Mutation Accumulation in Transfer RNAs: Molecular Evidence for Muller's Ratchet in Mitochondrial Genomes

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The accumulation of deleterious mutations is thought to be a major factor preventing the long-term persistence of obligately asexual lineages relative to their sexual ancestors. This phenomenon is also of potential relevance to sexual species that harbor asexually propagating organelle genomes. A comparative study of the transfer RNA genes in animal mitochondrial and nuclear genomes demonstrates that the former accumulate nucleotide substitutions much more rapidly than do the latter, and several lines of evidence are consistent with the idea that the excess substitutions are mildly deleterious. First, the average binding stability between complementary strands in the stems of mitochondrial tRNAs is less than half that in nuclear tRNAs. Second, most loop sizes in the mitochondrial tRNAs have experienced a net reduction in size over evolutionary time, and they are nearly 50 times more variable in the mitochondrial than in the nuclear genome. Third, although nearly 20% of the nucleotides in nuclear tRNA genes (particularly those involved in tertiary interactions) are invariant across all animal taxa and all tRNA species, there are no invariant sites in the mitochondrial tRNAs. These observations, as well as results from recent laboratory experiments, are consistent with the hypothesis that nonrecombining organelle genomes are subject to gradual loss of fitness due to the cumulative chance fixation of mildly deleterious mutations.

Introduction

In the absence of segregation and recombination, individuals cannot produce descendants with a reduced number of deleterious mutations, except in the rare case of back-mutations. Thus, in an asexual population, when the best class of individuals either produces no surviving offspring or only produces offspring that have acquired at least one new deleterious mutation, the population suffers an irreversible decline in fitness. The previously second-best class of individuals eventually will suffer the same fate, and so on. Muller (1964) first suggested that such mutational degradation is an inevitable consequence of asexuality. The phenomenon, now known as Muller's ratchet (Felsenstein 1974), is thought to be the predominant cause of extinction in obligately asexual lineages.

Once the cumulative mutation load in a population reaches a high enough level that the average individual cannot replace itself, the population size begins to decline. By enhancing the magnitude of random genetic drift, this leads to increasingly higher rates of accumulation of deleterious mutations and a rapid decline to extinction via a process that we call a mutational meltdown (Lynch and Gabriel 1990; Lynch et al. 1993; Lynch, Conery, and Bürger 1995*a*, 1995*b*). Because the deleterious mutation rate for higher organisms is on the order of one per nuclear genome per generation (Crow and Simmons 1983; Charlesworth, Charlesworth, and Morgan 1990; Johnston and Schoen 1994), this process appears to insure the extinction of most obligately asexual populations in a few hundred to a few thousand generations (Lynch and Gabriel 1990; Lynch et al. 1993), unless the population size is enormous (greater than the reciprocal of the back-mutation rate).

Recombination plays a pivotal role in the maintenance of low mutation load because it enables parents to produce progeny genotypes with reduced numbers of deleterious mutations (Muller 1964; Maynard Smith 1978; Pamilo, Nei, and Li 1987; Charlesworth, Morgan, and Charlesworth 1993; Lynch, Conery, and Bürger 1995*a*). For loosely linked genes, the recombinational mechanism of genome repair is so powerful that it insures that the nuclear genomes of large outcrossing populations are nearly invulnerable to a mutational meltdown on time scales shorter than hundreds of thousands of generations (Lande 1994; Lynch, Conery, and Bürger 1995*a*, 1995*b*). Nevertheless, the possibility of gradual mutational decay still exists in sexual species because the genomes of most higher organisms contain regions of reduced recombination. Rice's (1994) demonstration of rapid loss of fitness associated with nonrecombining, pseudo-Y chromosomes in experimental populations of *Drosophila* provides compelling support for this contention.

In animals, the stream-lined mitochondrial genome, which encodes for critical metabolic functions, has an essentially undetectable rate of recombination (Clayton 1992; Wolstenholme 1992). This raises questions about the efficiency with which animal mitochondria avoid Muller's ratchet, and by extension, about the long-term vulnerability of higher organisms to mutational meltdowns in organelle genomes (Takahata and Slatkin

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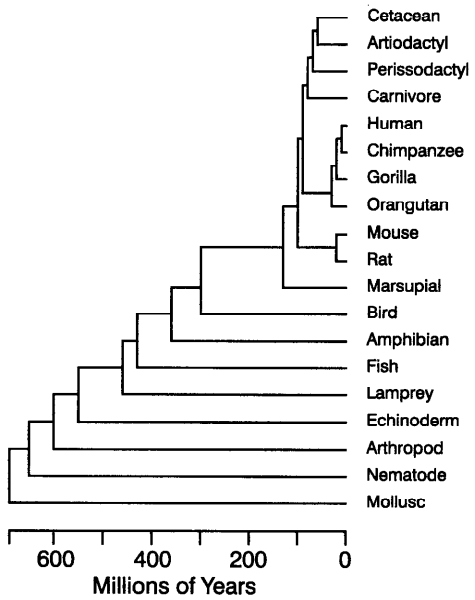

FIG. 1.—Phylogenetic tree assumed in the analyses.

1983; Hastings 1992; Gabriel, Lynch, and Bürger 1993; Reboud and Zeyl 1994). Some insight into this matter can be achieved by comparing the evolutionary properties of the mitochondrial and nuclear genomes of the same species. Ideally, such a comparison should involve genes whose products have similar functions in both genetic environments. The set of transfer RNA genes provides the most reasonable basis for comparison. The animal mitochondrial genome contains a set of 22 tRNA species (one for each amino acid except leucine and serine, which have two anitcodon types), analogous to the set found in the nuclear genome. All of the proteins with which animal mitochondrial tRNAs interact are encoded in the nuclear genome (Clayton 1992).

The extreme degree of evolutionary conservation of the primary, secondary, and tertiary structure of nuclear tRNAs among widely divergent taxa (Söll and RajBhandary 1995) implies that natural selection is successful in maintaining the optimal molecular architecture of these genes. One would expect to find a parallel situation in the mitochondrial genome unless, in this case, selection is less effective at eliminating deleterious mutations. The large number of mitochondrial and nuclear tRNA sequences that have recently become available allow a quantitative assessment of this issue.

Methods

Source of Data

The following analyses are based on sequence comparisons across 19 discrete taxonomic groups, ranging from various species of mammals to phyla of invertebrates (fig. 1). All mitochondrial and nuclear tRNA sequences that were available as of March 1995 were accessed from the Genbank and EMBL data repositories. The mitochondrial data set consists of 1,210 sequences, including those from 32 completely sequenced species. For this genome, at least one, and often several, sequences were available for all 19 taxonomic groups for each of the 22 tRNA classes.

The data for nuclear genes are more limited (a total of 201 sequences), but still cover a broad phylogenetic spectrum, including ungulates (cow), primates (human), rodents (rat and mouse), birds (chicken), frogs (*Xenopus laevis*), arthropods (*Bombyx mori* and *Drosophila melanogaster*), and nematodes (*Caenorhabditis elegans*), to a degree that varies among tRNA sets. Contrary to the situation in the mitochondrion, multiple anticodon-containing types exist in the nuclear genome for many of the 22 isoccepting sets of tRNAs. Thus, when the data existed, separate analyses were performed for each anticodon-containing set and averaged.

In the nuclear genome, a clover-leaf secondary structure, containing four stems and four loops, is conserved evolutionarily across all tRNAs. Exceptions can be found in animal mitochondria, where, for example, the Ser-AGY tRNA is missing the dihydrouridine stem-loop structure, and the TψC stem-loop structure is absent from most nematode and some molluscan mitochondrial tRNAs. Alignment of tRNA sequences is facilitated greatly after the individual sequences are subdivided into their secondary structural features. Taking this into consideration as well as the phylogenetic relationships among taxa (fig. 1), all sequences were aligned by eye within and among the 22 tRNA sets from both the mitochondrial and nuclear genomes. The entire aligned data set is available from the author. In the remainder of the text, dihydrouridine stems and loops will be referred to as D stems and loops, TψC stems and loops as T stems and loops, and variable loops as V loops. Uridine nucleotides will be symbolized as T.

Estimation of Nucleotide Substitution Rates

To estimate the rate of nucleotide substitution in the various structural components of the tRNA molecule, the method of Lynch and Jarrell (1993) was employed. This technique uses generalized least-squares analysis to regress measures of the number of substitutions per nucleotide site separating species on their times of divergence, and in doing so, it ameliorates statistical problems due to the nonindependence and heteroscedasticity of comparative data. Estimates of the substitution rate derived from a generalized least-squares analysis are generally quite similar to those obtained by ordinary least-squares. However, the latter approach usually leads to standard errors that are substantially downwardly bi-

ased because of the failure to account for the nonindependence of data.

Implementation of the generalized least-squares technique requires a phylogenetic tree with known times for the nodal taxa. In this study, a phylogenetic tree with divergence times similar to those employed by Lynch and Jarrell (1993), but with additional taxa, was used (fig. 1). The general topology of this tree and the dates of nodal taxa were inferred from data in the fossil record discussed in numerous references (Savage and Russell 1983; Lipps and Signor 1992; Novacek 1992; Forey and Janvier 1993; Morris 1993; Ahlberg and Milner 1994; Gingerich et al. 1994). The topology is consistent with a broad range of recent studies in molecular systematics (Field et al. 1988; Janke et al. 1994; Horai et al. 1995). Although errors in the *absolute* times of divergence are surely present, there is no reason to expect that they are biased in one direction. In any event, such errors should have little effect on the interpretation of the results of this study, since the focus is largely on the properties of mitochondrial genes relative to their nuclear counterparts.

As in any method for estimating substitution rates, the generalized least-squares technique requires that the observed pairwise comparative data (proportion of shared nucleotides) be corrected for multiple substitutions per site and converted into estimates of the actual number of substitutions per site prior to analysis. As a measure of substitutional distance between sequences, the statistic

$$D = \sum_{n=1}^{k} (I_\infty)^n \sum_{x=0}^{n} \frac{k^{(x)}}{(I_\infty)^x N^{(x)}} \cdot \frac{(n-1)!}{(n-x)!x!} \qquad (1)$$

was used, where N is the length of the sequence (in nucleotides), k is the number of differences between the sequences, $k^{(x)} = k(k-1)(k-2), \ldots, (k-x+1)$, $N^{(x)} = N(N-1)(N-2), \ldots, (N-x+1)$, and I_∞ is the asymptotic identity between two diverging sequences. This estimator is derived by Taylor expansion from the estimator (Equation 3) used in Lynch and Jarrell (1993), and asymptotically yields results that are identical to the previous estimator as $N \to \infty$. The use of equation (1) is motivated by arguments provided in Tajima (1993). The modified estimator reduces bias in estimates of genetic distance that can result with short sequences, and also prevents the generation of undefined estimates that can sometimes arise with such data. With the short stems and loops of tRNA molecules, neither of these issues is trivial.

For each of the four stem and four loop analyses, within both the mitochondrial and nuclear data sets, estimates of I_∞ were obtained by applying the nucleotide content of the sequences to Equations (7) and (8) in Lynch and Jarrell (1993), which fully account for the nonindependence of comparative data. The estimates of I_∞ employed in the evolutionary rate analyses were obtained by averaging over all 22 tRNA sets.

In all analyses involving pairwise comparisons between taxonomic groups, an average distance between all possible pairs of sequences across groups was used. For example, if three mammal and four bird sequences were available, the mammal-bird distance D would be an average of the twelve sequence-specific differences. The three anticodon nucleotides were excluded in the analyses of anticodon loops. Generalized least-squares estimation utilizes an iterative procedure, which occasionally does not converge on the final parameter estimates. For those few cases in which that occurred, ordinary least-squares analysis was performed.

Estimation of Stem Duplex Stability

The stability of RNA stem duplexes was estimated by use of the rules set forth in Freier et al. (1986) and later modified by Jaeger, Turner, and Zucker (1989). The estimated ΔG values do not include costs due to terminal loop sizes, but their inclusion would not qualitatively change the interpretation of comparative results, because the average loop sizes of both nuclear and mitochondrial tRNAs are similar.

Estimation of Loop Sizes

In the estimation of loop sizes, it was assumed that loops begin at the first non-Watson-Crick pair at the end of a stem, and that the minimum loop size consists of three nucleotides. For the average size of the variable loop in the nuclear genome, the Ser and Leu tRNAs were not included, as these are unusually large. For analyses of T loop sizes in mitochondrial genes, nematodes were not included, as they have lost this structure in most cases.

Estimation of Phylogeny-wide Means

In numerous analyses in this paper, phylogeny-wide means are reported, e.g., for the use of the four nucleotides at various sites, for the average stability of stem binding, and for average loop sizes. To account for the nonindependence of phylogenetic data, so as not to give excess weight to highly represented taxa, such means were always obtained by generalized least-squares, taking into account the phylogenetic relationships of species (as outlined in fig. 1). The general procedure follows Equation (8) in Lynch and Jarrell (1993).

Results

Rate of Sequence Evolution

There is no question that the average rate of nucleotide substitution in mitochondrial transfer RNA genes is substantially greater than that in the nuclear genome.

Table 1
Rates of Nucleotide Substitution per Site per Billion Years for Various Phylogenetic Groups, Averaged Over All tRNA Genes

Phylogenetic Group	Mitochondrion[a]	Nucleus
Rodent—primate	1.26 (0.05)	0.050 (0.010)
Cetacean—artiodactyl	1.23 (0.10)	—
Bird—basal mammal[b]	0.58 (0.02)	0.063 (0.059)
Fish—amphibian	0.39 (0.02)	—
Lamprey—fish	0.44 (0.02)	—
Nematode—arthropod	0.55 (0.02)	0.123 (0.016)
Mollusc—echinoderm	0.61 (0.02)	—

NOTE.—Substitution rates were estimated by use of a modification of Equation (3) in Lynch and Jarrell (1993): $\delta = (1 - I_\infty)(D - \Delta_0)/T$, where I_∞ is the asymptotic identity of two sequences that have diverged for an effectively infinite amount of time, $\Delta_0 = -\ln(1 - I_\infty)$, $D = -\ln(I - I_\infty)$ with I being the proportion of nucleotides shared by two sequences, and T is twice the time (in billions of years) to the common ancestor of the two species. With this approach, where I_∞ is obtained from nucleotide usage frequencies, correction is made for saturation in the degree of divergence that results when sufficient time has passed for multiple substitutions per site to occur; with large I (as in the case of nuclear genes in this study), $\delta = (1 - I)/T$.

[a] Standard errors are given in parentheses.

[b] The substitution rate on the external branch leading to birds was estimated by first computing the average proportion of nucleotides shared by bird and mammal sequences, then calculating the average substitution rate between birds and mammals (using the formula given above), and finally estimating the rate on the branch leading from birds to the bird-mammal common ancestor under the assumption that the rate on the mammal to bird-mammal common ancestor is the same as that obtained for pure mammalian lineages.

However, the degree of disparity differs widely among phylogenetic groups. At the simplest level, the total proportional sequence divergence can be used to estimate the average rate of substitution throughout the entire molecule (table 1). Averaging over all loci, for two independent lineages of mammals, the mean substitution rate in the mitochondrial genome is remarkably constant, approximately 1.24 substitutions/site/BY, and about 25 times greater than the average rate in the nuclear genome.

Most other major phylogenetic lineages, including birds, arthropods, nematodes, and molluscs, appear to evolve at the rate of approximately 0.58 substitutions/site/BY in the mitochondrial genome, i.e., at approximately half the mammalian rate. However, ectothermic vertebrates (lampreys, fish, and amphibians) evolve at a distinctly slower rate—approximately 0.42 substitutions/site/BY. This scaling does not extend to the nuclear genome, where the rate of evolution of tRNAs of invertebrates is actually double that in mammals. As a consequence, in invertebrates, the rate of base substitution in the mitochondrial genes is only about five-fold greater than that in the nuclear genes.

Separate analyses performed over the entire phylogeny reveal that the elevated rate of mitochondrial gene evolution applies to all four of the stems and loops (table 2). In the nuclear tRNAs, the anticodon loops (not including the anticodon sites) and D stems are particularly evolutionarily stable, both having an average substitution rate of only 0.01/site/BY, about 16 and 30 times lower than the average rates in the homologous structures in the mitochondrial genes. On average, in both genomes, the nucleotide sequences of stems diverge about 2.7 times faster than those of loops.

The heterogeneity in the evolutionary lability of various nucleotide positions can be revealed at a finer scale by considering the effective number of nucleotides employed per site throughout the animal phylogeny. For

Table 2
Average Rates of Nucleotide Substitution per Site per Billion Years for Loops and Stems of Transfer RNA Genes

		Mitochondrion[a,b]		Nucleus
Loops:	Dihydrouridine	0.123 (0.046)	>	0.044 (0.009)
	Anticodon	0.156 (0.021)	≫	0.010 (0.006)
	Variable	0.472 (0.074)	≫	0.103 (0.033)
	TψC	0.094 (0.051)		0.062 (0.018)
	Average[c]	0.201 (0.027)	≫	0.054 (0.009)
Stems:	Acceptor	0.625 (0.077)	≫	0.169 (0.040)
	Dihydrouridine	0.330 (0.059)	≫	0.011 (0.010)
	Anticodon	0.559 (0.049)	≫	0.180 (0.042)
	TψC	0.718 (0.070)	≫	0.149 (0.046)
	Average	0.575 (0.035)	≫	0.137 (0.020)
Total molecule:		0.450 (0.025)	≫	0.106 (0.013)

NOTE.—For each transfer RNA gene, the average substitution rate over the entire phylogeny was obtained by generalized least-squares analysis. The reported values are the averages over all genes.

[a] Standard errors are given in parentheses.

[b] Significant differences between nuclear and mitochondrial genes at the 0.05 and 0.01 levels are denoted by > and ≫, using absolute differences in excess of 1.96 and 2.56 standard errors as criteria for significance.

[c] Average loop and stem rates are obtained by weighting individual components by their mean sequence lengths.

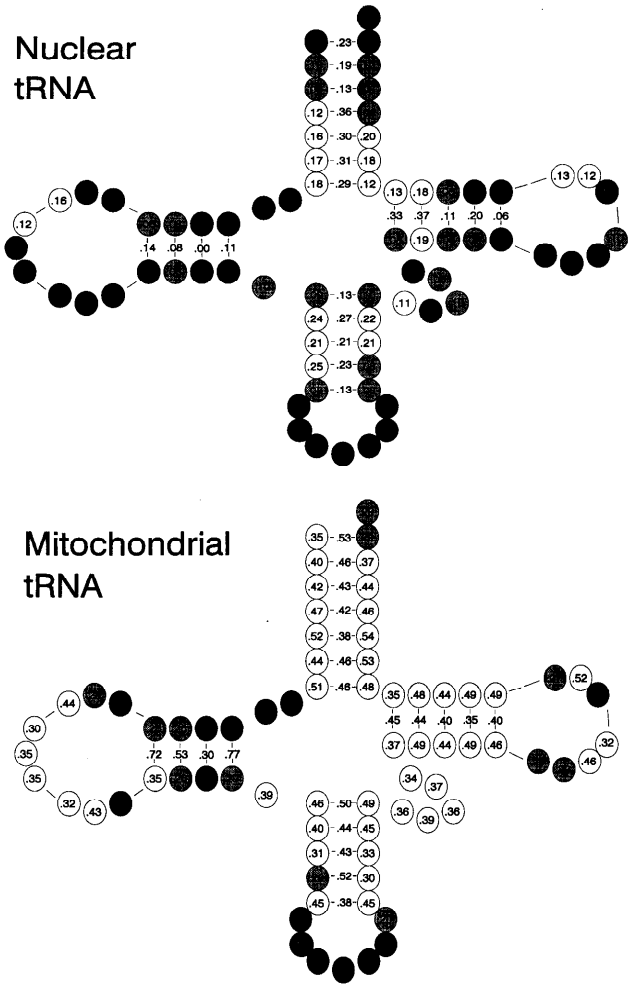

FIG. 2.—Estimates of average degrees of evolutionary lability for nucleotide sites (L_s) and stem pairs (L_p) in nuclear transfer RNA genes and for the homologous sites in mitochondrial genes. For the nuclear genes, sites with $0.0 \leq L_s \leq 0.05$ are shaded dark grey and those with $0.05 < L_s \leq 0.10$ are shaded light grey. For the mitochondrial genes, sites with $0.0 \leq L_s < 0.20$ are shaded dark grey and those with $0.20 \leq L_s \leq 0.30$ are shaded light grey. The thin lines in the D (left) and T (right) loops denote positions where insertions and deletions are common.

each base position, in each tRNA species, generalized least-squares estimates of the frequency of usage of each of the four nucleotides (p_A, p_C, p_G, p_T) was computed, and the effective number of nucleotides was estimated as

$$n_n = \frac{1}{p_A^2 + p_C^2 + p_G^2 + p_T^2}. \quad (2a)$$

The evolutionary lability of a site was then computed on a zero-to-one scale with the index

$$L_s = \frac{n_n - 1}{3}, \quad (2b)$$

where $L_s = 0$ when only a single nucleotide is used at the site for a particular tRNA species, and $L_s = 1$ when each of the four nucleotides is used with equal frequency throughout the phylogeny.

When the values of this index are averaged over each tRNA species at each site, a constraint map is obtained for the entire molecule (fig. 2). Using $L_s \leq 0.05$ as a criterion for defining a nearly-invariant site, 30 such sites (approximately 40% of the gene) are found in the nuclear transfer RNAs, and of these, thirteen are completely invariant across all taxa ($L_s = 0$). Such sites are found in every major structural component of the molecule, except the anticodon stem. On the other hand, only two nearly-invariant sites, one in the D loop and one in the anticodon loop, are found in the mitochondrial transfer RNAs. Every site in the mitochondrial molecule has a higher index of evolutionary lability than its homologue in the nuclear molecule.

Table 3
Average Binding Strength of Stems (in Free Energy; −ΔG in kcal/mol, as Estimated at 37°C, 1 M NaCl)

	Mitochondrion	Nucleus
Acceptor	3.67 (0.29)	8.43 (0.29)
Dihydrouridine	1.13 (0.22)	2.98 (0.46)
Anticodon	2.23 (0.20)	4.80 (0.22)
TψC	2.22 (0.20)	5.27 (0.46)

NOTE.—Generalized least-squares estimates were obtained for each gene, and the mean and standard errors (in parentheses) of these estimates are reported here. All differences between nuclear and mitochondrial genes are significant at the 0.01 level.

Stem Structure

On average, the binding strength between complementary strands of the stems in the nuclear tRNAs is about 2.4 times that for the mitochondrial genes (table 3). This is true for all four stems, and appears to be a consequence of two factors. First, G-C (and the reciprocal, C-G) pairs are nearly three times more common in nuclear tRNA stems than in those of mitochondrial tRNAs, whereas A-T (and the reciprocal, T-A) pairs are about 2.4 times more common in mitochondrial tRNA stems than in those of nuclear tRNAs. G-C/C-G pairs involve three hydrogen bonds, whereas A-T/T-A pairs involve only two. Second, mismatches (pairs other than A-T, C-G, G-T, and their reciprocals) are about six times more frequent in the stems of mitochondrial tRNAs.

To obtain further insight into the constraints on the use of various nucleotide pairs in stems, a second index of evolutionary lability was constructed in the following way. Let $p_{5A}, \ldots, p_{5T}$ denote the generalized least-squares estimates of the frequencies of usage of the four nucleotides at the 5′ position in a particular stem pair, and $p_{3A}, \ldots, p_{3T}$ denote the estimates of the frequencies of usage of the four nucleotides at the 3′ position in the

Table 4
Mean Loop Sizes and Variation in Loop Size (Measured as the Average Absolute Proportional Deviation from the Mean)

	Mean		Variation	
	Mitochondrion	Nucleus	Mitochondrion	Nucleus
Dihydrouridine	6.79 (0.23)	8.98 (0.25)	0.179 (0.013)	0.017 (0.008)
TψC	5.76 (0.15)	6.94 (0.15)	0.250 (0.023)	0.019 (0.018)
Variable	4.82 (0.10)	4.68 (0.12)	0.201 (0.019)	0.005 (0.003)
Anticodon	6.70 (0.16)	7.04 (0.04)	0.147 (0.017)	0.007 (0.005)

NOTE.—Generalized least-squares estimates were obtained for each gene, and the mean and standard errors (in parentheses) of these estimates are reported here. All differences between nuclear and mitochondrial genes are significant at the 0.01 level, except that for the mean size of the variable loop, which is not significant.

pair. Then, under the assumption that the nucleotides in both positions are randomly paired throughout a phylogeny, the equilibrium effective number of dinucleotide "alleles" would be

$$n_d = \frac{1}{\sum_{i,j=\mathrm{A},\cdots,\mathrm{T}} \sum (p_{5i}p_{3j})^2}. \quad (3a)$$

When each nucleotide is used in equal frequency at both sites in a pair, $n_d = 16$.

Letting $p_{\mathrm{AA}}, p_{\mathrm{AC}}, \cdots, p_{\mathrm{TT}}$ be the observed generalized least-squares estimates of the frequencies with which various nucleotide pairs are actually used, the observed effective number of alleles is

$$n_a = \frac{1}{p_{\mathrm{AA}}^2 + p_{\mathrm{AC}}^2 + \cdots + p_{\mathrm{TT}}^2}. \quad (3b)$$

Thus, taking into consideration the constraints on nucleotide usage at the two component sites, the evolutionary lability of pair usage at a stem site can be computed on a zero-to-one scale with the index

$$L_p = \frac{n_a - 1}{n_d - 1}, \quad (3c)$$

where $L_p = 0$ when only a single pair of nucleotides is used at the site for a particular tRNA species, and $L_p = 1$ when the frequency of usage of each nucleotide pair is determined entirely by the nucleotide frequencies at the component sites.

For all 21 stem pairs, L_p is greater in the mitochondrial genes than in the nuclear genes (fig. 2). The disparity is particularly pronounced in the D stem, where, for example, the nucleotide pairs used in the first and fourth positions in the mitochondrial genes are less constrained than those at any other stem site ($L_p > 0.70$), whereas those in the nuclear genes are tightly constrained ($L_p < 0.15$).

This structural analysis revealed an unusual feature of the mitochondrial stems—substantial asymmetry in the use of reciprocal Watson-Crick pairs. For example, in the acceptor stem, A-T pairs are used 34% of the time, whereas T-A is employed only 22% of the time; and G-C is used 19% of the time, whereas C-G is used only 10% of the time. Similarly, in the mitochondrial T stems, A-T and G-C pairs occur 32% and 34% of the time, whereas T-A and C-G pairs are only 14% and 8% frequent. Thus, there is a strong tendency for purines to appear on the 5′ side and pyrimidines on the 3′ side of the stem. Such asymmetry is not observed in the acceptor or anticodon stems of the nuclear genes. It does exist in the nuclear D and T stems, but this appears largely to be a consequence of the presence of invariant nucleotides at several positions in these structures, e.g., the universal use of C-G in the fifth position of the T stem, and the fixed usage of either C-G or T-A in the second position of the D stem for different tRNA species.

Loop Sizes

Although the average T and D loop sizes are approximately 1.5 and 1.9 nucleotides smaller in mitochondrial than nuclear tRNAs, the most pronounced difference in the two genomes with respect to loop size is the approximately 50-fold increase in variation around the mean in mitochondrial relative to nuclear genes (table 4). Loop sizes in nuclear tRNAs are nearly invariant, deviating by less than 1% from the expectation on average. On the other hand, the average absolute deviation of a mitochondrial tRNA loop from the phylogeny-wide mean is nearly 20%.

The increased variation in loop sizes in mitochondrial tRNAs is driven by an increase in the rates of insertion and deletion. To quantify these rates, the technique of Saitou and Ueda (1994) was employed. With this method, the principle of maximum parsimony is used to assign the evolutionary changes in loop sizes to the various branches of the phylogenetic tree, and the total number of changes is then divided by the total length of the tree to provide an estimate of the rate of insertion/deletion. Provided the length of the branches of the tree are small enough that the likelihood of mul-

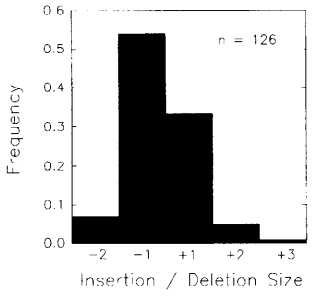

FIG. 3.—Frequency distribution for insertions and deletions in the loops of mitochondrial transfer RNAs.

tiple substitutions per branch is negligible, this method should provide essentially unbiased estimates. To insure that this was the case, the analysis for the mitochondrial genes was restricted to mammals. However, because the evolutionary rate of the nuclear tRNAs is so slow, insertions/deletions are essentially absent from the mammalian genes, so the analysis for the nuclear genome employed the total phylogeny.

In the mitochondrial tRNAs, nearly all insertion/deletion events involve one or two nucleotides, with those involving deletions (61%) and single nucleotides (87%) predominating (fig. 3). For the D, T, and V loops, insertion/deletion events occur at rates of 0.678 (0.140), 0.650 (0.133), and 0.057 (0.034)/nucleotide/BY. These rates are respectively 2.6, 3.3, and 0.06 times the rates of nucleotide substitution observed in the same structures. Thus, at least in mammals, in the D and T loops of mitochondrial tRNAs, the predominant mode of evolution is insertion/deletion, whereas in the V loop, it is nucleotide substitution.

As noted above, very few insertions or deletions occur in the nuclear tRNAs, so only approximate estimates of their rates of occurrence can be given. These are, for the D, T, and V loops respectively, 0.0078 (0.0059), 0.0006 (0.0006), and 0.0192 (0.0116)/nucleotide/BY. These rates are approximately 18%, 1%, and 19% of those for nucleotide substitution for the same structures. Moreover, the rates of insertion/deletion for the nuclear D and T loops are on the order of 1% and 0.1% of those observed in the mitochondrion.

Tertiary Structure

Most of the nucleotides observed to be invariant or nearly invariant in nuclear tRNAs (fig. 2) are known to be involved in nucleotide-pairing interactions that maintain the tertiary structure of the molecule (Kim 1978; Romby et al. 1987; Saenger 1988). These include four nucleotides in the D loop, two in the D stem, four in the T loop, three in the V loop, and both nucleotides in the connector between the acceptor and D stems. For example, the first nucleotide in the acceptor-D stem linker is known to bind with the first position in the D loop; in nuclear tRNAs, the nucleotides involved are always T and A, respectively. The fifth nucleotide in the D loop is known to bind with the second nucleotide in the T loop, and in nuclear tRNAs, the nucleotides involved are always G and T, respectively.

Because no invariant sites exist in the pool of mitochondrial tRNAs (fig. 2), there are no invariant nucleotide pairs underlying the tertiary structure of these molecules. In all cases, the most commonly used pairs in these genes are identical to the most commonly used pairs in the nuclear genes. However, the frequency at which the preferred pairs are used is greatly diminished in the mitochondrial genes. For example, for the first nucleotide in the acceptor-D stem linker and the first position in the D loop, T-A is used only 84% of the time. For the fifth nucleotide in the D loop and the second nucleotide in the T loop, G-T is used only 26% of the time.

Discussion

The preceding analyses suggest that the average rate of nucleotide substitution in a mammalian mitochondrial tRNA is elevated by a factor of 25 relative to the average rate for the same type of gene in the nuclear genome. For birds and for invertebrates, the mitochondrial rate is also inflated, but to a lesser degree, 9-fold and 4.5-fold respectively. Although highly significant, this extent of magnification of the evolutionary rate of mitochondrial tRNAs is much less than the 100-fold difference suggested in an earlier study based on a much smaller data set (Brown et al. 1982). The exceptionally high degree of disparity between mitochondrial and nuclear rates in mammals, relative to other animals, is attributable to two causes—mammals have an unusually high rate of substitution in the mitochondrial genome and an unusually low rate of substitution in the nuclear genome (table 1).

The rate of molecular evolution is a function of both the mutation rate and the efficiency of selection. To gain some insight into the ways in which these two factors determine genomic and taxonomic differences in substitution rates for tRNA genes, consider the rate of synonymous substitution in protein-coding genes. For mammals, the rates for mitochondrial and nuclear genes are approximately 38.9 and 3.5 substitutions/site/BY (Li and Graur 1991; Horai et al. 1995; Ohta 1995), whereas for *Drosophila,* the analogous rates are approximately 22.8 and 16.0 substitutions/site/BY (Sharp and Li 1989; Moriyama and Gojobori 1992; Ballard and Kreitman 1994; Rand, Dorfsman, and Kann 1994). To the extent that these estimates accurately reflect the neutral rate of substitution, they suggest that the mutation rate per nu-

cleotide in the nuclear genome is approximately 4.5 times greater in *Drosophila* than in mammals. A plausible, although by no means certain, explanation for this observation is that the occurrence of such mutation scales with generation number rather than with absolute time (Laird, McConaughy, and Hoyer 1969; Ohta and Kimura 1971; Li, Tanimura, and Sharp 1987; Gillespie 1991). Such scaling could be approximated if nuclear-gene mutations are generated predominantly by meiotic recombination events that occur during gametogenesis. By virtue of having shorter generation times, invertebrates would then have a higher rate of mutation per absolute time interval. On the other hand, the increase in the mitochondrial mutation rate in mammals, relative to *Drosophila* and most likely ectothermic vertebrates, may be a consequence of the elevated metabolic rate of mammals and its mutagenic repercussions for the mitochondrial genome (Martin and Palumbi 1993; Rand 1994).

Assuming that the synonymous substitution rate reasonably represents the mutation rate per nucleotide in a transfer RNA gene, the data cited above, in combination with the results in table 1, imply that selection on mutations in nuclear tRNA genes reduces the substitution rate to approximately 1.4% and 0.8% of the neutral expectation in mammals and invertebrates, respectively. Selection against mitochondrial mutations appears to be less effective—the substitution rates in mammals and invertebrates are, respectively, 3.1% and 2.5% of the neutral expectations. In both genomes, the apparent increased efficiency of purifying selection in invertebrates may be a simple consequence of the elevated effective population size in such species relative to vertebrates. In addition, the effective number of mitochondrial genomes transmitted between mother and offspring is known to be several-fold higher in *Drosophila* than in mammals (Rand and Harrison 1986; Solignac et al. 1987; Koehler et al. 1991; Howell et al. 1992), and this will further reduce the probability of fixation of a mildly deleterious allele by random genetic drift.

A reduction in the efficiency of selection against mitochondrial mutations, relative to nuclear mutations, also can be inferred for insertions and deletions. Such mutational changes are presumably very highly deleterious when they arise in transfer RNA stems, as they are nearly unobserved in the large number of existing sequences for both mitochondrial and nuclear genes. However, insertions and deletions in the D and T loops of mitochondrial tRNAs are quite common, accumulating at a rate of approximately 0.66/site/BY in mammals, which is substantially greater than the nucleotide substitution rates in these portions of the molecule (table 2).

Saitou and Ueda (1994) have suggested that the mutational rate of insertion/deletion in the mammalian mitochondrial genome is approximately 1.8/base/BY. If this is correct, then selection appears to reduce the rate of fixation of mitochondrial D and T loop insertion/deletions to approximately 37% of that expected under neutrality. On the other hand, for nuclear tRNAs, the average rate of insertion/deletion in the D and T loops is only 0.0042/base/BY, less than 1% of that observed for mitochondrial genes. Saitou and Ueda's (1994) estimate of the mutational rate of insertion/deletion for mammalian nuclear genes is 0.17/base/BY. This estimate may be too low for animals in general, if the elevated rate of substitutional mutation in mammals extends to insertion/deletion events. Thus, it appears that selection reduces the rate of fixation of insertion/deletion in the D and T loops of nuclear tRNAs to less than 2.5% of the neutral expectation. The efficiency of selection against insertion/deletions in the nuclear genes is substantially greater than that in the mitochondrial genes.

Because the rate of deletion is slightly higher than that of insertion, as observed in previous studies (de Jong and Ryden 1981; Saitou and Ueda 1994; Gu and Li 1995), the D and T loops of mitochondrial tRNAs are slowly experiencing a net reduction in size. For mammals, the estimated rate of size reduction is approximately 0.16 bases/site/BY. If it is assumed that the inflated rate of substitutional mutation observed in mammalian mitochondrial DNA applies to insertions and deletions as well, then the rate of size reduction in the tRNA loops of non-mammals is approximately $0.16 \times 22.8/38.9 = 0.094$ bases/site/BY. Further assuming that mitochondria arose approximately two billion years ago from bacterial endosymbionts (Knoll 1992; Schopf 1994), and noting that the mean size of the D and T loops of bacterial tRNAs are 9 and 7 bases, it can be inferred that the average D and T loops of mitochondrial tRNAs throughout the entire animal phylogeny should have lost approximately 1.7 and 1.3 bases respectively, relative to their ancestral states. Although crude, these estimates are very close to the observed changes of 2.2 and 1.2 bases.

The analyses in the preceding paragraphs have been presented as an attempt to separate the effects of the mutation rate and fixation probability on the differences in the evolutionary rates of mitochondrial and nuclear genes. Although there does appear to be a general increase in the mutation rate in the mitochondrial genome, all of the results also indicate that there is a reduction in the efficiency of selection against mutations that arise in mitochondrial tRNAs relative to those that arise in their nuclear counterparts. In the case of substitutional changes, the reduction is 2- to 3-fold, and it is at least 15-fold in the case of insertion/deletions. With

respect to both nucleotide content and structure, nuclear tRNA genes appear to be under extremely strong purifying selection. They evolve much more slowly than most other genes for which data are available (Kimura 1983; Li and Graur 1991), and the high proportion of invariant positions (throughout all eukaryotes) and the near absence of insertions and deletions point further to the stringency of selection against new mutations in nuclear tRNA genes.

The greater evolutionary rate of the transfer RNAs in the animal mitochondrion does not necessarily imply that the structural integrity of these molecules has been compromised, because compensatory mutations could prevent a net loss in fitness. However, while such mutations surely occur, several lines of evidence suggest that they have not kept pace with the deleterious mutations that have arisen in the mitochondrion: the lower stability of stem binding resulting from an increased incidence of A-T and non-Watson-Crick pairs, net reduction in mean loop sizes, increased variation in loop sizes, and absence of nucleotide conservation in sites involved in tertiary structure. Theoretical models have recently been suggested to explain how compensatory mutational changes in many mitochondrial genes may allow the maintenance of a reasonable L-shaped tertiary structure of the tRNA molecule (Steinberg and Cedergren 1994; Steinberg, Gautheret, and Cedergren 1994; Watanabe et al. 1994; Wolstenholme, Okimoto, and MacFarlane 1994). Nevertheless, these studies also indicate that structural instabilities remain in the mitochondrial genes that are not observed in their nuclear counterparts.

It has been argued that a reduction in the rate of elimination of mutant alleles in the mitochondrion might be due, in part, to a relaxation of functional constraints on mitochondrial tRNAs relative to their nuclear cognates (Brown et al. 1982; Kumazawa and Nishida 1993). This argument cannot be rejected with the current data. Nuclear tRNAs are known to have several functions beyond their involvement in biosynthetic pathways (Söll 1993) that are not known to occur in mitochondria. However, invocation of a reduction in the intensity of selection on mitochondrial genes strengthens, rather than weakens, the argument that the mitochondrial genome is vulnerable to gradual mutational degradation resulting from Muller's ratchet.

Nonrecombinogenic portions of the genome accumulate mildly deleterious mutations at a rate that is inversely proportional to the strength of selection because the fate of mutations with small effects is mostly influenced by random genetic drift (Haigh 1978; Charlesworth, Morgan, and Charlesworth 1993; Lynch et al. 1993; Stephan, Chao, and Smale 1993; Higgs 1994). Roughly speaking, deleterious mitochondrial mutations with selection coefficients in the neighborhood of the reciprocal of the effective population size cause the most rapid decline in fitness in populations. For such mutations, the strengths of selection and random genetic drift are approximately equal. As a consequence, the probability of fixation is relatively high and the impact of cumulative fixations on mean individual fitness is significant. Thus, mutations with small, but non-zero, individual effects provide the greatest cumulative threat of population extinction (Pamilo, Nei, and Li 1987; Gabriel, Lynch, and Bürger 1993; Lande 1994; Lynch, Conery, and Bürger 1995*a*).

Synergistic epistasis has been suggested as a potential mechanism that might greatly slow or even halt the ratchet in asexual genomes (Kondrashov 1994). This requires that consecutive deleterious mutations in a genome have progressively greater effects on fitness, until a point is reached eventually when the average mutational effect is so great that the likelihood of further fixations (and further fitness decline) is essentially zero. Because mutations in the truly neutral class can still go to fixation, such a scenario does not imply that molecular evolution must come to a halt. However, the epistasis hypothesis does predict that the rate of evolution will slow down over time. The data in this study are not entirely adequate to test this hypothesis, but the fact that the most recent evolutionary lineage in the analyses (mammals) exhibits the highest evolutionary rate is certainly not supportive of the idea that the rate of evolution of mitochondrial tRNAs is slowing down over evolutionary time. In addition, recent theoretical work (Butcher 1995) challenges the idea that the ratchet can be stopped by epistatic interactions between mutations, pointing out that Kondrashov's (1994) result depends critically on the assumption that mutations have fixed effects, which seems unlikely biologically.

Takahata and Slatkin (1983) have suggested an alternative mechanism that might reduce the rate of the Muller's ratchet in the mitochondrion. Like recombination, intracellular random genetic drift, resulting from the multiplicity of mitochondrial genomes per cell, can result in the production of progeny cells that carry fewer (or more) mutations than their parent cell. Their simulation results indicate that the degree to which this mechanism can slow the rate of Muller's ratchet depends on the multiplicity of genomes per cell. With large multiplicity, Takahata and Slatkin (1983) suggest that the ratchet might be stopped entirely. However, the cases in which they observed a cessation of the ratchet involved very strong selection (a 10% reduction in fitness of individuals fixed for a deleterious mutation). With lower average mutational effects, which seems to be supported by the existing data (Lynch, Conery, and Bürger 1995*b*),

the efficiency of the mechanism proposed by Takahata and Slatkin (1983) is likely to be reduced greatly.

A useful feature of tRNAs is that they are so small that they can be synthesized in the laboratory and their functional efficiency studied in vitro (Söll and Raj-Bhandary 1995). Thus, future experiments by molecular biologists have great potential for clarifying the validity of the basic assumptions underlying the theory of Muller's ratchet. Based on the above arguments, one would expect the functional efficiency of mitochondrial tRNAs to be lower than that of their nuclear counterparts. Some such experiments have, in fact, already been performed, and they support this contention. For example, bovine mitochondrial tRNAs have been shown to have unusually low melting temperatures compared to cytoplasmic tRNAs, with melting initiating below 25°C (Ueda, Ohta, and Watanabe 1985; Kumazawa et al. 1989). Such observations are consistent with the looser higher-order structure suggested by the descriptive analyses in this paper. Bovine mitochondrial $tRNA^{Phe}$ has been shown to have a lower V_{max} for amino-acylation when combined with bovine mitochondrial phenylalnyl-tRNA synthetase (a product of a nuclear gene) than does the cognate tRNA from *E. coli* (Kumazawa et al. 1989). Using an in vitro translation system containing bovine mitochondrial ribosomes and elongation factors, Kumazawa et al. (1991) found that the rate of incorporation of phenylalanine into polyphenylalanine was inflated approximately 10-fold when *E. coli* $tRNA^{Phe}$ was used instead of the bovine mitochondrial tRNA. These two observations imply that the rate of mitochondrial protein synthesis is substantially less efficient than that in its prokaryotic relatives.

In summary, population-genetic theory, descriptive analysis of gene-sequence data, and empirical studies support the idea that the animal mitochondrial genome is gradually accumulating mildly deleterious mutations that are otherwise purged from the nuclear genome. It seems unlikely that such mutation load is a significant source of extinction in many contemporary populations. Nevertheless, it remains a challenge to understand how or whether animal species can survive the inevitable decline in fitness of the mitochondrial genome on a longer time scale (perhaps hundreds of millions of years), in the absence of recombination. The hypothesis that mutation accumulation in mitochondria has contributed to the extinctions of select animal lineages in the fossil record is plausible.

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