The Neutral Theory of Molecular Evolution in the Genomic Era

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Abstract

The neutral theory of molecular evolution has been widely accepted and is the guiding principle for studying evolutionary genomics and the molecular basis of phenotypic evolution. Recent data on genomic evolution are generally consistent with the neutral theory. However, many recently published papers claim the detection of positive Darwinian selection via the use of new statistical methods. Examination of these methods has shown that their theoretical bases are not well established and often result in high rates of false-positive and false-negative results. When the deficiencies of these statistical methods are rectified, the results become largely consistent with the neutral theory. At present, genome-wide analyses of natural selection consist of collections of single-locus analyses. However, because phenotypic evolution is controlled by the interaction of many genes, the study of natural selection ought to take such interactions into account. Experimental studies of evolution will also be crucial.

INTRODUCTION

Positive (Darwinian) selection: natural selection that enhances the frequency of an allele relative to others

The neutral theory of molecular evolution has been controversial ever since it was proposed in the 1960s (43, 74, 79). The initial criticism came primarily from neo-Darwinian evolutionists, who were interested in studying morphological evolution (22, 99, 104, 146). However, as data on molecular evolution accumulated, it became clear that the general pattern of molecular evolution roughly agrees with that of the neutral theory, though there are some exceptions (76, 112). Even Mayr (101), who was a strong selectionist, accepted the concept of neutral evolution at the molecular level, though he stated that neutral evolution is of little interest for evolutionists. By contrast, Nei (112, 114) proposed that a substantial portion of morphological evolution is caused by neutral or nearly neutral mutations.

In recent years, however, many papers reporting detection of positive selection have been published. These papers have been reviewed by a number of authors (e.g., 2, 73, 103, 121) from the selectionist point of view. Some authors (e.g., 52, 144) have suggested that a majority of amino acid substitutions are due to positive selection and therefore a new theory of molecular evolution by natural selection should be developed. These papers are based on statistical analyses of genomic data under various assumptions that are not necessarily satisfied in the real world. It is therefore necessary to examine the validity of the assumptions and the statistical methods used. It is also important to examine their conclusions from the biological point of view. Some authors (e.g., 60, 64, 124) have already raised criticisms against the papers advocating selectionism. Controversy over the neutral theory has a long history, and it is important to know this history to avoid misunderstandings of the theory and unnecessary arguments. The readers who are not well acquainted with the early history are advised to read Lewontin (87), Kimura (76), and Nei (112, 113). Because of space limitation, we discuss the history only for a few cases important for our arguments.

The purpose of this review is to evaluate the neutral theory of molecular evolution from both the theoretical and empirical points of view. We are primarily concerned with the general features of molecular evolution rather than specific issues. Although we examine recent statistical tests of the neutral theory critically, our main interest is an evaluation of the legitimacy of the neutral theory to explain molecular and genomic evolution. During the past two decades, enormous progress has also occurred in the study of the molecular basis of phenotypic evolution and evolution of multigene families, which are highly relevant to the neutral theory. However, we shall not discuss these issues here because they have already been treated in recent review articles and books (e.g., 18, 19, 25, 114, 118, 149).

DEFINITION OF NEUTRAL THEORY OF MOLECULAR EVOLUTION

Neutral Mutations

In the early 1960s a number of molecular biologists sequenced small proteins such as insulins, cytochrome c, and hemoglobins from various groups of organisms and showed that the extent of sequence divergence between species increases as the divergence time increases, but the proteins in different species often have essentially the same function. For example, cow insulin appears functionally equivalent to human insulin, such that it could be used as a medication for diabetics. Similarly, *in vitro* experiments have shown that cytochrome c proteins are exchangeable among different mammalian species as long as the amino acids of the active sites of the protein remain the same (69). For these reasons, early molecular biologists concluded that amino acid substitutions outside the active sites are mostly neutral or nearly neutral (79). We believe this is a "biologically meaningful" definition of neutrality.

By contrast, population geneticists have often defined neutral mutations using mathematical theory. Fisher (38) and Wright (166) showed

that if the relative fitnesses (W_{ij}) of genotypes A_1A_1, A_1A_2, and A_2A_2 are given by $W_{11} = 1$, $W_{12} = 1 + s$, and $W_{22} = 1 + 2s$, respectively, the probability of fixation (u) of a new mutant allele (A_2) in the population is

$$u = 2s/(1 - e^{-4Ns}) \quad (1)$$

where N is the effective population size. Noting that u for $Ns = 1$ is approximately 50 times higher than that for $Ns = -1$, Fisher (38, p. 94) concluded that natural selection is very effective, because he believed N is of the order of 10^9. (Fisher did not have the concept of effective population size, which is often much smaller than the actual population size.) In other words, even selection coefficients (s) as small as $\pm 10^{-9}$ have significant effects on u. For this reason, he became a pan-selectionist, and there was no need for him to examine the possibility of neutral mutations. Interestingly, Kimura (74) used essentially the same definition of neutrality ($|2Ns| \leq 1$). However, because he knew the effective population size, he believed this definition of neutrality would be sufficient (76). Furthermore, the above definition was a mathematical formality for Kimura, and he was actually interested in neutral or nearly neutral mutations in the biological sense (74). Ohta (128–130) distinguished between neutral and nearly neutral mutations in terms of the value of $|2Ns|$, but this distinction is not very meaningful because she defined nearly neutral mutations as those with $0.2 \leq |2Ns| \leq 4$ and the effects on population fitness are very minor, as will be mentioned below.

From the biological point of view, Fisher's or Kimura's definition of neutrality is not very meaningful when N is very large, because the absolute value of u in Equation (1) is then very small. For example, when $N = 10^9$ and $Ns = 1$, s is 10^{-9}. Therefore, the absolute value of u is 2.04×10^{-9}. Similarly, when $N = 10^9$ and $Ns = -1$, u becomes 3.73×10^{-11}. The ratio of u for $Ns = 1$ to that for $Ns = -1$ is 54.6. However, how significant is the ratio of two very small numbers in reality? Furthermore, when $|s|$ is as small as in this case, it would be highly unlikely to remain constant from generation to generation in nature, and therefore this type of definition of neutrality would be meaningless. Note also that when $|Ns| = 1$ the expected time for a new mutation to be fixed in the population (t_F) is approximately $4N$ generations (77). This means that if $N = 10^9$ and the generation time is 1 year, as in the case of some insect species, $t_F = 4 \times 10^9$ years (longer than the history of life). Even for typical mammalian species, where N may be approximately 10^5 and the generation time is approximately 4 years, t_F would be 1.6×10^6 years. How can such a small s remain constant for the entire fixation period? Ohta (127) showed that when s fluctuates with generation and the mean $|\bar{s}|$ of $|s|$ is much smaller than the variance, u becomes practically equal to the probability of fixation of neutral mutations. That is, u is approximately equal to $1/2N$ whether s is positive or negative. It can be shown that this is true even with $N\bar{s} = 4$ and $N = 10^5$.

A more biologically relevant way of defining neutrality would be to consider a statistically significant difference between the mean fitness ($\bar{W}_M = 1 + 2s$) of the population fixed with the mutant allele and the mean fitness ($\bar{W}_A = 1$) of the population fixed with the ancestral allele. Considering a 30% significance level, Nei (113) presented a new definition of neutrality, which is given by $|s|\sqrt{2N} < 1$ approximately. [It should be noted that this definition is based on the assumption that the progeny size of an individual follows a Poisson distribution. Since the actual progeny size distribution is usually much more dispersed than the Poisson (24, 38), this definition should give a minimum $|s|$ value.] According to this definition, when $N = 10^5$, a mutation with $s = 0.001$ will be neutral because $1/\sqrt{2N} = 0.002$. Note that the effective population size of many vertebrate species appears to be of the order of $N = 10^4 \sim 10^6$ (115) and therefore s is highly unlikely to be constant over evolutionary time. For this reason, Nei (113) suggested that for practical purposes the definition of neutrality should roughly be $|s| < 0.001$ irrespective of population size in vertebrates, where s is taken to be the mean selection coefficient over evolutionary time. [In

Effective population size: the number of breeding individuals in an idealized population that has genetic diversity similar to that of the observed population

MHC: major histocompatibility complex

the bacteria *Escherichia coli*, N can be of the order of 10^9 (115).]

The above definition of neutrality is appropriate for directional selection. In the case of overdominant selection, even smaller s values may have significant effects, although in major histocompatibility complex (MHC) loci, where strong overdominant selection operates, the selection coefficient has been estimated to be 0.0007 to 0.042 (142). The selection coefficients that influence synonymous codon frequencies in the genome can be very small at individual nucleotide sites, yet the selection seems to be effective (1). This occurs because the frequencies of preferred and unpreferred codons are nearly the same for the majority of genes and selection occurs cumulatively over a large number of codons (113). In this review, however, we do not consider this type of problem, because we are interested in the function of genes rather than the chemical composition of the genome.

Difficulties in Defining and Estimating Genotype Fitnesses

In population genetics theory it is customary to measure the fitnesses of different genotypes in terms of the expected numbers of progeny sizes of the genotypes. When there is a pair of alleles, A_1 and A_2, at a locus, the relative fitnesses of the three genotypes A_1A_1, A_1A_2, and A_2A_2 are defined to be W_{11}, W_{12}, and W_{22}, respectively, as indicated above. Conceptually, this definition is very useful for predicting the evolutionary changes of allele or genotype frequencies in the population. However, if we try to estimate genotype fitnesses experimentally, we face enormous difficulties, as repeatedly emphasized by Lewontin (87, 88). For example, the genotype fitnesses in insects vary substantially with generation because of environmental changes (39, 91). **Table 1** shows the relative fitnesses of nine chromosomal genotypes possible for inversion types *ST* and *BL* of chromosome *CD* and *ST* and *TD* of chromosome *EF* in the grasshopper, *Maraba scurra*. The fitnesses for the genotype *BL/BL* of chromosome *CD* combined with the genotypes *ST/ST*, *ST/TD*, and *TD/TD* of chromosome *EF* are nearly the same for the three years 1956, 1958, and 1959. However, the fitnesses of other genotypes vary from year to year. In particular, the fitness of the genotype *ST/ST*; *ST/TD* was apparently 0 in 1958: none of the 732 individuals examined in that year had this genotype. Lewontin & White (91) showed that there is a significant

Table 1 Relative fitnesses (viabilities) for the nine genotypes with respect to two polymorphic inversion types, *BL* and *ST* of chromosome *CD*, and *TD* and *ST* of chromosome *EF*, in the Wombat population of the grasshopper, *Maraba scurra*, in southeastern Australia[1]

Year	Chromosome *CD* genotypes	Chromosome *EF* genotypes		
		ST/ST	*ST/TD*	*TD/TD*
1956	***ST/ST***	0.789	0.801	0.000
1958	***ST/ST***	1.353	0.000	0.000
1959	***ST/ST***	0.970	1.282	0.000
1956	***ST/BL***	1.000	0.876	1.308
1958	***ST/BL***	1.000	0.919	0.272
1959	***ST/BL***	1.000	0.672	1.506
1956	***BL/BL***	0.922	1.004	0.645
1958	***BL/BL***	0.924	1.113	0.564
1959	***BL/BL***	0.917	1.029	0.645

[1]Relative fitnesses were estimated by Haldane's (53) method. Fitness becomes 0 if no individuals with the genotype are observed. The fitness of genotype *ST/BL*; *ST/ST* was assigned to be 1 arbitrarily. From Lewontin & White (91).

chromosome × chromosome interaction with respect to fitness in the 1958 population. These observations make it difficult to define and estimate genotype fitnesses. For these reasons, Lewontin (87, p. 236) stated: "To the present moment no one has succeeded in measuring with any accuracy the net fitnesses of genotypes for any locus in any species in any environment in nature. . ." A similar conclusion was obtained by Endler (33), who examined hundreds of papers in which natural selection with respect to allozyme polymorphisms was studied. In this connection it is important to remember that natural selection occurs among different individuals or phenotypes rather than among different alleles or different nucleotides (100).

Recent progress in developmental biology has shown that the expression of genes is controlled by a variety of genetic factors including *cis*-regulatory elements, microRNAs, and epigenetic effects, as well as by environmental factors. Therefore, genotype fitness may vary with genetic background and environmental factors and is highly unlikely to be the same for all generations. These factors make the definition and estimation of genotype fitnesses even more difficult. It is therefore important to keep in mind that the mathematical definition and estimation of selection coefficients are crude attempts at representing nature and we should not give too much significance to mathematical theories dealing with small selection coefficients.

Definition of Neutral Theory

Partly for the above reasons, the neutral theory of molecular evolution has been defined in a flexible way. According to Kimura (76, p. 34), "The essential part of the neutral theory is not so much that molecular mutants are selectively neutral in the strict sense as that their fate is largely determined by random drift." To this definition, we now would like to add genomic drift as an additional form of random factor (114, 123).

It should also be noted that the neutral theory explicitly acknowledges the occurrence of a large number of deleterious mutations, which are eliminated by natural selection, as well as a small proportion of advantageous mutations. In this sense, Kimura's (74) mathematical definition of $|2Ns| \leq 1$ seems less suitable than Nei's (113) relaxed definition ($|s| < 0.001$). This relaxed definition will avoid many trivial controversies over the neutral theory, as will be shown below.

MOLECULAR CLOCKS

One of the interesting properties discovered by early molecular evolutionists is the approximate constancy of the rate of amino acid substitution in such proteins as hemoglobins, cytochrome c, and fibrinopeptides (29, 98, 181, 182). This notion of "molecular clocks" was immediately challenged by Simpson (146) and Mayr (99), who were authorities on morphological evolution. For these evolutionists, it was apparently unthinkable that any character evolves at a constant rate over a long period of time. However, the approximate constancy of evolutionary rate was later observed in many other proteins, though the molecular clock is not always very accurate (26).

One explanation for this puzzling observation is to assume that most amino acid substitutions are neutral and do not seriously change protein functions. Kimura (74) and King & Jukes (79) showed that if neutral mutations occur and are fixed by random genetic drift, the rate of amino acid substitution can be constant. Kimura (75) then took this rate constancy as support for the neutral theory.

Evolutionary Rate Under Purifying Selection

However, there were a few problems with this proposal. First, although the rate of molecular evolution was roughly constant for a particular protein, the rate varied considerably among different proteins. This puzzle was solved when Dickerson (27) showed that the variation is apparently caused by differences in functional constraints. For example, histones require a rigid structure for their function and this

cis-regulatory element: a region of DNA that is located adjacent to a gene and regulates the expression of the gene

Genetic drift: allele frequency change due to random sampling of alleles in a finite population

Genomic drift: random copy number changes of genes or other genetic elements in evolution; conceptually analogous to random genetic drift

structure is the same in animals and plants. For this reason, there are only a few amino acid differences between animal and plant histones. By contrast, fibrinopeptides have little functional constraint and evolve very fast, because they are cleaved from fibrinogen in the process of producing the functionally active fibrin required for blood clotting and have virtually no function themselves. In these cases if we assume that functionally important amino acid sites of a protein remain essentially unchanged in the evolutionary process but functionally unimportant sites change with a neutral rate, the rate of amino acid substitution (r) for the entire protein may be expressed by

$$r = fv \qquad (2)$$

where f is the proportion of functionally unimportant amino acid sites and v is the mutation rate (76). In reality, distinction between functionally important and unimportant sites may be difficult, but the above formula has a symbolic meaning and makes it easy to understand one of the important factors affecting the rate of amino acid substitution.

Evolutionary Rate and Generation Time

The second problem with the molecular clock concept was the fact that the rate of amino acid substitution was apparently constant per year rather than per generation. Because classical genetics had established that the mutation rate was approximately constant per generation in *Drosophila*, humans, and maize, the problem arose of how to reconcile these two sets of observations. Ohta (128) proposed that this dilemma can be resolved by assuming that most mutations are slightly deleterious and can be fixed in small populations, more easily by genetic drift than in large populations, and that large organisms such as mammals generally have smaller population sizes than small organisms such as *Drosophila*. In other words, a larger proportion of mutations may behave just like neutral alleles in large organisms as compared with in small organisms. Because large organisms tend to have a longer generation time (smaller number of generations per unit time) than small organisms, the rate of amino acid substitution per year may be similar for both large and small organisms if the mutation rate per generation is constant. However, this argument is quite unlikely to apply to all groups of eukaryotic and prokaryotic organisms, for which rough molecular clocks apply (59). Note also that if this argument were correct, the genomes of large organisms would be expected to deteriorate gradually, but in reality these organisms are more advanced in terms of organismal complexity than small organisms.

A much simpler solution to this problem is to assume that the rate of nondeleterious mutations is roughly constant per calendar year whereas the rate of deleterious mutations is approximately constant per generation (75, 111). Nei (111) noticed that the mutation rate in classical genetics was determined almost always by using highly deleterious mutations, many of which were homozygous lethal (107), and these mutations appeared to occur at the time of meiosis (96, 108). It is therefore understandable that classical Mendelian geneticists were led to believe that the mutation rate is constant per generation. However, some bacterial geneticists who studied phage resistance had reached the conclusion that the mutation rate is proportional to chronological time (122). Because phage-resistance mutations are nondeleterious, this observation suggests that nondeleterious mutations occur roughly at a constant rate per year. For these reasons, Nei (111) argued that the constancy of amino acid substitution per year is consistent with the theory that most amino acid substitutions are more or less neutral.

However, whether the mutation rate is constant per generation or per year has been controversial for a long time (30, 31, 47, 85, 163, 171). Kohne (81) argued that the evolutionary rate of hominoid genes should be lower than that of monkey genes because hominoids have a longer generation time than monkeys. The logic behind this argument is that if the generation time is long the number of cell divisions

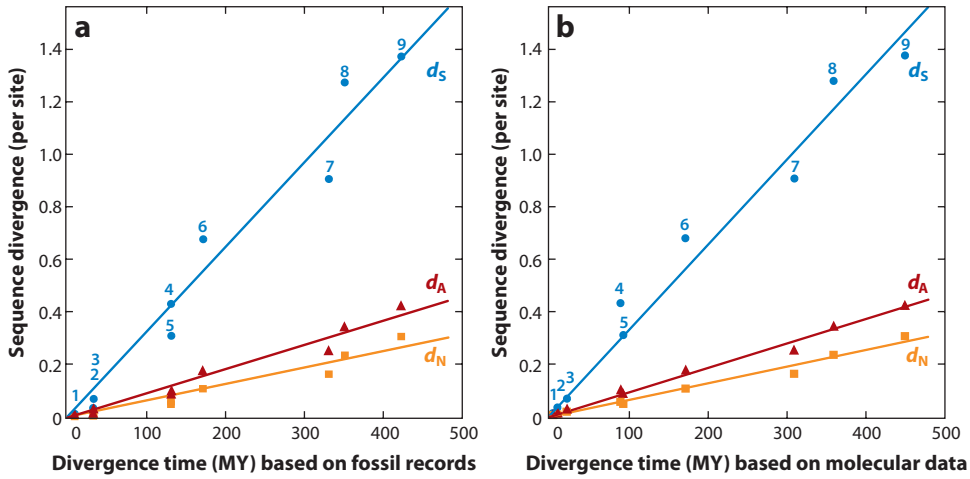

Figure 1

Linear relationships of the number of amino acid substitutions per residue (d_A) and the numbers of synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions per site, with divergence times based on the fossil record (*a*) and molecular data (*b*). The fossil record data are taken from Benton et al. (12), whereas the molecular data come from a small number (~10) of genes (58, 59). Each point represents the average sequence divergence of 4,198 nuclear genes with ≥100 codons from 10 vertebrate species (human versus 1 = chimpanzee, 2 = orangutan, 3 = macaque, 4 = mouse, 5 = cow, 6 = opossum, 7 = chicken, 8 = western clawed frog, 9 = zebrafish). Sequence and orthology data are from Ensembl (147). The d_A distance was computed by the Poisson correction method, whereas d_S and d_N were computed by the modified Nei–Gojobori method (178) with a transition/transversion ratio of 2.

per year in germline cells is small and therefore the evolutionary rate should be lower, assuming that mutation rate is proportional to the number of cell divisions. This is called the generation time hypothesis, and Li et al. (93) presented some data supporting this view [see also (68, 155)]. However, recent genome sequence data show that the rate of nucleotide substitution has been virtually the same for the hominoid and monkey lineages (44), suggesting that the generation time hypothesis may not be important for hominoid species. Because we now have the genomic sequences for many model organisms, we reexamined this problem using vertebrate genome sequence data. **Figure 1** shows the numbers of amino acid substitutions per residue (d_A) and synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions per site for humans and other vertebrate species in relation to divergence time. The d_A, d_S, and d_N values are the averages for 4,198 nuclear genes that appear to be orthologous among the 10 species used (human, chimpanzee, orangutan, macaque, mouse, cow, opossum, chicken, frog, and zebrafish). The evolutionary times in **Figure 1*a*** and ***b*** refer to the estimates obtained from the fossil record (12) and the molecular data based on a small number of genes (58, 59), respectively. In both cases the numbers of amino acid and nucleotide substitutions increase almost linearly with chronological time. Note that some researchers (93, 171) have suggested that rodent genes evolve much faster than other mammalian genes, but our data show that the evolutionary rate for mice is more or less the same as the rate for other species when a large number of genes are used (number 4 in **Figure 1**).

Functional Constraints of Proteins

However, the molecular clock does not necessarily hold true if we look at individual genes separately, and in some cases the rate of amino

Synonymous nucleotide substitution: a nucleotide substitution that does not lead to amino acid replacement

Nonsynonymous nucleotide substitution: a nucleotide substitution that results in amino acid change

acid or nucleotide substitutions varies considerably among different evolutionary lineages. One well known example is guinea pig insulin. Mammalian insulins are generally composed of 51 amino acids and highly conserved. Exceptions are those from hystricomorphic rodents such as guinea pigs and chinchillas; the insulins from these species have been shown to evolve more than 10 times faster than other mammalian insulins (79, 131). Initially, this high rate of evolution was thought to be due to positive selection (79), but Kimura (76) later proposed that it is instead due to relaxation of selection caused by the absence of the zinc ion in the insulin molecules. In fact, several studies have shown that the biological activity (balancing of blood glucose level) of insulins from these groups of species is only 3–30% of that of other mammalian species (6, 61).

Under certain conditions, functional constraints may be enhanced. A well-known example is histone H4 protein. This protein is known to evolve very slowly in animals and plants, but it evolves reasonably fast in protists (71). Therefore, it appears that the evolutionary rate of this protein decreased when animal and plant histones evolved.

Variation in Mutation Rate

Other factors can also cause variation in the evolutionary rate of proteins. One such factor is the change in mutation rate. Because synonymous substitutions are generally believed to be neutral, mutation rates are often studied by examining the rate of synonymous substitution. The average synonymous substitution rate of nuclear genes appears to be nearly the same for animals and plants (106, 165). However, the synonymous rate of animal mitochondrial genes is approximately 10 times higher than that of nuclear genes, whereas plant mitochondrial genes evolve approximately 10 times slower than nuclear genes (165). The fast evolutionary rate of animal mitochondrial genes was first thought to be due to Muller's ratchet effect, which would enhance the fixation of slightly deleterious mutations in asexual haploid populations because of the lack of recombination (95). However, this explanation is unsatisfactory because plant mitochondrial genes, which have the same mode of inheritance as animal mitochondrial genes, evolve very slowly as mentioned above. It now seems that the fast evolution of animal mitochondrial genes is due to a higher mutation rate partly attributable to lack of the DNA repair gene *RecA*, which is present in plant mitochondrial genomes (94).

However, the evolutionary rates of plant mitochondrial genes are known to vary enormously with gene or evolutionary lineage depending on the circumstance (106). For example, the mitochondrial genes *Atp1* and *Cox1* evolve 100 times faster in the genera *Pelargonium*, *Plantago*, and *Silene* of seed plants than those in most other genera. Interestingly, not all genes in the same species evolve at the same rate; some genes in these genera evolve as slowly as the genes in other species. Furthermore, phylogenetic analysis showed that this enhancement of evolutionary rate occurred only during the past 5 million years in the case of genus *Silene* (106). The reason why the rate varies so much among different species of plants is unclear, but the mutation rate apparently increased in the *Silene* lineage. The mutation rate of plant mitochondrial genes appears to vary according to the genetic background and the environmental condition. In nuclear genes, however, this type of extreme temporal variation in mutation rate seems to be rare.

Molecular Clocks and Neutral Theory

Kimura (75) believed that the molecular clock reflects accumulation of neutral mutations and therefore the clock can be used for testing the neutral theory. For this reason, a number of authors have attempted to disprove the neutral theory by finding cases where the molecular clock fails (e.g., 5, 47). However, because the evolutionary rate of a protein is affected by functional constraints as well as mutation rate, the relationship between neutral theory and molecular clocks is complicated. In other words, rejection of the molecular clock does not

necessarily imply rejection of the neutral theory. If the mutation rate varies with time, the molucular clock will be rejected, but the neutral theory may not.

Nevertheless, if the mutation rate and functional constraints for a locus remain the same over evolutionary time, the molecular clock is expected to apply for neutral mutations. In this case the expected evolutionary rate (r) is equal to the mutation rate (v) (79). By contrast, the expected rate of amino acid substitution for adaptive mutations is given by $r = 4Nsv$ approximately (79). Therefore, if N varies over time or s varies from mutation to mutation, the constancy of r or the molecular clock will be violated. In **Figure 1**, however, we have seen that both the numbers of amino acid (d_A) and nucleotide (d_S, d_N) substitutions increase more or less linearly with time when a large number of genes is considered. This linear increase is clearly consistent with the neutral theory in which neutral mutations are defined by $|s| < 0.001$. Of course, it is possible that a small proportion of nucleotide substitutions with greater s values are included in d_N or d_A.

EVOLUTION OF PROTEIN-CODING GENES

The mammalian genome generally consists of approximately 3×10^9 nucleotides, but the number of protein-coding genes has been estimated to be approximately 25,000, each of which has ~1.5 kb of coding sequence on average (86, 161). Therefore, >95% of the genome is noncoding. Previously, this portion of DNA was considered to be nonfunctional (126), but since many genetic elements that control gene expression reside in the noncoding regions, these regions are not all "junk DNA." Therefore, both protein-coding genes and regulatory elements in noncoding regions should be considered in the study of molecular evolution. However, because the function of most noncoding regions is still poorly understood, we will consider primarily the evolution of protein-coding genes.

General Properties of Evolution of Protein-Coding Genes

One of the salient features of the gene evolution is that new genes are generated by gene duplication but once the function of a gene is established it tends to maintain the same function for a long time, even when the total number of genes increases in conjunction with increasing organismal complexity. A typical example is the *RecA/RAD51* genes that are required for DNA repair. Both prokaryotes and eukaryotes have only a few copies of these genes and the gene structures have remained largely unchanged (94).

This conservative nature of gene evolution is universal except for a few groups of genes. **Figure 2** shows the distribution of w ($= d_N/d_S$) values among 15,350 pairs of human and mouse orthologous genes. Most of the genes (99.8%) have a w value of less than 1, and for 228 genes $w = 0$, indicating that no amino acid difference exists between the human and mouse proteins. These genes include highly conserved proteins such as histones, ubiquitins, elongation factors, tubulins, and ribosomal proteins. By contrast, only 33 genes have a value of $w \geq 1$. These

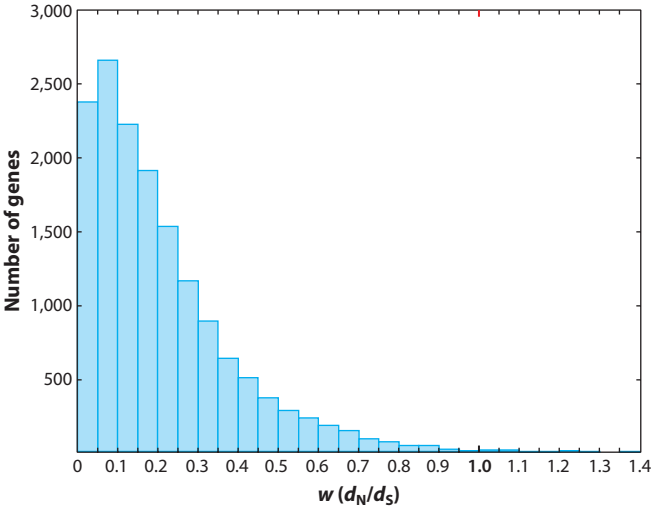

Figure 2

Distribution of w ($= d_N/d_S$) in human and mouse one-to-one orthologous genes. A total of 15,350 genes with ≥100 codons were used. Computational procedures are the same as those in **Figure 1**.

Purifying selection: a process of natural selection in which deleterious mutations are eliminated from the population; an important aspect of the neutral theory

OR: olfactory receptor

Neo-Darwinism: a theory of evolution by means of natural selection, with little effect of mutations and random genetic drift

divergent genes largely encode proteins involved in immune systems and reproductive systems. These results are similar to those found earlier (113). Although exceptionally conserved or divergent genes exist, the w values for the majority of genes are 0.05 to 0.4, and the average w for the entire set of genes is 0.21. This value is similar to that (0.25) obtained by the analysis of ~10,000 orthologous genes from the human, chimpanzee, and macaque genomes (44). If synonymous substitutions are approximately neutral, these results indicate that approximately 75–80% of nonsynonymous mutations are eliminated by purifying selection. We can therefore conclude that most mammalian genes are evolving under purifying selection. Because purifying selection is an important feature of the neutral theory, the results in **Figure 2** are consistent with the neutral theory.

Fast-Evolving Genes

Although a majority of genes is functionally constrained and many mutant genes are eliminated by purifying selection, a few groups of genes have a rather high w value. One such group of genes belongs to multigene families controlling physiological characters in which the gene products interact rather weakly with their ligands. An example is mammalian olfactory receptor (OR) genes (15). The human genome contains approximately 400 functional OR genes, whereas the mouse genome contains >1000 OR genes (117). In these organisms one odorant is perceived by several ORs, and one OR molecule identifies several different odorants (97). For this reason, the functional constraints of OR genes are apparently weak, particularly in primates, and the genes evolve relatively rapidly (e.g., 45, 48). (Several authors have reported positive selection for these genes, but their conclusions are questionable, as will be mentioned below.) The genes for pheromone and taste receptors appear to evolve in the same fashion (117).

A second group of fast-evolving genes is those involved in unimportant functions. For example, fibrinopeptides do not have important biological functions and evolve in a more or less neutral fashion as mentioned above. More clear-cut evidence for fast evolution of unimportant genes comes from recently derived pseudogenes, where the evolutionary rate is expected to be equal to the mutation rate (92, 105). If the acceleration of nonsynonymous substitution is caused by natural selection, why do pseudogenes evolve so quickly? The observations from pseudogenes are clearly inconsistent with neo-Darwinism but do support the neutral theory of molecular evolution.

Another class of protein-coding genes are those apparently subject to positive selection that generates a high degree of polymorphism. A good example is MHC class I and II genes, which have an unusually high number of polymorphic alleles (80). In these genes a segment which encodes the antigen-binding site has a w value that is significantly higher than 1. An examination of the pattern of nucleotide substitution suggested that the high degree of polymorphism is generated by natural selection to protect the host from newly invading pathogens (67). Similar positive selection is apparently operating in other immune system genes (63).

STATISTICAL METHODS FOR DETECTING POSITIVE SELECTION

The finding of positive selection in MHC genes stimulated a number of theoreticians to develop statistical methods for identifying positive selection during the past two decades. These statistical methods have been used by many biologists, and there are now a large number of papers reporting positive selection. In our view many of these methods do not have solid statistical and biological bases. We therefore examine the theoretical basis of these methods in some detail in this section.

Bayesian Methods for Identifying Positively Selected Codon Sites

In this class of methods, a special codon substitution model (e.g., 50, 109) is used, and

the value of w ($= d_N/d_S$) is assumed to vary from site to site according to a specific mathematical model (e.g., uniform and β distributions). Comparing several DNA sequences, one can then estimate w for each codon site using Bayesian statistical methods. If w for a given codon site is significantly higher than 1, the site is inferred to be under positive selection (e.g., 82, 172). Many different mathematical models of positive selection have been developed on an intuitive basis. In some cases only the existence of positive selection is tested, without identifying specific selected codon sites.

During the past 10 years, many biologists have used these methods and reported detecting positive selection in many different genes from various organisms, including humans, chimpanzees, and macaques (e.g., 7, 21, 83, 150, 156, 173). For example, analyzing a large number of genes from 10 vertebrate species, Uddin et al. (156) found signatures of human ancestry-specific adaptive evolution in 1,240 genes during their descent from the last common ancestor with rodents and suggested that adaptive evolution of these genes was important for human-specific morphological and physiological characters. The set of genes identified included 273 olfaction-related genes. Similar results were obtained by Clark et al. (21) in their analyses of human, chimpanzee, and mouse genes. In this study too, many olfaction-related genes were inferred to have evolved by positive selection.

However, recent theoretical and empirical studies have shown that these Bayesian methods are quite unreliable and generate a high proportion of false positives and false negatives (e.g., 65, 124, 125, 151, 164, 176, 180). There are two underlying problems. First, the likelihood ratio test (LRT) used in these methods is unreliable, partly because unrealistic mathematical models are used and partly because the number of nucleotide substitutions is often too small to obtain reliable results. Thus codon sites may be falsely identified as positively selected because of a high w value generated by chance. Second and more importantly, these methods depend on an assumption that all nonsynonymous substitutions change the function or fitness of the gene considered. However, this assumption is inappropriate, because only a small proportion of amino acid substitutions are known to affect protein function if we exclude deleterious mutations (79). In the cases of hemoglobins and color vision pigments, this proportion seems to be approximately 5% (132, 176).

For these reasons, it is very difficult to predict positively selected codon sites by using existing Bayesian statistical methods. Interestingly, it is now possible to infer the nucleotide sequences of ancestral organisms by using parsimony or Bayesian methods (40, 174) and reconstruct the ancestral proteins experimentally. One can then study the protein functions of ancestral and extant species and their evolutionary changes (70, 176, 177). Yokoyama and colleagues [see (175) for review] used this type of experiment to study the evolution of visual pigments. When they compared their experimental results with the adaptive sites predicted by Bayesian methods, the agreement between experimental results and statistical predictions was very poor (176) (**Figure 3**). Similarly poor agreement was obtained for other visual pigment (124, 125) and OR (180) datasets. We therefore conclude that the prediction of positive selection by use of the existing Bayesian methods is poor and the results should be verified by some form of experimental approach. This reservation applies to a large number of papers reporting positive selection detected by Bayesian methods. In the case of OR genes there is counter-evidence that they evolve in a more or less neutral fashion at the amino acid level (117).

LRT: likelihood ratio test

MK test: McDonald-Kreitman test

McDonald–Kreitman Test and Its Extensions

In recent years another class of statistical tests has been used extensively for detecting positive selection: the McDonald–Kreitman (MK) test (102) and its extensions. In this test the ratio of the number of nonsynonymous polymorphic sites (P_N) to the number of synonymous polymorphic sites (P_S) within species is

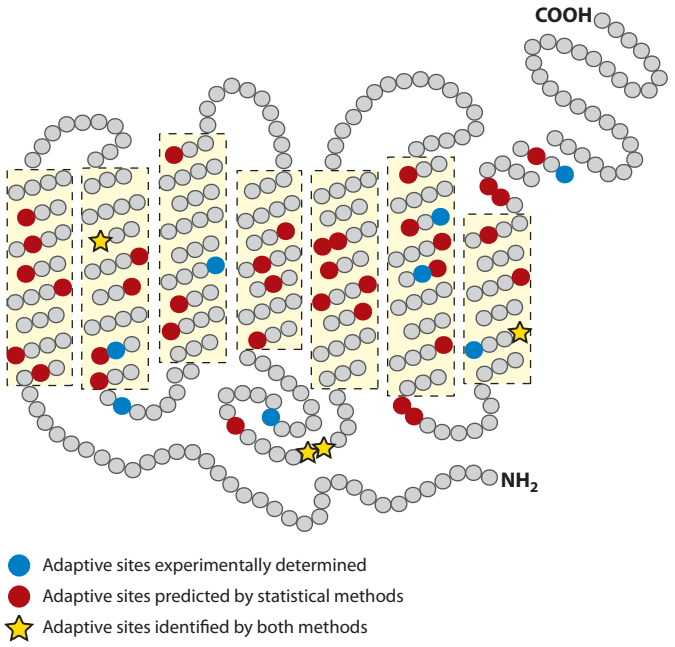

Figure 3

Structure of the bovine rhodopsin protein on which experimentally determined adaptive sites and statistically predicted adaptive sites in vertebrates are shown. The dashedline boxes indicate transmembrane regions. Original data from Yokoyama et al. (176) and Nozawa et al. (124).

compared with the ratio of the number of nonsynonymous nucleotide substitutions (D_N) to the number of synonymous substitutions (D_S) between species. If D_N/D_S is significantly greater than P_N/P_S, positive selection is inferred. In this approach, both P_N and P_S are assumed to represent neutral nucleotide polymorphisms. The argument for this assumption is that deleterious mutations are quickly eliminated from the population and advantageous mutations are quickly fixed, so that P_N as well as P_S represent mostly neutral mutations. In other words, it is assumed that only strongly advantageous, strongly deleterious, and neutral mutations occur. One can then measure the fraction of neutral polymorphisms by $f = P_N/P_S$ and the fraction of deleterious mutations by $1 - f$ (144). (In practice, $1 - f$ should include some advantageous mutations.)

Under this assumption, P_N/P_S is considered to represent the ratio of true numbers of nonsynonymous to synonymous neutral mutations, and the proportion of adaptive nonsynonymous substitutions between species is estimated by $\alpha = 1 - (P_N/P_S)/(D_N/D_S)$ (144, 148). With this interpretation α should be between 0 and 1, but in practice α can take any value between $-\infty$ and 1. Negative values are considered to reflect sampling errors or violations of the model, particularly the existence of deleterious nonsynonymous polymorphisms within a population (35). In fact, α is considerably affected by sampling errors, because the number of alleles used for studying polymorphisms is usually very small (<20 alleles). For example, when we estimated α values for 419 *Drosophila* genes studied by Shapiro et al. (145), the estimate of α varied from -35 to 1, and ~30% of the estimates were negative (**Figure 4**). For this reason, a weighted average ($\bar{\alpha}$) of α for many loci is often computed by

$$\bar{\alpha} = 1 - (\bar{P}_N/\bar{P}_S)/(\bar{D}_N/\bar{D}_S) \qquad (3)$$

where $\bar{D}_N$, $\bar{D}_S$, $\bar{P}_N$, and $\bar{P}_S$ are the average of D_N, D_S, P_N, and P_S for all loci, respectively, and this $\bar{\alpha}$ is used as a measure of the proportion of adaptive nonsynonymous substitutions between species.

In recent years many investigators have estimated the value of $\bar{\alpha}$ for a large number of genes in several different groups of organisms. In humans, *Arabidopsis*, and yeast, $\bar{\alpha}$ was either negative or positive but close to 0, so that D_N and D_S were thought to reflect neutral mutation (16, 28, 42, 49, 179). In nuclear genes of *Drosophila* species, however, many authors obtained estimates of $\bar{\alpha} = 0.25$–0.95 (4, 11, 13, 37, 143, 145, 148). For example, Shapiro et al. (145) obtained a statistically insignificant value of $\bar{\alpha} = -0.05$ when they studied 419 genes from *Drosophila melanogaster* and its sibling species. However, the authors believed that this result was attributable to the presence of deleterious polymorphisms in some genes, and when they eliminated nucleotide sites with low-frequency alleles, which are more likely to be deleterious, they obtained $\bar{\alpha} = 0.29$, which was significantly greater than 0. For this reason, they concluded that approximately 30% of amino acid differences between *D. melanogaster* and its sibling

species are caused by positive selection. Smith & Eyre-Walker (148) also eliminated genes with low frequency polymorphisms to obtain a significant value of $\bar{\alpha} = 0.43$. In another study, Begun et al. (11) obtained an estimate of $\bar{\alpha} = 0.54$, based on examining 10,065 genes from seven strains of *D. simulans* and a single strain of *D. melanogaster*. One of the most extreme $\bar{\alpha}$ values was obtained by Sawyer et al. (143) who considered 91 genes from *D. melanogaster* and *D. simulans*. This study suggested that approximately 95% of amino acid differences between *D. melanogaster* and *D. simulans* are caused by positive selection ($\bar{\alpha} = 0.95$). Studies of bacterial species generated both positive and negative $\bar{\alpha}$ values (20, 66). Similarly, animal mitochondrial genes showed that α can be significant in either the positive or negative direction (9, 103).

These results are very different from those derived from other molecular evolution studies over the past 40 years. Could they arise from intrinsic statistical properties of the MK test? First, we note that the MK test depends on several simplifying assumptions. For example, P_N is assumed to represent only neutral nonsynonymous polymorphisms. This assumption is clearly wrong, because every population contains some mildly deleterious nonsynonymous mutations (23, 35, 36). For this reason, some researchers eliminated low frequency polymorphisms (e.g., 37, 145, 148). However, elimination of genes (or nucleotide sites) with low frequency alleles is not justified, because most neutral mutations are also of low frequency and some slightly deleterious mutations can be of moderate frequency (167, 169, p. 385). In other words s is continuous, and it is very difficult to determine an appropriate threshold for removal of nucleotide sites with low frequency alleles. The assumption that no advantageous mutations are included in P_N is also incorrect, because the value of s for advantageous mutations is continuous and the frequency of mutations with small positive s is expected to be higher than those with large s (23). Many mutations with small positive and small negative s must be included in P_N as well as in D_N. This fact makes the interpretation of α very difficult.

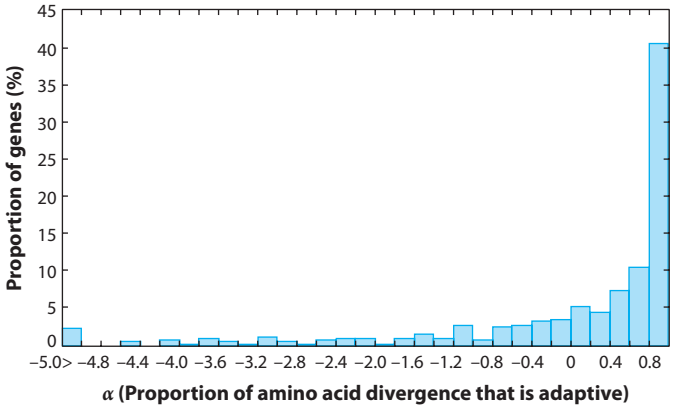

Figure 4

Distribution of the proportion (α) of putatively adaptive amino acid substitutions for 419 genes in *Drosophila*. Polymorphism data are from *D. melanogaster*, whereas divergence data are from comparison of *D. melanogaster* and *D. simulans* sequences. All sequence data were provided by Shapiro et al. (145). The α value was estimated as $1 - D_S P_N/[(D_N + 1)(P_S + 1)]$, where P_S and P_N are the numbers of synonymous and nonsynonymous polymorphic sites, respectively, and D_S and D_N are the numbers of synonymous and nonsynonymous differences between the two species, respectively [the values of D_N and P_S were incremented by 1 in this formula in order to avoid $\alpha = -\infty$ (148)]. Approximately 30% of α values were negative.

Second, there are alternative explanations for the positive α value in MK tests. Slightly deleterious mutations can be fixed in the population when population size fluctuates over evolutionary time. This may cause α to exceed 0 (35, 64, 102). Another factor that can make α positive is fluctuation of s for nonsynonymous mutations. In the real world s for a given mutation would almost certainly vary from generation to generation as was mentioned earlier. Variation of s would tend to cause fluctuation of allele frequencies (119, 168), and a mutant gene may appear to evolve as though it were neutral if the mean of s is effectively 0. However, the magnitude of P_N would be reduced substantially by random fluctuation of s, whereas P_S would not be affected (119). A partially inbred population or a substructured population may also reduce P_N/P_S relative to D_N/D_S, because inbreeding would decrease nonsynonymous mutations that are recessive. Thus, a positive α value does not necessarily imply positive selection. If D_N/D_S (or d_N/d_S) remains more or less constant over evolutionary time when a large number of loci

EHH: extended haplotype homozygosity

Single nucleotide polymorphism (SNP): the existence of two or more different nucleotides at a given site in DNA sequences from a population

are considered (**Figure 1**), α will be a measure of deficiency of nonsynonymous polymorphisms. Thus the MK test is not always for detecting positive selection.

Third, the biological meaning of $\bar{\alpha}$ is not clear, because nucleotides or amino acids are not the target of natural selection. If a sufficiently large number of polymorphic genes are studied and we can obtain a reliable estimate of α for each locus, we can consider the simple average ($\tilde{\alpha}$) of αs as a summary statistic for the entire set of genes or genome. If $\tilde{\alpha}$ is positive, there is an excess of interspecific divergence of nonsynonymous substitutions relative to intraspecific polymorphism for the entire genome, whereas a negative $\tilde{\alpha}$ value indicates a deficiency. If we know that different genes evolve differently, $\tilde{\alpha}$ is a more meaningful statistic than $\bar{\alpha}$. Interestingly, $\bar{\alpha}$ and $\tilde{\alpha}$ can give very different conclusions. As a simple example, consider the case where 50% of the genes have $P_N = 0$ and $P_S = 4$, whereas the remaining 50% of genes have $P_N = 1$ and $P_S = 2$. Suppose $D_N = 5$ and $D_S = 20$ for all genes. In this case the simple average ($\tilde{\alpha}$) of α will be 0, because $\alpha = 1 - (0/4)/(5/20) = 1$ for 50% of genes whereas $\alpha = 1 - (1/2)/(5/20) = -1$ for the remaining genes. By contrast, $\bar{\alpha}$ is 0.33, because $\bar{P}_N = (0 \times 0.5) + (1 \times 0.5) = 0.5$, $\bar{P}_S = (4 \times 0.5) + (2 \times 0.5) = 3$, $\bar{D}_N = 5$, and $\bar{D}_S = 20$. Thus, $\tilde{\alpha}$ leads to a conclusion of neutrality, whereas $\bar{\alpha}$ leads to the conclusion that 33% of nonsynonymous differences between species are due to positive selection. Although the above example is artificial, and $\tilde{\alpha}$ can be greater than $\bar{\alpha}$ under certain circumstances, it is clear that $\tilde{\alpha}$ and $\bar{\alpha}$ may give very different conclusions.

However, the most serious problem is the assumption that all excess nonsynonymous substitutions in D_N/D_S relative to P_N/P_S are adaptive and caused by positive selection. As mentioned above, only approximately 5% of amino acid substitutions seem to affect protein function. If this estimate applies to many other proteins, even an excess of 95% amino acid substitutions as estimated by Sawyer et al. (143) might not be important as a selective force. Therefore, this result is not necessarily inconsistent with the neutral theory.

In this connection, it is interesting to note that Sawyer et al. (143) estimated the value of Ns to be on average ~2.5 with a standard deviation of ~0.5 for amino acid substitutions. This result means that s is approximately 2.5×10^{-6} if $N = 10^6$. Andolfatto (4) obtained a similar average Ns value of 1.1 for nonsynonymous substitutions. According to Nei's (113) relaxed definition of neutrality mentioned above, such substitutions are essentially neutral, and the results are therefore compatible with the neutral theory even if $\bar{\alpha} = 0.25$–0.95.

Extended Haplotype Homozygosity and F_{ST} tests

Many other population genetics tests of positive selection exist. One group of tests consists of Tajima's (152) D statistic and its modifications. In these methods, the consistency of the intrapopulational nucleotide frequency distribution with the neutral expectation is tested by using various statistics. A typical example is Tajima's D statistic, which suggests balancing selection when $D > 0$ and purifying selection or directional positive selection when $D < 0$. Hudson et al. (62) proposed a method that examines the consistency of the nucleotide frequency distribution within and between species. The null hypothesis of these methods depends on the assumption that the population is in mutation-drift balance. In practice, this assumption is almost never satisfied, and therefore it is generally difficult to obtain definitive conclusions from this type of statistical test. Because these methods have been reviewed by many authors (73, 84, 113, 120, 170), we shall not go into the details. Below we discuss two other groups of methods that have become popular in recent years.

One group of methods is for examining regions of extended haplotype homozygosity (EHH). An increasing number of investigators are now using these methods to detect a signature of positive selection with single nucleotide polymorphism (SNP) data. The principle of

these methods is that if a particular nucleotide mutation is strongly selected, then SNP alleles closely linked with this mutation would also increase in frequency because of "hitchhiking"; these SNP sites may then display a high level of homozygosity for haplotypes carrying the selected mutation over an extended chromosomal region (139). By contrast, haplotypes associated with the ancestral (original) nucleotide are expected to show no enhanced homozygosity, because recombination will likely have occurred many times between the site with ancestral nucleotide and other nucleotide sites in the past. Therefore, SNP sites for the haplotypes associated with the mutant nucleotide are expected to show a high homozygosity for an extended chromosomal region compared with the haplotypes associated with the ancestral nucleotide. One may therefore be able to detect a signature of selection by comparing the extent of haplotype homozygosity for the mutant nucleotide (EHH_M) with that for the ancestral nucleotide (EHH_A). The ratio of EHH_M to EHH_A is called the relative EHH (rEHH) (139). This method worked well with the *G6PD* and *CD40* ligand (*TNFSF5*) genes, which presumably have been under positive selection.

Many investigators have used this type of statistical method to identify SNP sites or genomic regions that may be under positive selection (e.g., 51, 133, 141, 153, 159). For example, Voight et al. (159) suggested that 250 genomic regions are under positive selection in human populations. Similarly, Sabeti et al. (141) identified approximately 300 selected regions in the human genome. Some authors (55, 56, 160) using the linkage disequilibrium approach to look for selection in EHH data have reported that approximately 0.5 adaptive nucleotide substitutions per year, or 15 substitutions per generation, have occurred in the recent history of human populations. This rate is far above the upper limit to adaptive gene substitution suggested by Haldane (54).

There are some problems with these methods. First, it is difficult to specify a neutral hypothesis against which the selection hypothesis can be rigorously tested. For this reason, no statistical test of neutral evolution is conducted in many studies, and the genomic regions that show the top 1% or 5% of rEHH values are simply chosen as the regions under positive selection. If millions of SNP sites are tested, the top 1% may therefore include hundreds of genomic regions. Because rEHH (or any other statistic) is affected by random events such as mutation, recombination, gene duplication, and genetic drift, as well as the amount and quality of SNP data, the statistic used is subject to substantial errors. Therefore, a high rEHH value may not necessarily imply selection.

Second, these estimates have been obtained under the assumption that a particular SNP site or a set of SNP sites is positively selected and nearby SNP sites hitchhike to become homozygous because of the low recombination rate. In practice, this is a mere assumption, and no investigators have identified any driving nucleotide site except for a few sites for which natural selection had been suspected before the study. Wang et al. (160) showed that ~35% of selected SNPs are not within 100 kb of known genes. Have these SNPs really been affected by hitchhiking even though there is no known functional element? It is important to have some empirical evidence of selection for each putatively selected genomic region. Until this evidence is presented, the results of these studies remain mere speculations.

Another statistical method for predicting selected genomic regions, the F_{ST} statistic method, can be used when a population is divided into subpopulations. F_{ST} is computed for each locus or SNP site by $F_{ST} = V_x/[\bar{x}(1-\bar{x})]$, where V_x is the variance of allele frequency x among subpopulations and $\bar{x}$ is the mean of x over subpopulations. If all subpopulations are derived from a single parental population at the same time and gene migration subsequently occurs among them at a rate of m per generation following the island model (**Figure 5*a***), the equilibrium expectation for F_{ST} for neutral alleles is approximately $1/(1 + 4Nm)$ (166), where N is the effective population size of each subpopulation. If all loci are neutral and evolve independently, the variance of F_{ST} among

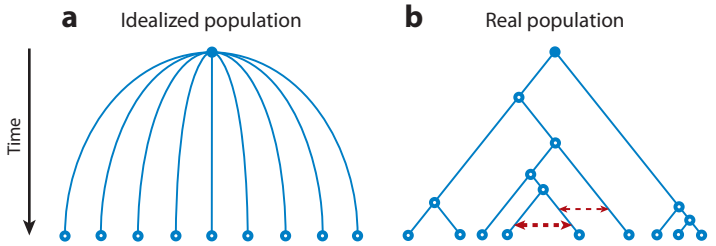

Figure 5

Simple examples of population structures. (*a*) An idealized model of population structure, in which subpopulations evolve independently after population splitting. (*b*) A realistic model of population structure, in which some subpopulations are more closely related than others. Partial gene exchange may also occur between some subpopulations (*red dashed lines with arrows*).

different loci is given by $k\bar{F}_{ST}^2/(n-1)$, where n is the number of subpopulations, $\bar{F}_{ST}$ is the average F_{ST} for the loci examined, and $k = 2$. Lewontin & Krakaur (89) proposed that this equation can be used for testing the neutral theory. However, this approach was criticized by Nei & Maruyama (116) and Robertson (137, 138), who showed that k can be much greater than 2 when some subpopulations are more closely related than others and the mutation rate varies with locus. In reality, the population differentiation is always more complicated than the idealized model assumes (**Figure 5*b***), so the Lewontin-Krakaur test was soon abandoned (90).

In recent years, however, a modified form of the Lewontin-Krakaur test has been proposed. In this modified form F_{ST} is computed for a large number of loci (SNP sites) for an organism, and the loci showing the highest or lowest 1% or 5% of F_{ST} values are assumed to be under positive selection (e.g., 3, 8, 110). Again, this outlier method is not justified, because there is no null distribution. Some authors have attempted to justify this method by using a computer-generated null distribution of F_{ST} obtained under a variety of gene migration models. However, the null distribution varies with the migration model so much (10, 34, 41, 135, 158) that it is difficult to decide which model should be used. Actually, as early as 1975 Robertson (138) stated "This increased variance of F_{ST} is a consequence of the genetic history of the species and cannot be overcome by any sophistication of sampling at the present time." This statement is still true. Nonetheless the modified Lewontin-Krakaur test has been applied to SNP data from human populations. For example, Akey et al. (3) identified 174 genomic regions as putatively selected sites using human SNP data, whereas Myles et al. (110) identified approximately 360 selected regions.

In recent years the number of loci that can be examined for evidence of positive selection has increased dramatically because of the increased availability of genome sequences and SNP data for model organisms. This situation has encouraged investigators to use the above statistical methods. However, because most of these methods do not test against a null hypothesis of neutral evolution, the test results are expected to include many false positives. In fact, computer simulation studies have shown that false-positive rates are quite high under many different conditions (72, 154).

The high false-positive rates are also revealed by comparing the putatively selected genomic regions obtained by different statistical methods in the same species. In humans, Nielsen et al. (121) compared 713 such genes included in the selected genomic regions identified by Voight et al. (159) with 90 genes identified by Wang et al. (160) and found that only 7 genes were shared between them. Akey (2) compared the positively selected regions identified by nine genome-wide studies (3, 17, 72, 78, 141, 153, 159, 160, 162). The total number of regions identified by these studies was 5,110, but only 722 regions (14.1%) were shared by at least two studies, 271 regions (5.3%) by at least three studies, and 129 regions (2.5%) by at least four studies. Enard et al. (32) also obtained similar results. These results indicate how unreliable these statistical methods are and imply that a large proportion of putatively selected sites are apparently false positives. This conclusion again raises questions about the results of genome-wide analysis.

To identify positive selection unambiguously, we must examine the biological functions of the predicted regions. In particular, putative positively selected amino acid differences at a

locus should be tested for functional differences using biochemical techniques.

DISCUSSION

Because genome sequences and SNP data are now available for many different organisms, it has become fashionable to scan all genes or all SNP sites available to find positively selected genomic regions. In general, however, this genome-wide analysis consists simply of single-locus analyses for a large number of loci. Although genome-wide analysis has the potential to give much information about natural selection, it may also give erroneous conclusions, as discussed earlier. When natural selection is studied for only a single locus, the study usually involves careful examination of the molecular nature of allelic differences and allele frequency changes. This can allow a solid conclusion for the locus in question. In a genome-wide analysis, however, every locus is treated equally in the statistical analysis, and therefore we tend to miss special features of individual loci.

Genome-wide analysis and single-locus analysis may give different conclusions even if the same statistical principle is used. We have seen this phenomenon in the case of MK tests. When a test designed for studying selection at a single locus is applied to the entire genome in a modified form, we may obtain a wrong conclusion. As we mentioned above, the theoretical basis of the MK test is not well established. The fact that only a small proportion of amino acid substitutions affect protein function also undermines the principle of the test. This conclusion is diametrically opposite to the claim of pervasive positive selection based on the MK test (e.g., 52, 144).

This does not mean that all statistical studies are inefficient. It is possible that EHH and F_{ST} methods may identify genes for which the allele frequencies are substantially differentiated due to natural selection. If we find improved methods for eliminating false-positive results, we may be able to choose the remaining genes for careful experimental studies. At the present time, however, many authors appear to be satisfied with finding signatures of positive selection. In our view, these signatures are not sufficient to infer selection and one needs to further demonstrate biological evidence for natural selection operating among different individuals (see **Table 1**). Furthermore, once we identify a particular mutation that undergoes selection, we should examine the nature of the mutation at the molecular level. It is important to know the molecular basis of mutational change, because mutation is the actual source of all phenotypic innovation and natural selection merely shifts allele frequencies (114).

Recent studies have led to the discovery of many new aspects of regulation of gene expression. We now know that noncoding DNA contains *cis*-regulatory elements, multiple promoters, regulators of alternative splicing, microRNAs, and epigenetic elements. These elements play important roles in phenotypic evolution (19, 25, 46, 163). Here we can see a large number of genes interacting in developmental and physiological processes. The evolution of these regulatory systems is one of the most important problems in evolutionary biology at present, but unfortunately this issue has not been studied in a comprehensive manner. A number of authors (e.g., 4, 11, 14, 57, 134, 136) have studied the evolution of *cis*-regulatory and other elements by using statistical methods and concluded that their evolution was aided by positive selection. However, this conclusion appears to contradict the fact that these elements are generally highly conserved. In fact, the estimates of $2Ns$ so far obtained for noncoding regions are within −0.2–2.5 (4), too small to be of biological significance. We believe that the evolution of these elements should be studied as a component of the entire gene expression system rather than as isolated elements. In such a study experimental approaches will play important roles.

In the study of human evolution the anthropocentric view is still prevalent. It is often assumed that many characteristics that distinguish humans from apes have evolved by natural selection (e.g., 140, 156, 157), and therefore the study of natural selection is essential to

understand human evolution. However, because there was no "purpose" for making humans when the human lineage first separated from the ape lineage, the human lineage must have evolved by fixation of mutations that happened to be advantageous or neutral in the niche to which human ancestors moved (114). There is no reason to believe that the human lineage has been subjected to more natural selection than the chimpanzee lineage. The ideal way of studying the evolution of phenotypic differences between humans and chimpanzees is to examine the expression of genes involved in a particular characteristic (e.g., brain size) in various developmental stages. One should then be able to identify specific mutations that generated the phenotypic difference. Of course, it would be difficult to do such an experiment with humans and chimpanzees, but it can be done with *Drosophila* or rodent species (19).

CONCLUSIONS

The neutral theory of molecular evolution has been tested for the past 40 years and is now widely accepted in the molecular biology community. In recent years, however, many population geneticists have challenged this view by reporting that a high proportion of amino acid substitutions are caused by positive selection. These results are mainly based on newly developed statistical methods such as Bayesian approaches for identifying positively selected codon sites and the MK test applied to genome-wide analysis. A critical review of these statistical methods has shown that their theoretical foundation is not well established and they often give false-positive and false-negative results. Correction of the deficiencies of these methods has shown that the results obtained by these methods are actually in conformity with the neutral theory. This also appears to be true with the methods based on EHH and F_{ST} analyses. At present, the genome-wide study of evolution largely consists of a collection of single-locus analyses, and sometimes misleading results are obtained. To understand genomic or phenotypic evolution, it is important to study the evolution of protein-coding genes and gene-regulatory elements as integrated units of gene function involved in developmental and physiological processes.

SUMMARY POINTS

1. The neutral theory of molecular evolution has been controversial for a long time, but the general pattern of molecular or genomic evolution is broadly consistent with the expectation from the neutral theory.
2. In recent years, however, many papers have been published that claim the prevalence of natural selection detected by new statistical methods.
3. One class of statistical methods that has been used extensively for detecting selection relies on Bayesian methods to identify selected codon sites. However, recent theoretical and empirical studies have shown that detectability of selection with these methods is poor. With this and other methods, we must consider the fact that most amino acid substitutions do not change protein functions.
4. A second class of popular methods is the MK test and its modifications. Here, the null hypothesis is poorly defined, so the overall efficiency of detecting selection is again poor. Some authors have estimated *Ns* values for putatively accelerated nonsynonymous substitutions, but the value is so small that the substitutions are practically neutral.
5. Two other classes of methods are the extended haplotype homozygosity (EHH) and F_{ST} tests. No clear null hypotheses exist in these tests, so they are likely to generate many false-positive results. Therefore, experimental study is necessary to confirm positive selection.

6. Recent genomic data based on more than 4,000 orthologous genes show that the numbers of nucleotide and amino acid substitutions increase almost linearly with divergence time in vertebrates. This result indicates that the overall pattern of nucleotide and amino acid substitutions is consistent with the neutral theory.
7. In general, however, almost all genes are under functional constraints. Therefore, the rate of amino acid substitution is considerably lower than the rate for unconstrained genes. This observation is also consistent with the neutral theory.
8. At the present time, genome-wide analysis of positive selection is a mere collection of single-locus analyses. In the future it is advisable to study the protein-coding genes and regulatory elements in an integrated way. Only in so doing can we understand the meaning of genomic evolution and the molecular basis of phenotypic evolution.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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176. Identified functionally important amino acid substitutions for rhodopsin genes by experimental methods and showed that ~95% of amino acid substitutions are more or less neutral.

182. Proposed the molecular clock hypothesis and showed that most amino acid substitutions outside the active site of a protein evolve at a constant rate per year.

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Errata

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