cesses and that the excess substitutions result from fixation of selectively advantageous mutations. This conclusion is based on a statistical test of the prediction that under neutrality "the ratio of replacement [nonsynonymous] to synonymous fixed substitutions should be the same as the ratio of replacement to synonymous polymorphisms." We believe that there are subtle but serious problems in McDonald and Kreitman's reasoning.

The prediction as derived from the neutral theory can be more precisely stated as a null hypothesis as follows: for strictly neutral mutations, the ratio of nonsynonymous to synonymous substitutions between species is equal to the ratio of nonsynonymous to synonymous substitutions between alleles within species. A proper test of this hypothesis requires estimates of the number of nucleotide substitutions per site that have occurred during the divergence of alleles within species and between species. However, the *G*-test used by McDonald and Kreitman does not compare rates of nucleotide substitution but instead is based on the ratio of synonymous to nonsynonymous sites that have been classified as polymorphic within or fixed between species. Such a classification of sites is arbitrary because fixation (or polymorphism) at a site in a population is a transient event in evolution. In addition, the number of polymorphic and fixed sites depends, to an unknown extent, on both number of sequences examined as well as the number of species studied. This second point is illustrated, for example, by site 816 in Table 1 of ref. 1, which would have been a fixed site if only *D. simulans* and *D. yakuba* were studied, but was classified as a polymorphic site because it was polymorphic in *D. melanogaster*. Although these effects may have only minor influence on the ratio of nonsynonymous to synonymous sites that are polymorphic or fixed in a sample, there is virtually no mathematical theory for predicting the distribution of the number of polymorphic and fixed sites across species under the neutral hypothesis. (Hey recently studied[5] the sampling distribution of fixed differences between two species.) Finally, since both the number of fixed and polymorphic sites are subject to large stochastic errors[4,5], the stochastic variances of these quantities should be taken into account in a valid test of the difference between the two ratios.

We suggest that as a general test of the null hypothesis of equal ratios one should evaluate the average nucleotide substitutions for all pairwise comparisons of sequences within and between species (π and d_A, respectively)[6]. Our test uses the frequency of nucleotides in the sample, accounts for multiple substitutions at sites, and incorporates stochastic errors in the evolutionary process. We first reanalysed McDonald and Kreitman's data for *D. melanogaster* and *D. yakuba*. We estimated d_A and the average π for the two species for both synonymous and nonsynonymous sites using the Nei–Gojobori method[6]. The standard errors of these quantities were computed by taking into account stochastic variances[6,7]. The results obtained were $d_A = 14.20 \pm 3.71$ and $\pi = 2.40 \pm 1.01$ per 100 synonymous sites and $d_A = 1.1 \pm 0.44$ and $\pi = 0.06 \pm 0.05$ per 100 nonsynonymous sites. Therefore, the ratio of nonsynonymous to synonymous substitutions is 0.077 ± 0.037 between species and 0.026 ± 0.024 within species. Although the former ratio is higher than the latter, the difference is not statistically significant ($Z = 1.2$; $P > 0.2$). (The smaller ratio within species could be partly due to excess synonymous substitutions caused by the balanced polymorphism of the *F* and *S* alleles in *D. melanogaster*[8].) The analysis for three species, which is somewhat more complicated, gives essentially the same results. Thus, these results do not support the conclusion that there is a significant excess of nonsynonymous substitutions resulting from adaptive fixation of mutations.

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MCDONALD AND KREITMAN REPLY — Graur and Li, and Whittam and Nei, point out that a polymorphism in one species may have arisen in an ancestral species; that different polymorphic sites have different allele frequencies; and that the number of polymorphisms will increase as more alleles and more species are sampled. For the neutral model of molecular evolution that we tested, all of these phenomena will affect neutral replacement substitutions and neutral synonymous substitutions equally. Therefore they do not affect the validity of our test of the neutral model, and they are not alternatives to adaptation as explanations for the ratio of replacement to synonymous substitutions being much greater for fixed differences than for polymorphisms at the *Adh* locus in three species of *Drosophila*[1].

The authors also question our method for counting a site which has one nucleotide fixed in one species, a different nucleotide fixed in a second species, and both nucleotides polymorphic in a third species. They suggest that we count such a site as one fixed difference and one polymorphism, rather than just as one polymorphism. Any rule for classifying substitutions as fixed or polymorphic will affect neutral replacement and neutral synonymous substitutions equally, and it is only important to apply the same rule to both. We choose to count as fixed substitutions only those that are fixed in every species in which they appear. This is because under the alternative hypothesis of adaptive fixation of replacement mutations, a replacement substitution that is adaptive in one species, and thus rapidly becomes fixed, will either be adaptive or maladaptive in other species, and thus is unlikely to be polymorphic in any species. There is also a practical reason for counting each substitution only once, rather than trying to estimate the number of times that the substitution has gone to fixation. Estimating the number of fixation events would require an accurate estimate of the species' phylogeny; we think it is an advantage of our test that it requires no such estimate.

The authors of the above letters suggest tests based on the gene diversity between and within species, rather than the numbers of fixed and polymorphic substitutions. These tests use estimates of the stochastic variance, which is the variation in gene diversity among loci resulting from the different coalescent times (times to the most recent common ancestor) of different loci[3,7]. For a single locus, however, replacement substitutions are intermingled with synonymous substitutions, and thus replacement and synonymous substitutions have the same coalescent time. Therefore only sampling variance needs to be considered. It would be interesting to see the results of a test using gene diversity that used the sampling variance, which is much smaller than the stochastic variance. Whatever the outcome of such a diversity-based test, however, our substitution-counting test remains valid. We suspect that any diversity-based test will be more complicated, will require more assumptions, and will be less statistically powerful than our method for detecting adaptive protein evolution.

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