periods of sudden changes. Thus Kimura thought that phenotypic evolution is based on neutral variation in coding regions that may be selected for when the environment changes. This idea was formally proposed as a four-stage scenario of macroevolution (Kimura 1990): (1) liberation from existing selective constraints; (2) a sudden increase of neutral mutations under relaxed selection; (3) some of the mutations turn out to be useful in the new environment; and (4) individual and intergroup selection lead to adaptive evolution. At the same time, Provine (1990) expressed his hope that ways might emerge to connect the neutral theory with phenotypic characters and even with mechanisms of speciation. Unfortunately, the four-stage scenario has not been pursued further and it remains as a conjecture, not as a testable hypothesis.

There is another line of thought, which has resurrected Thomas H. Morgan's mutationism for the occurrence of advantageous mutations. Following Morgan, Masatoshi Nei (1983, 1987, 2005) considered mutation as the main driving force of both molecular and phenotypic evolution. Unlike neo-Darwinism, which regards mutation as merely raw material and natural selection as the creative power, Nei's mutationism assumes that the most fundamental process for adaptive evolution is the production of functionally more efficient genotypes by mutation (especially birth and death of duplicated genes) and by recombination. Since a sufficiently large amount of genetic variation is presumably not maintained in a population or species, an inevitable fate of organisms under drastically changing environments is extinction unless indispensable mutations arise and are incorporated into the species in time. In this view, Nei considers the basic process of phenotypic evolution to be essentially the same as that of molecular evolution and the extent of neutral genetic changes of phenotypic characters to be as great as that of protein variation.

Although these views are not all mutually exclusive, they are different with respect to genetic material, rate and pattern of mutation, and roles of natural selection that are invoked in phenotypic evolution. In my opinion, mutationism would definitely prevail if we consider long-term evolution such as the ancestral lineage of jawed vertebrates. No doubt, in the common ancestral lineage, these organisms must have gained novel genetic systems for acquired immunity (Klein 1990) and tissue mineralization (Kawasaki and Weiss 2006), for example. However, this seems less likely as a way in short-term evolution, such as in the speciation process of humans, and mutational changes can be subtler and correspondingly less visible than the innovation of new genetic systems. Nei (1983) dared to speculate that a mutation for human-level intelligence probably occurred in the human lineage with an exceedingly small probability. It is possible that this mutation was a beneficial change that has altered the expression of existing forms of genes involved. We do not know of such a regulatory change. Alternatively, the mutation might be a change in the functional properties of proteins. One candidate is a mutation at the *ASPM* (abnormal spindle-like microcephaly associated) locus. The protein acts as a specific regulator of brain size and exhibits accelerated rates of nonsynonymous substitutions (Zhang 2003; Kouprina *et al.* 2004; Mekel-Bobrov *et al.* 2005). Another candidate may be a change in heat-shock protein (Hsp90) that can act as a "capacitor" of phenotypic evolution (Rutherford and Lindquist 1998; Queitsch *et al.* 2002). It is true that organisms must adequately buffer the influences of mutation and environmental challenge to create a trade-off between stability and the potential for changes. Hsp90 does this job. It can store hidden phenotypic variation and its change can produce a profusion of morphological changes, for instance, by affecting interaction with various proteins in signal-transduction pathways or by inducing epigenetic alterations in gene expression (Whitesell and Lindquist 2005). In any case, it is likely that the component of mutation responsible for phenotypic evolution follows different rules and depends heavily on the timescale of the evolutionary process under consideration.

It seems that phenotypic evolution is so opportunistic and mechanistically diverse that singling out one cause or rule is precluded. Yet, at the level of DNA and much of protein, a much simpler rule dominated by mutation *per se* has been governing their evolutionary fate. A molecular clock is a most remarkable manifestation and a tribute from nature to anyone who studies evolutionary biology.

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