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Strong Variations of Mitochondrial Mutation Rate across Mammals—the Longevity Hypothesis

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Mitochondrial DNA (mtDNA) is the most popular marker of molecular diversity in animals, primarily because of its elevated mutation rate. After >20 years of intensive usage, the extent of mitochondrial evolutionary rate variations across species, their practical consequences on sequence analysis methods, and the ultimate reasons for mtDNA hypermutability are still largely unresolved issues. Using an extensive cytochrome *b* data set, fossil data, and taking advantage of the decoupled dynamics of synonymous and nonsynonymous substitutions, we measure the lineage-specific mitochondrial mutation rate across 1,696 mammalian species and compare it with the nuclear rate. We report an unexpected 2 orders of magnitude mitochondrial mutation rate variation between lineages: cytochrome *b* third codon positions are renewed every 1–2 Myr, in average, in the fastest evolving mammals, whereas it takes >100 Myr in slow-evolving lineages. This result has obvious implications in the fields of molecular phylogeny, molecular dating, and population genetics. Variations of mitochondrial substitution rate across species are partly explained by body mass, longevity, and age of female sexual maturity. The classical metabolic rate and generation time hypothesis, however, do not fully explain the observed patterns, especially a stronger effect of longevity in long-lived than in short-lived species. We propose that natural selection tends to decrease the mitochondrial mutation rate in long-lived species, in agreement with the mitochondrial theory of aging.

Introduction

Mitochondrial DNA (mtDNA) has been the most widely used tool for reconstructing population and species histories, presumably in the first place for practical reasons: mtDNA is relatively easy to amplify, not duplicated, typically nonrecombining, supposedly nearly neutral, and highly variable between and within species. Several potential pitfalls associated with this marker, however, have been discussed in recent years, including evidence for recombination, adaptive evolution, and mutation hot spots (Eyre-Walker et al. 1999; Ballard and Whitlock 2004; Hurst and Jiggins 2005; Bazin et al. 2006; Galtier et al. 2006). Obviously, understanding the forces that drive the molecular evolution of mtDNA is necessary for a proper use of this molecule as a population genetic, phylogeographic, and phylogenetic marker. In this paper, we investigate one particularly intriguing feature of the animal mitochondrial genome, namely its hypermutability. The mitochondrial mutation rate is orders of magnitude higher than the nuclear one (Lynch et al. 2006). This hypermutability largely explains the high level of within-species mtDNA heterozygosity (Bazin et al. 2006) and the high amount of homoplasy detected in animal mtDNA phylogenetic analyses (Springer et al. 2001; Delsuc et al. 2003; Galewski et al. 2006). mtDNA hypermutability also causes numerous diseases in human (Florentz et al. 2003; Wallace 2005). The question of why such an elevated mutation rate has evolved in animals is open and debated (Rand 1994; Palmer et al. 2000; Hellberg 2006).

The mtDNA mutation rate is high on average and, additionally, appears variable between animal lineages, as indicated by evolutionary analyses (Martin et al. 1992; Martin and Palumbi 1993; Rand 1994; Mindell et al. 1996). Variations of amino acid substitution rates have been reported—a

well-known example is the accelerated evolution of the mitochondrial proteins of anthropoid primates (Grossman et al. 2004). Protein evolution, however, is partly governed by natural selection, so that such studies cannot be interpreted directly in terms of mutation rate. Focusing on synonymous positions would appear a better approach—under the assumption of neutral evolution, the substitution rate is equal to the mutation rate (Kimura 1968). Assessing the variation of synonymous substitution rates between lineages, however, is technically problematic because of saturation: the high mtDNA mutation rate can lead to the frequent occurrence of multiple hits at the same site, rapidly dissolving the phylogenetic and evolutionary signal (Spradling et al. 2001; Springer et al. 2001; Galewski et al. 2006), especially for fast-evolving lineages.

Achieving an accurate and unbiased estimation of the neutral mitochondrial substitution rate and its variations would benefit to many fields of evolutionary biology (Mindell and Thacker 1996). In phylogenetic studies, a correct assessment of the level of saturation in mtDNA data sets would help deal with homoplasy and long-branch attraction (Spradling et al. 2001; Springer et al. 2001; Galewski et al. 2006). An accurate estimation of the mtDNA mutation rate is also obviously required for molecular dating. The famous but rather rough “2% per million year” calibration of the molecular clock, proposed 3 decades ago for primate mtDNA (Brown et al. 1979), is still sometimes used for various mammalian or even bird taxa (e.g., Garcia-Moreno 2004; Weir and Schluter 2007). It is important to appraise whether such gross approximations are acceptable or not. Knowledge about the variations of the mtDNA mutation rate, finally, would also help interpret patterns of within-species diversity across animals, a topic recently debated in the literature (Bazin et al. 2006; Lynch et al. 2006; Nabholz et al. Forthcoming).

Besides these practical aspects, analyzing the variations of mtDNA mutation rate across species might shed light onto the fundamental issue of its determinism—and perhaps onto the ultimate reasons for mtDNA hypermutability. Several theories have been proposed to explain differences in rates of molecular evolution between lineages.

Key words: mutation rate, mitochondrial DNA, theory of aging, mammals.

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The generation time hypothesis (Li et al. 1987; Ohta 1993; Moers and Harvey 1994; Bromham et al. 1996) expresses that species with a shorter generation time experience a higher number of DNA replication rounds per year and thus a higher number of replication errors per time unit. This model applies to replication-dependent mutations only and assumes a constant replication error rate across species. It should affect the nuclear and organellar genomes with the same magnitude. It predicts an inverse relationship between generation time and neutral substitution rate.

The metabolic rate hypothesis (Martin et al. 1992; Martin and Palumbi 1993; Martin 1995; Nunn and Stanley 1998) links the mtDNA mutation rate to the by-production of mutagenic free radicals—the so-called reactive oxidative species (ROS)—during mitochondrial respiration (Wallace 2005). This hypothesis was recently put forward when Gillooly et al. (2005) proposed and tested a model explaining the relationship between mass-specific metabolic rate, which varies with body mass and temperature, and the mitochondrial substitution rate (Gillooly et al. 2005). This model applies to replication-independent mutations. It predicts a linear relationship between metabolic rate and neutral substitution rate.

Another body of literature, mainly from the biomedical field, suggests a distinct interpretation of the link between free radicals production and mutation rate, which we now formalize. In the mid 1950s, Denham Harman (1957) introduced the mitochondrial theory of aging, which states that the free radicals generated throughout the life span of an organism contribute to, and even cause, its senescence. This theory has been supported by the report of an inverse relationship between oxidative damage marker concentration and maximum life span in mammals (Barja and Herrero 2000). The mitochondrial theory of aging posits that somatic mutations occurring on mtDNA, by affecting the efficiency of respiratory proteins, result in an increase of the rate of free radical leakage, which in turn increases the mutation rate—a positive feedback loop eventually resulting in aging (Kujoth et al. 2007). It is therefore tempting to propose a selective hypothesis explaining the variations of the mtDNA mutation rate: the selective pressure for a reduced mutation rate should be higher in long-lived species than in short-lived ones. This model predicts a negative correlation between mutation rate and longevity.

This study, therefore, has 2 connected goals. First, we want to estimate as accurately as possible, and on a relatively wide taxonomical range, the lineage-specific neutral mtDNA substitution rates, taken as estimates of the mutation rate. This means overcoming the problem of saturation (i.e., frequent multiple hits) at synonymous positions in fast-evolving lineages and calibrating divergence times. Then we want to progress in our understanding of the determinism of mtDNA substitution rate variations by distinguishing between the 3 hypothesis evoked above, namely the generation time hypothesis, the metabolic rate hypothesis, and the longevity hypothesis.

We used mammals as our model taxon for 3 reasons. First, mtDNA sequence data are available in a very large number of mammalian species—more than one-third of the total number of living mammals. We chose to focus on 1 gene, cytochrome *b*, because it covers most of the spe-

cies for which mtDNA sequence data are available in GenBank and because its evolution appears representative of the whole mitochondrial genome (Waddell et al. 2007). The second advantage of mammals is that life history traits in this group are highly variable and well documented. Body mass was taken as an indicator of the metabolic rate—at constant temperature, the metabolic rate scales in quarter-power allometric relation with body mass (Gillooly et al. 2001, 2005). Generation time was estimated by the age of female sexual maturity. Life span, finally, was measured by the maximal recorded longevity. Mammals, finally, is an important and popular taxon for biodiversity and conservation studies, in which the mitochondrial marker has been extensively used. Properly characterizing the evolutionary dynamics of mtDNA in this group would appear a valuable and useful achievement.

Materials and Methods

Sequence Data

We extracted from National Center for Biotechnology Information/GenBank all the mammalian sequences for which the complete cytochrome *b* sequence was available. We selected 1 sequence per species. Accession number is available at <http://kimura.univ-montp2.fr/~benoit/data.html>. This was also done for 4 nuclear genes: interphotoreceptor retinoid-binding protein exon 1, (IRBP, 664 species), α adrenergic receptor 2B (151 species), breast cancer 1 (168 species), and von Willebrand factor (294 species). All 4 genes yielded qualitatively similar results but not always easy to compare because of differences in the number of available sequences. This is why we only provide the IRBP results.

Substitution Rate Estimation

The whole cytochrome *b* data set was split in groups within which sequence divergence is moderate. To achieve this, the GenBank taxonomic classification was traversed recursively, starting from mammalian orders and moving toward lower levels. For each traversed taxonomic group, we 1) gathered the corresponding third codon position sequences, 2) aligned sequences using ClustalW (Thompson et al. 1994), 3) built a maximum likelihood phylogenetic tree using PHYML (Guindon and Gascuel 2003), general time reversible + Gamma model of nucleotide evolution, and 4) calculated the pairwise patristic distances for every pair of species (defined as the sum of branch lengths in the path connecting the 2 species in the tree). When the median pairwise distance between species was lower than 0.4, the current taxonomic group was selected for further analysis, and the taxonomic traversal was stopped. Otherwise, the above procedure was applied to underlying taxonomic groups. The procedure was stopped at the genus level; even those genera that did not satisfy the divergence criterion were selected. This might lead to a slight underestimation of the evolutionary rate in the most fast-evolving lineages. The 0.4 threshold was obtained empirically by examining by eye the plot between uncorrected and maximum likelihood-estimated pairwise divergences—the relationship is more

Table 1
Fossil Calibration Dates Used in this Study (Myr)

Divergence	Maximal Date	Minimal Date	Reference
Monotrema/Theria	191.1	162.5	Benton and Donoghue (2007)
Didelphimorphia/Australidelphian	71.2	61.5	Benton and Donoghue (2007)
Diprotodontian/Dasyuromorphia	74	54	Drummond et al. (2006)
<i>Phascogale/Dasyurus</i>	10	24	Drummond et al. (2006)
<i>Bradypus/Dasybus</i>	Free	58	Delsuc et al. (2004)
Feliformia/Caniformia	63.8	42.8	Benton and Donoghue (2007)
<i>Equus/Rhinoceros</i>	58	54	Springer et al. (2003)
<i>Hippopotamus/Cetacea</i>	Free	52	Springer et al. (2003)
Pteropodidae/Rhinolophidae	60	43	Springer et al. (2003)
Soridae/Erinaceidae	Free	63	Springer et al. (2003)
<i>Bos/Sus</i>	65	48	Springer et al. (2003)
<i>Bos/Cervus</i>	23.8	16.4	Hassanin and Douzery (2003)
<i>Pan/Homo</i>	10	6.5	Benton and Donoghue (2007)
<i>Macaca/Homo</i>	33.9	23	Benton and Donoghue (2007)
Primates/Rodentia	100.5	61.5	Benton and Donoghue (2007)
<i>Apodemus sylvaticus/Apodemus mystacinus</i>	Free	7	Michaux et al. (2002)
<i>Gerbillus/Gerbilliscus</i>	9	7	Neumann et al. (2006)
<i>Xerus/Sciurus</i>	Free	36	Mercer and Roth (2003)
<i>Cavia/Octodon</i>	31	Free	Huchon and Douzery (2001)
<i>Mus/Rattus</i>	12.3	10	Benton and Donoghue (2007)
<i>Bolomys/Akodon</i>	5.3	3.5	Smith and Patton (1991)
<i>Neofiber/Ondatra</i>	4.3	2.2	Repenning (2001)

or less linear for corrected divergence values lower than 0.4. Sequence management and GenBank taxonomic exploration were achieved using homemade C++ programs based on the Bio++ libraries (Dutheil et al. 2006).

Within each selected group, the species-specific relative neutral substitution rate was estimated by applying MULTIDIVTIME (Thorne et al. 1998) to third codon positions (model F84). The Monte Carlo Markov Chain was run for 1 million generations after a burn-in of 200,000 generations that achieved stationarity. The rates assigned to terminal branches were taken as species-specific substitution rate estimates. This relative rate estimation was also performed using amino acid sequences for comparative purposes.

Groups were dated by applying MULTIDIVTIME to amino acid sequences (model mtREV + Gamma). The Monte Carlo Markov Chain was run for 200,000 generations after a burn-in of 200,000 generations. The 22 fossils calibration points we used are given in table 1. For computational reasons, the amino acid data set was made of 2 selected sequences per group—the whole data set was too large. The 2 representative species of each group were randomly chosen, with the constraint that their most recent common ancestor had to be the ancestral node of the considered group, thus avoiding the use of too closely related species. We manually generated the phylogenetic tree connecting these taxa according to the molecular phylogeny literature (Springer et al. 2003; Stepan et al. 2004; Teeling et al. 2005). The amino acid analysis provided 1 date estimate per group, allowing the conversion of relative lineage-specific substitution rates into absolute lineage-specific substitution rates.

We replicated the dating analysis 10 times using different pairs of representative sequences. The correlation of rate estimates between replicates was very high (R^2 between 0.84 and 0.98), indicating a good robustness to species sampling. Results were also essentially unchanged when we modified the prior distribution of the ancestral rate (mean and vari-

ance), or of the autocorrelation parameter (mean and variance), or when we used the MCMCTREE (Rannala and Yang 2007) implementation of the autocorrelated model of rate evolution. An analysis of mitochondrial whole-genome sequences in Cetacea and Carniformia, for which a good sample of complete sequences is available, yielded results very similar to the cytochrome *b* analysis.

Life History Data

Body mass, age of female sexual maturity, gestation duration, and longevity data were obtained from the AnAge database (de Magalhaes et al. 2005). Measuring the generation time is not straightforward because it depends on the age structure of species (Charlesworth 1994), about which data are lacking for most mammals. We took either female sexual maturity or the sum of female sexual maturity and gestation duration as rough approximations of the generation time. Both gave similar results. Life history data and rate estimation are available at <http://kimura.univ-montp2.fr/~benoit/data.html>.

Statistical Analyses

The relationships between life history traits and substitution rate variations were investigated using the general linear model implemented in R (R Development Core Team 2004). In order to assess the relative contribution of each variable, we performed these analyses using the species for which the 3 traits are documented ($N = 410$). Results obtained using the maximal number of species available for each life history trait were qualitatively unchanged (available at <http://kimura.univ-montp2.fr>). Substitution rates and life history traits were log transformed. We used the type II sum of squares in 2-way analysis of variance (ANOVA), as implemented in the CAR package (Fox

2002). This analysis is independent of the order in which factors are introduced; it is equivalent to testing 1 variable against all other variables in the type I ANOVA. Phylogenetic control was achieved using the phylogenetic contrast method (Felsenstein 1985) as implemented in the APE package (Paradis 2007).

Results

Estimating Mammalian mtDNA Third Codon Position Substitution Rates

The rate of evolution of a molecule is the average number of nucleotide substitutions occurring per time unit. To estimate this variable, say, for a pair of species, one should simply divide the number of changes having occurred during the divergence of the 2 species by twice the date of this divergence, typically obtained from the fossil record. In practice, we face several problems: 1) we want to consider the divergence of an arbitrary number of species, not just pairs of species; 2) the substitution rate can vary between lineages; 3) the fossil record is scarce, so that many divergence dates are unknown; and 4) estimating the number of changes having occurred in various lineages is difficult for high levels of sequence divergence, when saturation obscures the signal. Several methods have been developed to cope with these problems (Sanderson 1997; Yoder and Yang 2000). The sophisticated Bayesian methods (Thorne et al. 1998; Drummond et al. 2006; Rannala and Yang 2007) take into account the phylogeny of the analyzed species, model rate variations across lineages, incorporate constraints from the fossil record, and aim at correcting for multiple substitutions thanks to the use of Markov models of nucleotide evolution. In the case of mtDNA third codon positions, however, divergence levels can be so high that applying such methods to the whole data set would presumably lead to a severe underestimate of the fastest rates.

To address this specific problem, we used a 3-step method making use of the decoupled nonsynonymous (i.e., amino acid) and synonymous mtDNA evolutionary rates. At step 1, we partitioned the data set in monophyletic groups of sequences such that the typical divergence within a group is lower than some threshold. At step 2, we assigned a divergence date to each group. This was done by building a data set made of the amino acid sequences of 2 species per group and applying the MULTIDIVTIME program (Thorne et al. 1998) with appropriate fossil calibration. At step 3, we estimated the species-specific neutral nucleotide substitution rate. This was achieved by applying MULTIDIVTIME to third codon positions within each group, using the divergence dates inferred at step 2 as calibrations. So the relatively slow-evolving protein sequences were used to date the mammalian tree by propagating fossil information to every node of the tree, then these dates allowed us to estimate the substitution rates of fast-evolving third codon positions using recent, saturation-free divergences only.

The same strategy was applied to a nuclear marker for comparative purposes. The IRBP was chosen because it is the only nuclear gene available in a number of species comparable with the cytochrome *b* data set.

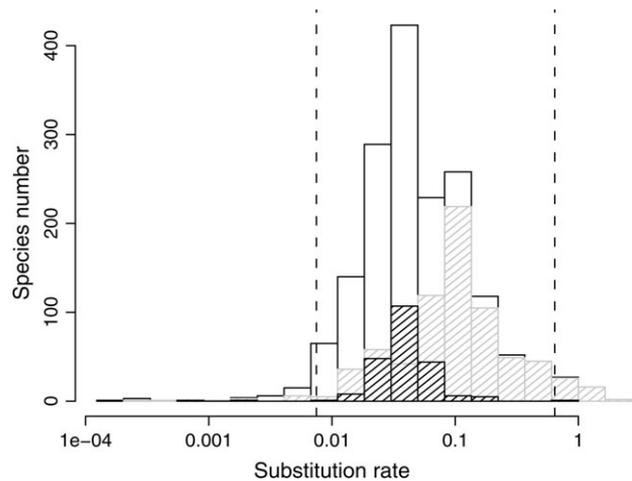


FIG. 1.—Distribution of the cytochrome *b* neutral substitution rate across 1,696 mammalian species. The substitution rate is log transformed. Black: whole data set; shading black: Chiroptera ($n = 222$); shading gray: Rodentia ($n = 734$); and vertical dotted lines: 2.5% and 97.5% percentiles. Substitution rates are in unit of substitution per third codon position per million year.

Extreme Variation of mtDNA Neutral Substitution Rate across Mammalian Species

We retrieved from GenBank the 1,938 mammalian species for which the complete cytochrome *b* sequence was available. They were partitioned in 175 groups, within which the median divergence between sequences was lower than 0.4 substitution per third codon position. These groups corresponded to 103 genera, 15 subfamilies, 43 families, 4 suborders, and 10 orders. The heterogeneity of these taxonomic levels is in itself suggestive of a large variation of substitution rate between lineages. Groups include a total of 1,696 species (species with no close relative having been removed), that is, nearly one-third of living mammals. Orders represented by more than 100 species are Rodentia (734), Cetartiodactyla (237), Chiroptera (222), Carnivora (131), Primates (113), and Eulipotyphla (104).

The mean estimated mammalian cytochrome *b* substitution rate at third codon positions was 0.098 substitution/site/Myr and the median 0.0427. The distribution of estimated mitochondrial substitution rates across species is approximately log normal and shows a very large variance (fig. 1, black histogram): the 2.5% and 97.5% percentiles are 0.007 and 0.6434 substitution/site/Myr, respectively. So even if we consider the 5% most extreme species as unreliable outliers, the estimated rates span 2 orders of magnitude, revealing an unexpectedly high level of variation of mtDNA third codon position substitution rate across mammalian lineages. To make this result more tangible, table 2 provides the detailed results for 6 pairs of closely related species for which the divergence date is known with good confidence—the first 3 are slowly evolving, the last 3 fast evolving. The Balushistan gerbil (*Gerbillus nanus*) evolves at a rate of 0.741 substitution/site/Myr (confidence interval [CI] 0.446–1.074), that is, >100 times faster than the Bryde's Whale (*Balaenoptera borealis*, 0.007 substitution/site/Myr, CI: 0.003–0.012). Cytochrome *b* third codon positions are renewed every 1–2 Myr, in average, in the

Table 2
Substitution Rate of Particularly Slow-Evolving (top) and Fast-Evolving (bottom) Species of the Data Set

Species 1	Species 2	Rate 1 ^a	Rate 2 ^a	Divergence	Reference
<i>Balaenoptera borealis</i>	<i>Balaenoptera brydei</i>	0.007 (0.003–0.012)	0.007 (0.003–0.012)	6.9 ± 2.2	Sasaki et al. (2005)
<i>Megaptera novaeangliae</i>	<i>Balaenoptera physalus</i>	0.008 (0.005–0.020)	0.008 (0.005–0.020)	15.3 ± 3.0	Sasaki et al. (2005)
<i>Homo sapiens</i>	<i>Pan Troglodytes</i>	0.011 (0.003–0.009)	0.008 (0.004–0.010)	8.25 ± 1.75	Benton and Donoghue (2007)
<i>Mus indutus</i>	<i>Rattus norvegicus</i>	0.223 (0.113–0.413)	0.098 (0.021–0.195)	11.5 ± 0.8	Benton and Donoghue (2007)
<i>Apodemus sylvaticus</i>	<i>Apodemus mystacinus</i>	0.220 (0.082–0.500)	0.258 (0.087–0.542)	7.9 ± 0.3	Michaux et al. (2002)
<i>Gerbillus nigeriae</i>	<i>Gerbillus nanus</i>	0.728 (0.087–1.170)	0.741 (0.446–1.074)	6.0 ± 0.92	Neumann et al. (2006) and 4.12 ± 0.9 Chevret and Dobigny (2005)

NOTE.—Divergence dates were taken from the cited references.

^a Rates are in unit of substitution per third codon position per million years. Confidence intervals are given within parenthesis.

fastest evolving mammals (e.g., mice, gerbils, and voles), whereas it takes >100 Myr in slow-evolving lineages (e.g., whales, kangaroos, and hominids).

A hierarchical ANOVA shows that the order taxonomic level explains 21% of the variance of substitution rate across species. The fastest evolving order, Rodentia, averages 0.176 substitution/site/Myr, which is 20 times faster than in Cetacea (average 0.010 substitution/site/Myr). Figure 2 shows the variation between orders. The family level, with 75% of explained variance, is the taxonomical level best explaining variations of substitution rate. Rodentia, for instance, include not only very fast-evolving families (e.g., Muridae) but also slow-evolving ones (e.g., Bathyergidae).

The average mammalian cytochrome *b* neutral substitution rate is nearly 60 times higher than the nuclear one, as estimated from IRBP (mean IRBP third codon position rate:

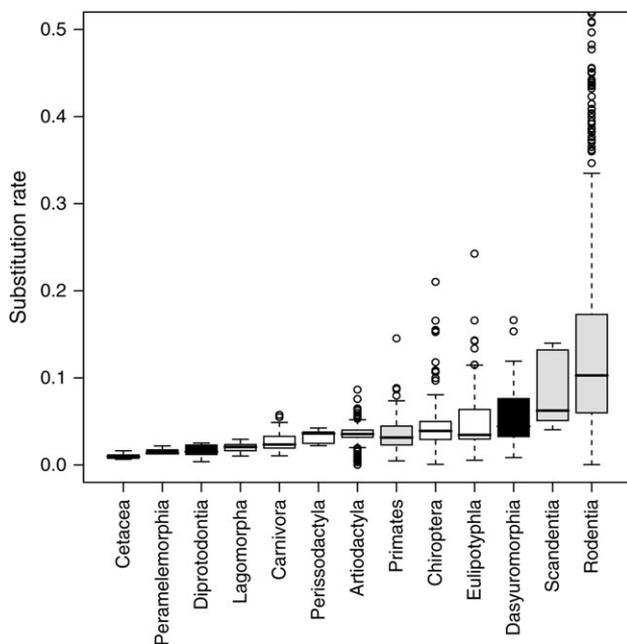


FIG. 2.—Within-order distributions of the cytochrome *b* neutral substitution rate. Orders represented by more than 5 species are shown. Light gray: Euarchontoglires; white: Laurasiatheria; and dark gray: Metatheria. Horizontal bars give the median of substitution rate distributions; boxes give the quartiles; whiskers extend to 1.5 times the interquartile range; and circles are for outliers. The Rodentia distribution includes values higher than 0.5, not shown for clarity. Substitution rates are in unit of substitution per third codon position per million year.

0.00083 substitution/site/Myr, 664 species). IRBP, in addition, does not show the extreme level of variation detected in mtDNA. Without paying any attention to the accuracy of estimates and the removal of outliers, the ratio of the fastest to the slowest estimated rate is only 9 as far as IRBP is concerned. The cytochrome *b* and IRBP rates are significantly correlated across species ($R^2 = 0.265$, P value < 0.0001, $N = 364$, not shown), but the relationship is not linear. In fast-evolving mammals, the mitochondrial substitution rate can be 100 times higher than the nuclear one (e.g., 136 in Muridae, Rodentia), whereas this ratio is lower than 20 in slow-evolving mammals (e.g., 15 in Hominidae, Primates).

Mitochondrial lineage-specific substitution rates were estimated from amino acid sequences for comparative purposes. Nonsynonymous and third codon position substitution rates were well correlated ($R^2 = 0.264$, P value < 0.0001). The median estimated rate was 0.003 amino acid substitution per site per million year. The Rodentia average was 0.088, that is, ~50 times faster than in Carnivora (0.0015). This shows that even nonsynonymous substitution rates are highly variable between mammalian lineages and that the saturation problem also applies at the amino acid level in fast-evolving groups.

Determinants of Substitution Rate Variations

Having resolved the problem of saturation, and estimated with reasonable accuracy the neutral substitution rate, we examined the possible causes of the detected variations by examining 3 life history traits: age of sexual maturity, body mass, and longevity. The cytochrome *b* substitution rate at third codon positions is strongly negatively correlated to each of these variables in log–log linear relationship (fig. 3, table 4). These relationships are in agreement with the 3 explanatory hypotheses evoked above. Female sexual maturity explains the highest fraction of substitution rate variance across species ($R^2 = 0.261$). Combining it with gestation duration—that is, using female sexual maturity + gestation duration as an estimate of generation time—did not improve significantly the relationship (results not shown). Body mass was the life history trait that explains the lowest amount of variance ($R^2 = 0.247$). We failed, moreover, to recover the slope predicted by Gillooly et al. (2005). These authors predicted that, at constant internal organism temperature, body mass and substitution rate should follow an allometric relationship with exponent equal to $-1/4$. Their analysis of 14 pairs of vertebrate

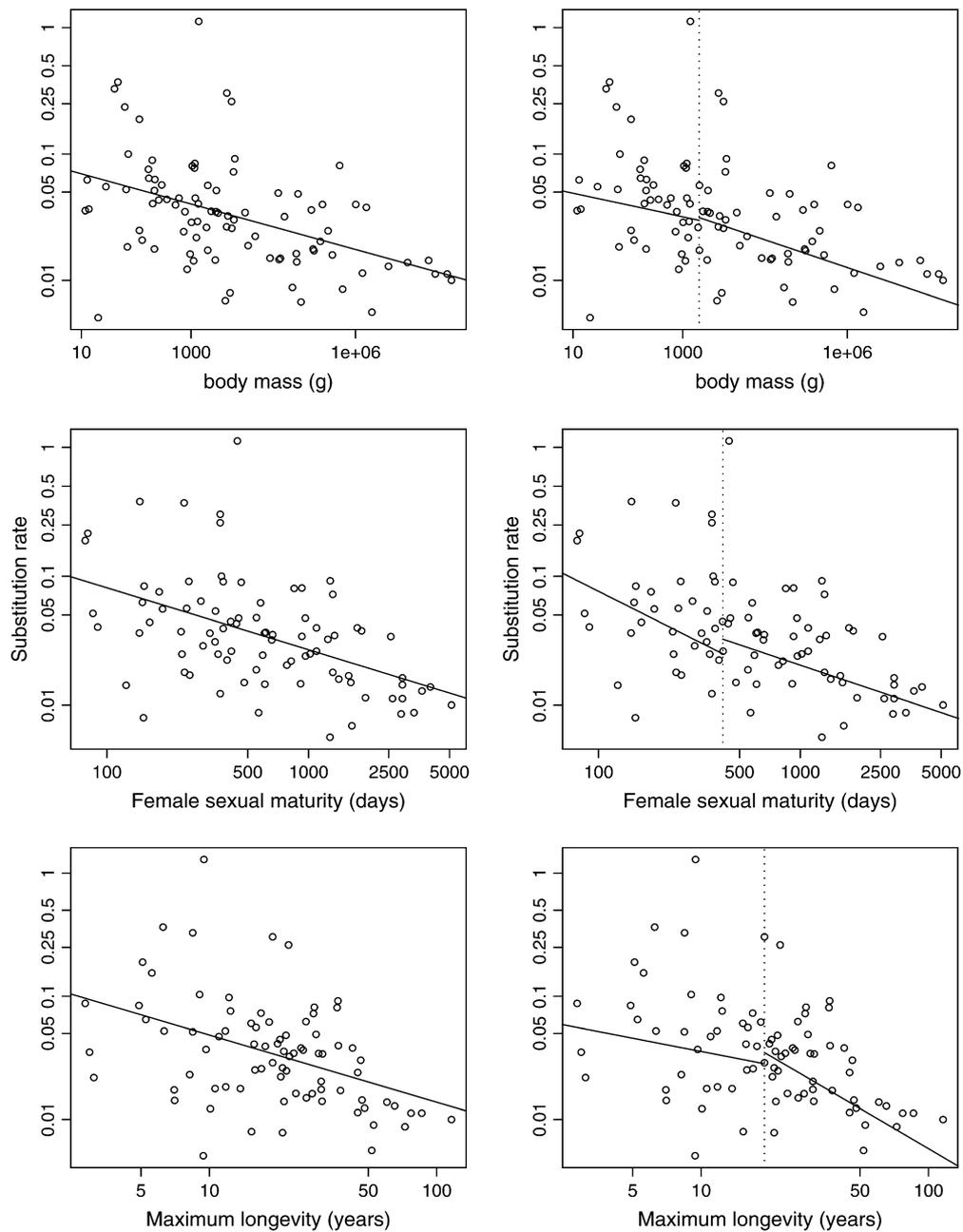


FIG. 3.—Relationship between substitution rate and life history traits. Each dot is for one family. Left panels: a single regression. Right panels: 2 independent regressions for families below versus above the median life history trait (vertical dashed lines). Substitution rates are in unit of substitution per third codon position per million year.

species was consistent with this prediction. Our 643-species data set yielded a slope of -0.132 ± 0.010 (type 1 regression) or -0.139 ± 0.010 (orthogonal regression) between log substitution rate and log body mass.

The 3 life history variables are highly positively correlated with each other, making the distinction between the relative contribution of each factor problematic (Bromham et al. 1996). We performed multiple regressions of substitution rate against every combination of 2 variables with interaction (see supplementary materials). The model best explaining substitution rate variations was $\log(\text{sexual maturity}) + \log(\text{longevity}) + \log(\text{body mass})$ ($R^2 = 0.296$,

P value < 0.0001). So despite the strong correlation between these 3 life history traits, they appear to have distinctive and cumulative effects on the determinism of substitution rate variations.

We checked whether these results were robust to the removal of phylogenetic effects. Phylogenetic inertia is suspected to be high in our data set because taxonomy explains a substantial fraction of mtDNA substitution rate variations and because the life history traits we use are known to be linked to the phylogeny. We therefore used the phylogenetic contrast method (Felsenstein 1985) to regress phylogeny out of the analyses. The effects of the 3 life history traits

on substitution rates were still significant after phylogenetic control (table 4). Their relative strengths, however, were different: the sexual maturity effect was decreased and even disappeared when combined with longevity in a multiple regression. Body mass was the variable explaining the largest percentage of substitution rate variance after phylogenetic control.

The longevity-related model we formalized (see Introduction) invokes strong selective pressure for reduced mtDNA mutation rate in long-lived mammals, for which too high a mutation rate could result in premature senescence, but relaxed selection in short-lived mammals, for which a larger range of mutation rates would be acceptable. To test this prediction with split the data set in 2 subsets of equal size according to species longevity (median: 19.8 years) and performed simple and multiple regressions separately for the 2 subsets (table 5). In agreement with our prediction, the effect of longevity was much stronger for long-lived ($R^2 = 0.198$) than short-lived ($R^2 = 0.098$) mammals, whereas the effects of body mass and sexual maturity were comparable on both sides of the longevity threshold (table 5). Figure 3 also shows a substantial shift in the slope of the substitution rate/longevity relationship between short-lived and long-lived species, whereas this effect is not detected for sexual maturity and body mass. Highly similar results were obtained when we split the data according to body mass or sexual maturity medians as threshold (data not shown). Interestingly, multiple regressions revealed that the major determinant of substitution rate variations is female sexual maturity in short-lived species but longevity in long-lived species (table 5).

The shift in slope and correlation coefficient for longevity between short-lived and long-lived mammals was robust to the phylogenetic control, to the removal of species with a longevity higher than 50 years, and to the removal of the particularly slow-evolving Cetaceans (results not shown). This result is consistent with the hypothesis of an optimized mitochondrial mutation rate in long-lived mammals—and uneasy to explain otherwise. The longevity-related selective hypothesis is congruent with the significant decrease of between-species variance in substitution rate with longevity observed in figure 3—the long-lived species would be under a stronger selective pressure according to this model. This effect is also revealed by introducing the squared longevity as an additional explanatory factor. Squared longevity explained the highest fraction of variance of substitution rate across species ($R^2 = 0.266$) when analyzed solely and withdrew the effect of longevity in a 2-way regression (data not shown), indicating that the effect of longevity is not linear. Finally, the effect of longevity on substitution rates was not higher, and was even lower, in long-lived ($R^2 = 0.0003$) than in short-lived ($R^2 = 0.1057$) species as far as the nuclear IRBP gene was concerned. So this result appears specific to mtDNA.

Relative Part of Transitions and Transversions in Substitution Rate Variation

Mammalian mtDNA undergoes much more transitions (A ↔ G, C ↔ T) than transversions. We investigated whether the variations in substitution rate we report are

due only to changes of the transition rate or to both transitions and transversions. We estimated the third codon position transversion rate variation by recoding the data set as purines and pyrimidines before applying the methodology described above. The average rate was 0.0059 transversion/site/Myr; transversions represent only 6% of all the substitutions. The transversion rate was correlated to the transition rate ($N = 1595$, $R^2 = 0.692$, P value < 0.0001). The variation of transition and transversion rates appears comparable. The house mouse *Mus musculus*, for instance, evolves ~30 times faster than the blue whale *Balaenoptera musculus* as far as mitochondrial transversions are concerned. Consequently, significant correlations between the transversion rate and life history traits were detected (sexual maturity, $R^2 = 0.280$; body mass, $R^2 = 0.136$; longevity, $R^2 = 0.262$).

Discussion

Reliability of Divergence Date Estimates

The estimation of substitution rates heavily relies on the accuracy of date estimates. We compared our estimated divergence dates with previous molecular studies which combined the information from several genes, including nuclear ones (Hasegawa et al. 2003; Springer et al. 2003; Steppan et al. 2004; Drummond et al. 2006). Our cytochrome *b* date estimates are largely congruent with published ones (table 3), especially for recent divergences (e.g., *Homo*—*Hylobates*, Hominidae—Cercopithecidae). Some older divergence dates appear overestimated by the cytochrome *b* analysis, however, like the primary divergence of Chiroptera or Eulipotyphla (table 3). It should be noted that the oldest divergences are not used to estimate substitution rates because third codon positions are only analyzed within groups of relatively recent origin. Standard deviations around the estimated dates are significantly larger in this study than in multigene analyses, presumably because a single gene is used. We incorporated as many fossil calibration points as possible (table 1) and by this way obtained reasonable estimates for recent dates.

A Highly Variable mtDNA Substitution Rate

According to our analysis, the mtDNA neutral substitution rate varies by 2 orders of magnitude across mammalian species. Previous studies had noticed that the mtDNA rate varies across lineages, but none detected the extreme levels of variations we report, for various reasons: smaller data set (Martin et al. 1992; Martin and Palumbi 1993), lack of a fossil information (Mindell et al. 1996; Bromham et al. 1996), or inappropriate treatment of saturation, even at the amino acid level (Gissi et al. 2000; Bininda-Emonds 2007). Strong substitution rate variations were revealed in this study because the estimation of dates and rates was obtained from essentially independent sources, namely amino acids and third codon positions, respectively. When we performed a single MULTIDIVTIME run on third codon positions using the whole data set and the same fossil calibration points, the ratio between the most extreme estimated substitution rates was only 10, that is, severely underestimated (data not shown).

Table 3
Comparison of Cytochrome *b* and Multilocus Divergence Date Estimates (Myr)

Divergence	Cytochrome <i>b</i>	Multilocus	Reference
Hominidae—Cercopithecidae	27.9 (23.2–33.5)	34.6 (33–36.2)	Springer et al. (2003)
<i>Homo</i> — <i>Hylobates</i>	16.1 (10.4–22.8)	21.7 (20.7–22.7)	Springer et al. (2003)
<i>Homo</i> — <i>Pan</i>	8.2 (6.6–9.9)	7.4 (6.7–8.1)	Springer et al. (2003)
<i>Mus</i> — <i>Rattus</i>	11.7 (10.4–12.3)	16.2 (15.8–16.6)	Springer et al. (2003)
Eulipotyphla ancestor	78.0 (63.9–101.6)	61 (57.9–64.1)	Springer et al. (2003)
Odontoceti—Mysticeti	34.4 (8.0–53.5)	25.5 (23.5–27.5)	Springer et al. (2003)
<i>Ceratotherium</i> — <i>Rhinoceros</i>	24.36 (12.6–39.5)	26.1 (23.8–28.4)	Springer et al. (2003)
Chiroptera ancestor	67.2 (52.6–83.5)	65.2 (62.3–68.1)	Springer et al. (2003)
Feliformia—Caniformia	53.0 (43.4–63.0)	49 (45.3–51.7)	Springer et al. (2003)
<i>Ochotona</i> — <i>Lepus</i>	45.5 (36.1–57.2)	51 (48–54)	Springer et al. (2003)
<i>Phascogale</i> — <i>Dasyurus</i>	20.2 (13.3–23.8)	14.4 (9.7–18.9)	Drummond et al. (2006)
Dasyuridae—Diprotodontia	59.0 (54.2–66.3)	64.2 (52.2–70.9)	Drummond et al. (2006)

This result has important practical implications in population genetics, molecular evolution, phylogeny, and molecular dating. Cytochrome *b* third codon positions should not be used for phylogenetic analyses in rodents, for instance, even at the family or subfamily level, whereas they can safely be trusted in Cetaceans. Molecular dating studies relying on mtDNA require a cautious calibration step. In the absence of fossil data, one should refrain from estimating divergence dates based on “generic” estimates such as 2% per million year. This can easily lead to an underestimation or an overestimation of divergence dates by a factor of 10. This and other studies, finally, highlight the necessity to account for variations of mutation rate when interpreting within-species diversity patterns. For instance, we would expect the average rodent species to be 10 times more polymorphic than the average primate species irrespective of population size, population structure, and selective effects.

It should be recalled, finally, that the substitution rate we estimated is an average over all cytochrome *b* third codon positions. Mutation hot spots have been detected in mammalian mitochondrial coding sequences, and the position of these hot spots appear highly variable in time and between species (Galtier et al. 2006). The dynamics of mtDNA mutation is only partly described by the lineage-specific averages we provide. Full-genome analyses of the between-sites and between-lineages variations would appear necessary for a better understanding of the molecular mechanisms underlying the frequent changes of mtDNA mutation rate we detect.

Understanding the Determinants

Having properly estimated the species-specific mtDNA substitution rate at third codon positions and detected an unexpectedly high level of variation across lineages, we examined possible determinants of this variation.

These changes being presumably neutral, we considered models relevant to the mtDNA mutation process, namely the metabolic rate hypothesis, the generation time hypothesis, and the longevity-related selected mutation rate hypothesis. These hypotheses are not mutually exclusive. They are, furthermore, uneasy to distinguish formally because the relevant life history traits—body mass, age of sexual maturity, and longevity—are strongly correlated across mammalian species.

It should be noted that life history traits have been measured on currently living specimen, whereas our evolutionary estimates of the mutation rate represent an average across the length of terminal branches of the tree—this is why using many species, and therefore reducing terminal branch lengths, is so important. It needs some time, furthermore, for a change of mutation rate to impact sequence divergence. Very recent changes of mutation rate have not yet substantially affected branch lengths and are not detectable by a phylogenetic approach. Consequently, when we restricted our correlation analyses to specific taxonomic groups, results were hardly ever significant at the genus or subfamily level, and significance increased when families, suborders or orders were analyzed. All these caveats mean that the absolute levels of correlation we report between life history traits and substitution rate probably underestimate the real effects.

Overall, our statistical analyses do not allow to refute either of the 3 explanatory hypothesis. Concerning the metabolic rate effect, we failed to recover the -0.25 power relationship reported by Gillooly et al. (2005) from a 14-species pairs analysis and predicted by their model. The implication of ROS in mtDNA point mutations in somatic cells is unquestioned (Richter et al. 1988), but only germ line mutations are relevant from an evolutionary point of view. It is unclear to us whether ROS production in female germ cells is proportional to the whole-body metabolic

Table 4
Effects of Life History Variables on mtDNA Substitution Rate

	Without Phylogenetic Control				With Phylogenetic Control			
	Slope	<i>F</i>	<i>R</i> ²	<i>P</i> Value	Slope	<i>F</i>	<i>R</i> ²	<i>P</i> Value
Body mass	-0.127	134.1	0.2474	<0.0001	-0.137	81.85	0.1674	<0.0001
Maturity	-0.441	144	0.2609	<0.0001	-0.342	59.82	0.1281	<0.0001
Longevity	-0.610	142.7	0.2592	<0.0001	-0.527	88.42	0.1322	<0.0001

P values under the 0.05 threshold are bold-faced.

Table 5
Effects of Life History Variables on mtDNA Substitution in Short-Lived (less than 19 years) versus Long-Lived (more than 19 years) Species

Variable 1	Variable 2	Short-lived				Long-lived			
		Slope	R^2	<i>P</i> Value 1	<i>P</i> Value 2	Slope	R^2	<i>P</i> Value 1	<i>P</i> Value 2
Maturity		-0.398	0.111	<0.0001		-0.435	0.168	<0.0001	
Body mass		-0.150	0.151	<0.0001		-0.080	0.104	<0.0001	
Longevity		-0.530	0.098	<0.0001		-0.812	0.198	<0.0001	
Maturity	Longevity		0.1281	0.0088	0.0495		0.213	0.0497	0.0008
Maturity	Body mass			0.0217	0.0001			<0.0001	0.054
Body mass	Longevity			0.2504	0.0002			0.1346	<0.0001

P values under the 0.05 threshold are bold-faced.

rate—experiments would be helpful here (see Lanfear et al. 2007). The fact that transition mutations are primarily involved in substitution rate variations could appear in agreement with the metabolic rate hypothesis—most mutations occurring through oxidative DNA damage are transitions (Wang et al. 1998). Replication-dependent mutations due to errors of the mtDNA polymerase, however, are mostly transitions as well (Kujoth et al. 2007), so the argument is weak.

Generation time is the strongest predictor of mtDNA substitution rate variations when variables are considered separately. One strong prediction of the generation time hypothesis, however, is that the effect should apply similarly to the nuclear and mitochondrial genomes. This is obviously not the case: the mtDNA (cytochrome *b*) mutation rate appears much more variable than the nuclear (IRBP) one, apparently rejecting the generation time hypothesis. One doubt remains, however, because of the distinct transmission modes of mitochondrial and nuclear DNA. If nuclear germ line mutations primarily occurred in males (see Li et al. 2002) and if the number of germ-line replications per generation was less variable across species in males than in females, then distinct mtDNA and nuclear patterns could be expected. Embryologic data about the male and female germ line pathways in various mammals would be required to clarify this point. Another argument speaking against generation time as the unique factor controlling substitution rate is the existence of a strong variation of base composition between mammalian lineages, especially the fastest evolving ones (Gibson et al. 2005, Urbina et al. 2006). Obviously, the synonymous substitution process is affected by other causes than just a per year/per generation scaling.

Our analysis, finally, brings support to the hypothesis of a selected decrease of substitution rate in long-lived species, in agreement with the evidence for a causal role of mtDNA mutations in aging (for a recent review see Kujoth et al. 2007). The mtDNA substitution rate is less variable, and better correlated to longevity, in long-lived than in short-lived mammalian species. Neither the metabolic rate nor the generation time hypothesis can explain these results. An independent argument for this model was provided by Samuels (2004) and Khaidakov et al. (2006). These authors showed that the total number of direct repeats in the mammalian mitochondrial genome was negatively correlated to species longevity. Direct repeats are known to contribute to somatic mtDNA damage, so this result was interpreted as reflecting a selective pressure for a decreased mtDNA so-

matic mutation rate in long-lived species. A group of particular interest for addressing the link between mutation rate and lifetime is Chiroptera (bats). Chiroptera live longer than nonflying eutherian mammals of comparable size (Brunet-Rossini and Austad 2004). The average body mass of Chiroptera species in our data set is 6.6 times lower than in Rodentia, but their average longevity is 2.4 times higher. It is interesting to note that Chiroptera evolve more than twice as slow as Rodentia, on average (fig. 1). Increasing the life history data set for Chiroptera would appear appropriate to progress with this comparison. Please note, finally, that this analysis, as most analyses of longevity in animals, relies on maximal life spans observed in captivity. Estimates from wild animals would be preferable, but such data are available in too few species.

The hypothesis of a selective constraint for a decreased mtDNA mutation rate in long-lived species is attractive, consistent with current knowledge about mitochondrial physiology and aging, and supported by our analysis. Additional arguments in favor of this model would be welcome. One natural perspective would be the analysis of the genetic systems controlling mtDNA mutation, that is, genes involved in mtDNA replication or oxidative stress reduction. According to our model, such genes should be under stronger selective constraint in long-lived than in short-lived mammals.

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