Rates of Nuclear and Cytoplasmic Mitochondrial DNA Sequence Divergence in Mammals

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Differential rates of nucleotide substitution among different gene segments and between distinct evolutionary lineages is well documented among mitochondrial genes and is likely a consequence of locus-specific selective constraints that delimit mutational divergence over evolutionary time. We compared sequence variation of 18 homologous loci (15 coding genes and 3 parts of the control region) among 10 mammalian mitochondrial DNA genomes which allowed us to describe different mitochondrial evolutionary patterns and to produce an estimation of the relative order of gene divergence. The relative rates of divergence of mitochondrial DNA genes in the family Felidae were estimated by comparing their divergence from homologous counterpart genes included in nuclear mitochondrial DNA (*Numt*, pronounced "new might"), a genomic fossil that represents an ancient transfer of 7.9 kb of mitochondrial DNA to the nuclear genome of an ancestral species of the domestic cat (*Felis catus*). Phylogenetic analyses of mitochondrial (mtDNA) sequences with multiple outgroup species were conducted to date the ancestral node common to the *Numt* and the cytoplasmic (*Cymt*) mtDNA genes and to calibrate the rate of sequence divergence of mitochondrial genes relative to nuclear homologous counterparts. By setting the fastest substitution rate as strictly mutational, an empirical "selective retardation index" is computed to quantify the sum of all constraints, selective and otherwise, that limit sequence divergence of mitochondrial gene sequences over time.

Introduction

Substitution rates in mitochondrial genes vary among phyletic lineages and among genes and gene regions within the same mitochondrial genome (Brown et al. 1982; Miyata et al. 1982; Britten 1986; Vawter and Brown 1986; Hasegawa and Kishino 1989; Palumbi 1989; Bulmer, Wolfe, and Sharp 1991; Hillis and Huelsenbeck 1992; Kondo et al. 1993; Li 1993; Lynch and Jarrell 1993; Rand 1994). Factors that can affect lineage-specific substitution rates include metabolic rates and body mass, generation time, differential fixation of slightly deleterious mutations, DNA repair mechanisms, and nucleotide composition (Wolfe, Sharp, and Li 1989; Li 1993; Rand 1994; Martin 1995). Wide heterogeneity in base composition can affect estimates of nucleotide substitution rate as well as phylogeny (Collins, Wimberger, and Naylor 1994; Jermiin et al. 1994; Galtier and Gouy 1996). Furthermore, heterogeneity in substitution rates can bias the reconstruction of species phylogenies when the algorithms for estimating distances make assumptions about the rate of nucleotide substitution (Felsenstein 1978, 1993; Swofford and Olsen 1990; Huelsenbeck and Hillis 1993). As an alternative to a univer-

sal clock, several studies have supported the hypothesis that local molecular clocks are more appropriate in describing the rates among multiple taxonomic lineages (Hasegawa and Kishino 1989; Bermingham and Lessios 1993; Li 1993).

MtDNA genes that have transposed to the nucleus provide the opportunity to directly analyze intracellular (paralogous) duplication events (Goodman 1981; Fukuda et al. 1985; Hardison and Gelinas 1986; Smith, Thomas, and Patton 1991; Arctander 1995; Collura and Stewart 1995; Zischler et al. 1995; Sunnucks and Hales 1996). The discovery of a transfer of mtDNA to the nuclear genome of ancestors of the domestic cat and other closely related feline species, termed *Numt* (Lopez et al. 1994), offers a unique opportunity to address some of the issues relating to substitution rate heterogeneity. The *Numt* locus on cat chromosome D2 represents a relatively intact transfer and amplification of nearly 50% or 7.9 kb of the feline mitochondrial genome, spanning sequences from the control region to cytochrome oxidase subunit II (COII) (Lopez et al. 1994; Lopez, Cevario, and O'Brien 1996). The complete mitochondrial DNA and *Numt* monomer sequence have been determined recently (Lopez, Cevario, and O'Brien 1996). It is likely that *Numt* became a pseudogene immediately after it transferred to the nucleus because of differences between nucleus and cytoplasm in their genetic codes (Barrell et al. 1980) as well as their regulatory factors. In addition, there are multiple indel mutations between cytoplasmic (*Cymt*) and *Numt* sequences (Lopez et al. 1994; Lopez, Cevario, and O'Brien 1996). As a pseudogene, the substitution rate of *Numt* is shown here to differ from that of *Cymt* because of selective constraints on cytoplasmic mitochondrial genes not operative on pseudogenes, differences in the abundance of superoxide radicals, and DNA repair efficiency differences be-

Table 1
Complete mtDNA Sequences Used

Common Name	Scientific Name	Accession No.	Reference
Human	*Homo sapiens*	V00662	Anderson et al. (1981)
Domestic cat......	*Felis catus*	U20753	Lopez, Cevario, and O'Brien (1996)
Harbor seal.......	*Phoca vitulina*	X63726	Arnason and Johnsson (1992)
Gray seal........	*Halichoerus grypus*	X72004	Arnason et al. (1993)
Fin whale	*Balaenoptera physalus*	X61145	Arnason, Gullberg, and Widegren (1991)
Blue whale	*Balaenoptera musculus*	X72204	Arnason and Gullberg (1993)
Cow.............	*Bos taurus*	V00654	Anderson et al. (1982)
Mouse...........	*Mus musculus*	V00711	Bibb et al. (1981)
Rat..............	*Rattus norvegicus*	X14848	Gadeleta et al. (1989)
Opossum.........	*Didelphis virginiana*	Z29573	Janke et al. (1994)

NOTE.—Except for domestic cat, these mammalian mitochondrial genomes were retrieved from GenBank (release 86, 12/1994) and EMBL (release 39, 6/1994).

tween organelles (Miquel 1992; Wallace 1992; Lopez, Cevario, and O'Brien 1996). Because nonfunctional *Numt* accumulates mutations stochastically in the nucleus, it serves as a "reference standard" for analyzing rate variation in active cytoplasmic mitochondrial genes.

To characterize variation in mitochondrial genes, we first examined the relative rates of mitochondrial gene divergence in 10 species from five orders of eutherian mammals. In addition, a phylogenetic approach was used to count the lineage-specific changes (i.e., nuclear vs. cytoplasmic), which were then used to estimate the divergence rates of feline mitochondrial gene sequences in *Numt* and *Cymt* since *Numt's* origin. These differential rates were used to calibrate mtDNA gene evolution in Felidae. Gene sequences included in the *Numt* segment evolve at a nearly equivalent and presumably selectively neutral "pseudogene" rate, while cytoplasmic genes diverge at different rates depending on the constraints of their physiological function.

Materials and Methods
Sequence Analysis

Representative mammalian mitochondrial genomes (table 1) were retrieved from GenBank (release 86, 12/1994) and EMBL (release 39, 6/1994). Complete cytoplasmic and nuclear mtDNA sequences of the domestic cat have been submitted to the GenBank database with accession numbers U20753 and U20754, respectively (Lopez, Cevario, and O'Brien 1996). We also obtained 376 bp of 12S rRNA (Janczewski et al. 1995; Masuda et al. 1996), about 371 bp of 16S rRNA (Johnson and O'Brien 1997), and 250–310 bp of the 5'ND2 gene (unpublished data) from several felids and carnivores. Tiger (*Panthera tigris*), lion (*P. leo*), and leopard (*P. pardus*) ND2 sequences were obtained using a Met-tRNA primer, 5'-GGTCAGCTAAATAAGCTATCGG-3' (positions 4717–4738 in domestic cat mtDNA), and the ND2 primer, ATGGATATTGTTAGGATTATTAGG, which is a reverse complement of nucleotides at feline positions 5236–5256 (Lopez 1995).

Sequences were aligned using gap creation penalty = 1 and gap extension penalty = 0.1 settings of PILE-UP of the University of Wisconsin Genetics Computer Group (UWGCG) (Genetics Computer Group 1994). Distance matrices, phylogenetic relationships, and branch lengths were estimated with PHYLIP version 3.5c (Felsenstein 1993), MEGA 1.01 (Kumar, Tamura, and Nei 1993), and Phylogenetic Analysis Using Parsimony (PAUP version 3.1.1) (Swofford 1993).

Divergence Rates and the Selective Retardation Index

Divergence (δ) between a pair of sequences is often expressed as a linear function of time (T),

$$\delta = 2\lambda T, \tag{1}$$

where λ is the absolute or intrinsic rate of divergence expressed as percent base pair divergence per million years (%bp/Myr). The simple modification

$$\delta = (\lambda_N + \lambda_C)T \tag{2}$$

is used when the two lineages, N and C, are thought to evolve at different rates. These expressions are most useful over time periods in which divergence is low and the stochastic accumulation of substitutions is approximately linear. We will use these expressions below to estimate λ's relevant to specific mtDNA genes in both *Numt* and *Cymt*, and to date the ancestral origin of *Numt*. We will also use expression (1) for the basis of the selective retardation index, as follows.

Consider two genes, A and B, in each of two taxa with divergence at locus A: $\delta_A = 2\lambda_A T$ and that at B: $\delta_B = 2\lambda_B T$. Noting that divergence time T is the same for two obligately linked mtDNA genes, we have the simple ratio $\delta_A/\delta_B = \lambda_A/\lambda_B$, which leads to

$$\lambda_A = (\delta_A/\delta_B)\lambda_B. \tag{3}$$

While we can construct this expression for any pair of genes sharing T, of particular interest are comparisons in which one of these genes is thought to evolve at a selectively neutral rate. In our application, we will assume that most mtDNA molecular evolution is by the stochastic accumulation of neutral and slightly deleterious mutations, with occasional fixation of advantageous mutations. Thus, we will identify the most divergent gene among taxa as the one evolving at a maximal

Table 2
Nucleic Acid Sequence Divergence of Mammalian Mitochondrial Genes

Gene	Size^c	I^a—Eutherian Mammals (pairwise average) δ (Tamura)	(δ̄, range)	Order^d	II—Cat/Harbor Seal δ	Order^d	III—Mouse/Rat δ	Order^d	IV—Harbor Seal/Gray Seal δ	Order^d	V—Fin Whale/Blue Whale δ	Order^d	VI^b—Eutherian Mammal/Opossum δ̄	Order^d
CR-3'^e	1,014	0.82	(0.54, 0.35–0.64)	1	0.27	4	0.35	1	0.03	13	0.03	17	1.06	1
CR-5'^e	595	0.79	(0.52, 0.33–0.66)	2	0.53	1	0.22	7	0.11	1	0.13	1	0.97	2
ATP8	212	0.44	(0.33, 0.25–0.43)	3	0.30	2	0.20	10	0.03	12	0.08	14	0.46	6
ND2	1,062	0.42	(0.32, 0.23–0.38)	4	0.26	5	0.29	2	0.05	6	0.11	3	0.52	5
ND6	544	0.39	(0.30, 0.19–0.37)	5	0.24	9	0.22	9	0.06	2	0.10	6	0.58	3
ND5	1,888	0.36	(0.29, 0.24–0.34)	6	0.29	3	0.25	3	0.05	5	0.09	8	0.43	7
ND4L	303	0.35	(0.27, 0.19–0.33)	7	0.25	7	0.23	6	0.02	18	0.10	5	0.43	9
ND4	1,433	0.35	(0.28, 0.21–0.32)	8	0.26	6	0.24	5	0.05	3	0.12	2	0.43	8
ND3	358	0.35	(0.28, 0.21–0.33)	9	0.23	10	0.24	4	0.02	15	0.10	7	0.38	11
ATP6	684	0.33	(0.27, 0.23–0.30)	10	0.24	8	0.18	14	0.03	10	0.10	4	0.38	10
ND1	958	0.30	(0.24, 0.19–0.28)	11	0.18	15	0.22	8	0.03	11	0.08	11	0.36	12
COII	708	0.29	(0.24, 0.18–0.30)	12	0.22	12	0.17	15	0.04	8	0.09	9	0.32	14
CYB	1,149	0.28	(0.23, 0.20–0.27)	13	0.23	11	0.19	13	0.05	4	0.08	10	0.33	13
CR-C^e	257	0.28	(0.25, 0.15–0.35)	14	0.14	16	0.12	17	0.03	14	0.01	18	0.55	4
COIII	848	0.26	(0.22, 0.19–0.25)	15	0.21	14	0.20	11	0.04	7	0.08	12	0.28	15
COI	1,552	0.25	(0.21, 0.19–0.22)	16	0.21	13	0.19	12	0.04	9	0.08	13	0.26	17
16S	1,676	0.23	(0.23, 0.17–0.31)	17	0.13	18	0.15	16	0.02	16	0.05	15	0.28	16
12S	1,006	0.23	(0.21, 0.18–0.26)	18	0.14	17	0.07	18	0.02	17	0.05	16	0.24	18

NOTE.—A UPGMA alignment algorithm with gap creation penalty = 1 and gap extension penalty = 0.1 was used. For difference calculations each gap was equated to one substitution.

^a Average Tamura distance (average percent difference, range) of all pairwise comparisons between mtDNA genes of species (human, domestic cat, fin whale, cow, and mouse) from each of five orders of eutherian mammals.

^b Average Tamura distance between opossum and each of five orders of eutherian mammals.

^c The size is in base pairs and represents the total alignment length from the five orders of eutherian mammals analyzed.

^d The order was determined using values calculated to the fifth decimal place. An order number of 1 was assigned to the most different comparison and 18 was assigned to the least different comparison.

^e The control region (CR) was split into three regions, the conserved region (CR-C) and the two variable regions located 5' and 3' to the conserved region (CR-5' and CR-3', respectively), relative to light strand replication.

and approximately neutral rate, and use it to gauge the rates of other loci. Note that we now have

$$\lambda_i = (\delta_i/\delta_M)\lambda_M \quad (4)$$

for the expression of gene i's rate relative to the maximum rate λ_M, which we assume to be neutral. Since λ_i is obligately less than or equal to λ_M, we view the expression $(1 - \delta_i/\delta_M)$ as the sum total of all selective, mutational, and other effects that make gene i evolve more slowly than gene M, and identify it as the selective retardation index

$$\sigma_i = 1 - \delta_i/\delta_M \quad (5)$$

so that

$$\lambda_i = (1 - \sigma_i)\lambda_M \quad (6)$$

by analogy to selection coefficients in classical population genetics theory (Wallace 1968; Wright 1969).

Results

Sequence Divergence Among mtDNA Genes in Mammals

Divergence rate variation among mitochondrial genes was first examined by pairwise comparison of aligned nucleotide sequences from 10 mtDNA genomes. In table 2 we present the extent of nucleotide sequence divergence for 13 protein-coding genes, 2 rRNA genes, and 3 control region segments (5', conserved central

"C" region, and 3') using 6 levels of comparison, each reflecting different evolutionary time periods. The six comparisons include Tamura's (1992) distance as well as the average percent sequence mismatch (δ, with gaps = 1 residue) for: (I) one sequence from each of five eutherian mammal orders (human–Primate, domestic cat–Carnivora, fin whale–Cetacea, cow–Artiodactyla, and mouse–Rodentia); (II) cat–harbor seal comparison reflecting two families (Felidae and Phocidae) within Carnivora; (III) mouse–rat, reflecting two rodent families; (IV) harbor seal–gray seal, within Phocidae family, between two genera; (V) fin whale–blue whale, two Cetacean species from the same genus; and (VI) average Tamura distance between marsupial (opossum) mtDNA genes and those of the five eutherian mammal species listed in level I. The results illustrate the range of sequence divergence among mitochondrial genes as well as the variation of divergence among the six different evolutionary levels. For example, among eutherian orders average divergence (Tamura 1992) ranged from 23% (12S rRNA gene) to 82% (control region 3' end) in analysis I.

The most consistently conserved genes were the 12S and 16S rRNA sequences, which ranked among the least divergent in all six analyses. The COI and COIII genes were also highly conserved except between seals (level IV). ND2, ND6, and ND5 were consistently among the most rapidly evolving sequences. The 5' con-

Table 3
Amino Acid Sequence Divergence of Mammalian Mitochondrial Protein-Coding Genes

| Gene | Size[c] | I[a]—Eutherian Mammals (pairwise average) | | | II—Cat/ Harbor Seal | | III—Mouse/ Rat | | VI[b]—Eutherian Mammal/ Opossum | |
		δ(PAM)	(δ̄, range)	Order[d]	δ	Order[d]	δ	Order[d]	δ̄	Order[d]
ATP8......	67	0.67	(0.45, 0.26–0.60)	1	0.36	1	0.24	4	0.91	2
ND6	175	0.51	(0.37, 0.15–0.48)	2	0.17	4	0.27	2	0.98	1
ND2	347	0.49	(0.37, 0.22–0.47)	3	0.29	2	0.32	1	0.68	3
ND5	606	0.36	(0.30, 0.18–0.37)	4	0.20	3	0.24	3	0.50	5
ND3	115	0.30	(0.26, 0.11–0.36)	5	0.12	6	0.15	5	0.44	6
ND4L.....	98	0.29	(0.27, 0.06–0.34)	6	0.06	11	0.15	6	0.51	4
ND4	459	0.27	(0.24, 0.12–0.33)	7	0.13	5	0.14	7	0.41	7
ATP6......	227	0.23	(0.22, 0.08–0.26)	8	0.08	9	0.05	10	0.32	9
ND1	318	0.22	(0.19, 0.08–0.23)	9	0.08	8	0.10	8	0.32	8
CYB	379	0.19	(0.17, 0.11–0.22)	10	0.11	7	0.06	9	0.25	10
COII	227	0.16	(0.15, 0.03–0.29)	11	0.04	12	0.01	13	0.21	11
COIII	261	0.12	(0.12, 0.07–0.15)	12	0.07	10	0.04	11	0.15	12
COI.......	514	0.07	(0.07, 0.02–0.09)	13	0.02	13	0.03	12	0.08	13

[a] Average PAM matrix distance (average percent difference, range) of all pairwise comparisons between mtDNA genes of species (human, domestic cat, fin whale, cow, and mouse) from each of five orders of eutherian mammals. Distance values were derived from the PROTDIST program in PHYLIP 35, using Dayhoff PAM substitution matrices (Felsenstein 1993; Doolittle et al. 1996).

[b] Average PAM distance between opossum and each of five orders of eutherian mammals.

[c] The size is in amino acid residues and represents the total alignment length from the five orders of eutherian mammals analyzed.

[d] The order was determined using values calculated to the fifth decimal place. An order number of 1 was assigned to the most different comparison and 13 was assigned to the least different comparison.

trol region segment (CR-5′) was generally one of the most divergent regions. CR-3′ was also highly divergent in all comparisons except levels IV and V, in which it was relatively highly conserved. Other sequences, like ATP8 (ranging from 2 to 14), ND4L (from 5 to 18), ND3 (from 4 to 15), and CR-C (from 4 to 18), were very unpredictable in terms of their rank order of divergence across levels.

In table 3 we display results from the same type of analysis applied to the 13 translated protein sequences of these genes. We have omitted levels IV and V from this table, since protein products were highly conserved (above 96% for level IV and 92% for level V). As with the nucleotide sequences, ND2, ND6, ND5, and ATP8 were generally the most divergent sequences. At the other extreme, COI, COII, and COIII were always among the most conserved, along with CYB. There was no dramatic variability in rank across levels as there was with nucleotide sequences. A possible exception is ND4L, which ranged from 4 to 11 in rank. The mtDNA and amino acid sequence divergence estimates (tables 2 and 3) indicate roughly consistent patterns in relative divergence rate that are useful for estimating relative divergence rates among genes selected for phylogenetic analysis.

Substitution Rate of Nuclear and Cytoplasmic mtDNA Homologues in Felidae

The cat family, Felidae, consists of 38 living species whose phylogenetic relationships have been investigated using a variety of morphological and molecular methods, including mtDNA gene divergence (Collier and O'Brien 1985; Wayne et al. 1989; Salles 1992; Janczewski et al. 1995; Johnson et al. 1996; Masuda et al. 1996; unpublished data). However, mitochondrial DNA analyses have been complicated by the occurrence in

some species of the *Numt* locus, a large ancestral insertion and tandem duplication of 7.9 kb of mtDNA sequences into the chromosomal DNA of small cats of the genus *Felis* (Lopez et al. 1994).

Several lines of evidence indicate that *Numt* is a pseudogene. First, comparison of protein-coding sequences between *Numt* and *Cymt* reveal termination codons as well as frameshifts in every open reading frame. In addition, *Numt*-specific transcripts were not apparent (Lopez 1995). Presumably, *Numt* can be regarded as a genomic fossil that is diverging as a selectively neutral pseudogene. In contrast, *Cymt* sequences are evolving under selective constraints dictated by their function.

Numt is found in the nuclear genomes of *F. catus* and several other species of *Felis* (*F. sylvestris, F. margarita,* and *F. chaus*) (Lopez et al. 1994; Johnson et al. 1996). We have previously estimated the date of origin of *Numt* in the genus *Felis* as 1.8–2.0 MYA based on partial sequence comparisons of *Numt* and *Cymt* (Lopez et al. 1994). The recent determination of the complete nucleotide sequence of *Cymt* from domestic cat (Lopez, Cevario, and O'Brien 1996) provides an unusual opportunity to examine the pattern and mode of mitochondrial gene divergence from a common ancestor of modern *Cymt* and *Numt* sequences over this 2-Myr interval.

In an attempt to allocate all changes in the divergence of *Numt* and *Cymt* from their common mtDNA ancestor, we used a phylogenetic analysis of three mitochondrial genes included in *Numt,* but which evolve at different rates in *Cymt*: slow (12S and 16S rRNA) and fast (ND2) (see table 2). The three mtDNA gene sequences from multiple individuals of each of 10 additional cat species plus a more distant outgroup species, hyena, *C. crocuta,* were determined and aligned with *Numt* and *Cymt* homologues. The phylogenetic relation-

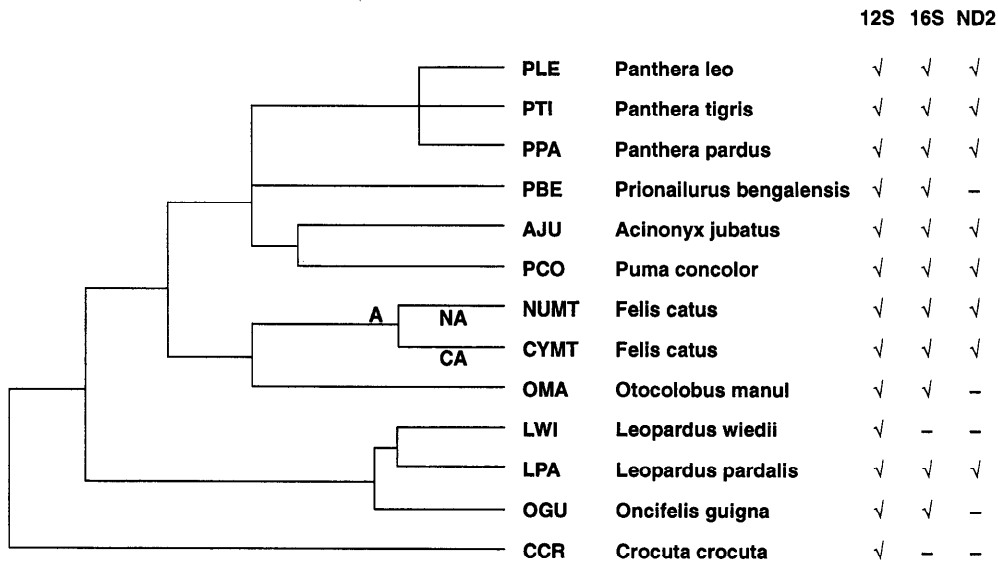|			12S	16S	ND2
PLE	Panthera leo		√	√	√
PTI	Panthera tigris		√	√	√
PPA	Panthera pardus		√	√	√
PBE	Prionailurus bengalensis		√	√	–
AJU	Acinonyx jubatus		√	√	√
PCO	Puma concolor		√	√	√
NUMT	Felis catus		√	√	√
CYMT	Felis catus		√	√	√
OMA	Otocolobus manul		√	√	–
LWI	Leopardus wiedii		√	–	–
LPA	Leopardus pardalis		√	√	√
OGU	Oncifelis guigna		√	√	–
CCR	Crocuta crocuta		√	–	–

FIG. 1.—Species used for estimation of relative divergence of *Numt* and *Cymt*. Point A is the common ancestor of *Numt* and *Cymt*, with branches NA and CA to *Numt* and *Cymt*, respectively. Phylogenetic topology is a summation of phylogenetic analyses reported elsewhere (Collier and O'Brien 1985; Wayne et al. 1989; Janczewski et al. 1995; Pecon Slattery et al. 1994; Masuda et al. 1996; Johnson and O'Brien 1997). Sequences used in this analysis are indicated to the right of species' names.

ship among these test species, established by previous analyses, is illustrated in figure 1. For both 12S and 16S, additional sequences were available from other species within the genus *Felis*.

The nucleotide sites with base pair differences between *Numt* and *Cymt* for the three genes are presented in figure 2, along with the homologous sequences from the outgroup species listed in figure 1. By comparing the *Numt/Cymt* differences to outgroups, we determined, based on the principle of maximum parsimony, whether the mutation was more likely to have occurred on the *Numt*-ancestor (NA) lineage or the *Cymt*-ancestor (CA) lineage (fig. 1). Of the 49 differences, 36 were unambiguously resolved. For example, in 12S rRNA position 1737, where *Cymt* is "A" and *Numt* is "C" and all the outgroups are "A," we assumed that the ancestral form was "A." Therefore the A→C substitution occurred on the NA limb of the phylogeny. The other 13 sites showed homoplasy, in that both alternatives seen in the *Numt/Cymt* comparison were also seen in the outgroups. However, appealing to the phylogenetic tree (fig. 1) allowed 10 of these to be resolved easily. For instance, at site 1438 of 12S, only LPA and LWI shared the C seen in *Numt*, while the others, including closely related OMA, all had T, suggesting a T→C transition on the NA lineage. In the case of ND2 homoplasies, in which outgroups were essentially equidistant, a majority rule was used. Finally, the remaining three *Numt/Cymt* differences were resolved by appealing to the other *Felis* sequences (not shown, Johnson et al. 1996; Masuda et al. 1996) as follows. Recognizing *F. nigripes* as an outgroup relative to the other *Felis* species, and also that *F. nigripes* apparently lacks *Numt*, the nucleotide seen in *F. nigripes* was taken to be ancestral. Through this process we were able to count the number of substitutions on the CA and NA phylogenetic limbs and use

these measures to estimate evolutionary rates for each of the three genes (fig. 2 and table 4).

The slowly evolving 12S rRNA gene had 23% of the *Numt/Cymt* divergence on the CA limb and 77% on the nuclear NA lineage. For the faster-evolving ND2 gene, 61% of the *Numt/Cymt* divergence occurred in the cytoplasmic CA limb and 39% occurred on the NA lineage. This reversal in relative divergence levels is readily explained by differences in divergence patterns on the two limbs. The nuclear (NA) divergence was relatively constant for all three genes with 2.7%, 1.1%, and 3.0% sequence divergence for 12S, 16S, and ND2, respectively (table 4). By contrast, the cytoplasmic lineage (CA) shows a marked range of divergence among genes, with 12S and 16S having relatively little change (0.8% and 1.3%, respectively), while ND2 had a 4.6% sequence divergence. Note that 17 of the 23 (74%) base changes for ND2 occur at third-base positions (fig. 2). On the CA lineage, the ND2 gene has accumulated mutations 5.7 times more rapidly than has the 12S rRNA and 3.5 times faster than has the 16S rRNA gene. By direct measurement, these results conform to the results inferred in table 2, which indicate that 12S and 16S are among the most highly constrained, slowly evolving genes, and ND2 is among the fastest.

Dating the *Numt–Cymt* Ancestor

Estimated rates of evolution for the mitochondrial genes along the CA and NA lineages were used to estimate the divergence date between *Cymt* and *Numt* as follows. First, nucleotide substitutions between *Numt* and *Cymt* (CN) were allocated into their respective branches (NA and CA, fig. 1) and converted to percent base pair divergence (%bp) for each gene (δ_N and δ_C, table 4). To calibrate *Cymt* rate estimates with fossil dates, we measured sequence divergence (δ) for the

12S

```
                1 1 1 1 1 1 1 1 1 1 1 1 1 1
                4 4 4 5 5 6 6 6 6 6 6 6 7 7
                3 4 5 6 8 1 1 1 6 8 8 9 0 3
                8 4 1 1 0 1 2 3 4 0 2 8 3 7
        Cymt    T T A A A A T * T C C C C A
        Numt    C C G G G C C A C T A T T C

                T C A A A A C * T C C T C A   PLE
                T C A A A A T * T C T T T -   PTI
                T C A A A A C * T C T T T -   PPA
                T T A A A A C * T C C T C A   PBE
                T C A A A A C * T C C T C A   AJU
                T C A A A A C * T C C T C A   PCO
                T C A A A A C * T C T T T A   OMA
                C C A A A A C * T C C T C A   LWI
                C C A A A A T * T C C T C A   LPA
                T C A A A A C * T C T T C A   OGU
                T C A A A A T * T C C T C A   CCR
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16S

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                2 3 3 3 3 3 3 3 3
                9 0 0 0 0 0 1 1 1
                6 6 6 6 6 9 3 6 8
                9 1 5 8 9 6 3 7 1 2
        Cymt    T * * G C C A T A C A
        Numt    G C T C T T G C C T G

                T * * C T C A T A T A   PLE
                T * * T T C A T A T A   PTI
                T * * C C T A T A C A   PPA
                T * * T C T A C A T A   PBE
                T * * T T C A C A T A   AJU
                T * * T T C A C A T A   PCO
                T * * T * C A C A T A   OMA
                T * * T T T A T A T A   LPA
                T * * C T C A T A T A   OGU
```

ND2

```
                4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5
                7 7 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 0 0 0 0 0 0 0 0 0
                8 9 0 0 0 1 4 7 8 9 0 2 3 3 3 5 6 7 0 0 0 0 0 0 0 0
                6 5 4 5 3 1 2 9 5 6 5 6 5 2 3 0 8 5 3 2 6 9 3 5 4
        Cymt    C C T A G T C T G C G * A A G C * T G G G C T A A
        Numt    A T C G A C A G A T A A₆ C G A A G C A A A T C G G

                A T C A A T A G A C A * A A A C * C - - - - - - - -   PLE
                A T C A A T A C A C G * A A A C * C A A G T C T -     PTI
                - - - - A T A C A C A * A A A C * C A A G T C T -     PPA
                - - - - A T A T A T A * A A A C * C A A G T C A -     AJU
                A T C A A T A G A C A * A A A C * C A A G T C A A     PCO
                A T C A A C A G A C A * A A A C * C A A G T T A A     LPA
```

FIG. 2.—Nucleotide differences between *Numt* and *Cymt,* with sequences at homologous positions of felid and hyena outgroups (see fig. 1 for species codes). Inferred changes relative to *Numt/Cymt* common ancestor are boxed. Coordinates with respect to Lopez, Cevario, and O'Brien (1996) are shown above the *Cymt* sequence. Since insertions relative to *Cymt* were not numbered in Lopez, Cevario, and O'Brien (1996), the coordinates given for each insertion (which are all *Numt*-specific) correspond to the *Cymt* base to the right of the insertion. Asterisks denote lack of corresponding sequence in *Cymt* and outgroups and dashes indicate lack of data. For ND2, third-base positions of codons are indicated (●) above the coordinate. Contiguous indels are treated here as a single site, but are ignored in all rate calculations. A single *Numt/Cymt* difference at position 1407 of the 12S sequence is not shown because of uncertain alignment among the outgroups.

three mtDNA genes among five (three for ND2) species of great cats, genus *Panthera* (*P. pardus, P. leo, P. tigris, P. onca,* and *P. uncia*), which radiated during a relatively short time period 2–3 MYA (Savage and Russell 1983; Wayne, Van Valkenburgh, and O'Brien 1991; Janczewski et al. 1995). For each of the three mtDNA

genes, we averaged the pairwise sequence divergence among *Panthera* species ($\bar{\delta}$, table 4). Using these in equation (1) we estimated a range of divergence rates for each of the three genes, (λ_i, in %bp/Myr) for the 2–3 Myr *Panthera* radiation. Then, with the rates estimated from *Panthera* and the measured divergence of the *Cymt* genes (δ_C), we estimated the date of the *Numt/Cymt* divergence (table 4). The time estimates ranged from 1.2–2.4 Myr for the origin of *Numt,* which overlaps previous estimates of the *Numt* origin as 1.8–2.0 Myr, obtained by using a more general rate calculation relating overall mitochondrial gene divergence in vertebrates to evolutionary time (Lopez et al. 1994).

In what follows we will use the date of 1.9 MYA, the midpoint of both estimates, as the date the *Numt/Cymt* lineages diverged. The gene-specific rates (λ_i) for the three cytoplasmic genes corresponding to $T = 1.9$ Myr and the δ_C values in table 4 are: $\lambda_{12S} = 0.42$, $\lambda_{16S} = 0.70$, and $\lambda_{ND2} = 2.43$, each expressed as %bp/Myr. For estimation of the nuclear pseudogene rate, percent divergence (δ_N) of the three genes on the NA branch ranged from 1.1–3.0 (table 4). Using the overall average of 2.3% for the three *Numt* gene sequences' divergence from the *Numt/Cymt* ancestor, we estimated the nuclear rate as $\lambda_{NA} = 1.2$%bp/Myr.

The cytoplasmic nucleotide substitution rates for the three mitochondrial genes can be used to estimate rates for other mitochondrial genes within the Felidae and, to some extent, among other mammals. The relative divergence rates of the three genes follow a similar pattern in both the CA lineage and *Panthera* estimates (table 4). The CA rate (λ_i) ratios for the 3 genes (12S : 16S : ND2) was 1:1.7:5.7, while the same ratio from the *Panthera* pairwise averages was 1:1.3:4.2, supporting the assertion that within cats (domestic cat lineage and *Panthera* lineage), mtDNA genes accumulate mutations at roughly equivalent rates. Further, the same order of relative rates is seen when sequence divergences among other mammals are compared. From table 2, analysis I (eutherian percent difference average), the ratio of 12S : 16S : ND2 divergence is 1:1.1:1.5, and for analysis VI (eutherian opossum difference) the ratio was 1:1.2:2.2 (Tamura's distance). Contributing to the difference among these ratios is the fact that ND2 is more prone to become saturated with substitutions when comparing species with more ancient divergences (~80 Myr) (Graybeal 1994).

By combining the average percent difference ($\bar{\delta}$) of mtDNA genes in table 2 with the gene-specific rates estimated for Felidae genes (table 4, last column), we can approximate evolutionary substitution rates for specific genes (λ_i) at least during the time period when divergence is proportionate to time. We attempt such a calibration for mtDNA genes based on each of the CA rates in table 5. The estimated rates are comparable when calibrated with 12S rRNA and 16S rRNA rates, but are three-fold greater when the ND2 gene is used. The more rapid evolutionary rate of ND2 likely plateaus more recently than the estimated 80 Myr since eutherian orders of mammals diverged, contributing to the apparent discordance.

Table 4
Relative Divergence of Genes Found in *Numt* and *Cymt*

Gene	Length (bp)	*Numt*/*Cymt* Divergence (no. differences (%bp))			Pairwise *Panthera* Divergence[a]			Age (*T*)[c] of *Numt*/*Cymt* Ancestor (Myr)	*Cymt* Divergence Rates (λ)[d] (%bp/Myr)
		CN (δ_{CN})	= NA (δ_N)	+ CA (δ_c)	δ̄ (%bp)	Range	λ[b] (%bp/Myr)		
12S.....	372	13 (3.5)	10 (2.7)	3 (0.8)	2.7	2.1–3.9	0.45–0.68	1.2–1.8	0.42
16S.....	374	9 (2.4)	4 (1.1)	5 (1.3)	3.5	2.7–4.8	0.58–0.87	1.5–2.3	0.70
ND2....	303	23 (7.6)	9 (3.0)	14 (4.6)	11.3	9.0–13.4	1.9–2.8	1.6–2.4	2.43
Total....	1,049	45 (4.3)	24 (2.3)	21 (2.0)	—	—	—	—	—

[a] From Janczewski et al. (1995), Johnson and O'Brien (1996), Masuda et al. (1996), unpublished data, and this study.

[b] λ, in %bp/Myr, is calculated as δ̄/4 and δ̄/6, assuming 2–3 Myr for *Panthera* divergence, from δ = 2λ*T* (eq. 1).

[c] Using the range of λ from *Panthera*: *T* = δ_c/λ, since δ_c is the fraction of the CN divergence specific to lineage CA.

[d] λ, in %bp/Myr, is calculated from δ_c/*T*, where *T* = 1.9 Myr is the midpoint of age *T* of *Numt*/*Cymt* ancestor estimated here and by Lopez et al. (1994).

Actual substitution rates are determined by the composite influence of mutation rate within the organelle, retardation of maximum divergence by selective constraints on gene function, base composition, and the tendency for rates to asymptote or plateau over evolutionary time. We will assume that the fastest observed divergence rate (λ_i) approaches the rate dictated by mutation rate alone in the absence of selective (or other) constraints that retard evolutionary divergence. Recognizing that the observed fastest gene rate may not actually achieve the maximum neutral mutation-driven rate (Nachman, Boyer, and Aquadro 1994; Ballard and Kreitman 1995) and also that control region divergence would likely give an inflated rate in the time frames examined in table 2, we set δ_M = δ_{ATP8} as the maximum divergence. Under this assumption, the divergence values in table 5 can be used to calculate the selective retardation index (σ_i), a coefficient that, in combination with the mutation rate, can be used to predict the observed nucleotide divergence rates (see eq. 6). We compute such a value for each gene as a first step toward quantifying the sum total of pressures, selective and otherwise, that influence gene divergence for these sequences (table 5).

Discussion

Our interest in *Numt* stems at least partially from its potential as a genomic fossil. Unlike most paleontological fossils, *Numt* is closely related to the ancestral cytoplasmic sequence, and most differences between the two modern versions (*Numt* and *Cymt*) can be specifically assigned to the relevant evolutionary branch CA or NA (fig. 1) by using outgroups, as we have done here (fig. 2). Furthermore, by using local molecular clocks for each gene region, we can estimate the time of origin of the *Numt* sequence. Our analyses of mammalian mitochondrial sequences provide an important context for this work. Sequence divergence estimates in table 2 represent a broad range of evolutionary times, from the very recent (levels IV and V) to the more ancient (levels I and VI).

If the mammalian mitochondrial genome is subject to major selective pressures, as more studies, including our own, seem to be indicating (Pietromonaco, Hessler, and O'Brien 1986; Saccone, Pesole, and Kadenbach 1991; Ballard and Kreitman 1994, 1995; Nachman, Boyer, and Aquadro 1994), then the compilation of an overall mammalian consensus sequence becomes increasingly important. As a practical issue, sequences that are invariant across a wide phylogenetic range will be useful for designing primers for studying mitochondrial sequences from novel taxa. Conversely, rapidly diverging sequences will prove useful for population genetic studies and studies of closely related taxa. Furthermore, the prospects of understanding the principal modes and constraints on mitochondrial sequence evolution will emerge as more sequences are added to the database.

The overall divergence of *Numt* and *Cymt* is 5.1% (Lopez 1995). If the average divergence of 2.3% for NA reported here is accurate, then the CA divergence is about 2.8%. This value is intermediate, as it should be, between the low values we estimated for 12S and 16S (0.8% and 1.3%, respectively), and the high value obtained (4.6%) for ND2. It is interesting to note that even though the majority of changes are accumulating in the *Cymt* lineage, such substitutions are predominantly at

Table 5
Intrinsic Divergence Rates (λ_i) of mtDNA Genes Estimated from Feline mtDNA Measured Divergence

	δ[a] (%bp)	λ_i (%bp/Myr)[b]			σ_i[c]
		12S rRNA	16S rRNA	ND2	
ATP8	0.33	0.66	1.00	2.51	0.00
ND2......	0.32	0.64	0.97	**2.43**	0.03
ND6......	0.30	0.60	0.91	2.28	0.09
ND5......	0.29	0.58	0.88	2.20	0.12
ND4L	0.27	0.54	0.82	2.05	0.18
ND4......	0.28	0.56	0.85	2.13	0.15
ND3......	0.28	0.56	0.85	2.13	0.15
ATP6	0.27	0.54	0.82	2.05	0.18
ND1......	0.24	0.48	0.73	1.82	0.27
COII......	0.24	0.48	0.73	1.82	0.27
CYB......	0.23	0.46	0.70	1.75	0.30
COIII.....	0.22	0.44	0.67	1.67	0.33
COI	0.21	0.42	0.64	1.59	0.36
16S.......	0.23	0.46	**0.70**	1.75	0.30
12S.......	0.21	**0.42**	0.64	1.59	0.36

[a] From table 2, level I (average %bp difference).

[b] Intrinsic rate estimates from *Cymt* are λ_{12S} = 0.42, λ_{16S} = 0.70, and λ_{ND2} = 2.43 (table 4). Equation (3), λ_i = (δ_i/δ_B)λ_B, was used, where *B* = 12S, 16S, ND2.

[c] From equation (5), setting δ_M = 0.33 for ATP8.

third codon positions—72% of all differences in protein-coding genes (Lopez, Cevario, and O'Brien 1996). Hence, it is reasonable to infer that *Cymt* has an intrinsically higher mutation rate than that for nuclear pseudogenes, but selection (e.g., functional constraints) confines substitutions to specific regions and residues of the mitochondrial genome that are less likely to affect function. In this regard, we note that Zischler et al. (1995) characterize the human nuclear insert of mtDNA as a slowly evolving fossil. While this may generally be true when comparing protein-coding genes, our work here shows that some *Cymt* genes evolve at or below the *Numt* rate.

The characterization of *Numt* as a genomic fossil allows us to better gauge the tempo and mode of evolution of *Cymt,* its cytoplasmic homologue. Clearly, the incipient evolution of mtDNA sequences needs more study to identify sources of constraints and patterns of mutation (Collura and Stewart 1995). In this regard, *Numt* offers additional potential on several fronts. First, we have only addressed regions of *Numt* for which we had multiple felid outgroups, slightly more than 1 kb pooled from three genes. Many additional protein-coding and tRNA genes are contained in *Numt*, so that relevant outgroup sequences could be used to allocate the *Numt/Cymt* differences to each lineage. As above, this allocation could be used to establish rates and patterns of change for additional *Cymt* genes. Second, versions of *Numt* exist in several additional species of the domestic cat lineage. Although probably due to a single event, that is, *Numt* insertion into the common ancestor of these species, a single origin has not been established. Further mtDNA insertions into the nuclear genomes of additional taxa, including more distantly related cats as well as other mammalian orders, have now been documented (Goodman 1981; Fukuda et al. 1985; Hardison and Gelinas 1986; Smith, Thomas, and Patton 1991; Zullo et al. 1991). Hence, estimating the origin of *Numt* is important, not only for these pursuits, but also because of the evolutionary recurrence of similar events (Blanchard and Schmidt 1996; Sunnucks and Hales 1996).

Acknowledgments

LITERATURE CITED

ANDERSON, S., A. T. BANKIER, B. G. BARRELL et al. (14 co-authors). 1981. Sequence and organization of the human mitochondrial genome. Nature **290**:457–465.

ANDERSON, S., M. H. L. DE BRUIJN, A. R. COULSON, I. C. EPERON, F. SANGER, and I. G. YOUNG. 1982. Complete sequence of bovine mitochondrial DNA (conserved features of the mammalian mitochondrial genome). J. Mol. Biol. **156**:683–717.

ARCTANDER, P. 1995. Comparison of a mitochondrial gene and a corresponding nuclear pseudogene. Proc. R. Soc. Lond. B **262**:13–19.

ARNASON, U., and A. GULLBERG. 1993. Comparison between the complete mtDNA sequences of the blue and fin whale, two species that can hybridize in nature. J. Mol. Evol. **37**: 312–322.

ARNASON, U., A. GULLBERG, E. JOHNSSON, and C. LEGJE. 1993. The nucleotide sequence of the mitochondrial DNA molecule of the grey seal, *Halichoerus grypus,* and comparison with mitochondrial sequences of other true seals. J. Mol. Evol. **37**:323–330.

ARNASON, U., A. GULLBERG, and B. WIDEGREN. 1991. The complete nucleotide sequence of the mitochondrial DNA of the fin whale, *Balaenoptera physalus.* J. Mol. Evol. **33**:556–568.

ARNASON, U., and E. JOHNSSON. 1992. The complete mitochondrial DNA sequence of the harbor seal, *Phoca vitulina.* J. Mol. Evol. **34**:493–505.

BALLARD, J. W. O., and M. KREITMAN. 1994. Unraveling selection in the mitochondrial genome of Drosophila. Genetics **138**:757–772.

———. 1995. Is mitochondrial DNA a strictly neutral marker? Trends Ecol. Evol. **10**:485–488.

BARRELL, B. G., S. ANDERSON, A. T. BANKIER et al. (15 co-authors). 1980. Different pattern of codon recognition by mammalian mitochondrial tRNAs. Proc. Natl. Acad. Sci. USA **77**:3164–3166.

BERMINGHAM, E., and H. LESSIOS. 1993. Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama. Proc. Natl. Acad. Sci. USA **90**:2734–2738.

BIBB, M. J., R. A. VAN ETTEN, C. T. WRIGHT, M. W. WALBERG, and D. A. CLAYTON. 1981. Sequence and gene organization of mouse mitochondrial DNA. Cell **26**:167–180.

BLANCHARD, J. L., and G. W. SCHMIDT. 1996. Mitochondrial DNA migration events in yeast and humans: integration by a common end-joining mechanism and alternative perspectives on nucleotide substitution patterns. Mol. Biol. Evol. **13**:537–548.

BRITTEN, R. J. 1986. Rates of DNA sequence evolution differ between taxonomic groups. Science **165**:349–357.

BROWN, W. M., E. M. PRAGER, A. WANG, and A. C. WILSON. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. **18**:225–239.

BULMER, M., K. H. WOLFE, and P. M. SHARP. 1991. Synonymous nucleotide substitution rates in mammalian genes: implications for the molecular clock and the relationship of mammalian orders. Proc. Natl. Acad. Sci. USA **88**:5974–5978.

COLLIER, G. E., and S. J. O'BRIEN. 1985. A molecular phylogeny of the Felidae: immunological distance. Evolution **39**:473–487.

COLLINS, T. M., P. H. WIMBERGER, and G. J. P. NAYLOR. 1994. Compositional bias, character-state bias, and character-state reconstruction using parsimony. Syst. Biol. **43**:482–496.

COLLURA, R. V., and C. B. STEWART. 1995. Insertions and duplications of mtDNA in the nuclear genomes of Old World monkeys and hominoids. Nature **378**:485–489.

DOOLITTLE, R. F., D.-F. FENG, S. TEANG, G. CHO, and E. LITTLE. 1996. Determining divergence time of the major kingdoms of living organisms with a protein clock. Science **271**: 470–477.

FELSENSTEIN, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. **27**: 401–410.

———. 1993. PHYLIP: phylogenetic inference package. Version 3.5c. University of Washington, Seattle.

FUKUDA, M., S. WAKASUGI, T. TSUZUKI, H. NOMIYAMA, and K. SHIMADE. 1985. Mitochondrial DNA-like sequences in the human nuclear genome. J. Mol. Biol. **186**:257–266.

GADELETA, G., G. PEPE, G. DE CANDIA, C. QUAGLIARIELL, E. SIBISA, and C. SACCONE. 1989. The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome: cryptic signals revealed by comparative analysis between vertebrates. J. Mol. Evol. **28**:497–516.

GALTIER, N., and M. GOUY. 1995. Inferring phylogenies from DNA sequences of unequal base compositions. Proc. Natl. Acad. Sci. USA **92**:11317–11321.

GENETICS COMPUTER GROUP. 1994. Program manual for Wisconsin package. Version 8. Madison, Wis.

GOODMAN, M. 1981. Decoding the pattern of protein evolution. Prog. Biophys. Mol. Biol. **38**:105–165.

GRAYBEAL, A. 1994. Evaluating the phylogenetic utility of genes: a search for genes informative about deep divergences among vertebrates. Syst. Biol. **43**:174–193.

HARDISON, R. C., and R. GELINAS. 1986. Assignment of orthologous relationships among mammalian alpha-globin genes by examining flanking regions reveals a rapid rate of evolution. Mol. Biol. Evol. **3**:243–261.

HASEGAWA, M., and H. KISHINO. 1989. Heterogeneity of tempo and mode of mitochondrial DNA evolution among mammalian orders. Jpn. J. Genet. **64**:243–258.

HILLIS, D. M., and J. P. HUELSENBECK. 1992. Signal, noise and reliability in molecular evolutionary analyses. J. Hered. **83**: 189–195.

HUELSENBECK, J. P., and D. M. HILLIS. 1993. Success of phylogenetic methods in the four-taxon case. Syst. Biol. **42**: 247–264.

JANCZEWSKI, D. N., W. S. MODI, J. C. STEPHENS, and S. J. O'BRIEN. 1995. Molecular evolution of mitochondrial 12S rRNA and cytochrome b sequences in the pantherine lineage of Felidae. Mol. Biol. Evol. **12**:690–707.

JANKE, A., G. FUCHUS-FELDIMAIER, W. KELLY THOMAS, A. VON HAESELER, and S. PAABO. 1994. The marsupial mitochondrial genome and the evolution of placental mammals. Genetics **137**:243–256.

JERMIIN, L. S., D. GRAUR, R. M. LOWE, and R. H. CROZIER. 1994. Analysis of directional mutation pressure and nucleotide content in mitochondrial cytochrome b genes. J. Mol. Evol. **39**:160–173.

JOHNSON, W. E., P. A. DRATCH, J. S. MARTENSON, and S. J. O'BRIEN. 1996. Resolution of recent radiations within three evolutionary lineages of Felidae using mitochondrial restriction fragment length polymorphism variation. J. Mamm. Evol. **3**:97–120.

JOHNSON, W. E., and S. J. O'BRIEN. 1997. Phylogenetic reconstruction of the Felidae using 16S and NADH-5 mitochondrial genes. J. Mol. Evol. (in press).

KONDO, R., H. SATOSHI, Y. SATTA, and N. TAKAHATA. 1993. Evolution of hominoid mitochondrial DNA with special reference to the silent substitution rate over the genome. J. Mol. Evol. **36**:517–531.

KUMAR, S., K. TAMURA, and M. NEI. 1993. MEGA: molecular evolutionary genetics analysis, version 1.01. The Pennsylvania State University, University Park, Pa.

LI, W.-H. 1993. So, what about the molecular clock hypothesis? Curr. Opin. Genet. Dev. **3**:896–901.

LOPEZ, J. V. 1995. Molecular evolution of *Numt*, a transposition and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. Doctoral thesis, George Mason University, Fairfax, Va.

LOPEZ, J. V., S. CEVARIO, and S. J. O'BRIEN. 1996. Complete nucleotide sequence of the domestic cat (*Felis catus*) mitochondrial genome and a transposed mtDNA tandem repeat (*Numt*) in the nuclear genome. Genomics **33**:229–246.

LOPEZ, J. V., N. YUHKI, R. MASUDA, W. S. MODI, and S. J. O'BRIEN. 1994. *Numt,* a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. J. Mol. Evol. **39**:174–190.

LYNCH, M., and P. E. JARRELL. 1993. A method for calibrating molecular clocks and its application to animal mitochondrial DNA. Genetics **135**:1197–1208.

MARTIN, A. P. 1995. Mitochondrial DNA sequence evolution in sharks: rates, patterns, and phylogenetic inferences. Mol. Biol. Evol. **12**:1114–1123.

MASUDA, R., J. V. LOPEZ, J. PECON-SLATTERY, N. YUHKI, and S. J. O'BRIEN. 1996. Molecular phylogeny of mitochondrial cytochrome b and 12S rRNA sequences in the Felidae: ocelot and domestic cat lineages. Mol. Phylogenet. Evol. **6**: 351–365.

MIQUEL, J. 1992. An update on the mitochondrial-DNA mutation hypothesis of cell-aging. Mutat. Res. **275**:209–216.

MIYATA, T., H. HAYASHIDA, R. KIKUNO, M. HASEGAWA, and M. KOBAYASHI. 1982. Molecular clock of silent substitutions: at least six-fold preponderance of silent substitutions in mitochondrial genes over those in nuclear genes. J. Mol. Evol. **19**:28–35.

NACHMAN, W., S. N. BOYER, C. F. AQUADRO. 1994. Nonneutral evolution at the mitochondrial NADH dehydrogenase subunit 3 gene in mice. Proc. Natl. Acad. Sci. USA **91**:6364–6368.

PALUMBI, S. R. 1989. Rates of molecular evolution and the fraction of nucleotide positions free to vary. J. Mol. Evol. **29**:108–187.

PECON SLATTERY, J., W. E. JOHNSON, D. GOLDMAN, and S. J. O'BRIEN. 1994. Phylogenetic reconstruction of South American felids defined by protein electrophoresis. J. Mol. Evol. **39**:296–305.

PIETROMONACO, S. F., R. A. HESSLER, and T. W. O'BRIEN. 1986. Evolution of proteins in mammalian cytoplasmic and mitochondrial ribosomes. J. Mol. Evol. **24**:110–117.

RAND, D. M. 1994. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. Trends Ecol. Evol. **9**:125–131.

SACCONE, C., G. PESOLE, and B. KADENBACH. 1991. Evolutionary analysis of the nucleus-encoded subunits of mammalian cytochrome c oxidase. Eur. J. Biochem. **195**:151–156.

SALLES, L. O. 1992. Felid phylogenetics: extant taxa and skull morphology (Felidae, Aeluroidae). Am. Mus. Novit. **3047**: 1–67.

SAVAGE, D. E., and D. E. RUSSELL. 1983. Mammalian paleofaunas of the world. Addison-Wesley, London.

SMITH, M. F., W. K. THOMAS, and J. L. PATTON. 1991. Mitochondrial DNA-like sequence in the nuclear genome of an akodintine rodent. Mol. Biol. Evol. **9**:204–215.

SUNNUCKS, P., and D. F. HALES. 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus Sitobion (Hemiptera: Aphididae). Mol. Biol. Evol. **13**:510–524.

SWOFFORD, D. L. 1993. Phylogenetic analysis using parsimony (PAUP). Version 3.1.1. Smithsonian Institution, Washington, D.C.

SWOFFORD, D. L., and G. J. OLSEN. 1990. Phylogeny reconstruction. Pp. 411–501 *in* D. M. HILLIS and C. MORITZ, eds. Molecular systematics. Sinauer, Sunderland, Mass.

TAMURA, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol. Biol. Evol. **9**:678–687.

VAWTER, L., and W. M. BROWN. 1986. Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in their molecular clock. Science **234**:194–196.

WALLACE, B. 1968. Topics in population genetics. Norton, New York.

WALLACE, D. C. 1992. Diseases of the mitochondrial DNA. Annu. Rev. Biochem. **61**:1175–1212.

WAYNE, R. K., R. E. BENVENISTE, D. N. JANCZEWSKI, and S. J. O'BRIEN. 1989. Molecular and biochemical evolution of the Carnivora. Pp. 465–494 in J. L. GITTLEMAN, ed. Carnivore behavior, ecology and evolution. Cornell University Press, Ithaca, N.Y.

WAYNE, R. K., B. VAN VALKENBURGH, and S. J. O'BRIEN. 1991. Molecular distance and divergence in carnivores and primates. Mol. Biol. Evol. **8**:297–319.

WOLFE, K. H., P. M. SHARP, and W.-H. LI. 1989. Mutation rates differ among regions of the mammalian genome. Nature **337**:283–285.

WRIGHT, S. 1969. Evolution and the genetics of populations. Vol. 2. University of Chicago Press, Chicago.

ZISCHLER, H., H. GEISERT, A. VON HAESELER, and S. PAABO. 1995. A nuclear "fossil" of the mitochondrial D-loop and the origin of modern humans. Nature **378**:489–492.

ZULLO, S., L. C. SIEU, J. L. SLIGHTOM, H. I. HADLER, and J. M. EISENSTADT. 1991. Mitochondrial loop sequences are integrated in the rat nuclear genome. J. Mol. Biol. **221**: 1223–1235.