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MILDLY DELETERIOUS MUTATIONS IN AVIAN MITOCHONDRIAL DNA: EVIDENCE FROM NEUTRALITY TESTS

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Abstract.—To determine whether mildly deleterious mutations (MDMs) are present in nonrecombining genomes such as avian mitochondrial DNA (mtDNA), I analyzed molecular data from 14 studies using the neutrality tests of Tajima (1989a) and McDonald and Kreitman (1991). The presence of MDMs in mtDNA is inferred from trends observed across species in estimates of heterozygosity (θ and π) and by comparisons of polymorphism and divergence using the neutrality index (NI). Assuming neutrality, θ equals π and NI equals one. In this study, however, θ is greater than π more often than expected by chance, which reflects an excess of low-frequency alleles, and NI values presented here and elsewhere are consistently greater than one, which suggests an excess of nonsynonymous mutations within species (polymorphism) relative to between species (divergence). These observations suggest that, within species, there is an excess of rare haplotypes and that these haplotypes are carrying MDMs. The excess rare haplotypes may need to be accounted for when estimating population genetic parameters that assume strict neutrality.

Key words.—McDonald-Kreitman test, mildly deleterious mutation, neutrality index, Tajima's *D*.

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Mitochondrial DNA (mtDNA) is used extensively as a molecular marker for making inferences about population parameters (e.g., phylogenetic relationships, gene flow, effective population size, and divergence times) in a wide variety of taxa. These and other inferential methods assume mtDNA is a strictly neutral marker (e.g., Kimura 1968, 1983). However, deviations from strict neutrality such as the presence of mildly deleterious mutations (MDMs; Ohta and Kimura 1971; Ohta 1973; Gillespie 1995) could bias some inferential methods.

MDMs are part of a class of mutations called "borderline" mutations, defined as those mutations with a selection coefficient close to the reciprocal of effective population size (i.e., $s \simeq 1/N_e$). MDMs are distinct from other mutations in that they are the fraction of "borderline" mutations that are deleterious, in contrast to the fraction of "borderline" mutations that are beneficial. MDMs can be distinguished from strictly neutral mutations by their different predictions. For example, a negative relationship between effective population size (N_e) and the probability of an allele fixing in a population is predicted for MDMs, whereas this probability is independent of N_e for strictly neutral mutations (Kimura 1968). Furthermore, by definition MDMs, have a slightly greater probability of being eliminated from a population than strictly neutral mutations and are rarely expected to appreciably increase in frequency if population size is large. Thus, if MDMs are common in mtDNA evolution, we might expect to observe them as a class of rare haplotypes: haplotypes held to low frequency by their mildly deleterious effects, but not eliminated from the population immediately because they are not highly deleterious. Statistical tests of neutrality have the potential to reveal such a class.

Neutrality tests can be divided into two categories: those based on measures of heterozygosity or allele frequencies within a single species (e.g., Watterson 1978; Tajima 1989a), and those based on nucleotide variation between species (e.g., Hudson et al. 1987; McDonald and Kreitman 1991). I used Tajima's test and the McDonald and Kreitman (M-K) test because they are relatively powerful tests within each category (Simonsen et al. 1995; Akashi 1999).

Tajima's (1989a) method uses two different estimators of the parameter $4N_e\mu$, where μ equals the mutation rate. The first, θ, is defined as the number of segregating sites (S) divided by $\Sigma^1/_j$, where j equals one to $N - 1$ and N is the sample size (Watterson 1975). The second is π, which is defined as the average number of differences in pairwise comparisons (i.e., average genetic divergence; Tajima 1983). Assuming neutrality and equilibrium for mutation and drift, $\theta = \pi = 4N_e\mu$. (Watterson 1975; Tajima 1983), which forms the basis of Tajima's (1989a) test. This test produces a difference-statistic (D_{Taj}) with mean value zero. The value of D_{Taj} is known to be sensitive to factors other than selection such as small sample sizes (Simonsen et al. 1995); recent bottlenecks, which inflate the number of segregating sites (θ) relative to genetic divergence (π; Tajima 1989b); or population subdivision, which inflates π relative to θ (Tajima 1989a; Rand 1996).

Unlike Tajima's test, the M-K test is thought to be relatively insensitive to demographic history (McDonald and Kreitman 1991). This test has been applied to studies of animal mtDNA (e.g., Nachman 1998; Rand and Kann 1998) and consistently revealed an excess of amino-acid replacement (nonsynonymous) mutations within species (polymorphism) relative to nonsynonymous mutations between species (divergence). This excess is measured by the neutrality index (NI; Rand and Kann 1996, 1998), described as the ratio of polymorphism to divergence for nonsynonymous substitutions ($r_{pd}N$) divided by the same ratio for silent (synonymous) mutations ($r_{pd}S$). The NI has an expected value of one, with values greater than this reflecting excess nonsynonymous polymorphism relative to nonsynonymous divergence.

Because some neutrality tests may have been biased by

TABLE 1. Species, neutrality test results, and references used in this study. See text for definition of variables. The variance estimates for θ and π are available from the author. NI is neutrality index (Rand and Kann 1996) and is calculated from values used in the M-K test. None of the M-K tests were significant (i.e., $P > 0.05$ for all tests).

Species	Data	N	S	θ	π	D_{Taj}	NI	Reference
Agelaius phoeniceus	RFLP	127	28	5.16	1.67	−1.9863*	—	Ball et al. 1988
Aerodramus maximus	cyt-*b*	5	1	2.08	0.40	−0.8165	0.00	Lee et al. 1996
Ammodramus caudacutus	RFLP	29	12	3.05	3.08	0.0262	—	Rising and Avise 1993
Ammodramus nelsoni	RFLP	43	10	2.31	1.45	−1.0958	—	Rising and Avise 1993
Baeolophus griseus	cyt-*b*	28	1	0.25	0.07	−1.1541	—	Cicero 1996
Baeolophus inornatus	cyt-*b*	25	3	0.79	0.98	0.5798	—	Cicero 1996
Brachyramphus marmoratus	cyt-*b*	43	14	4.32	0.69	−2.4452*	1.41	Friesen et al. 1996
Collocalia esculenta	cyt-*b*	4	2	1.83	1.16	0.5915	0.50	Lee et al. 1996
Melospiza melodia	RFLP	170	25	4.37	3.23	−0.7223	—	Zink and Dittmann 1993a
M. melodia.	CR	95	43	8.38	3.49	−1.8432*	—	Fry and Zink 1998
M. melodia	cyt-*b*	6	7	3.06	2.60	−1.5205*	6.50	Zink and Blackwell 1996
Passerella iliaca	RFLP	272	77	12.45	8.63	−0.9157	—	Zink 1994
P. iliaca	cyt-*b*	11	17	5.92	5.45	−0.2697	1.30	Zink and Blackwell 1996
Pomatostomus temporalis	cyt-*b*	16	20	6.02	6.56	0.3599	—	Edwards and Wilson 1990
Quiscalus quiscula	RFLP	35	15	3.64	1.47	−1.9416*	—	Zink et al. 1991
Spizella passerina	RFLP	55	26	5.68	1.56	−2.3450*	—	Zink and Dittmann 1993b
Average	—	60.2	18.8	4.09	2.65	−0.9686	—	—

* Significant at $P < 0.05$ where P is the probability of observing a value that extreme under the neutral mutation hypothesis (Tajima 1989a).

demographic history, I have taken a comparative approach and look for trends in the values of θ, π, and NI across species, in an attempt to infer the role MDMs play in mtDNA evolution. Because there are differences among taxonomic groups (e.g., birds vs. insects) in genetic code and codon bias (among others), I restricted the analysis to a well-defined taxonomic group, the class Aves. Birds provide a convenient group for such analysis because mtDNA has figured prominently in studies of intraspecific genetic variation in birds and these data are available in published or electronic (e.g., GenBank) form.

The present data were collected to address the prediction that if mildly deleterious mutations are important in avian mtDNA evolution, then estimates of $4N_e\mu$ from θ should consistently exceed estimates from π. This should result in negative D_{Taj} values and, if mildly deleterious mutations are also nonsynonymous, I expect to consistently observe NI values that are greater than one.

MATERIALS AND METHODS

Sequence and RFLP Data

Tajima's (1989a) test and the M-K test were performed on seven restriction fragment length polymorphism (RFLP), eight cytochrome-*b* (cyt-*b*) and one control-region (CR) sequence dataset (Table 1). Information regarding GenBank accession numbers and the grouping of individuals into populations and subspecies is available from the author. In this study, RFLP, cyt-*b*, and CR sequences were compared because mtDNA genes are physically linked and are similarly effected by selection at any other mtDNA locus.

θ, π, and the Neutrality Index

I used Tajima's (1989a) neutrality test on data from 16 published studies representing 13 avian species (Table 1). Significance of D_{Taj} was determined using the confidence intervals of Tajima (1989a, table 2). Several statistical tests were used to compare values of θ and π as well as a regression analysis to determine if the observed relationship between θ and π differs significantly from the relationship expected under neutrality (i.e., $\theta = \pi$). To avoid including a species more than once, only one dataset per species was used. This dataset was determined by comparing the difference between θ and π for each dataset and choosing the one with the smallest difference (i.e., closer to neutrality).

The M-K test was used on cyt-*b* data from five species (Table 1). Two-way contingency tables were constructed and Fisher's exact test (Sokal and Rohlf 1995) was used to determine significance. An NI (Rand and Kann 1996), obtained by dividing $r_{pd}R$ by $r_{pd}S$, was computed for each M-K test.

RESULTS

θ and π, Estimators of $4N_e\mu$

Six (28%) of the neutrality tests (Table 1) reject the null hypothesis of neutral molecular evolution. Sixteen D_{Taj} values were calculated, and only six reject neutrality. However, all significant test results are due to a D_{Taj} that is less than zero and, overall, 12 of 16 tests produced a negative D_{Taj}. When multiple tests from a single species are excluded, nine of 13 D_{Taj} are negative and 5 are significant. The probability of observing 9 negative D_{Taj} in a sample of 13 tests is less than 14%. Negative D_{Taj} values result when $\theta > \pi$, and here $\theta > \pi$ more often than expected by chance (sign-test, $P = 0.046$). Despite the preponderance of $\theta > \pi$ observed here, the relationship between θ and π within taxa is actually quite close to relationship expected under neutrality (i.e., $\theta = \pi$). The sample means of θ and π are not different (Kruskal-Wallis = 3.31; $P = 0.068$; χ^2-approximate) because most of the variation is found within θ and π (86.7%) and not between them (13.3%). When θ is regressed on π using unweighted least squares and the intercept is forced through the origin, the slope of the regression function is 1.29 (SE = 0.1648). This slope differs from zero ($P < 0.0001$), but does not differ from one, its expectation under neutrality ($t = 1.02$, df = 11, $P > 0.20$).

TABLE 2. Comparison of D_{Taj} values for avian species. NI, neutrality index (Rand and Kann 1996).

Species	CR sequence	Cyt-*b*	RFLP	NI
Melospiza melodia	−1.8432*	−1.5205*	−0.7223	6.5
Passerella iliaca	—	−0.2697	−0.9157	1.3

* Significant at $P < 0.05$.

D_{Taj} values calculated from different data, but from the same species, are available for two species (Table 2). As expected, sequence and RFLP data from *Passerella iliaca* are concordant regardless of the test performed. However, RFLP data from *Melospiza melodia* does not reject neutrality, but CR sequence data does. Surprisingly, the *M. melodia* cyt-*b* data does not reject neutrality by the M-K test, while Tajima's test rejects neutrality using the same cyt-*b* data.

The Neutrality Index

None of the five M-K tests (Table 1) produced a statistically significant result (all $P > 0.05$). However, three NI values are greater than one, the expected value. These results are consistent with observations of NI values greater than one for a wide range of taxa (Nachman 1998; Rand and Kann 1998).

DISCUSSION

Mildly Deleterious Mutation in Avian mtDNA

Two trends in these data suggest MDMs may play an important role in avian mtDNA evolution. First, estimates of heterozygosity using θ are consistently greater than estimates using π; second, NI values are consistently greater than one.

Negative D_{Taj} (i.e., $\theta > \pi$) values suggest an excess of rare haplotypes in a population; an interpretation consistent with the presence of MDMs. If mutations arise that are mildly deleterious, by definition, they are less likely to reach fixation than strictly neutral mutations. These MDMs are expected to remain rare due to their deleterious effects and are eventually eliminated from the population by the combination of weak purifying selection and drift. Before elimination, however, these rare alleles inflate polymorphism by contributing to the number of segregating sites (θ), but do not contribute proportionally to genetic divergence (π) because of their relative rarity.

The molecular signature for such a biased contribution to polymorphism would be an excess of segregating sites (θ) relative to average divergence (π). This excess is evident in the observation that θ is greater than π more often than expected by chance (sign-test, $P = 0.046$). When θ is regressed on π, however, the observed relationship between θ and π does not differ significantly from the relationship expected under neutrality (i.e., $\theta_{\text{predicted}} = 0 + 1\pi$). This contrast suggests that MDMs are segregating in avian mtDNA genomes, but overall avian mtDNA is evolving near neutrality.

The NI values also support the presence of mildly deleterious mutations in avian mtDNA. None of the M-K tests (Table 1) reject neutrality, although all five produce NI values that are greater than one. These NI values suggest an excess of nonsynonymous mutations within species relative to the number of nonsynonymous mutations that are fixed between species. Because MDMs are held at low frequency due to weak selection against them, they should rarely occur as fixed differences between species (assuming large population size). If nonsynonymous mutations tend to also be MDMs, then we expect to observe an excess of such mutations within species, relative to between species. The signature of such an excess would be if NI values were consistently greater than one. Because so few M-K tests were performed here, statistical analysis of trends in NI values for avian mtDNA is not possible. However, the observations presented here (three of five NI values greater than one) are consistent with observations reported by Nachman (1998) and Rand and Kann (1998) for an additional 30 M-K tests, although these were mostly non-avian taxa). Seventeen of their tests reject neutrality; of these, 14 have NI values greater than one.

It is important to note potential sources of bias in estimates of θ and π. A recent selective sweep or bottleneck can produce an excess of segregating sites relative to genetic divergence. The rationale is that after such an event, the only mutations in the population are new mutations, which are by definition rare, and rare mutations contribute more to polymorphism (θ) than to genetic divergence (π). Although it cannot be ruled out, it seems unlikely that the nine taxa in Table 1 for which $\theta > \pi$ all experienced a selective sweep or bottleneck recently enough to produce an excess of rare haplotypes, but not so recently that no new genetic variation has accumulated. Small sample size could also bias test results. Sample sizes were quite variable (e.g., *Collocalia* or *Passerella*; Table 1). Simonsen et al. (1995) suggested that the power of D_{Taj} to reject neutrality is compromised if sample size is less than about 50 individuals (fig. 3). I found no relationship ($r = 0.19$, $P = 0.52$) between the sample size and the power of Tajima's test, as measured by the absolute value of D_{Taj}. Alhough this is a very rough way to determine whether the tests were compromised by small sample size, it is worth noting that three of the five tests involving the largest sample sizes ($\bar{N} = 143.8$) produce significant D_{Taj} values and all five are negative. These results suggest sample size may not have seriously biased inferences made in comparisons across taxa.

It is difficult to draw general conclusions about mtDNA evolution when looking at individual neutrality tests. Almost half (40%) of the taxa failed a neutrality test and whether neutrality is rejected seems to depend on the test used and the data type (e.g., *M. melodia*). Sources of bias such as selective sweeps or bottlenecks could play a role in whether a particular test produces a significant value of D_{Taj} or is simply negative, as seems to be the trend. The observations that θ is consistently greater than π and NI is consistently greater than one suggests an excess of rare haplotypes in avian populations and that these haplotypes are carrying mildly deleterious, nonsynonymous mutations. Whether an excess of rare haplotypes in most populations could bias some estimators of population genetic parameters (e.g., effective population size, gene flow, migration rate coalescence time) should be investigated.

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