Rapid evolution of animal mitochondrial DNA

(primates/restriction endonuclease cleavage maps/gel electrophoresis/DNA melting)

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ABSTRACT   Mitochondrial DNA was purified from four species of higher primates (Guinea baboon, rhesus macaque, guenon, and human) and digested with 11 restriction endonucleases. A cleavage map was constructed for the mitochondrial DNA of each species. Comparison of the maps, aligned with respect to the origin and direction of DNA replication, revealed that the species differ from one another at most of the cleavage sites. The degree of divergence in nucleotide sequence at these sites was calculated from the fraction of cleavage sites shared by each pair of species. By plotting the degree of divergence in mitochondrial DNA against time of divergence, the rate of base substitution could be calculated from the initial slope of the curve. The value obtained, 0.02 substitutions per base pair per million years, was compared with the value for single-copy nuclear DNA. The rate of evolution of the mitochondrial genome appears to exceed that of the single-copy fraction of the nuclear genome by a factor of about 10. This high rate may be due, in part, to an elevated rate of mutation in mitochondrial DNA. Because of the high rate of evolution, mitochondrial DNA is likely to be an extremely useful molecule to employ for high-resolution analysis of the evolutionary process.

Functional considerations lead one to expect slow evolutionary change in the genes of animal mitochondria. This expectation is based on a widely accepted generalization concerning rates of molecular evolution: The more important the function of a gene or protein, the more slowly it undergoes evolutionary change in primary structure (1–3). Mitochondria have extremely important cellular functions. Because the life of animals is crucially dependent on mitochondrial functions, one would expect mitochondrial evolution to be highly constrained. The mitochondrial genome might be expected to share in these constraints. The genome of animal mitochondria is small and relatively uniform in size among vertebrate and invertebrate animals. The implication is strong that this genome was reduced at an early stage of animal evolution to the minimum size compatible with function. However, the expectation, based on functional considerations, that mitochondrial DNA (mtDNA) should have evolved slowly during the remainder of animal evolution is not borne out. Past studies have indicated that the rate of evolution of mtDNA is at least as fast as that of single-copy nuclear DNA (4–8). Our results indicate that mtDNA has been evolving much more rapidly than single-copy nuclear DNA in higher animals. To explain this high rate of evolution, we discuss evidence that mtDNA could have an unusually high rate of mutation.

MATERIALS AND METHODS

Tissues and Cell Lines. Liver samples from one Guinea baboon (*Papio papio*) and two rhesus macaques (*Macaca mulatta*) were obtained from the California Primate Research Center, University of California, Davis, CA. The established

lines of human (*Homo sapiens*) and guenon (green monkey, *Cercopithecus aethiops*) cells used were, respectively, HeLa (strain S3) and BSC-1.

Preparation of mtDNA. The preparation of mtDNA from cultured cells was as described (9). For preparation from liver, the samples (either fresh or frozen) were first minced, then homogenized at high speed in a Waring blender in 5 vol of cold 10 mM NaCl/10 mM Tris/1 mM EDTA buffer (pH 7.8) and then treated in the same manner as the cultured cell homogenates. All mtDNA samples were purified by two cycles of sedimentation equilibrium centrifugation in propidium diiodide/cesium chloride gradients, with an intervening sedimentation velocity step, as described (9).

Restriction Endonuclease Digestion. The 11 restriction endonucleases employed (obtained from New England Bio-Labs) are listed in the legend of Fig. 1. The bacterial strains from which they were isolated and the digestion conditions employed are given by Roberts (10).

Gel Electrophoresis of DNA Fragments. The restriction endonuclease digests of mtDNA were analyzed after electrophoresis in 1%, 1.2%, and 2% agarose slab gels as described (11). Estimates of fragment sizes were obtained by comparison of fragment mobilities with those of the *Eco*RI fragments of bacteriophage λ DNA (12) and of the *Hin*dIII fragments of bacteriophage PM2 DNA (13, 14), which has a genome size of 10,000 ± 300 base pairs as established by comparison with bacteriophage φX174 DNA (unpublished data).

Electron Microscopy and Absorbance Melting of DNA. Electron microscopy of intact mtDNA and of the restriction endonuclease fragments was performed and the results were analyzed with the equipment and in the manner described by Brown and Vinograd (9). The preparation, isolation, and annealing of the respective complementary strands of mtDNA and the absorbance melting buffer, apparatus, and means of analysis employed have been described by Brown *et al.* (11).

Cleavage Mapping with Restriction Endonucleases. The determination of the positions of restriction endonuclease sites relative to the origin and direction of animal mtDNA replication has also been described elsewhere (9), and a review of cleavage mapping methods employing fragment size estimates obtained by gel electrophoresis is available (15). In all cases we were able to determine the location of sites unambiguously, using data from multiple enzymatic digests alone.

When comparing the cleavage maps for different species, we assumed that the resolving power of the mapping technique was ±1 map unit (1 map unit equals 1% of the genome, or 165 base pairs). Thus, if an enzyme cuts mtDNA from one species at a site that lies within 1 map unit of a site cleaved by that enzyme in another species, the two sites are considered to be at homologous positions on the two maps. Whenever possible, a site in one species that appeared to be within 5 map units of a corresponding site in another species was analyzed by co-electrophoresis of additional double digests to improve the accuracy of the estimates of site location on the maps.

Table 1.   Mitochondrial genome sizes for some primate and rodent species

Species	Genome size,* base pairs
Primates	
Human, *Homo sapiens*	16,500 ± 300 (73)
Chimpanzee, *Pan troglodytes*	16,400 ± 300 (19)
Guenon, *Cercopithecus aethiops*	16,400 ± 500 (36)
Rhesus, *Macaca mulatta*	16,500 ± 800 (G)
Baboon, *Papio papio*	16,500 ± 800 (G)
Talapoin, *Miopithecus talapoin*	16,500 ± 800 (G)
Woolly monkey, *Lagothrix cana*	16,300 ± 400 (47)
Bush baby, *Galago senegalensis*	16,500 ± 300 (19)
Rodents	
House mouse, *Mus musculus*	16,300 ± 400 (67)
Norway rat, *Rattus norvegicus*	16,400 ± 300 (74)
Golden hamster, *Mesocricetus auratus*	16,300 ± 500 (21)

* Genome sizes are given ± 1 SD. Unless otherwise noted, size estimates were obtained from contour length measurements with bacteriophage φX174 replicative form DNA as an internal size standard. The size of φX174 replicative form DNA is taken as 5375 base pairs (18). Numbers in parentheses indicate the number of molecules measured. Size estimates followed by (G) were obtained from agarose gel electrophoresis and have an estimated SD of 5%. All estimates except those for rhesus and baboon are from ref. 8.

Calculation of Sequence Divergence from Map Comparisons.

Estimates of the degree of sequence difference between pairs of mtDNAs can be obtained by comparing their cleavage maps. The calculations involved in making these estimates rest on several assumptions: (*i*) that each change in base sequence is due to the substitution of one base pair (i.e., that no sequence rearrangements, deletions, or additions occur); (*ii*) that the patterns of methylation of cytosine residues do not change; and (*iii*) that all base pair positions in the sequence are equally likely to undergo substitution. The validity of these assumptions is discussed later in this article.

We start by calculating s, the fraction of shared cleavage sites—i.e., the number of sites shared (z) divided by the total number of sites compared. If there are x cleavage sites in one species and y cleavage sites in another species, the total number of sites compared is $x + y - z$. The fraction of sites common to two species is therefore given by

$$s = z/(x + y - z). \qquad [1]$$

This method of calculating s differs from that used by Upholt and Dawid (16).

One may also calculate $1 - s$, which is not only the fraction of unshared cleavage sites but also the minimum number of base substitutions per site compared. Then, by taking into account the fact that there are n base pairs per cleavage site (for the restriction enzymes used in this study, $n = 6$), one calculates m, the *minimum* number of base substitutions per base pair by which the two species differ in the sites compared

$$m = (1 - s)/n. \qquad [2]$$

The quantity $100m$ is the minimum percent sequence difference at the sites compared.

From s, one also calculates p, the *estimated* number of base substitutions per base pair by which the two species differ at the cleavage sites compared

$$p = (-\ln s)/n. \qquad [3]$$

The derivation of this equation has been given in detail by Upholt (17). This equation corrects for multiple substitutions at the same cleavage site. Later we refer also to $100p$, which is
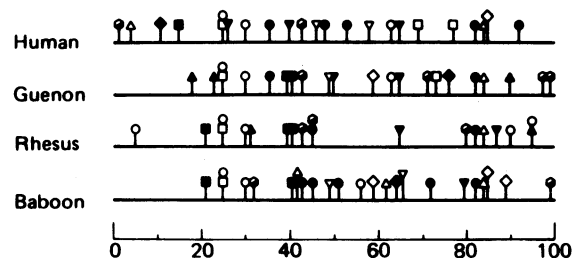

FIG. 1.   Cleavage maps for mitochondrial DNA of human, guenon, rhesus, and baboon. Cleavage sites for each restriction endonuclease are identified by vertical lines topped with the following symbols: □, *Eco*RI; ■, *Bam*HI; ○, *Hind*III; ●, *Xba* I; ▽, *Pst* I; ▼, *Hpa* I; △, *Kpn* I; ▲, *Bgl* II; ◊, *Pvu* II; ◆, *Xho* I; ◒, *Sst* I. The linear maps have been derived from the original, circular maps by assigning the 0 position to the origin of DNA replication; length is given in percent of total. The direction of DNA replication is to the right.

the estimated percent sequence difference at the cleavage sites compared.

RESULTS

Genome Size. The mitochondrial genome did not differ in size among the species compared. Table 1 gives the results of size determinations for eight primate species and three rodent species. None of the values differs significantly from 16,400 base pairs.

Cleavage Maps. Mitochondrial DNA from 4 primate species was treated with 11 restriction enzymes. By measuring the sizes of the DNA fragments produced by single enzymes as well as by pairs of enzymes, we constructed the cleavage maps shown in Fig. 1. The maps are based on analysis of several hundred fragments. Because the number of fragments is so large, the details of the analysis involved in constructing the maps will be published elsewhere (W. M. Brown, M. George, Jr., S. Ferris, H. M. Goodman, and A. C. Wilson).

The four cleavage maps differ from one another at many restriction sites. The sites appear to be distributed in a random manner throughout each genome. No clustering of sites is evident, although there is a notable lack of sites in the nonhuman genomes from 0 to about 20 map units. Only 5 of the 57 positionally distinct sites in the four maps are common to all.

Table 2 summarizes the results of pairwise comparisons of the maps. The fraction of sites (s) shared by each pair ranges from 0.18 to 0.28. From these s values we calculated p and m, respectively the estimated and minimal number of base substitutions per base pair since each pair of species diverged. The average m value for the pairwise comparisons, 0.13, is exceeded by the average p value, 0.25. Thus, the primate mtDNAs

Table 2.   Quantitative comparison of cleavage maps for mtDNA of higher primates*

Species compared	Restriction sites compared	Fraction of sites in common, s	Substitutions per base pair	
			Minimum, m	Estimated, p
Baboon–rhesus	34	0.24	0.127	0.24
Baboon–guenon	37	0.24	0.126	0.24
Rhesus–guenon	32	0.28	0.120	0.21
Human–baboon	39	0.18	0.137	0.29
Human–rhesus	35	0.20	0.133	0.27
Human–guenon	36	0.25	0.125	0.23

* Values have been calculated by the use of Eqs. 1, 2, and 3 after comparison of the cleavage maps shown in Fig. 1.

compared are estimated by the p method to differ by an average of 25% in nucleotide sequence.

Melting of Heteroduplex DNA. The results of a thermostability experiment are consistent with the above results. Within a limited range, the degree of sequence difference between related DNAs can be estimated by measuring the temperature at which a heteroduplex DNA (formed by annealing the related DNAs together) melts and comparing this temperature with the melting temperature for the homoduplexes. In practice, the temperature at which the duplexes are 50% melted, the t_m, is measured because this point can be determined with maximum precision. The difference between the t_m values of the heteroduplex and homoduplex DNAs, the Δt_m, is related to the percent of mismatched bases between the sequences compared.

Mammalian mtDNA is ideally suited to this analysis, because it is possible to prepare isolated complementary single strands due to an unequal distribution of the guanine and thymine residues between the strands. For example, the ratio of guanine in the "H" and "L" strands of mtDNA is >2.2:1 and of thymine >1.2:1 for both human and guenon (8). The H strand of one species can be annealed to the L strand of a second species, and the t_m of the resultant heteroduplex can be compared to the t_m values of the parent homoduplexes. This has been done for human and guenon mtDNAs (8). The absorbance melting profiles for the homo- and heteroduplexes, Fig. 2, indicate no difference in t_m between the human and guenon homoduplexes but a lowering of 21.5°C for the t_m of the heteroduplex. Moreover, the transition from the duplex to the single-stranded state occurs within a temperature range of ≈6°C for the homoduplexes, but the range for the heteroduplex is >35°C, indicating that mismatched bases are distributed in a nonuniform manner in the genome, causing some regions (those most extensively mismatched) to melt early and others (those most extensively matched) to melt late. Similar results have been obtained in a comparison of mtDNAs from two species of frogs (5). Finally, the heteroduplex profile is a relatively smooth, apparently monophasic curve, indicating the absence of detectable segments of the genome that have been completely conserved, also in agreement with the frog study (5). The calculated value for the heteroduplex t_m represents a maximum estimate, because the hyperchromicity at 25°C had not reached a minimum value, as shown by the positive slope of the curve at 25°C (Fig. 2).
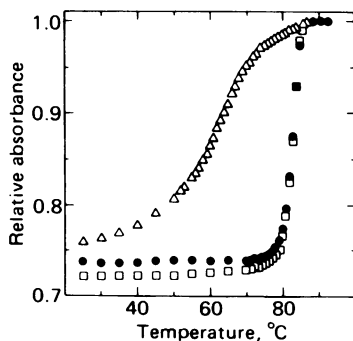

FIG. 2. Thermal stability of heteroduplex and homoduplex mtDNAs. The ordinate value is calculated by dividing the absorbance (at 260 nm) at a given temperature by the absorbance at 90°C. The heteroduplex (△) was formed by annealing equimolar amounts of the human H strand with the guenon L strand. The human (●) and guenon (□) homoduplexes were similarly reconstituted from their respective H and L strands. No difference in t_m was observed between native nicked-circular homoduplexes and homoduplexes reconstituted from the separate strands. All data are from ref. 8.

From the thermostability experiment, it is calculated that human and guenon mtDNA differ by at least 22% in base sequence. The calculation is based on the empirical observation that percent sequence mismatch is approximately equal to Δt_m (19). This result is in agreement with the estimated value, 23%, calculated from the cleavage map comparisons (Table 2).

DISCUSSION

Are the Sample Sizes Adequate? The results we present have been derived from the analysis of mtDNA from only one or two individuals of each species. This is justifiable for this study because there is an approximately 100-fold difference between the magnitude of intraspecific variation and the magnitude of the interspecific variations reported here. In a comparison of 68 cleavage sites in mtDNA from 21 racially diverse humans, the average value for p was 0.002 (unpublished data). The average value for p among the comparisons reported here is 0.25. Even a value of intraspecific variation as high as $p \approx 0.01$, reported in a comparison of mtDNA among three goats and between two sheep (16), would not contribute significantly to the results of the interspecific comparisons reported here. In comparisons among mtDNA from closely related species, however, this justification may not be valid. Individual variation should be assessed in such cases.

Mitochondrial DNA Evolves Fast. Mitochondrial DNA appears from restriction endonuclease and thermostability analyses to evolve unusually rapidly. Species pairs that show little divergence in their single-copy nuclear DNA sequences show extensive divergence in their mtDNA sequences. Table 3 is a comparison of the sequence difference in mtDNA, inferred from restriction maps, with the sequence difference in the single-copy fraction of nuclear DNA, inferred from thermostability studies. For the pairs of primates examined, the mtDNA difference exceeds the nuclear DNA difference by an average of 5-fold, based on m, and 10-fold, based on p. This implies that mtDNA evolves 5 to 10 times faster than single-copy nuclear DNA.

The above estimates for the relative rate of mtDNA evolution are probably conservative. The estimate based on m is a minimum and underestimates the degree of sequence dissimilarity when the probability of a substitution per base pair approaches $1/n$. The value given by p underestimates the difference between two sequences proportionally as the difference between the two sequences increases, and becomes unsatisfactory for sequences that differ at 80% or more of their restriction sites (17). Furthermore, the estimate provided by p is based on the assumption that all restriction sites are equally susceptible to

Table 3. Comparison of the extent of sequence divergence in mitochondrial and nuclear DNA

Species compared	% sequence difference		
	mtDNA*		Single-copy DNA†
	Minimum	Estimated	
Baboon–rhesus	12.7	24	1.4
Baboon–guenon	12.6	24	2.2
Guenon–rhesus	12.0	21	2.0
Human–baboon	13.7	29	4.7
Human–guenon	12.5	23	6.3

* Based on the m and p values in Table 2.
† Calculated from thermostability of heteroduplexes with the assumption that percent sequence difference equals Δt_m. The Δt_m values are from refs. 20 and 21. Some of the nuclear DNA comparisons involving either baboon or guenon were done with other species of the same genus, rather than with *Papio papio* or *Cercopithecus aethiops*.

evolutionary change (17). Evidence that this assumption is invalid comes from thermostability studies, in which the greatly increased breadths of the melting transitions of heteroduplex mtDNAs indicate that a wide range of susceptibility to evolutionary change exists in the mitochondrial genome (see *Results*, Fig. 2, and ref. 5). There is evidence that the *Hin*dIII sites located at map positions 25 and 30, Fig. 1, are highly conserved (ref. 9; unpublished data). As stated by Upholt (17), failure of this assumption results in a value of p that underestimates the amount of substitution.

Further information about the rate of mtDNA evolution may be extracted from Fig. 3, in which p values for 20 species pairs are plotted against times of divergence. We have used the initial slope of the curve to estimate the absolute rate of evolutionary change in this DNA. The rate estimate is 0.02 substitutions per base pair per million years (broken line, Fig. 3), which is 10 times higher than the rate estimate for single-copy nuclear DNA (dotted line, Fig. 3).

Fig. 3 also indicates that p is most accurate for mtDNA comparisons between species that have diverged in the last 5 million years and becomes increasingly less accurate for greater divergence times. By 25 million years (the estimated divergence time of the line leading to humans from that leading to Old World monkeys) the value of p underestimates the degree of substitution predicted from the initial rate by ≈50%. This ex-
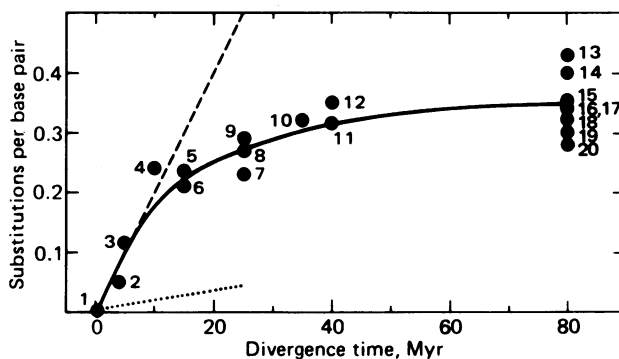

FIG. 3. Dependence of sequence divergence in mtDNA upon time of divergence. On the ordinate, the estimated number of base substitutions that have accumulated per base pair (p) is given for each pair of species compared. This number is calculated from cleavage map comparisons by use of Eqs. 1 and 3. The rate of substitution for mtDNA is obtained from the initial slope of the curve, indicated by the broken line. The rate for single-copy nuclear DNA is obtained from the slope of the dotted line, plotted with the data from Table 3. Each point on the graph corresponds to a comparison of two species or of individuals within a species: 1, mean difference among humans (unpublished data); 2, goat and sheep (16); 3, human and chimpanzee (unpublished data); 4, baboon and rhesus; 5, guenon and baboon; 6, guenon and rhesus; 7, human and guenon; 8, human and rhesus; 9, human and baboon; 10, rat and mouse (22); 11, hamster and mouse (22, 23); 12, hamster and rat (22, 23); 13–20, rodent–primate species pairs (comparison of data from this study with the data in ref. 22). The numbers of restriction sites compared are: 1, 68; 2, 9; 3, 12; 4, 34; 5, 37; 6, 32; 7, 36; 8, 35; 9, 39; 10, 36; 11, 19; 12, 17; 13–20, 23 (mean). Both fossil and protein data were used to estimate the times of divergence. The evidence that protein sequences change at sufficiently constant rates to allow estimation of divergence times has been reviewed (1). The reliability with which fossil evidence can be used to estimate divergence times has also been discussed (24). Until recently, the most uncertain and controversial time in this figure was 5 million years for the chimpanzee–human divergence. This date, based on protein comparisons (1, 25), has now been confirmed by new interpretations of fossil evidence (26). The divergence times among rodents are also controversial; precedence has been given in these cases to the protein evidence (1, 27). For the remaining divergence times, there is satisfactory agreement among estimates based on protein evidence (1, 25) and fossil evidence (see legend to figure 1 of ref. 1; refs. 28, 29).

plains why the apparent mtDNA substitution rate is 12 times the single-copy nuclear DNA rate for comparisons among the three Old World monkeys, but only 5 times that rate for the comparison between Old World monkeys and humans. An increasing deflection of the relationship between p and divergence time is expected (17), but the degree of deflection observed may be accentuated by the presence of highly conserved sites.

Mitochondrial Gene Arrangement Appears Stable. In making the comparisons, we have assumed that no sequence rearrangements (inversions, transpositions, deletions, additions) have occurred and that all changes observed are due to point mutations alone. This assumption is supported by data showing that the arrangement of the mitochondrial origin of replication and the small and large ribosomal RNA (rRNA) genes, relative to both the direction of mtDNA replication and rRNA transcription, are the same in organisms as divergent as humans (30), mouse (31), rat (32), and frogs (33), and a similar arrangement for these elements may also occur in fruit flies (33–35). These studies have also shown that, except for a few transfer RNA (tRNA) genes, the transcribed genetic information carried by mtDNA is contained in one strand only (the strand with the higher buoyant density in a CsCl gradient). In addition, the more dense strand of mtDNA from one species always hybridizes exclusively with the less dense strand from a second species (8). This is true for species as distantly related as humans and frogs (8), thus indicating that the genetic information in common between their mitochondrial genomes has been retained in the same mtDNA strand for approximately 350 million years (36). Finally, the identical sizes of the mtDNAs compared (Table 1) indicate that no additions or deletions have occurred. It thus seems likely that the assumption of no sequence rearrangements in the primate mtDNAs is a reasonable one.

Why Does Mitochondrial DNA Evolve Fast? To explain why the mitochondrial genome evolves so rapidly, one must first recall that evolution results from two basic processes: the occurrence of mutations in DNA and the fixation of mutations in populations of organisms. The rate of evolution (E) is the product of the mutation rate per population (M) and the fraction of mutations fixed (F),

$$E = MF. \qquad [4]$$

In principle, the high rate of mtDNA evolution could be due to a high rate of mutation, to a high rate of fixation, or to both.

The possibility of a high mutation rate deserves consideration. The presence of an average of five ribonucleotides per strand in animal mtDNA, inferred from alkaline and enzymatic digestion data (37), suggests that the editing function of the mtDNA replication complex may be inefficient or lacking. The absence of an enzymatic function capable of the excision and repair of thymine dimers has been well documented (38). The ability of animal mitochondria to repair other types of DNA damage has, by inference, also been shown to be inefficient, if not lacking (39). These factors alone could contribute greatly to a high mutation rate. In addition, mtDNA has a higher turnover rate than nuclear DNA in tissues (40), thus providing more rounds of replication during which errors could be generated.

The possibility of an enhanced chance of fixation also deserves consideration. This could arise from low functional constraints on the mitochondrial gene products. At present, the only genes from animal mtDNA with known functions are those coding for tRNA and rRNA. Thermostability analysis indicates that the genes for the mitochondrial rRNAs change appreciably faster than the corresponding nuclear rRNA genes (5, 8). The

faster rate could be a result of lower functional constraints on the mitochondrial rRNAs. It is as likely, however, that the slower nuclear rRNA rate results from the tandemly repeated structure of these genes and has nothing to do with functional constraints on the rRNAs themselves.

Another factor that could contribute to a high chance of fixation is dispensability, which refers to the probability that an organism will survive and reproduce if the gene is missing or inactive (1). If a given mitochondrial gene were inactive, the organism might survive because the organism is, in a sense, polyploid for mitochondrial genes; each cell contains many mitochondria, each containing at least one copy of the mitochondrial genome (41). A mutation inactivating a given gene in one genome might therefore have little or no effect on the fitness of the organism. However, because little is known either about the genetic consequences of this genome multiplicity or about the functions of the animal mitochondrial genes, except those coding for rRNA and tRNA, it is not yet possible to assess the relevance of the above factors to the fast rate of evolution.

Regardless of the reasons for its high evolutionary rate, mtDNA will be an extremely useful molecule for evolutionary biologists to use in assessing relationships among species and populations that diverged rather recently—e.g., within the past 5–10 million years. By quantitatively defining the genetic distances among such closely related organisms, one will gain deeper insight into the mechanism of speciation, the process by which new species arise.

Note Added in Proof. Masatoshi Nei (personal communication) has developed an alternative method for calculating degree of divergence in nucleotide sequence from cleavage map comparisons. For the species compared in our study, the two methods give similar *p* values.

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