Annu. Rev. Genet. 2001. 35:539–66

DOES NONNEUTRAL EVOLUTION SHAPE OBSERVED PATTERNS OF DNA VARIATION IN ANIMAL MITOCHONDRIAL GENOMES?

Anne S. Gerber[1], Ronald Loggins[1], Sudhir Kumar[2], and Thomas E. Dowling[2]

[1]Department of Biology, University of North Dakota, Grand Forks, North Dakota 58202-9019 and [2]Department of Biology, Arizona State University, Tempe, Arizona, 85287-1501; e-mail: thomas.dowling@asu.edu

Key Words selection, neutrality, mtDNA, Tajima's D, MK test

■ **Abstract** Early studies of animal mitochondrial DNA (mtDNA) assumed that nucleotide sequence variation was neutral. Recent analyses of sequences from a variety of taxa have brought the validity of this assumption into question. Here we review analytical methods used to test for neutrality and evidence for nonneutral evolution of animal mtDNA. Evaluations of mitochondrial haplotypes in different nuclear backgrounds identified differences in performance, typically favoring coevolved mitochondrial and nuclear genomes. Experimental manipulations also indicated that certain haplotypes have an advantage over others; however, biotic and historical effects and cyto-nuclear interactions make it difficult to assess the relative importance of nonneutral factors. Statistical analyses of sequences have been used to argue for nonneutrality of mtDNA; however, rejection of neutral patterns in the published literature is common but not predominant. Patterns of replacement and synonymous substitutions within and between species identified a trend toward an excess of replacement mutations within species. This pattern has been viewed as support for the existence of mildly deleterious mutations within species; however, other alternative explanations that can produce similar patterns cannot be eliminated.

CONTENTS

INTRODUCTION

Evolutionary research has been greatly aided by technical advances in molecular biology, allowing for more direct characterization of genetic variation. One marker system in particular, mitochondrial DNA (mtDNA), has revolutionized evolutionary studies of animals (reviewed in 5, 6). Since its initial application in the 1970s, this molecule has been widely used to examine evolutionary genetic questions such as population structure, patterns of hybridization, and evolutionary relationships of a variety of animal taxa. Although the advent of the polymerase chain reaction (PCR) created the potential for examination of a much large number of genes encoded in the nucleus, mtDNA has remained the molecule most often used in evolutionary studies.

Several attributes of mtDNA were responsible for its rise to prominence and continued popularity as a tool for evolutionary studies (reviewed in 5, 6). These characteristics were initially derived from the study of a relatively small number of vertebrate taxa, particularly mammals (reviewed in 17). Small size, duplex, circular conformation, and high copy number allowed for isolation of a sufficient quantity of mtDNA for use in direct characterization with restriction endonucleases (e.g., 9, 19). Gene content and arrangement were thought to be conserved across most animal lineages with few or no intervening noncoding sequences (reviewed in 17). Studies of transmission indicated that mtDNA was inherited only through maternal parents without recombination (e.g., 44, 65). Despite conservation of form, rates of nucleotide substitution were much higher than for nuclear genes (4, 18, 126). These properties made mtDNA an ideal marker system, and scientists utilized it to examine a more diverse group of organisms.

Later studies identified the lack of generality of many of these features in nonmammalian taxa (reviewed in 5, 6, 80). These deviations were so significant that their impact on the utility of mtDNA as a marker for evolutionary studies required additional assessment. As more information of major invertebrate and vertebrate

taxa accumulated, considerable variation in gene arrangement was found (e.g., 15, 104, 109). While rearrangements among basal groups are common, order is sufficiently stable within major groups (but see for example, 15, 64, 67) to allow for production of universal primers for PCR amplification of specific fragments and genes (e.g., 58, 91). Several instances of paternal transmission have been identified (e.g., 43, 45, 59), and evidence for recombination has also been reported (e.g., 10, 32); however, levels of paternal leakage and recombination appear in most instances to be so low that they can be safely ignored (6, 63). Rates of mtDNA evolution (e.g., 72, 93, 114) and base composition (e.g., 62) were found to be significantly different among lineages, making local calibrations essential for proper use of a molecular clock.

Even considering these caveats, the general attributes of mtDNA still make it one of the premier marker systems for analysis of population genetic (e.g., measurement of gene flow and population subdivision, estimation of female effective population size) and phylogenetic (e.g., reconstruction of relationships, estimation of divergence times) questions. Most of these applications have taken place under the assumption (explicit or otherwise) that sequence variation in mtDNA is selectively neutral, but the basis and evidence supporting this assumption have not been evaluated.

Considerations of the Neutral Theory

The Neutral Theory has long been debated by evolutionary biologists, especially the role played by mildly deleterious mutations (55, 60, 90, 101). Here we present no new information relevant to the resolution of this dispute; however, we provide the necessary context within which we interpret information.

The neutral theory of molecular evolution (53, 55) attempts to explain the amount and pattern of DNA and protein polymorphism observed in natural populations. It proclaims that "a large proportion of molecular variation within populations is neutral or nearly neutral" (86, p. 3). Since the "polymorphism [within species] is just a transient phase of molecular evolution [among species]" (55), this proclamation applies to molecular sequence diversity among species as well as to within-population molecular variation. Therefore, the neutral theory applies to patterns of observed variation within and among taxa.

Considerable confusion exists in the literature about the evolutionary processes responsible for neutral evolution and their relationship with the neutral theory. To resolve this confusion, let us examine the evolution of protein-coding genes. Consider the subset of third codon positions containing fourfold degenerate sites. At these positions, the fate of almost all mutations will be governed by chance [neutral mutation-random drift (55)] because DNA sequence mutations do not manifest themselves at the protein sequence level and have no effect on fitness. In fact, fourfold degenerate sites are often considered to be completely neutral in nature. Therefore, observed variation at these sites is explained by the neutral theory. Now consider second codon positions. All second codon positions are 0-fold degenerate

(i.e., all mutations produce an amino acid change); however, only a small fraction of mutations persist at such sites as purifying selection will quickly eliminate all other mutations. Because of the action of purifying selection, observed variation at 0-fold degenerate sites will be predominantly neutral or nearly so and governed mainly by neutral processes. When we contrast patterns of variation between these two categories, the overall fraction of mutations that are selectively neutral at 0-fold degenerate sites is much smaller than that at fourfold degenerate sites under the neutral theory. Likewise, the average nucleotide sequence diversity at 0-fold sites (π_0) would be much less than fourfold sites (π_4). The observed pattern of sequence variation ($\pi_0 < \pi_4$) is explained by the neutral theory, but the process generating this pattern is purifying selection that weeds out deleterious mutations. Kimura (54) specifically developed this line of reasoning in the framework of the Neutral Theory: "In my opinion, various observations suggest that as the functional constraint diminishes the rate of evolution converges to that of synonymous substitutions. If this is valid, such a convergence (or plateauing) of molecular evolutionary rates will turn out to be strong supporting evidence for the neutral theory."

The popular use of the phrase neutral evolution confounds these two aspects as it is often taken to mean that the fate of all mutations is governed by random chance alone in all codon positions. This perspective (strict-neutrality) is accurate only when considering the evolution of pseudogenes or nonfunctional genes. Therefore, the neutral theory framework (55) explains the patterns of sequence variation at different types of sites (in codons or parts of the gene) and clearly allows for the existence of purifying selection in the process. There has also been considerable debate over the incorporation of slightly deleterious mutations under the Neutral Theory (60, 90). In this paper, we interpret neutrality (or neutral evolution) in the broadest sense (90). Evolutionary processes such as positive selection or adaptive evolution produce nonneutral patterns of molecular variation and are responsible for nonneutral evolution, whereas pattern shifts due to drift, mutation, and/or purifying selection are consistent with the neutral theory.

Given these general considerations, the issue is not whether selection acts upon mtDNA, as it clearly does [the rate of synonymous substitution is many times larger than the rate of nonsynonymous (replacement) substitution]. The important questions are: Which processes act and how do they affect patterns of variation (e.g., number and types of substitutions, base composition, etc.) observed in natural populations? Is variation in mtDNA nucleotide sequences driven by positive selection or does it reflect the interaction of mutation, drift, and shifting selective constraints?

Early Justification for Neutral Evolution of Animal Mitochondrial DNA

Support for the assumption of neutral evolution of mtDNA was at first inferential, based on contrasts of evolutionary rates of nuclear and mitochondrial gene

evolution. In one of the earliest reports of rapid evolution of mtDNA, Brown et al. (18) hypothesized that the accelerated rate of sequence change (relative to nuclear genes) could arise from low functional constraints on mitochondrial gene products. They also provided other alternatives that implicated a higher rate of spontaneous mutation of mtDNA or a slowdown in the mutation rate of nuclear DNA in the observed discrepancy. Cann & Wilson (22) and Cann et al. (21) suggested that the probability of fixation of mildly deleterious mutations might be higher in mtDNA owing to hitchhiking through linkage to adaptive variants. However, Cann et al. (21) concluded, "... the high rate of evolutionary change in mammalian mtDNA is probably the result of the blending of two forces. One could be increased mutation pressure and/or lack of recombination, both of which would enhance the frequency of change at all positions. The other could be relaxed translational constraints, which would have a major effect on the components of the translational machinery and a minor effect on the mitochondrial genes coding for proteins." Therefore, increased rates of mtDNA evolution were hypothesized to result from a higher mutation rate, relaxation of purifying selection, or a combination of these two factors.

Avise (4) evaluated the available information on causes for the rapid rate of mtDNA evolution and the issue of relaxed selective constraints. He concluded, "... there is currently no compelling reason to suppose that most of the mtDNA variants routinely assayed cannot be interpreted as neutral markers of the female lineages in which they occur. This has been the working assumption in most population surveys of mtDNA." Avise et al. (7) continued along this line of reasoning. Since the majority of substitutions in mtDNA were found to be synonymous or insertion/deletions in noncoding regions, they stated "... most of the particular mtDNA genotypic variants segregating in populations probably have, by themselves, absolutely no differential effect on organismal fitness." Avise et al. (7) added the caveat that "... mechanistically neutral mtDNA variants may, through linkage to selected mtDNA mutations, have evolutionary histories that are at times influenced or even dominated by effects of natural selection."

Given that the rapid rate of evolution was the original basis for assumed neutrality of mtDNA, variation in rates of evolution become pertinent to the discussion of neutrality. Several studies have demonstrated slowdowns in the rate of mtDNA evolution for various lineages (e.g., 8, 72). Templeton (116) demonstrated how evolutionary rates could vary in mtDNA because of the impact of different modes of cladogenesis, even in relatively closely related taxa such as Hawaiian *Drosophila*. In a comparison of rates of mitochondrial and nuclear DNA evolution between sea urchins and primates, Vawter & Brown (119) argued that the relatively rapid rate of vertebrate mtDNA evolution was due mainly to fluctuations in rates of nuclear DNA evolution. Findings such as these indicate that the assumption of rate constancy of mtDNA evolution is not valid.

Recently, many authors have tested for selection on mtDNA, and results of these studies have generated concern over the use and interpretation of mtDNA as a neutral marker (reviewed in 13, 83, 96, 100). Given these developments, it is

important that evidence for selection on mtDNA be evaluated to allow for continued informed use of this molecular marker. Here we review statistics that have been used to test neutrality of mtDNA and hypotheses examined by these tests. We also review recent studies that have examined the role of selection on mtDNA evolution and evaluate their ability to discriminate among competing hypotheses. The extensive literature makes it difficult to incorporate all publications on this subject. Therefore, we focus our efforts on statistics and experimental approaches most commonly used to examine the role of selection on mtDNA evolution.

STATISTICAL TESTS FOR DETECTING DEPARTURES FROM NEUTRALITY

A number of tests have been developed over the past 25 years to examine whether observed patterns of DNA or protein polymorphism are consistent with predictions of the Neutral Theory (for a recent review, see 61). These measures are generally based upon the relationship of different estimates of diversity within and between populations, including the number of segregating sites, heterozygosity, gene diversity, and the ratio of synonymous and replacement substitution rates (see 55, 66, 86, 87). Here we present a brief summary of some of these tests, and discuss the null hypotheses tested when they are applied for the mtDNA analysis.

Single-Locus Tests of Protein and DNA Polymorphism Within a Species

Watterson (121, see also 29) developed a test that contrasts observed heterozygosity for a given locus with the theoretical distribution of single-locus heterozygosity given the observed number of alleles. If the fit of the observed and theoretical distributions is adequate, the Neutral Theory cannot be rejected. Such tests are extremely conservative (86). Of course, rejection of the null hypothesis of neutrality in these tests could also occur if one or more of the underlying assumptions (e.g., constant population size) are violated (111).

Tajima's (110) D statistic is widely used to examine the distribution of DNA polymorphisms within species. It is based on the examination of the relationship between the estimates of the population parameter $\theta (= N_e\mu$, where N_e is the effective population size and μ is the mutation rate), which can be calculated from the number of segregating sites (θ_S) and the nucleotide diversity (θ_π) in a sample of sequences from a panmictic population. The difference between these two estimates ($\Delta\theta = \theta_\pi - \theta_S$) and its variance provides a test for neutrality. Both estimates of θ are expected to be the same when a population achieves a neutral mutation-drift equilibrium ($\Delta\theta = 0$). Significant positive values of $\Delta\theta$ are consistent with balancing selection that may leave its signature in the form of increased allele frequencies. The existence of many deleterious mutations in the population will produce an excess of rare alleles, which will lead to a negative value for $\Delta\theta$. Interpretation of observed departures from neutrality are only valid if the assumption

of neutral mutation-drift balance is satisfied. It is now well appreciated that the sign of $\Delta\theta$ depends on the population history if a population has recently undergone a bottleneck (111). Therefore, Tajima's test measures skew of allele frequency spectra (61). Fu & Li (38) developed a test similar to Tajima's D in which polymorphisms were separated into recent and ancestral categories based on a phylogenetic tree; however, it has been used less frequently for mtDNA.

Comparing Variation Within and Between Species for Two Loci

This approach takes advantage of the Neutral Theory prediction that "polymorphism [within species] is just a transient phase of molecular evolution [among species]" (55). The best example is the Hudson-Kreitman-Aguade (HKA) test (46), which compares within-species polymorphism and between-species divergence at two (or more) loci simultaneously. If the two loci are evolving at different rates, then the locus with a higher rate is expected to exhibit higher levels of polymorphism under the Neutral Theory. Kreitman (61) noted that the requirement of free recombination between loci makes this test simply a test of neutral substitution rate differences between loci when genes from mtDNA or Y-chromosome are compared, because they evolve as a linked unit. Therefore, its utility is limited to comparisons between mitochondrial (or Y-chromosome) and nuclear loci.

Comparing Synonymous and Replacement Substitutions Within or Between Species

In protein coding genes, we can estimate the rates of synonymous (r_S) and nonsynonymous (r_N) substitutions. Because synonymous substitutions are usually free from selection, r_S is often equated with the neutral substitution rate (76). For neutral evolution in general, $r_N < r_S$ because a large fraction of replacement mutations is likely to be eliminated by purifying selection (55). Therefore, a gene showing $r_N > r_S$, a pattern of molecular variation opposite from that expected under neutrality, is considered to be caused by positive Darwinian selection (47, 55, 87). Since r_N and r_S are both computed from the same set of sequences, they share the same evolutionary history and time of divergence. Therefore, a comparison of r_N and r_S can be conducted by comparing d_N and d_S, where d_S is the number of synonymous substitutions per synonymous site and d_N is the number of replacement substitutions per replacement site. These tests can be applied in the same way for within-species polymorphism as well as between-species comparisons.

Specifically, we test the null hypothesis of $d_N = d_S$ in a one-tailed test, as the alternative hypothesis being tested is $d_N > d_S$. A common practice for conducting this test is to compute the difference Δd $(= d_N - d_S)$ and construct a normal deviate test by using either analytical or bootstrap variance of Δd (reviewed in 87). Illustrated examples and relative usefulness of different methods for computing d_N and d_S are given in Nei & Kumar (87). If the sequences compared are closely related (e.g., individuals from the same population), the number of synonymous and

replacement differences observed might be small and the large-sample assumption made in the normal deviate test is not satisfied. This may make the normal deviate test liberal in rejecting the null hypothesis (131). Under these circumstances, Fisher's Exact Test is more appropriate, typically performed as a 2×2 contingency table in which the nucleotide sites are partitioned into two pairs of site categories, synonymous and replacement sites with and without substitutions (87, 131).

Comparing Synonymous and Replacement Substitutions Within Species (Polymorphisms) and Between Species (Fixed Differences)

Tests in this category are built on the same principle as that for the HKA test and others: Neutral evolution within species is expected to show the same pattern as that observed between species. When substitutions are partitioned into synonymous and replacement types, then estimates of d_N/d_S within a population should be similar to that observed between species if the given gene is evolving in the same manner between species and within populations. This is exactly what the McDonald-Kreitman [MK test (75)] test examines. In this case, a 2×2 contingency table is constructed that contains the numbers of synonymous and replacement sites showing fixed differences between two species and polymorphisms within a population. Rejection of the null hypothesis of the same evolutionary pattern suggests shifts in patterns consistent with either neutral or nonneutral evolution. This can be examined by comparing the ratio of replacement to synonymous substitutions (R) computed from the elements of the 2×2 table for within-population (R_P) with that obtained from between-species analysis (R_F). The null hypothesis tested in the MK test is simply that $R_P = R_F$. The ratio R_P/R_F is the Pattern-Shift Index (PSI) that provides an estimate of the extent of evolutionary pattern shift observed within a population relative to that observed between species. PSI is exactly the same as the neutrality index of Rand & Kann (99).

There are several potential interpretations of MK test results. If a gene is evolving under positive selection among species as well as within populations, then $d_N > d_S$ ($R_F > 1$ and $R_P > 1$) for both among-species and within-population analyses. In this case, the nonneutral nature of sequence variation is obvious irrespective of results of the MK test.

For mtDNA, the value of d_S is rarely smaller than d_N, and, therefore, the application of the MK test usually leads to test of the shifts in neutral evolutionary patterns over time. In some instances, one may find $R_F > R_P$, which indicates an excess of replacement between species as compared to the population polymorphism data. If R_P is assumed to be the ratio expected under neutrality, one may invoke positive selection as the cause behind the increased number of replacement substitutions between species. However, if we assume that R_F is the neutral expectation, then an R_P lower than R_F potentially indicates depression of the replacement substitution rate or an increase in synonymous substitution rates within populations.

The other possible outcome, $R_P > R_F$, may indicate depression of the replacement substitution rate or an increase in synonymous substitution rates between species if we assume that R_P is the neutral expectation. The most popular interpretation of the MK test results assumes that R_F reflects the neutral ratio. Therefore, when $R_P > R_F$, there is an excess of replacement variants within species that is often equated with the overabundance of mildly deleterious mutations within populations. In this case, existence of a few newly arisen, positively selected replacement mutations may also lead to an overabundance of replacement substitutions within populations. This does not necessarily mean that the evolutionary patterns within species are nonneutral under Kimura's (55) framework, as the majority of replacement mutations may be still neutral in nature (2).

PAST STUDIES AND AVAILABLE EVIDENCE

Even as mtDNA was becoming an important marker for use in evolutionary genetic studies, neutrality of the molecule was called into question (e.g., 129). Several early studies focused on selective differences among length variants (e.g., 98, 126), possibly due to faster replication of smaller molecules. Because such variation appears not to affect long-term fitness (e.g., 3, 23, 132), selection on length variation is not considered further. Instead we focus on how selection may shape the distribution of observed nucleotide sequence variation within and among species.

Studies of neutrality of nucleotide sequence variation fall into three broad categories: (*a*) observed differences in performance among haplotypes, (*b*) quantification of shifts in haplotype frequency in experimental populations, and (*c*) comparison of observed patterns of nucleotide sequence variation with those expected under the neutral model. These categories are simply designated for convenience of discussion, as some overlap exists among them.

Observed Differences in Performance Among Haplotypes

Several lines of evidence have been used to examine and/or infer differential performance among mtDNA haplotypes. Some of the strongest evidence for purifying selection comes from studies of disease, with well-known disorders in humans and mice resulting from mutations in mtDNA (120). However, some of these diseases appear late in life and may have limited repercussions on the reproductive contribution of affected individuals (e.g., 77). Other diseases appear to have different fitness effects in males and females (e.g., 103), potentially leading to persistence of deleterious mutations (36).

In some instances, selection between haplotypes is inferred from patterns of variation, with limited support. Malhotra & Thorpe (69) found parallel patterns of morphological and mtDNA variation and ecological gradients in lizards from two Caribbean islands. Based on high levels (>12%) of sequence divergence at cytochrome *b* (cyt*b*) and lack of plausible vicariant events, the authors concluded that observed patterns resulted from natural selection. In a study of hybridization

between arctic char (*Salvelinus alpinus*) and brook char (*S. fontinalis*), Glémet et al. (40) found that introgressive hybridization had resulted in complete replacement of brook char mtDNA by that of arctic char in one river subdrainage in Québec. Physiological studies indicated nonequivalence of thermal sensitivity of cytochrome oxidase and pyruvate oxidation by red muscle mitochondria from introgressed and nonintrogressed brook char; however, tests of individual fish failed to identify a significant advantage for either haplotype (cited in 40). Duvernell & Aspinwall (28) found that haplotypic variation in the cyprinid fish *Luxilus chrysocephalus* was consistent with geography and concluded that observed patterns of variation were determined by selection. Dowling et al. (27) noted that distribution of haplotypes is affected by introgression and glacial history, making it difficult to eliminate a role for historical factors in explaining patterns of haplotypic variation.

The direct impact of mitochondrial haplotypes on various performance attributes has also been examined. Schizas et al. (105) quantified differential survivorship to pesticide exposure among three haplotype lineages (designated I, II, III) of the marine copepod, *Microarthridion littorale*. When a random mixture of adults containing these three lineages was exposed to pesticides, lineage I exhibited a significant increase in frequency over control lines, whereas lineages II and III showed nonsignificant reductions in frequency relative to the controls. These results were consistent with frequencies of these haplotypes at clean and toxic sites, suggesting a relationship between haplotype and persistence in natural populations. Studies of the association between mtDNA haplotypes and size-associated parameters (e.g., body weight, growth rate) in rainbow trout yielded mixed results (25, 33). Some haplotypes exhibited significantly enhanced growth; however, there was considerable variation among strains, leading the authors to hypothesize that time of spawning may also play a role in growth dynamics.

These studies demonstrate the significance of mtDNA gene products for organismal fitness; however, it can be difficult to eliminate the impacts of cyto-nuclear interactions in producing the observed patterns of variation. The significance of cyto-nuclear interaction has been demonstrated through examination of polymerase function in cell lines or manipulated eggs (reviewed in 80). These generally indicated improved performance when mitochondrial and nuclear gene products were derived from the same or similar species, indicating coevolution of nuclear and mitochondrial gene products. King & Attardi (56) used human cell lines depleted of mitochondria to demonstrate that source of exogenous mitochondria influenced cytochrome oxidase (COX) activity. Additional studies of human cell lines indicated that it is possible to replace human mtDNA with that of apes; however, human mtDNA always comes to predominate in lines initiated through fusion of ape cytoplasts with human cells, even when the human mtDNA has large deletions (79). The impact of cyto-nuclear interactions on performance has also been demonstrated in other organisms. Burton et al. (20) examined the interaction between mitochondrial cytochrome oxidase genes (COI, COII) and nuclear cytochrome *c* sequences in the copepod *Tigriopus californicus*. Crosses between

natural populations from different regions resulted in decreased COX activity by the F_2 generation, whereas intrapopulation crosses undergoing the same treatment yielded an increase in COX activity.

Nevertheless, certain haplotypes may perform better regardless of nuclear background. Takeda et al. (113) used heteroplasmic mice created through reciprocal cytoplast transfer of two lines (C57BL/6 and RR) to examine temporal shifts and tissue-specific segregation of mtDNA haplotypes with different control regions. While equivalent amounts of the two forms were detected in early stage embryos, RR became abundant in most tissues regardless of nuclear background, possibly due to a replicative advantage of RR during development and differentiation.

Experimental Manipulation and Frequency Variation

Early experimental tests of fitness and neutrality of mtDNA were first conducted on lab populations of *Drosophila*. Although labor-intensive, this approach allows for direct examination of fitness effects due to mtDNA variation. In one of the earliest of these studies, MacRae & Anderson (68) tracked frequencies of two mtDNA haplotypes (*bogota* from Colombia, South America, and AH from Apple Hill, California) over 32 generations in one line of *D. pseudoobscura*. Initially present at 30%, the *bogota* haplotype increased to ~80% by generation 4, where it remained for 18 generations. At generation 22, addition of more AH haplotypes reduced the frequency of *bogota* to below 60%; however, the population returned to the pre-perturbation frequency within one generation. Attempts to replicate these results were unsuccessful, although mtDNA frequencies were observed to change significantly during the course of 10 generations in other experiments reported in the same paper. MacRae & Anderson concluded that nonneutral forces controlled mtDNA haplotype frequencies and suggested that sporadic selection can favor one haplotype over the other, possibly as a result of cyto-nuclear interactions. Selection was also invoked in other tests of *D. subobscura* (34) because haplotypes went to fixation faster in large populations. Haplotypes within their own nuclear background were positively selected, but in a mixed nuclear background, one particular haplotype predominated in all instances.

In early tests implicating selection, other explanations were forwarded to explain deviations from neutral behavior in mtDNA frequencies. Singh & Hale (107) note that studies of *D. pseudoobscura* employed haplotypes derived from different subspecies and that observed variation was consistent with known variation in reproductive compatibility. Similar results and conclusions were presented in studies of *D. simulans* populations established to detect selection on mtDNA (89), except that partial reproductive isolation due to maternally transmitted *Rickettsia* was considered to be sufficient to account for apparent departures from neutrality. Jenkins et al. (49) examined the potential impacts of assortative mating and maternally transmitted cytoplasmic incompatibility factors in *D. pseudoobscura* and concluded that neither of these factors could explain patterns of frequency change observed in the earlier experiments of MacRae & Anderson (68).

Several sets of experiments have been conducted that were specifically designed to account for the effects described above. Nigro (88) examined frequency variation in transplasmic lines of *Drosophila simulans* treated for bacterial infection and found that the *si*II haplotype was positively selected when placed in the nuclear background of its own strain but negatively selected when in the *si*III nuclear background. DeStordeur (26) used microinjection to study partitioning of mitochondrial haplotypes in heteroplasmic *D. simulans* and *D. mauritiania* and found results consistent with those of Nigro (88). In a study involving *D. pseudoobscura* and *D. persimilis*, Hutter & Rand (48) found asymmetric cyto-nuclear fitness associations. García-Martínez et al. (39) performed experiments on experimental populations of *D. subobscura*. One haplotype went to fixation in all four cages, yet tests of neutrality found no departure from neutral expectations (see below). Results such as these have generally been seen as evidence for selection on mtDNA haplotypes as well as cyto-nuclear interactions.

Not all experiments identified fitness differences among haplotypes. Kilpatrick & Rand (52) established population cages with two divergent, reproductively compatible strains of *D. melanogaster* in replicate populations. Mitochondrial frequencies were examined on each of the two nuclear backgrounds, as well as a hybrid nuclear background. Haplotype frequencies did not change on the three nuclear backgrounds, and perturbation to test for departures from drift revealed no evidence for purifying or positive selection of one mtDNA haplotype over the other, regardless of the nuclear environment. This result was not restricted to *Drosophila*, as Khambhampati et al. (51) also found no evidence for selection among mtDNA haplotypes in similar experiments on caged populations of the mosquito *Aedes albopictus*.

Nucleotide Sequence Variation and Neutral Theory Expectations

As characterization of DNA sequences became easier, mtDNA neutrality could be tested by statistical analyses of sequence data. Because of the general availability of PCR primers, much of the early data comes from humans and associated commensal taxa (e.g., *Drosophila* and mice). Development of universal PCR primers allowed for sequencing of noncommensal species, providing additional data for analyses of patterns of evolution.

DROSOPHILA Ballard & Kreitman (12) examined evolution of cyt*b* in *Drosophila melanogaster*, *D. simulans*, and *D. yakuba*. MK tests identified a significant excess of replacement polymorphisms within all species pooled ($R_P > R_F$), whereas the HKA test [using the nuclear genes alcohol dehydrogenase (*Adh*) and *period*] only identified departures from neutrality in *D. simulans*. The Watterson test identified an excess of rare haplotypes in *D. melanogaster* and was suggestive of such excess in *D. simulans*. The authors hypothesize recent selective sweeps in both species to explain these results.

Rand et al. (97) examined sequence variation in subunit five of the NADH dehydrogenase gene (ND5) of *D. melanogaster* and *D. simulans* from diverse localities worldwide. Three tests were employed in this study: MK, HKA (using the nuclear genes *Adh*, *Pgd*, and *period*), and Tajima's D. Although this paper is often reported as supporting the case for nonneutrality of mtDNA, neutrality was not rejected using the MK test except for one region of the gene in both species. HKA test results also were not significant using DNA sequences, whereas Tajima's D failed to reject neutrality for all tests except for the carboxy-terminal end of the ND5 gene in *D. simulans*. Rand & Kann (99) also examined sequence variation in the ND3 and ND5 genes among many lines of *D. melanogaster* and *D. simulans* sampled from around the world. As in their earlier studies, applications of the MK test failed to detect significant departures from neutrality in these data, and Tajima's D was consistent with neutrality except for replacement sites in the ND5 gene. Although these tests were not significant, the authors noted a consistent excess of amino acid polymorphism over that which they expected from neutrality ($R_P > R_F$) and attributed this pattern to selection. García-Martínez et al. (39) examined 984 bases of the ND5 gene from 45 haplotypes of *D. subobscura* sampled from a diversity of localities. Tajima's D, Fu & Li's D and F (38), and the MK test did not identify significant departures from the null expectation, inconsistent with fitness tests on observed changes in haplotype frequences.

PRIMATES In the earliest tests of selection on human mtDNA, examination of allelic variation detected by RFLPs (125) found 71% of the diversity values from comparisons of allele frequency distributions to expected distributions fell within the range expected under the Neutral Theory. The greatest deviations from neutrality were found in protein-coding loci. Explanations for departures from neutrality were recent range expansion and historical purifying selection. Excoffier (30) found that all African human populations examined conformed to Neutral Theory expectations, but Oriental and Caucasoid populations did not. Diversity-reducing factors in Oriental and Caucasoid population dynamics, including population expansion, were proposed to account for these nonneutral patterns. Rogers & Harpending (102) found that the distribution of pairwise nucleotide differences for human mtDNA does not conform to the neutral model, due to either a rapidly expanding population size or a bottleneck caused by a selective sweep.

Nachman et al. (85) analyzed mtDNA sequence variation in humans, chimpanzees, and gorilla in several ways. First they sequenced the ND3 gene from humans, chimpanzees, and one gorilla. Using the MK test they could not reject neutrality within humans. They did reject neutrality within the chimpanzee data but concluded that this was likely due to inclusion of more than one subspecies in the sample. Using RFLPs from the whole genome and other published mtDNA sequences, they identified departures from neutrality within humans using chimpanzee and gorilla as comparative taxa. Tajima's D was also computed for the human RFLP-derived data, and significant departures from neutrality were found among the non-African human samples. Templeton (117) examined sequences

from COII in hominoid primates. Contingency tests were used to detect departures from neutrality in mtDNA sequences from humans, chimpanzees, pygmy chimpanzees, gorillas, and an orangutan. Unlike the MK test, these contingency tests (first proposed in 116) allowed for examination of the ratio of replacement to silent substitutions between species among a number of categories, including "older" to "younger" haplotypes as represented by interior and tip haplotypes, respectively, from phylogenetic networks. Replacement mutations in the cytosolic regions of the COII gene appeared to be deleterious, whereas replacement mutations from the transmembrane region and all silent mutations behaved according to neutral expectations.

A similar nested contingency method was employed by Wise et al. (128) to examine variation in the ND2 gene of humans and chimpanzees. Contingency tests based on parsimony networks of haplotypes from humans and chimpanzees identified a significant excess of replacement polymorphisms within the transmembrane regions in humans but not in chimpanzees. Tajima's D and the Fu & Li tests also rejected neutrality in the human data, but not in the chimpanzee data.

MICE Nachman et al. (84) examined sequence data from the ND3 gene from *Mus domesticus*, *M. musculus*, and *M. spretus*. Using the MK test, a significant excess of replacement mutations was found in each species. However, frequencies of protein variants were not significantly different from neutral expectations when tested by the methods of Watterson and Tajima's D, leading the authors to conclude, "it is possible that some of the amino acid substitutions in our sample are strictly neutral."

NONCOMMENSAL SPECIES Initial results from studies discussed above have been used to argue for rejection for neutrality (e.g., 12, 85, 97, 128). The tendency toward an excess of replacement polymorphism within species ($R_P > R_F$) has been generally interpreted as evidence for the persistence of mildly deleterious mutations. Because selection against these variants is weak, replacement polymorphisms are observed within species but do not persist long enough to become fixed differences between species.

Concerns were raised by the possibility that relaxation of selection in *Homo sapiens* (112) could be responsible for the apparent excess of replacement polymorphisms in humans and their commensals, specifically *Drosophila* and mice. To determine whether the findings for humans and their commensals represent a general phenomenon, sequences of numerous noncommensal species have been reviewed and analyzed using the MK test (83, 100, 101, 123). In these analyses, the MK test was generally performed on species whose DNA was collected for phylogeographic analyses and included mammals, birds, reptiles, amphibians, fishes, and invertebrates (summarized in Table 1).

Additional comparisons in noncommensal species have subsequently been reported (also included in Table 1). Some recent reports include the data for MK tests (e.g., 14, 118), whereas others provide only discussion of the outcome

TABLE 1 Summary of MK test results

Species for polymorphism[a]	N	Species for divergence	N	Gene	bp	Polymorphic		Fixed		P^d	PSI	Reference
						R^b	S^c	R^b	S^c			
Invertebrates												
Alpheus lottini	21	*A. formusus*	7	COI	564	2	131	0	27	0.55	NC[e]	99
Habronattus pugillis	81	*H. geronimoi*	1	ND1	440	7	71	3	15	0.39	0.5	73
Haemonchus contortus	37	*H. placei*	31	ND4	459	27	94	16	15	0.00	0.3	14
Heliconius erato	52	*H.telesiphe*	NR[f]	COI, COIII	819	16	70	2	24	0.24	2.7	83
Heterorhabditis marelatus	4	*H. bacteriophera*	1	ND4	474	1	14	13	45	0.27	0.3	14
Lutzomyia longipalpis (within clades)	34	*L. longipalpis* (among clades)	34	ND4	618	7	35	2	56	0.03	5.6	118
Mytilus edulis F lineage	9	*M. edulis* M lineage	10	COIII	396	21	53	12	47	0.23	1.6	95
All European *Mytilus* (F lineage)	26	American *M. trossulus* (F lineage)	4	COIII	396	21	48	0	27	0.00	NC[e]	95
Mytilus galloprovincialis (Atl.) (F lineage)	6	*M. galloprovincialis* (Atl.) (M lineage)	5	COIII	396	23	42	11	46	0.04	2.3	95
Mytilus galloprovincialis (Med.) (F lineage)	9	*M. galloprovincialis* (Med.) (M lineage)	10	COIII	396	45	79	8	31	0.05	2.2	95
Mytilus M lineage (European)	24	*M. trossulus* M lineage (American)	3	COIII	396	29	77	4	18	0.30	1.7	95
Teladorsagia circumcincta	39	*T. boreoarcticus*	8	ND4	390	14	65	11	18	0.04	0.4	14
Fishes												
Gadus morhua	236	*Melanogrammus aeglefinus*	NR[f]	cyt*b*	275	0	16	5	25	0.15	0.0	83
Gadus morhua	41	*G. ogac*	1	cyt*b*	300	3	22	0	10	0.25	NC[e]	100
Gila cypha	18	*G. elegans*	16	ND2	758	1	5	16	47	0.54	0.6	[g]
Gila elegans	16	*G. cypha*	18	ND2	758	1	5	16	47	0.54	0.6	[g]
Gila robusta	68	*G. cypha*	18	ND2	370	5	4	4	22	0.03	6.9	[g]

(*Continued*)

TABLE 1 (*Continued*)

Species for polymorphism[a]	N	Species for divergence	N	Gene	bp	Polymorphic Fixed				P^d	PSI	Reference
						R^b	S^c	R^b	S^c			
Gila robusta	68	*G. elegans*	16	ND2	370	4	4	6	16	0.23	2.7	g
Luxilus chrysocephalus	27	*L. cornutus*	18	ND2	1047	36	128	8	41	0.26	1.4	h
Luxilus cornutus	18	*L. chrysocephalus*	27	ND2	1047	16	50	10	37	0.45	1.2	h
Ptychocheilus oregonensis	28	*P. grandis*	20	cyt*b*	639	1	9	1	42	0.35	4.7	I
Ptychocheilus oregonensis	28	*P. umpquae*	16	cyt*b*	639	1	9	0	10	0.50	NC[e]	I
Reptiles and Amphibians												
Ambystoma jeffersonianum	6	*A. laterale*	NR[f]	cyt*b*	307	4	3	0	27	0.00	NC[e]	83
Ambystoma laterale	11	*A. jeffersonianum*	5	cyt*b*	238	3	4	0	22	0.00	NC[e]	100
Bufo marinus (E + W Andes)	27	*B. marinus* (between E and W Andes)	27	ND3	336	1	19	2	18	>0.1	0.5	108
Dendrobates pumilio	12	*D. speciosus*	NR[f]	cyt*b*	292	0	6	4	6	0.23	0.0	83
Emoia impar Group II	8	*E. impar* Group I	NR[f]	cyt*b*	779	3	4	12	81	0.07	5.1	83
Ensatina eschscholtzii	24	*Plethodon elongatus*	NR[f]	cyt*b*	684	38	171	21	27	0.00	0.3	83
Phyllobates lugubris	8	*Dendrobates pumilio*	NR[f]	cyt*b*	292	11	59	0	19	0.06	NC[e]	83
Tarentola delalandii	30	*T. boettgeri*	1	cyt*b*	369	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	NR[f]	42
Birds												
Aerodramus maximus	5	NR[e]	NR[f]	cyt*b*	NR[f]	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	0.0	37
Brachyramphus marmoratus	14	*B. brevirostris*	5	cyt*b*	1041	3	24	0	50	0.02	NC[e]	100
Brachyramphus marmoratus	43	NR[e]	NR[f]	cyt*b*	NR[f]	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	1.4	37
Collocalia esculenta	4	NR[e]	NR[f]	cyt*b*	NR[f]	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	0.5	37
Fringella coelebs ssp.	15	*F. teydea*	1	cyt*b*, atp6, nd5	1283	13	63	6	39	0.60	1.3	71
Grus antigone	9	*G. rubicunda*	NR[f]	cyt*b*	1143	7	10	1	30	0.00	21.0	83
Grus antigone	9	*G. canadense*	4	cyt*b*	1140	10	25	2	49	0.00	9.8	100
Melospiza melodia	11	*Passerella iliaca*	NR[f]	cyt*b*	431	5	2	10	26	0.04	6.5	83

Melospiza melodia	6	NR[e]	NR[f]	cyt*b*	NR[f]	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	6.5	37
Passerella iliaca	19	*Melospiza melodia*	NR[f]	cyt*b*	431	5	10	10	26	0.74	1.3	83
Passerella iliaca	11	NR[e]	NR[f]	cyt*b*	NR[f]	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	1.3	37
Pomatostomus temporalis	35	*P. isidori*	NR[f]	cyt*b*	282	0	17	8	18	0.01	0.0	83
Mammals												
Holochilus brasiliensis	82	*H. vulpinus*	21	ND3	348	8	16	7	49	<0.05	3.5	50
Isothrix bistriata	10	*I. pagurus*	NR[f]	cyt*b*	798	16	108	6	38	0.99	0.9	83
Isothrix bistriata	10	*I. pagurus*	1	cyt*b*	798	15	103	4	33	0.76	1.2	100
Mesomys hispidus	29	*M. stimulax*	NR[f]	cyt*b*	798	36	123	0	20	0.01	NC[e]	83
Mesomys hispidus	29	*M. stimulax*	2	cyt*b*	798	30	126	0	14	0.08	NC[e]	100
Mesomys hispidus clade 1	10	*M. hispidus* clade 2	19	cyt*b*	798	30	112	0	9	0.13	NC[e]	100
Microtis longicaudus	72	*M. pennsylvanicus* + *M. montanus*	NR[f]	cyt*b*	1143	NR[f]	NR[f]	NR[f]	NR[f]	>0.05	NR[f]	24
Pan troglodytes	19	*Homo sapiens*	21	ND2	1041	7	32	10	82	0.40	1.8	128
Sciurus aberti	20	*S. niger*	1	cyt*b*	1140	12	38	18	146	0.03	2.6	100
Ursus arctos	166	*Helarctos malayanus*	NR[f]	cyt*b*	1140	11	44	15	68	0.83	1.1	83
Ursus arctos	28	*U. americana*	1	cyt*b*	1137	11	44	15	81	0.50	1.4	100

[a]In some instances, within-species values were calculated from both groups pooled.

[b]Replacement sites.

[c]Synonymous sites.

[d]Level of significance determined by G-test, Fisher's Exact Test, or Monte Carlo.

[e]Undefined because of 0 in the denominator.

[f]Not reported.

[g]AS Gerber, CA Tibbets, TE Dowling, in press.

[h]TE Dowling, unpublished data.

[i]DG Buth, CA Tibbets, TE Dowling, manuscript in preparation.

(e.g., 24, 37, 70). An additional 28 MK test comparisons (26 significant) were reported by Peek et al. (92) on COI sequences from deep-sea clams. These results are not included in Table 1 as they represent all pairwise comparisons among small samples of eight species (from three different genera). Some comparisons in Table 1 contain partial replicates across studies; however, they were never reported as identical [e.g., *Isothrix bistriata* and *Mesomys hispidus* (83, 100)]. Of the 53 comparisons in Table 1, the MK test was significant in only 18 instances and marginally significant ($0.05 < P < 0.10$) in 3 others. In most cases with defined values, the pattern shift index was greater than 1, but was not significant (24 of 38 nonredundant comparisons, one-tailed binomial test, $P = 0.072$). Potential bias in these samples (e.g., nonindependence of comparisons) makes this result difficult to interpret. We tentatively conclude that there is a trend toward an excess of replacement mutations within species relative to replacement substitutions between species, with further analyses required to rigorously test this hypothesis.

In addition to the MK test, Tajima's and Fu & Li's approaches have been applied in recent studies (42, 92, 118). These tests generally failed to detect deviations from neutrality, with rare rejections reported in the study of deep-sea clams [one of eight tests (92] and geckos [combined clades but not independent population samples (42)].

CONCLUSIONS

Opinion on the subject of neutrality of mtDNA appears to have shifted over the past decade, with the general assumption in the literature that mtDNA has been proven to evolve nonneutrally. Studies that present evidence for nonneutral behavior in mtDNA generate attention because so many evolutionary biologists have used mtDNA loci for population genetic and phylogeographic studies under the assumption (explicit or implicit) that this marker is neutral and evolves in a clock-like fashion. The conclusion of nonneutral evolution, largely based upon results from ratio comparisons, has clearly been convincing to many.

There can be no doubt that some forms of selection act on mitochondrial DNA. Evidence from performance experiments suggests that certain haplotypes have a selective advantage; however, it is difficult to eliminate a role for historical and ecological factors as well as cyto-nuclear interactions. In fact, most evidence is consistent with coevolution of mitochondrial and nuclear gene products. Experimental tests of differential fitness among haplotypes (mostly in insects) have suggested a role for selection; however, it is difficult to exclude the influence of mating preference, cytoplasmic effects imposed by *Wolbachia*, and, most importantly, cyto-nuclear interactions.

Statistical analyses of sequences have been the most influential and have been most often cited in support of nonneutral evolution of mtDNA. Early studies focused on humans and their commensals; however, relaxation of selection pressure was proposed as an explanation for apparent departures from neutrality. This

explanation was further scrutinized through analyses of DNA sequences from a variety of noncommensal organisms reflecting a diversity of animal taxa. Sequences used in these analyses were typically drawn from GenBank, often having been collected for analysis of population structure and phylogenetic relationships. Test results have been mixed, sometimes yielding patterns consistent with nonneutral evolution. One regular outcome of these comparisons has been an excess of replacement mutations within species relative to the number of replacement substitutions between species, leading to $R_P > R_F$ in contrast to patterns seen in nuclear genes (123).

Although published sequences have been an important resource for evolutionary biologists, tests of neutrality rely upon assumptions that may not be met by these data. If this happens, rejection of the null hypothesis may not mean that selection is responsible but may reflect violation of one or more of these assumptions (reviewed in 122). For example, significantly negative values for Tajima's D have been interpreted as evidence for selection; however, the same pattern can result from recent changes in population size or structure (61, 111). Other biotic factors can produce patterns that mimic selection, as exemplified by the impact of *Wolbachia* (11). Many of these tests assume that intraspecific samples are drawn from a panmictic population; however, this assumption is rarely met as samples were often drawn from several populations sampled for phylogeographic analyses. In addition, sample sizes may be too small to effectively test the null hypotheses. Simonsen et al. (106) suggested that the power of Tajima's D to detect nonneutrality is weakened in sample sizes less than 50 individuals (but see 37).

The MK test has become widely used because of its simple, elegant design, and the conclusion of nonneutrality of mtDNA is largely based on analyses using this test. However, a number of potential problems with the test have been identified. Maynard Smith (74) noted that differences in the mutation rate of transitions and transversions and codon biases can produce patterns that mimic selection (in his case positive selection) and suggested that ". . . the potential dangers of using his method [MK test] uncritically should be recognized." Graur & Li (41) argued that rules for designating sites as fixed or polymorphic may underestimate the amount of between-species variation. This has led to the development of several methods for counting sites, the choice of which may influence the result (66, 95, 100). The number of individuals used may also affect the results of the MK test. In addition to typical difficulties associated with small samples sizes (see replicate *Brachyramphus* and *Melospiza* comparisons in Table 1), use of too few individuals in one or both species may result in an overestimate of fixed sites (41).

Another potential difficulty is created by divergence among species compared (100, 122, 130) because multiple substitutions at the same site are not accounted for in the MK test (41, 124). To avoid the effects of saturation, the MK test is expected to be most useful when used between closely related taxa (75, 82). This problem is expected to be more severe for animal mtDNA as it often evolves much faster than nuclear genes, particularly for vertebrates. To illustrate the potential influence of divergence on the MK test, ND2 sequences from fishes of the North

TABLE 2 Summary statistics for MK tests of *Luxilus*

Species for polymorphism	N	Species for divergence	N	bp	Divergence[a]	Polymorphic Fixed					
						R[b]	S[c]	R[b]	S[c]	*P*[d]	PSI
L. chrysocephalus	27	*L.* sp.	2	1047	0.064	36	128	5	31	0.391	1.74
L. chrysocephalus	27	*L. cornutus*	1	1047	0.110	36	128	9	47	0.337	1.47
L. chrysocephalus	27	*L. cerasinus*	1	1047	0.154	36	128	13	78	0.270	1.69
L. chrysocephalus	27	*L. cardinalis*	1	1047	0.164	36	128	9	91	0.005	2.84

[a]Jukes-Cantor estimate of sequence divergence.

[b]Replacement sites.

[c]Silent sites.

[d]Level of significance determined by Fisher's Exact Test.

American minnow genus *Luxilus* (T.E. Dowling, unpublished data) were aligned and tested (Table 2). As in many of the previous examples, samples were collected for phylogeographic and phylogenetic analyses and do not represent samples from panmictic populations. This specific analysis includes geographic samples of *L. chrysocephalus*, a closely related undescribed species from the Central Highlands region in Arkansas (*L.* sp.), *L. cornutus*, *L. cerasinus*, and *L. cardinalis*. In these comparisons, the taxon being examined for polymorphism (*L. chrysocephalus*) was held constant, while several different outgroup species were applied sequentially to examine the impact of levels of divergence on outcome of the analysis. When less divergent taxa (e.g., *L.* sp., *L. cornutus*, and *L. cerasinus*) were used to identify fixed differences, the MK test failed to reject the null hypothesis (Table 2); however, use of the most divergent taxon as the between-species reference identified a significant excess of replacement substitutions within *L. chrysocephalus*. Since within-species polymorphism has been held constant among these comparisons, this result must reflect a shift in the ratio of replacement and synonymous substitutions among species, and therefore a change in the perceived null hypothesis.

In most instances, the number of fixed replacement substitutions tends to be smaller than the number of polymorphic replacement mutations, leading to $R_F < R_P$. This has generally been interpreted as support for the existence of mildly deleterious mutations within species; however, this conclusion requires that R_F reflect the ancestral condition. Alternatively, the observed patterns could be due to a downward bias in R_F away from the ancestral condition. This bias could result from relatively rapid accumulation of fixed synonymous sites that have arisen within populations during divergence. Because most replacement mutations are expected to be deleterious, this should lead to a relative increase in the number of fixed synonymous sites and deflate R_F erroneously. Comparison of distantly related taxa will add to the bias toward finding an excess of changes within species (122), leading to incorrect inference about shifts in patterns that do not reflect the impact of selection. Under these circumstances, R_F will not reflect the ancestral condition, making this comparison inappropriate.

This difficulty is further exacerbated by shifts in base composition and codon usage (1, 31). MtDNA is notorious for shifts in GC content (reviewed in 104). In insect mtDNA, there is a strong bias toward A + T, and the GC content at the third position varies among species (e.g., 115, 127). Vertebrates also show considerable variation in GC content (e.g., 57, 71, 104). The strength of compositional asymmetry at synonymous positions and between replication strands supports the conclusion that bias is driven by mutation pressure (35, 78), possibly due to asymmetric replication of the molecule (reviewed in 104). Under this scenario, shifts in ratios examined may not be indicative of selection but may instead identify shifts in mutational patterns or base compositional biases (31, 74).

Problems in interpreting MK test results and PSI reflect challenges in determining whether there are significant differences in ratios obtained from within-population and between-species comparisons. As noted by Nei & Kumar (87), "the ratio of two quantities is disturbed more easily by different factors than a difference." In general, caution is essential when drawing inferences from statistically significant results from any statistical test that compares ratios of synonymous and replacement substitutions from two phases of molecular evolution (see also 16, 81, 87).

The above example and discussion clearly demonstrate the difficulties that high levels of divergence and variation in base composition pose for the MK test. As in the *Luxilus* example, the MK test may be more likely to reject neutrality when used between divergent taxa. Many of the previously reported significant tests (e.g., human-chimpanzee, *Drosophila* species) involve divergent taxa (by necessity), making these results difficult to interpret.

Where Do We Go From Here?

In the mid-1980s, it was widely assumed that mtDNA was evolving neutrally, leading many investigators to use this locus for population-level and systematic studies. It now appears that we have exchanged one set of assumptions (neutrality of mtDNA loci) for another (nonneutrality of mtDNA). Positive selection almost surely plays some role in the evolution of mtDNA; however, if positive selection were infrequent the evidence of such events would disappear rapidly. Therefore, it would appear that existing patterns of polymorphism in mtDNA reflect some combination of mutation, drift, and selection perhaps on mildly deleterious mutations.

The available data and statistical approaches make it difficult to assess the relative significance of selection on the evolution of mitochondrial DNA. To appropriately address this question, samples need to be collected in such a way as not to violate assumptions of statistical methods used. At this time, evidence for nonneutral evolution of mitochondrial DNA (i.e., process) has been inferred from the ratios of replacement and synonymous variants within and between taxa (i.e., pattern). In order to truly make the connection between pattern and process, it is essential that two major issues be addressed: (*a*) the influence of biases in the data on outcome of statistical analyses and (*b*) the elimination of alternative explanations

consistent with the anticipated pattern shifts. Until this is achieved it will not be possible to conclude that mitochondrial DNA evolves in a nonneutral manner.

Regardless of one's interpretation of neutrality and patterns of mtDNA evolution, we must understand the impact selection will have on phylogenetic and population genetic studies. Unfortunately, the influence of selection on questions such as these has not been specifically considered for mtDNA. Positive selection will clearly be disruptive to evolutionary studies, but the rapid rate of mtDNA evolution will likely reduce the time period of significant impact (if we assume that such bouts are relatively infrequent). Purifying selection (weak as well as strong) on a single site would have limited effect on the length and shape of a phylogenetic tree; however, recurrent mutation of multiple, strongly deleterious alleles will reduce the length of the tree but not affect the topology (reviewed in 94). Even though the tree is consistent with neutral expectations, estimates of S and π are influenced by weak purifying selection on single sites. Therefore, phylogenetic reconstruction is not likely to be impacted by patterns of selection generally observed for mtDNA, but estimates of divergence time and effective population size could be affected, depending upon the number of sites selected and the intensity of selection.

This still leaves us with the question: Is it appropriate to use mtDNA sequences for evolutionary studies? The answer is a qualified yes. Previous cautionary notes sounded by Ballard & Kreitman (13), Rand and coworkers (96, 100), and Nachman (83) (to name but a few) have provided a valuable service to the scientific community by forcing closer examination of the assumptions made when testing hypotheses. Clearly, further studies need to be conducted to address the impact of selection on population genetic and phylogenetic studies. Regardless, we always need to maintain a state of vigilance over assumptions behind analytical methods used to characterize evolutionary patterns and processes of molecular markers such as mitochondrial DNA.

ACKNOWLEDGMENTS

We thank Dan Garrigan, Phil Hedrick, Mark Miller, Masatoshi Nei, and Susan Masta for insightful discussion and/or critical evaluation of this manuscript. Research support has been provided by the National Science Foundation (SK, TED), National Institutes of Health (SK), US Bureau of Reclamation (TED), Burroughs-Wellcome Fund (SK), and the University of North Dakota Faculty Research Fund (ASG).

LITERATURE CITED

1. Akashi H. 1995. Inferring weak selection for patterns of polymorphism and divergence at "silent" sites in *Drosophila* DNA. *Genetics* 139:1067–76

2. Akashi H. 1999. Within- and between-species DNA sequence variation and the 'footprint' of natural selection. *Gene* 238: 39–51

3. Allegrucci G, Cesaroni D, Venanzetti F, Cataudella S, Sbordoni V. 1998. Length variation in mtDNA control region in hatchery stocks of European sea bass subjected to acclimation experiments. *Genet. Sel. Evol.* 30:275–88
4. Avise JC. 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. *Philos. Trans. R. Soc. London Ser. B* 312:325–42
5. Avise JC. 1994. *Molecular Markers, Natural History, and Evolution*. New York: Chapman & Hall
6. Avise JC. 2000. *Phylogeography: The History and Formation of Species*. Cambridge: Harvard
7. Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, et al. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* 18:489–522
8. Avise JC, Bowen BW, Lamb TA, Meylan AB, Bermingham E. 1992. Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Mol. Biol. Evol.* 9:457–73
9. Avise JC, Lansman RA, Shade RO. 1979. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. I. Population structure and evolution in the genus *Peromyscus*. *Genetics* 92:279–95
10. Awadalla P, Eyre-Walker A, Maynard-Smith J. 1999. Linkage disequilibrium and recombination in hominid mitochondrial DNA. *Science* 286:2524–25
11. Ballard JWO. 2000. Comparative genomics of mitochondrial DNA in *Drosophila simulans*. *J. Mol. Evol.* 51:64–75
12. Ballard JWO, Kreitman M. 1994. Unraveling selection in the mitochondrial genome of *Drosophila*. *Genetics* 138:757–72
13. Ballard JWO, Kreitman M. 1995. Is mitochondrial DNA a strictly neutral marker? *TREE* 10:485–88
14. Blouin MS. 2000. Neutrality tests on mtDNA: unusual results from nematodes. *J. Hered.* 91:156–58
15. Boore JL. 1999. Animal mitochondrial genomes. *Nucleic Acids Res.* 27:1767–80
16. Brookfield JF, Sharp PM. 1994. Neutralism and selectionism face up to DNA data. *Trends Genet.* 10:109–11
17. Brown WM. 1983. Evolution of animal mitochondrial DNA. In *Evolution of Genes and Proteins*, ed. M Nei, RK Koehn, pp. 62–88. Sunderland: Sinauer
18. Brown WM, George M, Wilson AC. 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 76:1967–71
19. Brown WM, Vinograd J. 1974. Restriction endonuclease cleavage maps of animal mitochondrial DNAs. *Proc. Natl. Acad. Sci. USA* 11:4671–721
20. Burton RS, Rawson PD, Edmands S. 1999. Genetic architecture of physiological phenotypes: empirical evidence for coadapted gene complexes. *Am. Zool.* 39:451–62
21. Cann RL, Brown WM, Wilson AC. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. *Genetics* 106:479–99
22. Cann RL, Wilson AC. 1983. Length mutations in human mitochondrial DNA. *Genetics* 104:699–711
23. Clark AG, Lyckegaard EMS. 1988. Natural selection with nuclear and cytoplasmic transmission. III. Joint analysis of segregation and mtDNA in *Drosophila melanogaster*. *Genetics* 118:471–81
24. Conroy CJ, Cook JA. 2000. Phylogeography of a post-glacial colonizer: *Microtus longicaudus* (Rodentia: Muridae). *Mol. Ecol.* 9:165–75
25. Danzmann RG, Ferguson MM. 1995. Heterogeneity in the body size of cultured Ontario rainbow trout with different mitochondrial DNA haplotypes. *Aquaculture* 137:231–44
26. DeStordeur E. 1997. Nonrandom

partition of mitochondria in heteroplasmic *Drosophila*. *Heredity* 79:615–23
27. Dowling TE, Broughton RE, DeMarais BD. 1997. Significant role for historical effects in the evolution of reproductive isolation: evidence from patterns of introgression between the cyprinid fishes, *Luxilus cornutus* and *Luxilus chrysocephalus*. *Evolution* 51:1574–83
28. Duvernell DD, Aspinwall N. 1995. Introgression of *Luxilus cornutus* mtDNA into allopatric populations of *Luxilus chrysocephalus* (Teleostei: Cyprinidae) in Missouri and Arkansas. *Mol. Ecol.* 4:173–81
29. Ewens WJ. 1972. The sampling theory of selectively neutral alleles. *Theor. Popul. Biol.* 3:87–112
30. Excoffier L. 1990. Evolution of human mitochondrial DNA: evidence for departure from a pure neutral model of populations at equilibrium. *J. Mol. Evol.* 30:125–39
31. Eyre-Walker A. 1997. Differentiating between selection and mutation bias. *Genetics* 147:1983–87
32. Eyre-Walker A. 2000. Do mitochondria recombine in humans? *Philos. Trans. R. Soc. London Ser. B* 355:1573–80
33. Ferguson MM, Danzmann RG. 1999. Inter-strain differences in the association between mitochondrial DNA haplotype and growth in cultured Ontario rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 178:245–52
34. Fos M, Domínguez MA, Latorre A, Moya A. 1990. Mitochondrial DNA evolution in experimental populations of *Drosophila subobscura*. *Proc. Natl. Acad. Sci. USA* 87:4198–201
35. Frank AC, Lobry JR. 1999. Asymmetric substitution patterns: a review of possible underlying mutational or selective mechanisms. *Gene* 238:65–77
36. Frank SA, Hurst LD. 1996. Mitochondria and male disease. *Nature* 383:224
37. Fry AJ. 1999. Mildly deleterious mutations in avian mitochondrial DNA: evidence from neutrality tests. *Evolution* 53:1617–20
38. Fu Y-X, Li W-H. 1993. Statistical tests of neutrality of mutations. *Genetics* 133: 693–709
39. García-Martínez J, Castro JA, Ramón M, Latorre A, Moya A. 1998. Mitochondrial DNA haplotype frequencies in natural and experimental populations of *Drosophila subobscura*. *Genetics* 149:1377–82
40. Glémet H, Blier P, Bernatchez L. 1998. Geographical extent of arctic char (*Salvelinus alpinus*) mtDNA introgression in brook char populations (*S. fontinalis*) from eastern Québec, Canada. *Mol. Ecol.* 7:1655–62
41. Graur D, Li W-H. 1991. Neutral mutation hypothesis test. *Nature* 354:115–16
42. Gubitz T, Thorpe RS, Malhotra A. 2000. Phylogeography and natural selection in the Tenerife gecko *Tarentola delalandii*: testing historical and adaptive hypotheses. *Mol. Ecol.* 9:1213–21
43. Gyllensten UB, Wharton D, Joseffson A, Wilson AC. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature* 352:255–57
44. Gyllensten UB, Wharton D, Wilson AC. 1985. Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *J. Hered.* 76:321–24
45. Hoeh WR, Blakley KH, Brown WM. 1991. Heteroplasmy suggests limited biparental inheritance of *Mytilus* mitochondrial DNA. *Science* 251:1488–90
46. Hudson RR, Kreitman M, Aguade M. 1987. A test of neutral molecular evolution based on nucleotide data. *Genetics* 116:153–59
47. Hughes AL. 1999. *Adaptive Evolution of Genes and Genomes*. Oxford: Oxford Univ. Press
48. Hutter CM, Rand DM. 1995. Competition between mitochondrial haplotypes in distinct nuclear genetic environments: *Drosophila pseudoobscura* vs. *D. persimilis*. *Genetics* 140:537–48
49. Jenkins TM, Babcock CS, Geiser DM,

Anderson WW. 1996. Cytoplasmic incompatability and mating preference in Colombian *Drosophila pseudoobscura*. *Genetics* 142:189–94
50. Kennedy R, Nachman MW. 1998. Deleterious mutations at the mitochondrial ND3 gene in South American marsh rats (*Holochilus*). *Genetics* 150:359–68
51. Khambhampati S, Rai KS, Verleye DM. 1992. Fequencies of mitochondrial DNA haplotypes in laboratory cage populations of the mosquito, *Aedes albopictus*. *Genetics* 132:205–9
52. Kilpatrick ST, Rand DM. 1995. Conditional hitchhiking of mitochondrial DNA: frequency shifts of *Drosophila melanogaster* mtDNA variants depend on nuclear genetic background. *Genetics* 141:1113–24
53. Kimura M. 1968. Evolutionary rate at the molecular level. *Nature* 217:625–26
54. Kimura M. 1977. Preponderance of synonymous changes as evidence for the neutral theory of molecular evolution. *Nature* 267:275–76
55. Kimura M. 1983. *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge Univ. Press
56. King MP, Attardi G. 1989. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 246:500–3
57. Kocher TD, Conroy JA, McKaye KR, Stauffer JR, Lockwood SF. 1995. Evolution of NADH dehydrogenase subunit 2 in east African cichlid fish. *Mol. Phylogenet. Evol.* 4:420–32
58. Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, et al. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* 86:6196–200
59. Kondo R, Matsuura ET, Ishima H, Takahata N, Chigusa SI. 1990. Incomplete maternal transmission of mitochondrial DNA in *Drosophila*. *Genetics* 126:657–63
60. Kreitman M. 1996. The neutral theory is dead. Long live the neutral theory. *BioEssays* 18:678–83
61. Kreitman M. 2000. Methods to detect selection in populations with applications to the human. *Annu. Rev. Genom. Hum. Genet.* 1:539–59
62. Kumar S. 1996. Patterns of nucleotide substitution in mitochondrial protein coding genes of vertebrates. *Genetics* 143: 537–48
63. Kumar S, Hedrick P, Dowling T, Stoneking M. 2000. Questioning evidence for recombination in human mitochondrial DNA. *Science* 288:1931a
64. Kumazawa Y, Nishida M. 1995. Variations in mitochondrial tRNA gene organization of reptiles as phylogenetic markers. *Mol. Biol. Evol.* 12:759–72
65. Lansman RA, Avise JC, Heuttel MD. 1983. Critical experiment to test the possibility of "paternal leakage" of mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 80:1969–71
66. Li W-H. 1997. *Molecular Evolution*. Sunderland: Sinauer
67. Macey JR, Larson A, Ananajeva NB, Fang Z, Papenfuss TJ. 1997. Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. *Mol. Biol. Evol.* 14:91–104
68. MacRae AF, Anderson WW. 1988. Evidence for non-neutrality of mitochondrial DNA haplotypes in *Drosophila pseudoobscura*. *Genetics* 120:485–94
69. Malhotra A, Thorpe RS. 1994. Parallels between island lizards suggests selection on mitochondrial DNA and morphology. *Proc. R. Soc. London Ser. B* 257:37–42
70. Malhotra A, Thorpe RS. 2000. A phylogeny of the *Trimeresurus* group of pit vipers: new evidence from a mitochondrial gene tree. *Mol. Phylogenet. Evol.* 16:199–211
71. Marshall HD, Baker AJ. 1998. Rates and patterns of mitochondrial DNA sequence evolution in Fringilline finches (*Fringilla*

spp.) and the greenfinch (*Carduelis chloris*). *Mol. Biol. Evol.* 15:638–46
72. Martin AP, Naylor GJP, Palumbi SR. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. *Nature* 357:153–55
73. Masta S. 2000. Phylogeography of the jumping spider *Habronattus pugillis* (Araneae: Salticidae): recent vicariance of sky island populations? *Evolution* 54:1699–711
74. Maynard Smith J. 1994. Estimating selection by comparing synonymous and substitutional changes. *J. Mol. Evol.* 39:123–28
75. McDonald JH, Kreitman M. 1991. Adaptive protein evolution at the *Adh* locus in *Drosophila*. *Nature* 351:652–54
76. Miyata T, Yasunaga T, Nishida T. 1980. Nucleotide sequence divergence and functional constraint in mRNA evolution. *Proc. Natl. Acad. Sci. USA* 7:7328–32
77. Moilanen JS, Majamaa K. 2001. Relative fitness of carriers of the mitochondrial DNA mutation 3243 A > G. *Eur. J. Hum. Genet.* 9:59–62
78. Mooers AO, Holmes EC. 2000. The evolution of base composition and phylogenetic inference. *TREE* 15:365–69
79. Moraes CT, Kenyon L, Hao H. 1999. Mechanisms of human mitochondrial DNA maintenance: the determining role of primary sequence and length over function. *Mol. Biol. Cell* 10:3345–56
80. Moritz C, Dowling TE, Brown WM. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annu. Rev. Ecol. Syst.* 18:269–92
81. Moriyama EN, Powell R. 1996. Intraspecific nuclear DNA variation in *Drosophila*. *Mol. Biol. Evol.* 13:261–77
82. Moriyama EN, Powell JR. 1997. Synonymous substitution rates in *Drosophila*: mitochondrial versus nuclear genes. *J. Mol. Evol.* 45:378–91
83. Nachman MW. 1998. Deleterious mutations in animal mitochondrial DNA. *Genetica* 102/103:61–69
84. Nachman MW, Boyer SN, Aquadro CF. 1994. Nonneutral evolution at the mitochondrial NADH dehydrogenase subunit 3 gene in mice. *Proc. Natl. Acad. Sci. USA* 91:6364–68
85. Nachman MW, Brown WM, Stoneking M, Aquadro CF. 1996. Nonneutral mitochondrial DNA variation in humans and chimpanzees. *Genetics* 42: 953–63
86. Nei M. 1987. *Molecular Evolutionary Genetics*. New York: Columbia Univ. Press
87. Nei M, Kumar S. 2000. *Molecular Evolution and Phylogenetics*. Oxford: Oxford Univ. Press
88. Nigro L. 1994. Nuclear background affects frequency dynamics of mitochondrial DNA variants in *Drosophila simulans*. *Heredity* 72:582–86
89. Nigro L, Prout T. 1990. Is there selection on RFLP differences in mitochondrial DNA? *Genetics* 125:551–55
90. Ohta T. 1996. The current significance and standing of neutral and nearly neutral theories. *BioEssays* 18:673–77
91. Palumbi SR. 1996. Nucleic acids II: The polymerase chain reaction. In *Molecular Systematics*, ed. DM Hillis, C Moritz, BK Mable, pp. 205–47. Sunderland: Sinauer. 2nd. ed.
92. Peek S, Gaut BS, Feldman RA, Barry JP, Kichevar RE, et al. 2000. Neutral and nonneutral mitochondrial genetic variation in deep-sea clams from the family Vesicomyidae. *J. Mol. Evol.* 50:141–53
93. Powell JR, Caccone A, Amato GD, Yoon C. 1986. Rates of nucleotide substitution in *Drosophila* mitochondrial DNA and nuclear DNA are similar. *Proc. Natl. Acad. Sci. USA* 83:9090–93
94. Przeworski M, Charlesworth B, Wall JD. 1999. Genealogies and weak purifying selection. *Mol. Biol. Evol.* 16:246–52
95. Quesada H, Warren M, Skibinski DOF. 1998. Nonneutral evolution and differential mutation rate of gender-associated

mitochondrial DNA lineages in the marine mussel *Mytilus*. *Genetics* 149:1511–26
96. Rand DM. 1996. Neutrality tests of molecular markers and the connection between DNA polymorphism, demography and conservation biology. *Conserv. Biol.* 10:665–71
97. Rand DM, Dorfsman M, Kann LM. 1994. Neutral and non-neutral evolution of *Drosophila* mitochondrial DNA. *Genetics* 138:741–56
98. Rand DM, Harrison RG. 1986. Mitochondrial DNA transmission genetics in crickets. *Genetics* 114:955–70
99. Rand DM, Kann LM. 1996. Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes from *Drosophila*, mice and humans. *Mol. Biol. Evol.* 13:735–48
100. Rand DM, Kann LM. 1998. Mutation and selection at silent and replacement sites in the evolution of animal mitochondrial DNA. *Genetica* 102/103:393–407
101. Rand DM, Weinrich DM, Cezairliyan BO. 2000. Neutrality tests of conservative-radical amino acid changes in nuclear- and mitochondrially-encoded proteins. *Gene* 291:115–25
102. Rogers AR, Harpending H. 1992. Population growth makes waves in the distribution of pairwise differences. *Mol. Biol. Evol.* 9:552–69
103. Ruiz-Pesini E, Lapena A-C, Díez-Sánchez C, Pérez-Martos A, Montoya J, et al. 2000. Human mtDNA haplogroups associated with high or reduced spermatazoa motility. *Am. J. Hum. Genet.* 67:682–96
104. Saccone C, DeGiorgi C, Gissi C, Pesole G, Reyes A. 1999. Evolutionary genomics in metazoa: the mitochondrial DNA as a model system. *Gene* 238:195–209
105. Schizas NV, Chandler GT, Coull BC, Klosterhaus SL, Quattro M. 2001. Differential survival of three mitochondrial lineages of a marine benthic copepod exposed to a pesticide mixture. *Environ. Sci. Technol.* 35:535–38
106. Simonsen KL, Churchill GA, Aquadro CF. 1995. Properties of statistical tests of neutrality for DNA polymorphism data. *Genetics* 141:413–29
107. Singh RS, Hale LR. 1990. Are mitochondrial DNA variants selectively neutral? *Genetics* 124:995–97
108. Slade RW, Moritz C. 1998. Phylogeography of *Bufo marinus* from its natural and introduced ranges. *Proc. R. Soc. London Ser. B* 265:769–77
109. Staton JL, Daehler LL, Brown WM. 1997. Mitochondrial gene arrangement of the horshoe crab *Limulus polyphemus* L.: conservation of major features among arthropod classes. *Mol. Biol. Evol.* 14:867–74
110. Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585–95
111. Tajima F. 1989. The effect of change in population size on DNA polymorphism. *Genetics* 123:597–601
112. Takahata N. 1993. Relaxed selection in human populations during the Pleistocene. *Jpn. J. Genet.* 68:539–47
113. Takeda K, Takahashi S, Onishi A, Hanada H, Imai H. 2000. Replicative advantage and tissue-specific segregation of RR mitochondrial DNA between C57BL/6 and RR heteroplasmic mice. *Genetics* 155:777–83
114. Takezaki N, Gojobori T. 1999. Correct and incorrect vertebrate phylogenies obtained by the entire mitochondrial DNA sequences. *Mol. Biol. Evol.* 16:590–601
115. Tamura K. 1992. The rate and pattern of nucleotide substitution in *Drosophila* mitochondrial-DNA. *Mol. Biol. Evol.* 9:814–25
116. Templeton AR. 1987. Genetic systems and evolutionary rates. In *Rates of Evolution*, ed. KSW Campbell, MF Day, pp. 218–34. Allen & Unwin: London
117. Templeton AR. 1996. Contingency tests of neutrality using intra/interspecific gene trees: the rejection of neutrality for

the evolution of the mitochondrial cytochrome oxidase II gene in the hominoid primates. *Genetics* 144:1263–70
118. Uribe Soto SI, Lehmann T, Rowton ED, Velez BID, Porter CH. 2001. Speciation and population structure in the morphospecies *Lutzomyia longipalpis* (Lutz & Neiva) as derived from the mitochondrial ND4 gene. *Mol. Phylogenet. Evol.* 18:84–93
119. Vawter L, Brown WM. 1986. Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science* 234:194–96
120. Wallace DC. 1999. Mitochondrial diseases in man and mouse. *Science* 283: 1482–88
121. Watterson GA. 1977. Heterosis or neutrality? *Genetics* 85:789–814
122. Wayne ML, Simonsen KL. 1998. Statistical tests of neutrality in the age of weak selection. *TREE* 13:236–40
123. Weinreich DM, Rand DM. 2000. Contrasting patterns of nonneutral evolution in proteins encoded in nuclear and mitochondrial genomes. *Genetics* 156:385–99
124. Whittam T, Nei M. 1991. Neutral mutation hypothesis test. *Nature* 354:115–16
125. Whittam TS, Clark AG, Stoneking M, Cann RL, Wilson AC. 1986. Allelic variation in human mitochondrial genes based on patterns of restriction site polymorphism. *Proc. Natl. Acad. Sci. USA* 83:9611–15
126. Wilson AC, Cann L, Carr SM, George M, Gyllensten UB, et al. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol. J. Linn. Soc.* 26:375–400
127. Wirth T, LeGuellec R, Veuille M. 1999. Directional substitution and evolution of nucleotide content in the cytochrome oxidase II gene in earwigs (dermapteran insects). *Mol. Biol. Evol.* 16:1645–53
128. Wise CA, Sraml M, Easteal S. 1998. Departure from neutrality at the mitochondrial NADH dehydrogenase subunit 2 gene in humans, but not in chimpanzees. *Genetics* 148:409–21
129. Wolstenholme DR, Clary DO. 1985. Sequence evolution of *Drosophila* mitochondrial DNA. *Genetics* 109:725–44
130. Yang Z, Bielawski JP. 2000. Statistical methods for detecting molecular adaptation. *TREE* 15:496–503
131. Zhang J, Kumar S, Nei M. 1997. Small-sample tests of episodic adaptive evolution: a case study of primate lysozymes. *Mol. Biol. Evol.* 14:1335–38
132. Zouros E, Pogson GH, Cook DI, Dadswell MJ. 1992. Apparent selective neutrality of mitochondrial DNA size variation: a test in the deep-sea scallop *Placopten magellanicus*. *Evolution* 46:1466–76