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Nonneutral evolution at the mitochondrial NADH dehydrogenase subunit 3 gene in mice

(mtDNA/neutral theory/slightly deleterious/selection/*Mus domesticus*)

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ABSTRACT **The neutral theory of molecular evolution asserts that while many mutations are deleterious and rapidly eliminated from populations, those that we observe as polymorphisms within populations are functionally equivalent to each other and thus neutral with respect to fitness. Mitochondrial DNA (mtDNA) is widely used as a genetic marker in evolutionary studies and is generally assumed to evolve according to a strictly neutral model of molecular evolution. One prediction of the neutral theory is that the ratio of replacement (nonsynonymous) to silent (synonymous) nucleotide substitutions will be the same within and between species. We tested this prediction by measuring DNA sequence variation at the mitochondrially encoded NADH dehydrogenase subunit 3 (ND3) gene among 56 individual house mice, *Mus domesticus*. We also compared ND3 sequence from *M. domesticus* to ND3 sequence from *Mus musculus* and *Mus spretus*. A significantly greater number of replacement polymorphisms were observed within *M. domesticus* than expected based on comparisons to either *M. musculus* or *M. spretus*. This result challenges the conventional view that mtDNA evolves according to a strictly neutral model. However, this result is consistent with a nearly neutral model of molecular evolution and suggests that most amino acid polymorphisms at this gene may be slightly deleterious.**

To understand the genetic basis of evolutionary change, we must understand the extent to which selection governs the amount and distribution of genetic variation in natural populations. Considerable debate has centered on whether most naturally occurring genetic variants are strictly neutral (1), slightly deleterious (2), or advantageous (3). While these different views imply relatively small differences in selection coefficients, these differences can still have profound consequences for how molecular evolution occurs.

The neutral theory, in its strictest form, asserts that while many mutations are strongly deleterious and therefore rapidly eliminated from populations, those that we observe within populations are equivalent with respect to fitness (1). The level of genetic variation within populations is determined by the neutral mutation rate and the effective population size. The amount of divergence between species is determined by the neutral mutation rate and the time since divergence. Under strict neutrality, the amount of variation within species is expected to be correlated with the rate of divergence between species for different genes or gene regions.

A modification of the strictly neutral model, known as the nearly neutral or slightly deleterious model (2, 4), proposes that observed variants have a distribution of selective effects focused around neutrality. The extent to which these nearly neutral variants are affected by selection is a function of population size. Variants will behave as neutral if their effect on fitness is less than $1/2N$ in a diploid population of N individuals. Thus, in a large population, a larger fraction of nearly neutral mutations will be affected by selection. According to this model, the level of polymorphism within species and the rate of divergence between species depend on the population size.

In addition to these two views, there are a variety of models that describe how balancing selection can maintain variability within populations (e.g., ref. 5) and how directional selection can fix substitutions within populations (e.g., ref. 6).

One of the appealing features of the strictly neutral model is its simplicity and mathematical tractability. Because it provides a number of straightforward predictions, it has served as a useful null hypothesis for understanding the forces shaping genetic variation within and between species. One prediction of the strictly neutral model, formulated into a test by McDonald and Kreitman (7), is that the ratio of silent (synonymous) to replacement (nonsynonymous) nucleotide substitutions will be the same within and between species (7, 8).

Mitochondrial DNA (mtDNA) is one of the most widely used genetic markers in evolutionary studies and is traditionally assumed to evolve according to a strictly neutral model (9–11). Because mtDNA is transmitted essentially as a haploid locus, balancing selection through overdominance is impossible, although other types of balancing selection are theoretically possible (12). The mitochondrial genome is extremely small and may therefore present relatively few targets for directional selection. Despite the intuitive appeal of a strictly neutral model for the mitochondrial genome, there have been few attempts to empirically test the neutrality of mtDNA. Claims for the nonneutrality of mtDNA variants in *Drosophila pseudoobscura* (13) have met with controversy (14, 15).

Here we present a test of the strictly neutral model for mtDNA by comparing the ratios of silent to replacement nucleotide substitutions at the gene encoding NADH dehydrogenase subunit 3 (ND3) within and between species of mice. The data are incompatible with a strictly neutral model but are consistent with a slightly deleterious model of molecular evolution for this gene.*

MATERIALS AND METHODS

Fifty-six individual *Mus domesticus* were wild caught from within their native range in Western Europe. The regions sampled are indicated in Table 1; precise localities have been published elsewhere (16). Two *Mus musculus* were wild caught in Prague, Czechoslovakia, and a single *Mus spretus* was obtained from The Jackson Laboratory. Wild-caught animals were preserved as museum specimens and have been

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Abbreviation: ND3, NADH dehydrogenase subunit 3.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U09637–U09639).

deposited in the collections of the Museum of Zoology at The University of Michigan. Livers and spleens from all animals were removed, stored in liquid nitrogen, and used for preparation of DNA according to standard techniques (17).

A 534-bp fragment encompassing the ND3 gene was PCR amplified (18) in all individuals. Amplification primers were based on the "universal" primers of Kocher *et al.* (19) and slightly modified to correspond to the mouse mitochondrial sequence (20). The letters in the primer designations that follow correspond to the light (L) or heavy (H) strand of mtDNA, and the numbers correspond to the position of the 3′ base of each primer in the sequence of Bibb *et al.* (20): L9385 (5′-ACGTCTCCATTTATTGATGAGG-3′) and H9876 (5′-GAGGTTGAAGAAGGTAGATGGC-3′). Amplification conditions have been described (16). Direct sequencing of double-stranded PCR products was done using the dideoxy chain-termination method (21) with Sequenase 2 enzyme and kit (United States Biochemical) according to the protocol supplied by the manufacturer with slight modifications as described elsewhere (16). Both strands were sequenced. The sequences from *M. domesticus* represent a subset of a larger survey of mtDNA variation in this species, published elsewhere (16).

DNA sequences were aligned by hand and the numbers and positions of nucleotide substitutions were counted. The McDonald–Kreitman test is based on the number of segregating sites within species and the number of fixed differences between species (7). We compared the number of segregating sites within *M. domesticus* to the number of fixed differences between *M. domesticus* and *M. musculus*. We also compared the number of segregating sites within *M. domesticus* to the number of sites that were fixed within *M. domesticus* and different between *M. domesticus* and the single sequence from *M. spretus*. McDonald and Kreitman (7) presented a single two-by-two contingency table in which polymorphism data from several species were pooled and in which fixed differences among several species were also pooled. We chose not to pool our data in this manner because such combined samples have the potential to obscure differences among lineages. Replacement and silent sites were counted as described (22). Corrections for multiple hits were calculated using the Kimura two-parameter model (23); this correction takes into account the transition/transversion bias known to occur in mtDNA. Both corrected and uncorrected values are presented. Corrected values probably reflect more accurately than uncorrected values the actual number of substitutions that have occurred between species; however, only uncorrected values were used in the statistical tests. This ensures that all observations are independent and results in highly conservative tests since observed synonymous divergence values are underestimates. Fisher's exact tests were used (24) to test the null hypothesis that the proportion of replacement differences is the same within and between species. Relationships among sequences were deduced using the parsimony algorithm, PAUP (25), as well as the distance-based algorithms, UPGMA, Fitch–Margoliash, and Neighbor-Joining in the PHYLIP package (26).

RESULTS

The nucleotide and amino acid polymorphisms observed within *M. domesticus* are summarized in Table 1. Within *M. domesticus*, 16 DNA haplotypes and nine protein variants were observed, and 13 silent and 11 replacement polymorphic nucleotide sites were observed. A parsimony network based on amino acid substitutions of the nine protein variants within *M. domesticus* is shown in Fig. 1. In Fig. 1, the size of the circle representing each variant is proportional to the frequency of that variant in our total sample. As can be seen in Table 1 and Fig. 1, in the total sample, there is a single variant present in high frequency (P1 is in 60.7% of the mice surveyed) and eight minor variants (P2–P9) present in frequencies of 12.5% or less. Three of the variants (P7–P9) were found in just one individual (1.8%). Most of the variants are separated from each other by a single amino acid substitution, although one branch in the network contains two

Table 1. Polymorphic nucleotide and amino acid sites at ND3 within *M. domesticus*

Haplotype	*N*	Locality	Polymorphic nucleotides	Protein variants	*N*	Polymorphic amino acids
			999999999999999999999999			99999999999
			444444555555556666667777			44555557777
			677889001123370234490239			78011230239
			189487341380985455728185			84413898185
			* * **** * ****			
			CTCTTAAATCCTATATTTATTTGA			IITILLMSIVT
1	15	I, G, S	.....	P1	34	.....
2	11	I	.....C.....			
3	2	G	T.....C.....			
4	2	I	.....G.....			
5	2	I	.....CC.....			
6	2	G	.....C.C.....			
7	3	S	.....G.....T...G.....	P2	7	..A.....
8	3	S, B	....C..G.....T.....			
9	1	I	.....G.....T.....			
10	4	B	.....T.T.....C.CA.	P3	4	.....FI.TI.
11	4	B	.....T.....C.CA.	P4	4	.....I.TI.
12	2	B	.....A.C.C.....	P5	2	....M.....
13	2	Sw	.CT.....	P6	2	T.....
14	1	I	...C..G.....	P7	1	.T.....
15	1	S	.....C.....G	P8	1	...T.....A
16	1	I	.....C...	P9	1	.....P...

Localities are as follows: I, Italy (*N* = 26); B, Great Britain (*N* = 12); G, Greece (*N* = 10); S, Spain (*N* = 6); Sw, Switzerland (*N* = 2). Asterisks (*) refer to replacement nucleotide polymorphisms. Numbers refer to positions in the published mouse sequence (20). The reference sequence under these numbers is the consensus for all *M. domesticus* sampled. Dots indicate identity to the consensus; only differences are shown. Amino acids are denoted with single-letter codes.

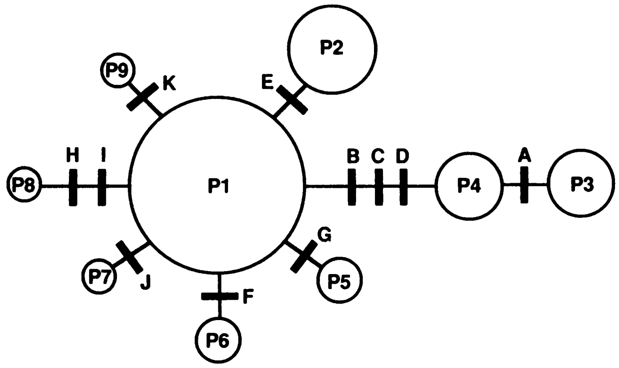

FIG. 1. Network relating the nine protein variants in *M. domesticus*. Marks and letters along branches represent replacement sites as follows: A, 9528; B, 9539; C, 9721; D, 9738; E, 9504; F, 9478; G, 9513; H, 9511; I, 9795; J, 9484; K, 9708. The size of each circle is proportional to the frequency of the variant.

substitutions and another branch contains three substitutions (Fig. 1).

There appears to be substantial geographic structure to the variation, as discussed in detail elsewhere (16). For example, the most common variant in the total sample, P1, is entirely absent in the sample from Great Britain. Furthermore, variants P3 and P4, which are three or more steps diverged from all other variants, were found only in Great Britain, although other protein variants were also found in Great Britain.

No variation was observed within *M. musculus* at ND3; the two mice sampled had identical sequences.

Protein and DNA comparisons among *M. domesticus*, *M. musculus*, and *M. spretus* are shown in Fig. 2. *M. domesticus* and *M. musculus* are thought to have diverged ≈0.35 million years ago, and *M. domesticus* and *M. spretus* are thought to have diverged ≈1.1 million years ago (27). The average uncorrected level of sequence divergence at ND3 is 3.2% between *M. domesticus* and *M. musculus* and 8.4% between *M. domesticus* and *M. spretus*. When corrected for multiple hits (23), these divergence estimates (3.7% and 11.7%, respectively) are similar to those obtained from the entire molecule based on restriction fragment length polymorphism data (4.3% and 10.0%, respectively) (27).

Table 2 summarizes the numbers of replacement and silent differences observed within and between species. Although there are many more silent than replacement differences between species, there are roughly equal numbers of silent and replacement polymorphisms observed within *M. domesticus*. We have tested the hypothesis that the ratio of silent to replacement differences is the same within and between species (7) in comparisons involving *M. domesticus* and either *M. musculus* or *M. spretus*. This hypothesis is rejected in both tests (Table 2) as we observe an excess of amino acid polymorphisms in *M. domesticus* over that predicted by the comparison to either *M. musculus* or *M. spretus* under a strictly neutral model of molecular evolution.

The following additional McDonald–Kreitman comparisons were made involving *M. domesticus* and *M. spretus* using Fisher's exact test. (*i*) Variants P3 and P4 were excluded from the sample because they are particularly divergent and the test was then calculated. (*ii*) To see if the pattern is restricted geographically, the test was done using only animals from Great Britain. (*iii*) Likewise, the test was done using only animals from mainland Europe. (*iv*) Because there was some debate following publication of the initial McDonald–Kreitman test (28, 29) about the proper statistical approach for the test, differences between species were calculated in a slightly different manner—namely, as the average number of replacement and synonymous differences between *M. spretus* and *each* of the *M. domesticus* sequences [a slight modification of the approach of Whittam and Nei (28)]—instead of counting only those differences that are fixed. (*v*) We assigned all mutations to branches on a phylogenetic tree using a parsimony criterion. We then counted all mutations

Table 2. Number of replacement and synonymous nucleotide differences within and between species

	Polymorphism in *M. domesticus*	Differences* *M. musculus*	Differences* *M. spretus*
Replacement	11	0 (0)	2 (2)
Synonymous	13	8 (9)	23 (31)

Polymorphisms were counted as sites segregating within *M. domesticus*. Differences between *M. domesticus* and *M. musculus* are fixed. Differences between *M. domesticus* and *M. spretus* are fixed within *M. domesticus* and different between *M. domesticus* and the single sequence from *M. spretus*. Observed numbers of differences are given first followed by values corrected for multiple hits (23) rounded to the nearest integer, given in parentheses. Fisher's exact tests were used (24) to test the null hypothesis that the proportion of replacement differences is the same within and between species. The null hypothesis was rejected in the comparison between *M. domesticus* and *M. musculus* ($P < 0.05$) and in the comparison between *M. domesticus* and *M. spretus* ($P < 0.005$).
*Differences to either species.

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Ms  . . . . I I . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Mm  . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . .
Md  I N L Y T V I F I N I L L S L T L I L V A F W L P Q M N L Y S E K A N P Y E C
Md ATcAACCTGTACACTGTTAtcTTCAtTAAtATTTTATTaTCCCTaaCGCTAAtTcTAGTTGCATTCTGAcTtCCCCAAATaAATCTGTACTCAGAAAAAGCAAATCCATATGAATGC
Mm ..T.......................................T..G.A..G..................................................................
Ms .............T.A.C.............................A......T.G...........G...............T.A..T..............C............
   9459                                                                                                            9572

Ms  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Mm  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Md  G F D P T S S A R L P F S M K F F L V A I T F L L F D L E I A L L L P L P W A
Md GGATTtGACCCTACAAGCTCTGCACGTCTACCaTTCTCAATAAAATTTTTCtTGGTAGCAATtACATTTCTAtTaTTTGACCTAGAAATTGCTCTTCTACTTCCACTACCATGAGCA
Mm ..........................C...................................C........G.........T..........C........................
Ms ..T.................C.....A.................................................................A.....C........G..C......
   9573                                                                                                            9689

Ms  . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . .
Mm  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Md  I Q T I K T S T M M I M A F I L V T I L S L G L A Y E W T Q K G L E W T E END
Md ATtCAAACAATTAAAACCtCTACTATAATAAtTATAGCCTTTATTCTAgTCACAATTCTATCTCTAGGCCTAGCATATGAATGAACACAAAAAGGATTAGAATGAaCAGAGTAA
Mm ..............................................................................................................A...
Ms ..C............................CC.......................C.....C..............C..............G........G..G.....A...
   9690                                                                                                         9803
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FIG. 2. Aligned ND3 protein and nucleotide sequences of *M. spretus* (Ms), *M. musculus* (Mm), and *M. domesticus* (Md). Single-letter codes are used for amino acid sequences (upper three lines in each row). Nucleotide sequences are the lower three lines in each row. The sequence of *M. domesticus* is the consensus from 56 individuals. Polymorphic sites within *M. domesticus* are in lowercase letters. Numbers correspond to positions in Bibb *et al.* (20). The underlined codon is absent in the published mouse sequence (20).

that appear on within-species branches and counted all mutations that appear on between-species branches. In each of these five additional tests, the null hypothesis was rejected ($P \leq 0.05$ for all cases).

DISCUSSION

The data presented here provide a clear rejection of the null hypothesis that the mitochondrial ND3 gene is evolving according to a strictly neutral model of molecular evolution in *M. domesticus*. This result holds if mice from Great Britain or mice from mainland Europe are considered separately. The result is also obtained if the divergent variants P3 and P4 are excluded. In addition, the pattern is observed in two different interspecific comparisons (*M. domesticus–M. musculus* and *M. domesticus–M. spretus*).

In the original McDonald–Kreitman test, rejection of the null hypothesis was attributed to an excess of replacement substitutions between species (7). Our data show a deviation in the opposite direction. What could account for the observed pattern?

One formal possibility is that some form of balancing selection is maintaining amino acid variability at ND3 in mice. It is noteworthy that, in humans, ND3 exhibits the greatest diversity of restriction fragment length polymorphism haplotypes relative to neutral expectations of all mitochondrial loci (30). Mitochondrial genes may be potential targets of balancing selection because of the possibility of strong cytoplasmic–nuclear interactions (13). However, the conditions necessary for maintaining a stable balanced polymorphism in a clonal system such as mtDNA may be complicated (12). One prediction concerning balanced polymorphisms is that variants will be maintained in the population, on average, longer than neutral variants. We have constructed a phylogenetic tree (Fig. 3) relating the 56 *M. domesticus* mtDNAs using the data presented here as well as data from the control region (16). Branch depths on this tree are approximately proportional to sequence divergence. There is no overall tendency for amino acid substitutions to be associated with deeper mtDNA lineages; four replacement polymorphisms (positions 9504, 9539, 9721, and 9738) out of 11 are found along deep branches (>0.5% sequence divergence) of the phylogeny. While this result may be consistent with some forms of balancing selection, it is inconsistent with a simple balanced polymorphism of ancient alleles at intermediate frequencies.

A more likely explanation for the observed pattern is that many of the amino acid polymorphisms at ND3 are slightly deleterious. Slightly deleterious mutants may persist within populations for brief periods, but they are unlikely to rise in frequency or become fixed (1, 2). Thus slightly deleterious mutants may contribute more to polymorphism within species than to differences between species (1, 2). A slightly deleterious model of molecular evolution has previously been invoked to explain patterns of mtDNA divergence among lineages of Hawaiian *Drosophila* (31).

M. domesticus is commensal with humans, and two aspects of this commensal association may have contributed to the maintenance of slightly deleterious mutations within populations. First, the small, isolated demes characteristic of commensal mice may result in higher levels of heterozygosity for slightly deleterious mutations as a result of population subdivision (32). Second, it is possible that the change in ecological niche accompanying the evolution of commensalism has resulted in a recent relaxation of selective constraint on the ND3 gene.

Nonetheless, two observations suggest that perhaps not all of the amino acid substitutions we observe are slightly deleterious. First, some protein variants (P2, P3, P4) and some replacement polymorphisms (9504, 9539, 9721, 9738)

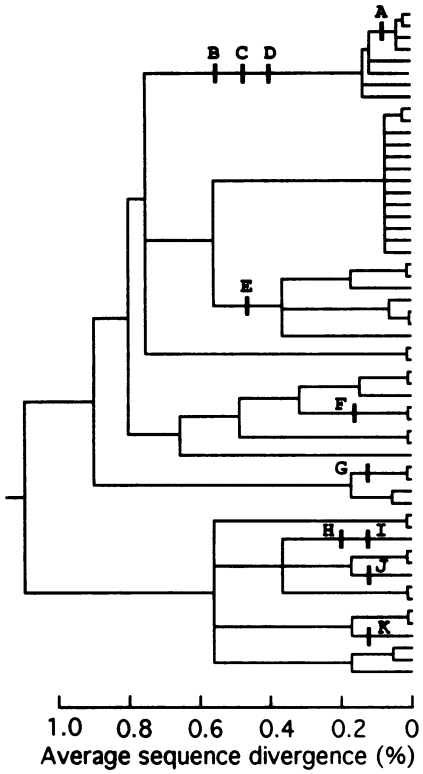

FIG. 3. Phylogenetic tree depicting relationships among the 56 *M. domesticus* mtDNAs. Vertical marks across lineages show the position of amino acid changes in the tree. Replacement sites are indicated by letters as in Fig. 1. The tree is from Nachman *et al.* (16) and is based on 1449 nucleotides sequenced from PCR-amplified mtDNA encompassing the ND3 gene and the control region in 56 *M. domesticus* and 2 *M. musculus* (used as an outgroup and not pictured above). The 120 variable sites were analyzed cladistically using PAUP (25). The tree shown is a strict consensus tree of all (520) equally parsimonious solutions found in 10 different runs using the heuristic search option of PAUP with random addition of sequences. A nearly identical tree is obtained if the replacement polymorphisms are excluded as characters in the analysis. A nearly identical topology is also obtained with three different distance-based algorithms: UPGMA, Fitch–Margoliash, and Neighbor-Joining, which were all run using the PHYLIP program (26). An entirely congruent though less-resolved topology is obtained when only the ND3 data or only the control region data are analyzed alone, using either PAUP or the distance-based algorithms in PHYLIP.

are represented at moderately high frequencies in the total sample, although these frequencies are not significantly different from neutral expectations using several tests (33–35). Second, one-third of the protein variants have accumulated more than one amino acid substitution relative to the most common variant (Fig. 1). The most divergent protein sequences, P3 and P8, differ from each other at six residues. Indeed, it is possible that some of the amino acid substitutions in our sample are strictly neutral.

The generality of our result remains to be seen. A similar pattern has recently been reported for a different mitochondrial gene (ATPase 6) in *Drosophila melanogaster* (36). The rejection of a strictly neutral model in favor of a slightly deleterious model for mtDNA is unexpected and has important implications. It suggests that by studying genetic variation within species, we are not necessarily looking at a representative sample of the differences that will ultimately distinguish species. In addition, the rate of molecular evolution under a slightly deleterious model is dependent on the effective population size (2). Thus mtDNA may not evolve in a clock-like manner among lineages with similar generation times but different effective population sizes.

mtDNA has become established as a powerful tool for making a variety of evolutionary inferences that are based either explicitly or implicitly on the assumption that it evolves according to a strictly neutral model (9–11). For example, mtDNA has been used for estimating gene flow (37), for estimating changes in population size (38), and as a molecular clock for dating events within and between species (39, 40). The data presented here indicate that the assumption of strict neutrality for mtDNA does not hold and that models of mtDNA evolution that incorporate selection may be more appropriate.

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