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METHODS TO DETECT SELECTION IN POPULATIONS WITH APPLICATIONS TO THE HUMAN

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■ **Abstract** The development of statistical tests of natural selection at the DNA level in population samples has been ongoing for the past 13 years. The current state of the field is reviewed, and the available tests of selection are described. All tests use predictions from the theory of neutrally evolving sites as a null hypothesis. Departures from equilibrium-neutral expectations can indicate the presence of natural selection acting either at one or more of the sites under investigation or at a sufficiently tightly linked site. Complications can arise in the interpretation of departures from neutrality if populations are not at equilibrium for mutation and genetic drift or if populations are subdivided, both of which are likely scenarios for humans. Attempts to understand the nonequilibrium configuration of silent polymorphism in human mitochondrial DNA illustrate the difficulty of distinguishing between selection and alternative demographic hypotheses. The range of plausible alternatives to selection will become better defined, however, as additional population genetic data sets become available, allowing better null models to be constructed.

INTRODUCTION

Kimura's immensely influential formulation of the neutral theory of molecular evolution, which came in 1968, was based primarily on an argument about the magnitude of the genetic load that would be imposed by positive selection if it were the only driving force in protein evolution (56). The availability of α- and β-hemoglobin sequences from a variety of primate and mammalian species allowed him to estimate a rate of amino acid substitution and to model this substitution process under two opposing hypotheses, selection and neutrality. Kimura argued that the load was too great under selection, whereas it was practically nonexistent under near-neutrality. In this remarkable note to *Nature*, Kimura also considered protein (allozyme) polymorphisms within species under these alternative models, because data for allozymes were readily available at that time,

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and he came essentially to the same conclusion in favor of neutrality. So, in this short paper, Kimura not only formulated his neutral theory of molecular evolution, he also pointed to a future direction in empirical testing of selection and drift hypotheses—relational analysis of within-species polymorphism and between-species divergence. "Polymorphism," he poignantly stated some years later, "is just a transient phase of molecular evolution" (57). Kimura did even more in this paper. He also deduced a major role for selection by showing that the rate of amino acid substitution in hemoglobin was far lower than that predicted from reasonable estimates of the nucleotide mutation rate. He argued from this result that the prevalent form of natural selection acting on proteins was selective constraint, the elimination of deleterious mutations, and not positive selection. So auspiciously began the modern field of testing for neutrality and selection from protein and DNA sequences.

The detection of positive selection in DNA sequences poses an immense challenge. The genetic material can be likened to a device that faithfully records every informative event (i.e. a mutation) but then over time proceeds to either erase (by back mutation) or obscure (by parallel mutation) some of the recorded information. Furthermore, there is not simply one recorder playing at any one time, but a whole population of them (the gene pool), and each records a slightly different, but correlated, version of history. However, only one of these recordings or, more accurately, a very heavily spliced (i.e. recombined) version gets saved for posterity (the future ancestral sequence). Which spliced snippets get saved depends on innumerable chance events, ranging from the relatively benign drift of a neutral mutation in a large population to the strong directional shifts in allele frequencies at sites linked to an adaptive mutation. So, even though every functionally important mutational event in the history of a species is, by definition, recorded in the DNA sequence of a species, these informative mutations are likely to be embedded in a sea of less meaningful ones (selectively neutral and nearly neutral mutations) and are likely to be associated with stochastic events that can result in many possible configurations of linked variation or change. The challenge of detecting selection at the level of DNA is the challenge of finding its signal in a leaky, lossy medium.

This chapter provides an overview of statistical approaches for detecting natural selection in DNA sequences. As already indicated, the distinction between a nucleotide variation that distinguishes two alleles from within a species, commonly referred to as *polymorphism*, and a nucleotide variation that distinguishes two alleles from different species, sometimes called the *divergence* or *fixed differences*, can be a subtle one. Polymorphism and divergence data can be viewed as providing information about evolution at different time depths in the process. The substitution differences between species are the set of mutations that have successfully reached fixation, through genetic drift, direct selection, or indirect selection (genetic hitchhiking). Polymorphisms, in contrast, are mutations whose final disposition, either fixation or loss, has yet to be determined by these same processes.

In general, the polymorphisms that are found within a species are considerably younger than the differences found between species, but this is not always the case.

Polymorphisms can be maintained by balancing selection for very long periods of time, so that the most recent common ancestor of two alleles can predate speciation events. Neutral variants that are tightly linked to a site under selection will also be maintained by this selection, albeit passively, and the age of the balanced polymorphism will promote neutral divergence at linked sites (46, 53). The alleles of the classical class I and II loci of the major histocompatibility complex (59, 86) provide many excellent examples of synonymous and presumably neutral divergence between selectively maintained alleles. Human leukocyte antigen (HLA) alleles taken from two different primate species can be more similar than two alleles chosen from within a species (66, 69). For these alleles, it is not possible to view the polymorphism within species and the differences between species as distinctly different entities.

Statistical tests can capitalize on the multiple time depths provided by within- and between-species variation, and some tests use both kinds of information. This review of methods to detect selection, accordingly, does not restrict itself to only one arena. The strongest inferences about selection can be made, however, when data sets include multiple kinds of information—the complete sequence of alleles along a contiguous stretch of DNA in a population sample, the haplotypes defined by these variants, and homologous sequences from closely related species.

The review has two components. The first is an exposition of available methods for testing data for evidence of selection. Some tests apply equally well to between-species and within-species sequence comparisons; some require both kinds of data, whereas others focus specifically on within-species polymorphism. Some tests are quite general in that they can detect departures from neutrality that arise from many possible causes, whereas other tests have been designed to contrast neutrality with a specific selection alternative. Because of the ad hoc nature of the tests described, there is no single best test. Therefore, appropriate applications for each type of test are identified.

The second component of this review deals with specific applications of statistical tests of neutrality to human data. I make no attempt to be comprehensive, but instead highlight studies that either illustrate the use of a test or that raise interesting issues about the evolutionary forces governing human variability. These issues are ones that are likely to receive greater attention in coming years.

THE NULL HYPOTHESIS IN TESTS TO DETECT SELECTION

Kimura's theory of neutrally evolving mutations is the backbone for evolutionary analysis of DNA sequence variation and change for three reasons. First, a substantial fraction of nucleotide polymorphism and evolutionary substitution occurs in regions of the genome that are least functional—noncoding, nontranslated, and degenerate positions of codons—and these changes may in many instances be successfully modeled as selectively neutral mutations. Second and more important,

selective neutrality is a useful null hypothesis against which to test for evidence of selection. The relative simplicity of the theory, together with its breadth of predictive scope, has facilitated the development of readily testable null hypotheses. Third, selectively neutral mutations segregating as polymorphisms in a population are susceptible to the influences of selection at linked sites. This selection, as detailed below, can cause deflections away from the neutral-equilibrium situation, and characteristics of these deflections can be predicted for various forms of selection. This means that the statistical analysis of (potentially) neutral variation in a gene (or other region of the genome) can be informative about selection acting at linked sites, including balancing and directional selection. So, for many reasons, the neutral theory plays a critical role in the detection of selection.

Clues from Other Species

Many statistical tests of neutrality were formulated and first applied to data from *Drosophila* species, not only because *Drosophila* species are highly polymorphic at the nucleotide level, but also because this group is species rich, thus allowing taxonomic comparisons across a large range of phylogenetic distances. Although this review concentrates on findings in humans, major findings in *Drosophila* species cannot be avoided. To the contrary, contrasts between the two species can be very informative. For example, the most recent compilation of the nucleotide polymorphism levels in *Drosophila* species (76) shows that heterozygosity at noncoding and/or synonymous sites (the probability that a nucleotide site is polymorphic in a pair of randomly chosen sequences) can be as high as 1%–2%. Experience with *Drosophila* species also shows, however, that levels of noncoding nucleotide polymorphism vary across the genome and correlate quite strongly with recombination rates (3, 8, 61; see 14, 103 for theoretical treatments of possible selective mechanisms).

In humans, nucleotide heterozygosity has been estimated to be ~0.1% (11, 67), and this value shows up widely in the recent literature as being representative of the human genome, but this conclusion is premature and almost certainly wrong. The correlation between nucleotide polymorphism levels and recombination rates is likely to be driven by very general genetic and selective mechanisms—genetic hitchhiking caused by the fixation of adaptive mutations or the elimination of deleterious mutations—and so the pattern is likely to be present in humans as well as flies. A recent report finding evidence of a recombinational context for human nucleotide polymorphism levels should come as no surprise (78).

This example also illustrates how the discovery of an unexpected pattern of nucleotide polymorphism in *Drosophila* species can prompt theoretical investigation of the underlying genetic and evolutionary mechanisms. Once understood, many of these mechanisms turn out to be fundamental and therefore pertinent to other organisms. These discoveries promote not only the development of new statistical tests of neutrality, but also better interpretation of significant departures from neutrality. They also foster better experimental design. The knowledge, for

example, that neutral polymorphism levels have a recombinational context means that statistical tests of selection that involve the comparison of polymorphism levels across loci should be between loci that lie in regions with similar rates of recombination. Theoretical insights, statistical tests, the interpretation of test results, and experimental design all have benefited from discoveries made in *Drosophila* species.

Differences Between "Them" and "Us"

The human species differs from many other species as a population-genetic entity in one important respect—the species population size has expanded dramatically in the past 10,000–100,000 years, from as few as thousands of individuals to the present population size of ~10 billion individuals (5, 19, 41, 55, 90, 97, 98). The human population is not, therefore, at a stationary equilibrium for neutral variation, and this is known with factual certainty. This situation raises important issues about the proper formulation of the null (i.e. neutral) hypothesis because, among other things, statistical signatures of positive selection and an expanding population can be similar (92). Statistical inferences about positive selection, therefore, may be more difficult in humans than in other species. The discrimination of selective sweeps and population expansion are further discussed below. The history of human migration is an additional factor that has influenced nucleotide polymorphism. Population subdivision and recent migration can cause the configuration of allelic variation at a locus to depart from the selectively neutral equilibrium predicted for a panmictic population and can easily be misinterpreted as evidence for selection.

Interpreting Departures From Neutrality

When a statistically significant departure from selective neutrality is found in a gene (or a region of the genome), unfortunately there are often two equally plausible alternative hypotheses in accord with the data, one involving natural selection and one involving one or more of the demographic or population factors. This situation raises the issue again of an appropriate null hypothesis against which to test data for evidence of natural selection. Unlike other species, we have considerable information about the history of our own species, ranging from paleontology to linguistics. How much of the known history of human demography and population movement should be incorporated into the null model? The answer is not yet known.

STATISTICAL TESTS OF SELECTION

All of the ad hoc tests involving polymorphism data share the following undesirable characteristic: the information content of the data is condensed into summary statistics, and, as a consequence, information is thrown out. A simple example of

this condensational loss of information can be seen in the use of the number of segregating sites S in a sample to estimate the neutral parameter θ (22, 102; see Equation 1 below). Consider two data sets of 20 sequences—one with only two equally frequent alleles that differ at 20 nucleotide sites and one with 20 unique alleles, each one differing from another by one nucleotide site. In both data sets, $S = 20$, and both data sets yield the same estimate for θ. However, the distinctness of two data sets is lost in this summary statistic because S does not retain information in the data about the frequency of each segregating site in the sample. Yet, S has desirable statistical properties under the neutral model, making it a useful summary statistic of the variation contained in a sample of DNA sequences.

There has been a recent surge of interest in methods conditioned on all of the information contained in population genetic data, that is, the specific haplotypic configuration of segregating nucleotides in a sample, to fit data to models of neutral evolution (32–35, 63, 64, 84). These efforts draw heavily on applications of coalescent theory, a subject that is beyond the scope of this review, but pertinent to the development of many tests. Useful reviews of the subject can be found elsewhere (21, 30, 43, 99). This approach requires extensive computer simulation to explore the likelihood of the data for a given model and set of parameters and, at present, can handle only relatively small data sets in which there is at most modest recombination (101). Furthermore, the coalescent maximum-likelihood approach to model testing has not yet been extended to include models with selection. This may change, however, with the recent emergence of coalescent models with selection (62, 82). An excellent example of the likelihood approach can be found in Harding et al's analysis of β-hemoglobin nucleotide polymorphism (38).

Tests to Compare Two Categories of Sites in the Same Gene

Table 1 is a summary of leading statistical tests of selection. Kimura suggested a method to detect positive selection in protein differences between species soon after the advent of DNA sequencing. It was based on the notion that the neutral rate of nucleotide substitution, which can in some cases be estimated by comparing noncoding or synonymous differences between two sequences, provides a benchmark that can only be exceeded when positive selection also contributes to the substitution process. The test statistic that is commonly used is the ratio of the estimated number of amino acid replacements per site (K_a) to the estimated number of synonymous changes (K_s) in the same protein. The criterion for selection is

$$K_a/K_s > 1.$$

This is an extremely stringent criterion for inferring the action of positive selection and is likely to miss the majority of cases in which positive selection is operating. The most useful applications of this test have been those restricted to specific functional domains of a protein, domains for which there are a priori reasons to expect positive selection. The canonical example of this approach in a human gene

TABLE 1 Statistical tests of selection[a]

Test	Type	Designed to detect	Best use	Caveats	Reference(s)
HKA	Within vs between spp. (two loci)	Differences in variation levels not accountable by constraints	Balancing selection; recent selective sweeps or other variation-reducing forces	High recombination rates may reduce effectiveness of test	49
McDonald (run test)	Within- vs between-spp. (contiguous region)	Regions with non-neutral patterns of poly. and div.	Equilibrium balancing selection	Has some advantages over the HKA test	71, 72
McDonald Kreitman G	Within- vs between-spp. (syn. vs nonsynon.)	Adaptive evolution	Adaptive protein evolution; mutation/selection	Selection on codon usage can seriously jeopardize test	73
Tajima's D	Within sp.	Skew in frequency spectrum	General purpose test of frequency spectrum skew	See reference 27 for situations in which the test performs poorly	96
Fu & Li's D	Within sp.	Recent vs ancient mutations	General purpose test of frequency spectrum skew	Fu's more recent tests may be more powerful	29
Fu W	Within sp.	Departures in frequency spectrum	Population subdivision	Hudson's Gst test is more powerful for detecting subdivision	27
Fu Gη	Within sp.	Departures in frequency spectrum	Population subdivision, shrinkage, and overdominance selection	Little power against excess number of rare alleles 28	27
Fu Gξ	Within sp.	Departures in frequency spectrum	Population subdivision, shrinkage, and overdominance selection	Little power against excess number of rare alleles	27
Fu F_s	Within sp.	Excess or rare alleles (one sided)	Population growth, genetic hitchhiking, and background selection	May be best overall test for detecting genetic hitchhiking and population growth	28
Hudson	Within sp. and allele	Unexpectedly low variation within an allele class	Directional selection	A good test for young alleles driven to high frequency	45
Wall B ans Q	Within sp.	Linkage disequil. between adjacent segregating sites	Population subdivision, balancing selection	Q is more powerful when there is substantial recombination	100
Andolfatto's S_k	Within sp. (sliding window)	Non-neutral haplotype structure	Balancing and directional selection; pop. subdivision	Interpretation may be difficult	2

[a]Abbreviations: HKA, Hudson-Kreitman-Aguadé; syn., synonymous; nonsynon., nonsynonymous; disequil., disequilibrium; poly., polymorphism; div., divergence; pop., population.

is the binding cleft of the HLA molecule, which determines its antigenic specificity (50–52). This example also illustrates the blurred distinction between polymorphic and fixed differences—Hughes and coworkers showed that $K_a/K_s > 1$ is true for both intraspecies and interspecies allelic comparisons.

An interesting example of accelerated protein evolution revealed by this test is the human (and primate) protamines, Prm-1, Prm-2, and Tnp-2 (105). These proteins are spermatid associated, and all of them follow a similar trend toward very fast rates of protein evolution, leading to the suggestion that sperm competition among males and/or sexual selection is the driving force in their rapid evolution. However, the commentary on this paper by Clark & Civetta proposes an alternative explanation for rapid protein evolution (17).

The $K_a/K_s > 1$ test is an example of a statistical test that compares changes in two categories of DNA sites, replacement and synonymous, in the same gene. In this test, the synonymous mutation rate is assumed to be a proxy for neutrally evolving DNA. In principle, this rate could be estimated from synonymous mutations in other genes or even from noncoding regions. The reason it is not so estimated in mammalian genomes is that synonymous rates of evolution have an isochore context dependency; synonymous rates between isochores are known to vary two- or threefold (9, 68). There may also be a recombinational context to synonymous polymorphism levels, as explained above (78). To the extent that differences in the synonymous substitution rate reflect differences in regional mutation rates or effective population sizes and not differences in selection intensity on synonymous mutations (23, 85), then the most appropriate data against which to test for accelerated protein evolution are synonymous substitutions of the same gene.

A second class of statistical tests of neutrality, the McDonald-Kreitman tests, also compare the ratio of variability in replacement and synonymous sites, but do so for both within-species polymorphism and between-species divergence (73). Under completely neutral evolution, the variability within a species and the rate of evolution between species (i.e. the substitution rate) are each linearly related to the neutral mutation rate. The ratios of variability in the two categories of sites, as estimated from polymorphism data and from fixed differences between species, are expected to be the same under a model of completely neutral evolution, and this expectation can be tested in a two-by-two contingency table. Under the alternative model of adaptive protein evolution, there is relatively more replacement substitution between species than replacement polymorphism within a species. The aforementioned protamine genes, which have evolved very rapidly in the phylogenetic lineage leading to humans, exhibit a significant excess of fixed replacement substitutions on this lineage compared with replacement polymorphisms within our species as measured by the MK test, which is consistent with the positive-selection hypothesis (105).

One of the more interesting findings from applications of the MK test is that mitochondrial genes, including those of humans, often exhibit a relative excess of replacement polymorphism compared with replacement divergence (77, 79, 80, 89). The widespread occurrence of this pattern of departure from neutrality suggests

that deleterious mutations are an important component of polymorphic amino acids in mitochondrial proteins; they accumulate as polymorphisms in populations but tend not to become fixed, owing to deleterious selection. A similar pattern has not emerged for nuclear genes.

A more sophisticated version of the MK test categorizes the changes in one type of site [such as degenerate positions in codons (synonymous sites)] based on the direction of mutational change (1, 6). Akashi (1), for example, studied weak selection in codon usage in Drosophila by categorizing synonymous mutations as being favored if they mutated from a nonpreferred codon to a preferred codon and as being unfavored if they mutated from a preferred codon to an unpreferred codon. The direction of mutational change was inferred from outgroup species sequences. Akashi showed that the within- and between-species ratios of these two categories of mutational change departed significantly from the neutral expectation in the direction predicted if preferred mutations were selectively favored and unpreferred mutations were selected against. A similar test of synonymous (and noncoding) mutations in human and primate HLA genes (23), with the categories of change being from A/T bases mutating to C/G or from C/G bases mutating to A/T, shows a significant departure from neutrality. The author argued for selection favoring G/C in high G/C-rich isochores. This conclusion flies in the face, however, of the more conventional interpretation of isochore structure of the vertebrate genome, that of mutational bias (26, 95, 104), and more work on this issue is needed.

Direct Comparison of Mutation Frequencies in Two Different Categories

The advent of large-scale SNP data sets for human gene loci provides an opportunity to directly compare within-population allele frequencies (and between-population differences in allele frequencies) for synonymous and replacement polymorphisms. Previous work on allele frequency distributions for allozymes, a proxy for amino acid replacement variants, found little evidence for a departure from neutral equilibrium predictions for allele frequencies, the number of alleles, or heterozygosities (reviewed in 81). The analysis of allozyme frequencies for 139 species, including humans, for example, showed them to be largely in accord with neutral predictions, although an excess of low-frequency alleles was found in a fraction of the studies (13). The excess of low-frequency alleles was attributed not to deleterious selection but to population bottlenecks.

This issue can now be reexamined with greater resolution by using large-scale SNP data, and the first indication favors deleterious selection against protein variants (11). Cargill et al (11) found that amino acid replacement polymorphisms were at consistently lower frequencies than synonymous polymorphisms in the same gene. This analysis of allele frequencies will be even more interesting once the ancestral alleles are determined from interspecies sequence comparisons (36).

Another interesting comparison can be made from large-scale SNP studies: the ratio of synonymous and replacement polymorphisms in different species.

Silent polymorphism levels appear to be ≥10- to 20-fold lower in humans than corresponding levels in *Drosophila* spp. (see 88 for a current compilation of human polymorphism estimates and see 76 for *Drosophila* polymorphism estimates). Yet allozyme studies revealed only a much smaller difference of approximately twofold (40). The question is whether this discontinuity will hold up. If so, then a plausible case can be made for selective maintenance of protein polymorphism.

Tests to Compare Polymorphism Levels in Different Genes

In the previous tests, the categories of sites (or change) are physically intertwined in the same region of DNA, usually a single coding sequence. How does one go about comparing polymorphism levels in two nonoverlapping regions of DNA, such as the variability in two different genes? One might want to know, for example, whether the synonymous variability in nonclassical *HLA* genes is significantly lower than that in the classical HLA genes. This comparison, it turns out, is considerably more difficult than the comparison of replacement and synonymous variability in one gene, and it is important to understand why this is the case.

Under the infinite-sites–neutral-equilibrium model, the expected nucleotide variation for a diploid is given by the neutral parameter $\theta = 4N_e\mu$, where N_e is the evolutionary effective population size and μ is the mutation rate. The problem of comparing levels of variation in sequence samples of two genes, in particular silent variation, can be reformulated as the problem of comparing two estimates of the neutral parameter.

Individual realizations of the neutral evolutionary process can yield levels and configurations of neutral polymorphism far from expected values, owing to the highly stochastic nature of the process. This variability can be viewed as arising from two causes, the stochastic nature of the mutation process itself (the number of mutations is generally modeled as a Poisson process) and stochasticity in the genealogical history of the alleles (coalescent times under the Wright-Fisher model are exponentially distributed). The relative contribution of the latter, variability in the gene tree, to the overall stochasticity in polymorphism levels is dependent on the recombination rate across the locus. For example, an unbiased estimator of the neutral parameter θ can be calculated from the number of segregating sites S_n, in a sample of size n, and it is not dependent on the extent of intralocus recombination,

$$\hat{\theta} = S_n/K_{n-1}, \quad K_n = \sum_{i=1}^{n} 1/i.$$

The variance is dependent on recombination, and it will be between the values given for no recombination and free recombination, that is, between

$$\theta K_{n-1}^{-1} \quad \text{and} \quad \theta K_{n-1}^{-1} + \theta^2 \left\{ 1 + \frac{1}{4} + \cdots + (n-1)^{-2} \right\} K_{n-1}^{-2}.$$

The recombination rate is generally not known, and, although it can be estimated from sequence polymorphism data itself, the available estimators are poorly

behaved statistically (101). A promising approach is to simultaneously estimate the neutral parameter and recombination rate parameter in the neutral model by using the complete data and maximum likelihood (32, 65, 84). But these methods are computationally not yet feasible for large data sets, or when there is anything more than a low level of recombination.

In comparing levels of polymorphism in two genes, it is common to use the most conservative assumption about recombination—no recombination within a locus and free recombination between loci—but this has the unfortunate effect of maximally weakening the power of the test comparison. In practice this means that large differences in polymorphism levels are required to obtain statistical significance. A discussion about statistical methods for directly comparing polymorphism levels by comparing estimates of the neutral parameter is given in Kreitman & Hudson (60). One ad hoc approach to improve the power of the test is to use a conservative estimate of the recombination rate, based on the minimum number of recombination events seen in a data set (2, 100).

Tests to Compare the Variation Within and Between Species

A more sophisticated but maximally conservative method for comparing levels of polymorphism between two loci (or in contiguous regions) is the Hudson-Kreitman-Aguadé test (49). This test attempts to control for differences in neutral mutation rates between two loci (or sequenced intervals) that might be caused by differences in the level of selective constraint acting in each locus. The test compares within-species polymorphism and between-species divergence at two or more loci; it assumes no recombination within loci and free recombination between loci.

It should be noted that the comparison of polymorphism at two loci when there is no recombination either within or between them, such as might occur for two mitochondrial or two Y-linked genes, needs only to consider the stochasticity associated with the mutation process, because the loci (and alleles) share a single common genealogy. A goodness-of-fit test (60) or the likelihood ratio test (34, 63) can be used to compare the variability in this case. The application of such a test would be to determine whether the neutral mutation rates differ between the two loci.

More sophisticated versions of the HKA test have been developed by McDonald (71, 72) for comparing the levels of polymorphism and divergence along one contiguous stretch of DNA. Rather than arbitrarily dividing the region of interest into subintervals to compare levels of polymorphism by the HKA test, McDonald's tests scan the polymorphism and divergence in a window of defined length as it is slid across the entire region. A run test or a Kolmogorov-Smirnov statistic is then used to evaluate the statistical significance (compared with equilibrium neutral expectations) of heterogeneity in the ratio of polymorphism and divergence. The tests account for nonindependence of the variability contained in overlapping windows, multiple tests, and uncertainty about the recombination rate.

The within- and between-species family of tests can be useful for detecting the presence of a balanced polymorphism. The region surrounding a site under balancing selection will have enhanced neutral variation, distributed mainly between (rather than within) the selected alleles. This enhanced variation will fall off with distance from the site under selection, and it will depend on the magnitude of the product of the recombination rate and population size (46, 53). In regions of high recombination in *Drosophila* species, the window of enhanced variability can be only ~100 bases (60) and so may not leave an easily detectable signature of polymorphism.

Tests to Compare the Frequency Spectrum of Polymorphism Within a Locus

In many instances an investigator is interested only in determining whether the polymorphism contained in a within-species data set for a single locus violates neutral equilibrium expectations. A family of ad hoc tests has been developed for this purpose. Tajima's D was the first such test (96), and it remains a popular test. This test compares the difference between two estimators of the neutral parameter, the number of segregating sites in a sample, S, and the average pairwise difference, π, in the number of nucleotides. Only the latter incorporates information about the frequency of variants in the sample. Positive and negative values of the test correspond to departures from equilibrium neutral expectations in the direction of having the frequency spectrum skewed towards too many intermediate-frequency polymorphisms or too many low-frequency polymorphisms, respectively. Significantly positive values of Tajima's D test are consistent with balancing selection for two or more alleles, but they can also indicate the presence of population subdivision. Significantly negative values of Tajima's D test are consistent with a recent selective sweep of a linked mutation or, in the human context, a population bottleneck and/or recent expansion in population size (92).

Several additional test statistics have been developed to examine allelic and nucleotide polymorphism configurations for their consistency with neutral expectations (27–29). The power of these tests, as well as Tajima's D, against non-neutral alternatives has been examined (28). One of Fu's test statistics, F_s, may be the best overall test for detecting genetic hitchhiking (i.e. selective sweeps) and population growth.

Deciphering the Cause of "Reduced" Variation: Selective Sweep or Background Selection?

In *Drosophila* species and probably in humans, levels of silent nucleotide polymorphism are positively correlated with rates of recombination (3, 8, 78). Two alternative mechanisms have been proposed to account for reduced neutral variation, genetic hitchhiking caused by selective sweeps (54, 70, 103) and background selection against deleterious mutations (14, 15, 44, 47, 48). The two alternative

hypotheses to account for reduced variation can, in principle, be distinguished by using frequency spectrum tests, at least for a sweep scenario in which only a single adaptive mutation may be present in a population at a given time (10, 14, 28, 91). Selective sweeps, but not background selection, deflect the frequency spectrum of neutral mutations in the neighborhood of a recent selective sweep toward low frequency. Evidence for both mechanisms can be found in the analysis of polymorphism levels and frequency spectrum skews in *Drosophila* genes (10, 85, 94). Further complicating the situation, some models of positive selection may not give rise to detectable departures from neutral equilibrium expectations, and therefore will not be detectable by these tests (31).

The Duffy blood group locus in humans may represent an example of a recent selective sweep of an adaptive allele in part of the species range. This locus segregates for three alleles, FY^*A and FY^*B, which differ by a single amino acid, and FY^*O, which corresponds to the absence of the Fy antigen. The FY^*O allele is very nearly fixed in sub-Saharan African populations but rare elsewhere (12). Individuals who are homozygous for the FY^*O allele are resistant to *Plasmodium vivax* malaria (75), thus providing a strong selective advantage to the allele in vivax malaria-prone localities. HKA tests reveal "reduced" variation in sub-Saharan populations in both the locus and in a region 4–5 kilobases upstream from the locus, supporting the positive-selection hypothesis (37). Evidence is equivocal, however, for a frequency spectrum shift toward low-frequency mutations (37). The situation may be complicated by the fact that the Hy^*O allele exists on two haplotypes, which may have arisen before positive selection.

Additional Haplotype Tests to Detect Departures from Neutrality

A scenario in which positive selection has recently pushed a new mutation and the haplotype on which it resides up to a relatively high frequency in a population can lead to a configuration in which the selectively favored allele harbors very little (or no) within-allele variation in the region surrounding the selectively favored mutation. A test of this scenario was developed (45) that is particularly applicable in cases in which the alleles in a sample can be divided into two classes, a priori, such as when the locus is known to be segregating for two protein variants. The test uses a coalescent simulation of neutral genealogies, conditioning on allele frequency and the number of segregating sites in the total sample, to estimate the probability of observing a sample data set as extreme as the one observed.

A refinement of this haplotype test approach allowed Kirby & Stephan to investigate the haplotype structure in different segments of a sample of sequenced alleles by using a sliding window (58). They investigated the probability of observing zero polymorphisms in subsets of the data. Further refinement of this sliding window haplotype test approach was made by Andolfatto et al (2). This test defines S_k to be the largest number of consecutive segregating sites in a

sample of size n that contain only k different haplotypes ($1 < k < n$, where n is the sample size). An empirical distribution of S_k is determined by a neutral coalescent simulation approach that is conditioned on n and S. The test calculates the proportion of simulated data sets that contain at least one stretch of S_k-segregating sites with $\leq k$ haplotypes. The test corrects for multiple tests and window sizes.

Other Tests of Selection

Statistics based on the linkage disequilibrium between adjacent pairs of segregating sites can be used to test for balancing selection (100). The tests are based on the principle that neutral polymorphisms that accumulate in the region surrounding a site under selection will mostly be segregating between the selected alleles, thus establishing permanent linkage disequilibrium. This paper also uses simulated data to investigate the power of eight different test statistics to reject an equilibrium-neutral panmictic-population model against a symmetric migration island model alternative. Wall (100) found that all tests have low power when the recombination rate is as high as or higher than the mutation rate.

Alleles can be maintained as balanced polymorphisms by a number of different mechanisms, including unconditional overdominance, frequency-dependent selection, and local deme selection. The latter two mechanisms can, in principle, be distinguished with nucleotide polymorphism data from more than one deme (16). Balancing selection can be distinguished from local selection by determining whether a peak of polymorphism surrounding a site under selection is found within or between demes. Evidence for frequency-dependent selection can also be found in the analysis of within-allele polymorphism levels in relation to allele frequencies (93).

Caveats

With the availability of so many ad hoc statistical tests to detect selection, it is not unlikely that one or another of the tests will support a departure from neutrality. The statistical significance of a test can be interpreted in the context of the multiple testing problem, but, because the tests are not entirely independent, reassessing a particular test's significance would require, at the very least, empirical investigation in simulated data sets. In practice, researchers do not report all of the tests they have carried out on the data, but rather focus on the statistically significant ones. This bias is likely to be exacerbated by the tendency to publish "significant" results.

Biases in the ascertainment of polymorphism data are also likely to foster misinterpretation of statistical tests of selection. For example, a SNP survey that, for technical reasons, identifies only variants that are above a critical threshold frequency will produce highly biased polymorphism data sets. The best data sets are ones in which all nucleotide variants have been identified, independently of their frequencies in a sample, but another form of ascertainment bias will result even when "complete" data sets are obtained if the region is chosen because it surrounds

a known SNP of a given frequency. Ascertainment biases, if they are known, can likely be incorporated into tests of neutrality (Y-X Fu, personal communication).

A deeper problem exists, however, in interpreting a test's departure from the equilibrium-neutral model prediction, specifically the existence of alternatives that don't involve natural selection at all. The human species, as we know, has rapidly expanded in number, and levels of nucleotide variation are not at equilibrium with respect to the species' current size. Human population history—its major epochs of migration and expansion, instances of geographic isolation, the mixing of subpopulations, and major and minor historical population bottlenecks—is rich enough to assure the existence of a plausible alternative to any selection hypothesis to explain a single-locus departure of nucleotide polymorphism from equilibrium-neutral predictions.

An instructive example of this problem lies in the interpretation of human mitochondrial nucleotide polymorphism. In a very insightful paper, Di Rienzo & Wilson reported that the genealogy of mitochondrial sequences in non-Africans was more starlike in shape than might be expected under neutrality and that the distribution of pairwise differences was Poisson shaped (20; also see 74). Di Rienzo interpreted this apparent departure from neutrality as an indication of recent population expansion. Theoretical treatment of the problem provided additional support for the expansion hypothesis (90), but a bottleneck at ~50,000–100,000 years ago, possibly caused by the selective sweep of a favorable allele, could not be rejected.

Mitochondrial DNA has been assumed to be nonrecombining (but for evidence of recombination, see 4, 24); the sweep of a favorable mutation anywhere in the mitochondrial genome will cause the fixation of a single haplotype. Support for the selection hypothesis has come from the analysis of nuclear encoded genes. The nuclear genome shows little evidence for a skew towards rare alleles (18, 37, 38, 42, 83, 106), and thus towards a negative Tajima's *D*, as predicted under the population expansion hypothesis.

Theoretical investigation of bottlenecks and subsequent expansions (25) shows, however, that Tajima's *D* can be negative or positive depending on the size of the bottleneck and the timing and magnitude of an expansion. Given that the mitochondrial genome has a smaller effective population size (being maternally inherited and effectively haploid) than the nuclear genome, the conflicting portraits of polymorphism in the two genomes may be consistent with a population bottleneck (25). The exciting possibility of a selective sweep in the modern mitochondrial genome remains, unfortunately, an unresolved issue.

THE PRESENT AND FUTURE OF HUMAN MOLECULAR POPULATION GENETICS

The only current safeguard against gross misinterpretation of test results vis-à-vis selection vs historical demography is to have an a priori hypothesis about the type and direction of selection that are expected for the locus under investigation. The

previously described work on Duffy provides a good example of this approach (37). There are two reasons to hope, however, that the situation for analyzing human polymorphism data sets will improve. First, as additional data sets accumulate, a reduction in the number of plausible historical demographic scenarios will be possible. The specific range of parameter values, for example, allowing mitochondrial genes but not nuclear genes to differ in the observed frequency spectrum of mutations may be shown to be unrealistic. Second, population history, whether it involves ancient bottlenecks, recent expansions, or specific population movements, affects the polymorphism of all nuclear genes equally. From a practical perspective, this means that the common signatures of human history on genetic variation should yield to the avalanche of data expected in future polymorphism studies. Better data mining techniques and sharper theoretical predictions are needed, however, to make this a reality.

It should be possible, in principle, to construct a realistic neutral model of human variation that takes into account major features of human history. Such a model would then serve as a null hypothesis, a selectively neutral backdrop, against which to look for evidence of natural selection in individual genes. In no other organism is this possibility likely to be achieved at the high level of resolution possible for humans. Our species, despite its low levels of nucleotide polymorphism, issues in ethical sampling of native populations, and the inability to control matings, may thus replace *Drosophila* species as the poster child for molecular population genetics.

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