Three Perspectives on Neutrality and Drift in Molecular Evolution

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This article offers three contrasting cases of the use of neutrality and drift in molecular evolution. In the first, neutrality is assumed as a simplest case for modeling. In the second and third, concepts of drift and neutrality are developed within the context of population genetics testing and the development and application of the molecular clock.

1. Introduction. The importance of random drift for the field of molecular evolution is undeniable, even if its relative significance has been the subject of intense controversy. Random drift was given a leading role in the development of molecular evolution by Motoo Kimura, Tomoko Ohta, Tom Jukes, Jack King, and others. Beginning in the late 1960s, advocates of neutral molecular evolution presented it as a radical alternative to the omnipotence of natural selection in biological evolution. Jack King and Tom Jukes even dubbed neutral molecular evolution "non-Darwinian," in a successful attempt to provoke organismal evolutionary biologists (Dietrich 1994, 1998). According to the neutralists, random genetic drift at least partially displaced selection at the molecular level because a large number of observed molecular changes were postulated to be free from natural selection or are very weakly selected—that is to say, a large number of mutations were understood to be neutral or nearly neutral (Kimura 1968; King and Jukes 1969). Although advocates of neutral molecular evolution set off a long-standing controversy with selectionists, hypotheses and models of neutrality and drift fundamentally shaped the field of molecular evolution (Ohta and Gillespie 1996).

In this article I will argue that considerations of neutrality and drift entered into the study of molecular evolution first as the simplest cases

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in stochastic modeling. In the context of the neutralist-selectionist debates after 1970, concepts and models of neutrality and drift were articulated in two parallel enterprises: one arising from population genetics, concerning the development of statistical tests of neutrality, and another arising from comparative biochemistry, concerning the constant rate of substitution at the molecular level known as the molecular clock. Rather than try to present a seamless, comprehensive narrative of the rise of neutral molecular evolution, I am going to condense out of this history three contrasting episodes. In the end, I will point to some more philosophical questions that I think these episodes raise.

2. Assumed Neutrality. The idea that there might be mutations that have no selective value, either positive or negative, was common in population genetics in the mid-twentieth century (Dobzhanksy 1955; Crow 1985). Neutral alleles and the random drift of their frequencies in a population were familiar features of many population genetics models. Early work by Sewall Wright, R. A. Fisher, and J. B. S. Haldane developed models of neutral alleles in order to understand processes of drift, especially in relation to processes of selection and migration. These early neutral models allowed population geneticists to make comparative judgments about the efficacy of selection, for instance. Assuming neutrality also had the advantage of allowing modelers to work with mathematically simpler models. Different aspects of a model could be explored using the assumption of neutrality and then made more realistic and complicated by adding in selection.

Motoo Kimura entered evolutionary biology as an expert on stochastic population genetics. Throughout the 1950s, he developed a method of analysis using diffusion equations, which he applied to a range of stochastic problems including the random assortment of chromosomes in paramecium, the interaction of selection and drift in finite populations, and the probability of fixation of a neutral allele (Kimura 1957, 1985). In the early period, before 1966, Kimura saw neutral mutations as a possibility, but not as a reality. The introduction of molecular data into evolutionary genetics and the early development of the study of molecular evolution in the 1960s convinced Kimura and others that systems of neutral alleles did exist. This reification fundamentally altered the interpretation of stochastic models of neutral alleles and made their outcomes much more relevant to the practice of experimental evolutionary genetics and molecular evolution.

This shift in status of neutral assumptions is beautifully illustrated by the case of the infinite alleles model presented by Motoo Kimura and James Crow in 1964. The infinite alleles model was constructed in the context of the classical balance debate over the relative proportions of

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homozygous and heterozygous loci in natural population. James Crow was an advocate of the classical position, that is, that there was a large amount of homozygosity in natural populations maintained by purifying selection. As such, he was interested in trying to predict the number of different alleles that could be maintained in a population. In other words, he wanted to be able to predict the homozygosity of a population. But he also wanted to assume that every new mutation was a mutation to a new allele—so mutation did not create any new homozygosity. In 1958, he set this problem for Motoo Kimura, who had already been recognized as one of the best stochastic modelers in population genetics (Crow 1989). Kimura sent a solution a few months later for the case of neutral alleles. Crow and Kimura collaborated two years later to produce a more complete characterization of what became known as the infinite alleles model (Kimura and Crow 1964).

In their 1964 paper, Kimura and Crow examined some of the population consequences of three different allele systems: "(1) A system of selectively neutral isoalleles whose frequency in the population is determined by the mutation rate and by random drift. (2) A system of mutually heterotic alleles. (3) A mixture of heterotic and harmful mutants" (1964, 725). The systems of heterotic alleles (heterozygote superior or overdominant alleles) and heterotic and harmful alleles were developed to argue against the balance position's assertion that large numbers of heterozygous combinations were maintained in natural populations. For both of these cases, Kimura and Crow constructed an equilibrium model that allowed them to calculate the proportion of homozygous loci, the effective number of alleles maintained in a population, and the segregational load. A segregational load occurs when the most fit genotype is the heterozygote and Mendelian segregation insures that in each generation inferior homozygous combinations will be formed. The segregation load is the decrease in the fitness of the population that occurs as the result of the formation of the less fit homozygotes. It is important to note that the paper's main argument is intended to cast doubt on assertions made by advocates of the balance position. Specifically, they argued against Bruce Wallace's assertion that *Drosophila* are heterozygous at 50% of their loci. Using their model, Kimura and Crow calculated the segregational load that would be produced if there were 10,000 loci total. The resulting load was very high and suggested that most loci are homozygous (Kimura and Crow 1964, 736).

The empirical application of the selected cases of the infinite alleles model stands in contrast to the neutral case. The neutral model was acknowledged as "unrealistic," but Kimura and Crow thought that it could "help provide some insight as to what situations were possible or likely in a natural population" (1964, 735). In the neutral case, its formulas

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allowed them to calculate homozygosity for different parameter values. The results were then interpreted as representing the "upper limit for heterozygosity in a population" because introducing directional selection or relaxing the infinite allele assumptions would increase the proportion of homozygosity. The result is only about the relationship posited within the model for an imagined situation. Kimura and Crow explicitly noted that they did not want to argue for the plausibility of neutral alleles.

The shift to the advocacy of the neutral theory and the existence of neutral alleles after 1968 then involves realizing and advocating the fact that the simplest mathematical case may in fact hold in nature. With the advent of the neutral theory, the mathematical treatment of the neutral case became much more important than the argument against the balance position. In fact, now the 1964 paper is remembered for making predictions important for testing the neutral theory (Crow 1989). At the time, the consequences of the neutral case were seen as idealized limits, not as empirical predictions about natural systems.

3. Tests of Neutrality and Selection. While Kimura advocated the importance of neutral mutations and random drift, he never advocated that all mutations were neutral. The neutralist-selectionist debate was extremely polarized during the 1970s and 1980s, but it was not an all-or-nothing debate pitting drift against selection. Like virtually all major controversies in evolutionary genetics, the neutralist-selectionist controversy was a relative-frequency controversy where the relative contributions of drift and selection were at issue (Beatty 1995). The neutral theory gave important roles to both selection and drift. In fact, selection played an indispensable role determining the fate of most mutations. In order to appreciate this you have to understand that Kimura carefully distinguished between the process of mutation and the process of substitution (Kimura 1983).

When Kimura argued for the existence of large numbers of neutral alleles, he was making a claim about observed substitutions. Kimura, Ohta, and others acknowledged that there was a range of mutations that included a small number of advantageous mutants, some portion of neutral and near neutral mutants, and a substantial number of deleterious mutants. While there is some discussion about the relative proportions of these mutant classes, they agree that directional selection will quickly eliminate a large proportion of the deleterious mutants and quickly fix the truly advantageous mutants. The remaining mutants will be clustered around the completely neutral class of mutants. These mutants will be subject to either weak selection or no selection. Neutral and very weakly selected or nearly neutral mutants would be subject to random drift. What Kimura meant by random drift was that allele frequencies in a population

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would randomly fluctuate as a result of gamete sampling in reproduction each generation (Kimura 1983). So neutral and nearly neutral mutants would drift until they became either fixed in a population or lost. The neutralists' claim is that the neutral mutations may represent only a fraction of the total number of mutations when they occur, but "most mutants that spread into a species are neutral" (Kimura and Ohta 1971, 469).

The distinction between neutral mutants and substitutions is epistemically driven. Neutral mutation cannot be observed; substituted differences can. Actually, what stage of the substitution process is represented by observed molecular differences is not perfectly clear (see below). The point of the distinction between mutation and substitution is that observed differences have been subject to selection and drift before we have observed them. Observed molecular differences then are the outcomes of a random process of mutation, which Kimura understood as produced largely from DNA replication error, processes of directional selection, and processes of random drift produced by gamete sampling. We can detect mutants that have been fixed or are in the process of spreading through a species. Because the time to fixation for a neutral allele can be very long, these transient alleles appear as polymorphisms in a population. Understood from this perspective, then, Kimura and Ohta are claiming that most protein polymorphisms are transient—they are neutral alleles on their meandering path toward fixation or loss. In terms of their histories, both fixed differences and polymorphisms have gone through the similar processes of mutation, selection, and drift; polymorphisms are just not as far along as are fixed differences.

Kimura and Ohta claimed that one of the principle virtues of the neutral theory was that it generated testable predictions, and in so doing promised to "emancipate" biologists from "naïve panselectionism" (Kimura and Ohta 1971, 469). Using the diffusion equation method, Kimura and Ohta generated a number of quantitative predictions for neutral alleles. For instance, in 1969, Kimura and Tomoko Ohta calculated the average number of generations until a neutral mutant was either lost or fixed in a finite population (Kimura and Ohta 1969). The average time to fixation was $4N_e$. These kinds of quantitative predictions, coupled with the promise of electrophoretic data in the late 1960s, spurred Crow and others to argue in favor of the neutral theory—not as a correct theory, but as a highly testable theory (Crow 1969). Selectionist critics such as G. Ledyard Stebbins, Richard Lewontin, and Francisco Ayala were less convinced that decisive tests could be conducted and deployed Popperian ideas of falsifiability to assail neutralist hypotheses (Stebbins and Lewontin 1972; Ayala et al.1974). Both attitudes were justified. The neutral theory did indeed make a large number of testable quantitative predictions, but most

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of the tests from 1970 until 1985 to detect neutrality and selection were not considered decisive.

Testing the neutral theory ran into two main problems. On the one hand, statistical tests, such as the one proposed by Warren Ewens in 1972, were very promising, but they turned out to have little statistical power. It was not possible to reject the neutral null hypothesis using the Ewens test, for instance. On the other hand, nonstatistical tests were disputed and considered indecisive. For instance, Francisco Ayala's group used data about electrophoretic variability in natural populations of *Drosophila* to test a range of neutralist predictions. Using a measure of heterozygosity predicted using an infinite alleles model, Ayala and his coworkers noted that the frequency distribution of heterozygous loci was significantly different. Instead of the predicted distribution clustered around the average heterozygosity of 0.177, the distribution was fairly even except for an excess of loci with very little heterozygosity. This excess of rare alleles was seen as an explanatory failure of the neutral theory (Ayala et al. 1974, 378). In response, Jack King noted that many of the assumptions made in the infinite alleles model could be the source for the rare alleles discrepancy. Others noted that the infinite alleles model was not appropriate for electrophoretic data, since electrophoretic classes probably encompassed many allelic differences (King 1976). Ayala and his coworkers were sensitive to these criticisms and discussed mutation models for electromorphs as well as the models' assumptions. In the course of the give and take over these models, Kimura and Ohta began to advocate a larger role for slightly deleterious mutants whose frequencies would still be subject to drift. Others, such as Masatoshi Nei, began to emphasize shifting population dynamics such as those resulting from population bottlenecks. These kinds of results were influential in that they drove revisions of both the neutralist and selectionist models, but they were not decisive in terms of resolving the dispute between neutralists and selectionists.

The availability of DNA sequence data after 1985 represents a significant turning point for the neutral theory and its tests. Using DNA sequence data, Martin Kreitman and others devised statistical tests that could statistically distinguish between neutrality and selection. These statistical tests subject a neutral null hypothesis to rejection (Kreitman 2000). The success of this method has been hailed by Jim Crow as one of the most important events in the history of molecular evolution. However, it is important to note that the frequent rejection of neutral null hypotheses does not necessarily imply the demise of the neutral theory. The relationship between statistical null hypotheses and the neutral theory is complicated by the introduction of new neutral models of sequence evolution, new data about DNA sequences, and new methods, such as coalescents, which make neutral assumptions. Most important, however, the popu-

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lation genetic tradition of testing was only one avenue of support for the neutral theory.

4. Comparative Molecular Evolution and the Molecular Clock. Molecular evolution emerged from the interaction of a number of different disciplines (Dietrich 1994). Neutralists, such as Kimura and Ohta, had strong backgrounds in population genetics, but readily made use of the data and techniques emerging from biochemistry that used comparisons of various biochemical properties of molecules to draw evolutionary conclusions. At the forefront of these efforts were biochemists such as Tom Jukes, Emile Zuckerkandl, Morris Goodman, and Alan Wilson (Dietrich 1994; Hagen 1999).

In 1965, Emile Zuckerkandl and Linus Pauling articulated what has been referred to as "the most significant result of research in molecular evolution" (Wilson et al. 1977). By comparing the amino acids sequences of proteins from different species, Zuckerkandl and Pauling found that the sequence differences were "approximately proportional in number to evolutionary time" (Zuckerkandl and Pauling 1965, 148). In other words, the rate of amino acid substitution was approximately constant. In 1965, they christened this constancy the molecular clock (Zuckerkandl and Pauling 1965; Morgan 1998). Zuckerkandl and Pauling invoked both selection and drift to explain the constancy of the clock (Morgan 1998). After 1968, however, Motoo Kimura, Allan Wilson, and others used the neutral theory of molecular evolution to explain the mechanism of the clock

Rate constancy at the molecular level followed easily from the basic theoretical commitments of Kimura and the neutralists. According to the neutralists, the rate per generation of mutant substitutions in a population is equal to the mutation rate per gamete. The rate of evolution for selectively advantageous mutants, in contrast to neutral mutants, is dependent on both population size and selection pressure. Thus, in order for a selectionist model to account for the constancy of the rates of evolution, it must show how constancy is possible when the rates are strongly dependent on the environment, as represented by the selection coefficient s and the measure of population size N, which can be quite variable. Under the selectionist model, the rates of molecular evolution should show nearly the same variability as the rates of phenotypic evolution. Unfortunately for the neutralists, the rates of molecular evolution were found to be far from uniform.

The rate of amino acid substitution was known from the beginning (1965) to vary among different proteins. The neutralists explained this difference in terms of different proteins having different fractions of neutral mutants; the number of neutral mutants depends on the functional constraints for each protein. So, for instance, fibrinopeptide A has a much

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higher rate of substitution than histone IV, which is highly constrained (King and Jukes 1969, 792). But even within protein families variation was observed. So, for instance, insulins in the line leading to guinea pigs seem to have evolved faster than insulins in other lines (King and Jukes 1969; Ohta and Kimura 1971, 19). The neutralists needed a way to explain these deviations from the intrinsic rate of molecular evolution.

In 1971, Ohta and Kimura analyzed these variations in proteins statistically. When they did this for different alpha and beta hemoglobins, and for cytochrome *c*, they found that observed variance in the beta hemoglobin and the cytochrome *c* were significantly larger than expected. From this they concluded that "the variations in evolutionary rates among highly evolved animals are larger than expected from chance" (Ohta and Kimura 1971, 21). Ohta and Kimura did not take this as a reason to give up the neutral theory. The increased variance in substitution rates was chalked up to a small fraction of advantageous mutations that affect the molecule's function but do not interfere with the constancy of the overall rate of substitution (1971, 23).

After Ohta and Kimura's paper in 1971, a tremendous amount of empirical research was done on the molecular clock (Wilson et al. 1977). Charles Langley and Walter Fitch, for instance, used a procedure based on minimum phyletic distances to test whether or not the process of nucleotide substitution was a constant Poisson process. They concluded that "it is clear that the *total* rate of substitution (as observed through the minimum phyletic distance procedure) varies markedly in geological time and among divergent lines of descent" (Langley and Fitch 1974, 174). Similar conclusions stressing the nonuniformity of rates in hemoglobin and a slowdown of rates in primate lineages were offered by Goodman, Moore, and Matsuda (1975). In 1981, Morris Goodman used the variability in the clock to argue against the existence of the molecular clock and against Kimura's explanation of it in terms of the neutral theory. By 1983, even Kimura himself admitted that the rate of molecular evolution was not perfectly uniform (Kimura 1983, 79), but, in his opinion, "emphasizing local fluctuations as evidence against the neutral theory, while neglecting to inquire why the overall rate is intrinsically so regular or constant is picayunish. It is a classic case of 'not seeing the forest for the trees'" (Kimura 1983, 85).

The tension between variability and constancy in the molecular clock is in part unavoidable. As a statistical object, the molecular clock is an aggregate characterized by a central tendency (most commonly an average or mean) and dispersion from that tendency. How much variability can be tolerated depends on how the constancy of the clock itself is interpreted. Some biologists thought of the molecular clock as an average: it referred to the average rate of substitution for a given population of molecules

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or molecular sequences. Other biologists, however, thought of it as more than an average. They thought of the clock as an intrinsic property of individual molecules. For them, averaging over a population of molecules, each with their own individual rate, was a means of discerning the underlying intrinsic rate of that type of molecule.

This subtle difference in the interpretation of the statistical constitution of the molecular clock reflected profound differences in the kinds of mechanisms proposed for the clock (selective vs. neutral) as well as in the value of formulating general principles or laws for evolutionary processes. For instance, Allan Wilson extended Kimura's argument concerning "the rule of the molecular clock" and its exceptions by framing it in terms of a larger conceptual divide (Wilson, Ochman, and Prager 1987). Wilson and his coauthors admit that there will be exceptions to the rule of the molecular clock, but, like Kimura, they emphasize its so-called intrinsic rate. Faced with the twin phenomena of a statistical mean and variation about that mean, Wilson et al. argued that one had to choose either the perspective of a naturalist or a biochemist. The naturalists cherish each individual molecule and its unique historical trajectory. As a result, they emphasize variability rather than the mean. Wilson believed that naturalists needed to adopt the biochemists' perspective. In his words, "Biochemists can agree with naturalists that every nucleotide position has a unique history, as does every atom of gas. But, they also recognize that the universal gas law ($PV = nRT$) was not discovered by the detailed analysis of the behavior of individual atoms. Bringing together molecular biology and natural history in the search for general laws of evolution requires, as many naturalists now recognize, a willingness to transcend 'microscopic' analysis" (Wilson et al. 1987). From the biochemists' perspective, the constancy of the rate of molecular evolution was the important phenomenon, not the variability. Neutralists, like Kimura and Wilson, argued that different types of molecules had characteristic intrinsic rates of evolution. So, all cytochrome *c* molecules, for instance, shared the same intrinsic property of evolving at approximately the same rate. The rate of each molecule type was determined by the rate of mutation and the distribution of conserved and nonconserved sites. Highly conserved sites were inferred to remain unchanged because alterations would hamper the molecule's function and so be selected against. Nonconserved sites changed freely and so were considered to be free from selection.

It is important to note that rate constancy is produced by the same processes thought to inform neutral models of populations, such as the infinite alleles model. The difference is in the form of the outcome (Millstein 2002). So , while population genetics tests were plagued by low power, the molecular clock was considered to be strong evidence in favor of the neutral theory (Kimura and Ohta 1971). This does not mean that the

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clock has not been subject to challenge and revision. High variability in the molecular clock for many molecules is now widely accepted. This high variability has forced neutralists to revise their explanations for rate constancy and has provided an opening for selectionist explanations that can explain both patterns of constancy and variability (Takahata 1987; Gillespie 1991).

5. Conclusion. There is no other field in biology where random drift is as important as it is in the field of molecular evolution. While neutral molecular evolution has been hotly contested, the controversy that marked the 1970s and 1980s has significantly depolarized. The reality of neutral substitutions and random drift is accepted by all molecular evolutionists, even if they still quibble about how much neutrality there is, how best to detect it, and which outcomes can be ascribed to it.

In this article, I have presented three perspectives on neutrality and drift drawn from the history of molecular evolution. While there are many more instances that I could have discussed, the three perspectives presented here raise a number of questions that I believe merit consideration. The changing status of neutral assumptions in the infinite alleles models highlights the possibility of both instrumentalist and realist uses of neutrality and random drift in biology. Efforts to test the neutral theory and to explain the molecular clock speak to real historical difficulties of distinguishing neutralist and selectionist outcomes. I have tried to argue that three different processes (random mutation, directional selection, and random genetic drift) contributed to many different observable neutralist outcomes. The problem of distinguishing processes using outcomes is one of producing a reliable association between a specific outcome and the contribution of processes individually, jointly, or in contrast to some different process. The history of the neutral theory demonstrates that this is not easy or straightforward, but that it is possible in some cases, such as with statistical tests of neutrality using DNA sequences.

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