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Complete Nucleotide Sequences of the Domestic Cat (*Felis catus*) Mitochondrial Genome and a Transposed mtDNA Tandem Repeat (*Numt*) in the Nuclear Genome

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The complete 17,009-bp mitochondrial genome of the domestic cat, *Felis catus*, has been sequenced and conforms largely to the typical organization of previously characterized mammalian mtDNAs. Codon usage and base composition also followed canonical vertebrate patterns, except for an unusual ATC (non-AUG) codon initiating the NADH dehydrogenase subunit 2 (ND2) gene. Two distinct repetitive motifs at opposite ends of the control region contribute to the relatively large size (1559 bp) of this carnivore mtDNA. Alignment of the feline mtDNA genome to a homologous 7946-bp nuclear mtDNA tandem repeat DNA sequence in the cat, *Numt*, indicates simple repeat motifs associated with insertion/deletion mutations. Overall DNA sequence divergence between *Numt* and cytoplasmic mtDNA sequence was only 5.1%. Substitutions predominate at the third codon position of homologous feline protein genes. Phylogenetic analysis of mitochondrial gene sequences confirms the recent transfer of the cytoplasmic mtDNA sequences to the domestic cat nucleus and recapitulates evolutionary relationships between mammal species.

INTRODUCTION

Genes coding for essential components of oxidative phosphorylation and electron transfer in vertebrate and many invertebrate cells are carried on compact double-stranded, circular mitochondrial genomes (Brown, 1985; Hatefi, 1985; Wolstenholme, 1992; Wallace *et al.*, 1993). In most mammals, this genetic code encompasses a minimal repertoire of 13 protein-coding, 22 tRNA, and 2 ribosomal rRNA genes, with a basic genomic organization conserved since the eutherian–metatherian split (Janke *et al.*, 1994). The more variable noncoding control region (CR)[2] or D-loop regulates transcription and replication of the major coding DNA strand of the mitochondrial genome (Clayton, 1991). The CR typically exhibits the greatest amount of sequence divergence and length variation in mtDNA (Aquadro and Greenberg, 1983; Greenberg *et al.*, 1983; Brown, 1986; Saccone *et al.*, 1991; Hoelzel *et al.*, 1994).

The model of relative structural constancy or "economy" for metazoan mitochondrial genomes (Attardi, 1985) has been unsettled recently by numerous examples of mtDNA heteroplasmy (Buroker *et al.*, 1990; Rand, 1993; Hoelzel *et al.*, 1993, 1994) and genetic transfer between mitochondria and nuclear chromosomes in various animal taxa (Fukuda *et al.*, 1985; Zullo *et al.*, 1991; Smith *et al.*, 1991; Lopez *et al.*, 1994). A dramatic recent example is the *macrosatellite*-like locus, *Numt*, a transposition and tandem amplification (38–76×) of 7.9 kb of mtDNA into the nuclear genome of the domestic cat, *Felis catus*, and several closely related species of the genus *Felis*.

Ten complete mammalian mitochondrial genome sequences (human, mouse, cow, Norway rat, fin whale, blue whale, harbor seal, grey seal, horse, and American opossum) have been published to date (Anderson *et al.*, 1981, 1982; Bibb *et al.*, 1981; Gadeleta *et al.*, 1989; Arnason *et al.*, 1991, 1993; Arnason and Johnsson, 1992; Arnason and Gullberg, 1993; Xu and Arnason, 1994; Janke *et al.*, 1994). In this paper, we report the complete DNA sequence of the mitochondrial genome of the domestic cat and compare the complete sequence of one complete *Numt* monomer (Lopez *et al.*, 1994). Feline mtDNA represents the first sequence of a terrestrial carnivore mitochondrial genome in current sequence databases, although certain feline mtDNA gene

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. U20753 and U20754.

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[2] Abbreviations used: CR, control region; indel, insertion/deletion; nt pos., nucleotide position; *Numt*, nuclear mitochondrial DNA; OLR, origin of light-strand replication; ORF, open reading frame; MYA, million years ago; PCR, polymerase chain reaction; RS, repetitive sites; TAS, termination-associated sequences; Ti, transitions; Tv, transversions.

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sequences have been reported (Lopez *et al.*, 1994; Janczewski *et al.*, 1995; Arnason *et al.*, 1995). The addition of two distinct forms of feline mtDNA—one cytoplasmic and one nuclear—to the databases should aid the study of molecular dynamics and evolution of genomes (e.g., selfish DNA, rate heterogeneity, C-value paradox), mammalian phylogeny, and conservation genetics (Miklos, 1985; Li and Graur, 1991; Charlesworth, 1994; O'Brien, 1994a,b; Pecon Slattery, 1994; Avise, 1994).

MATERIALS AND METHODS

Cloning and sequencing. Total nuclear and cytoplasmic nucleic acids (DNA and RNA) were extracted from fresh lymphocytes of one domestic cat (FCA 65) according to standard procedures (Sambrook *et al.*, 1989; Lopez *et al.*, 1994). All of the nuclear and cytoplasmic mtDNA sequences shown were encompassed in three *Eco*RI fragments (c.a. 12.0, 7.9, and 5.0 kb), which were purified from preparative agarose gels. Isolation and characterization of the original 7.9-kb nuclear (pNumt.1) and two cytoplasmic clones (pCmt.12 and pCmt4.8) containing all of the sequences presented in this paper were described by Lopez *et al.* (1994). Sequences of both heavy and light strands were determined by either (a) subcloning 1.0- to 2.0-kb fragments into M13 mp18/mp19 single-stranded phage vectors (Sambrook *et al.*, 1989) or (b) walking in both 5′ and 3′ directions along the original intact λ phage or pBlueScript phagemid (Stratagene) clones of pNumt.1, pCmt.12, or pCmt4.8. For walking, forward and reverse primers were designed at approximately 300-bp intervals and synthesized on an Applied Biosystems Inc. (ABI) automated 394 DNA/RNA synthesizer. The three original clones served as templates for cycle sequencing reactions run on an automated DNA sequencer 373A (ABI) using a fluorescence-labeled dideoxynucleotide termination method (Dye terminator). Some regions (about 30%) of *Numt* DNA were read manually by polyacrylamide gel electrophoresis using [^{35}S]dATP in Sequenase reactions (U.S. Biochemical). Verification of sequence data was evaluated with ABI analysis software, the Sequencher 2.1 program (Gene Codes Corp., 1994), or by visual inspection of chromatograms. Genetic regions that contained any ambiguous or unreadable nucleotides were sequenced again.

Sequence analysis. Feline sequences were analyzed by programs of the University of Wisconsin Genetics Computer Group (GCG) (1994). Phylogenetic analyses were performed with PHYLIP 3.5c (Felsenstein, 1993) and Phylogenetic Analysis Using Parsimony (PAUP, Version 3.1.1) (Swofford, 1993) on VMS VAX mainframe and Macintosh computers. Secondary structures were predicted with FOLD by Zuker and Steigler (1981) on GCG. The CMATRIX program developed at the LVC/NCI-FCRDC was used to calculate total percentage similarities in nucleotide or amino acid sequences of mtDNAs. CMATRIX imposed a penalty of 1.0 for each gap encountered and did not evaluate varying degrees of chemical similarity between DNA and amino acid residues. Therefore, our usage of the term "similarity" will be commensurate with the common use of sequence "identity" in the literature (GCG Manual, 1994). Multiple sequence alignments were created by either PILEUP or PRETTY in GCG. Most of the alignments to determine homology and gene boundaries within feline mtDNA were made with either cow or the harbor seal, *Phoca vitulina*, which encompass the mtDNA sequences phylogenetically nearest to cat available (Arnason and Johnsson, 1992; Li *et al.*, 1990). The numbering system used for cat cytoplasmic mtDNA follows the harbor seal convention (Arnason and Johnsson, 1992).

To determine the context of feline mtDNA sequences within mammalian evolution, phylogenetic analyses and pairwise comparisons of percentage sequence similarity were performed with previously determined mammalian mitochondrial genomes retrieved from GenBank (Release 86, December, 1994) and EMBL (Release 39, June, 1994)—harbor seal (*Phoca vitulina*), grey seal (*Halichoerus grypus*), fin whale (*Balaenoptera physalus*), blue whale (*Balaenoptera musculus*), human (*Homo sapiens*), cow (*Bos taurus*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*). The American opossum (*Didelphis virginia*) genome was primarily used as an outgroup taxon. The *Numt* sequence was submitted to GenBank in the form of the *in vivo* pNumt.1 clone, isolated by Lopez *et al.* (1994). GenBank Accession Nos. for the feline cytoplasmic genome and *Numt* are U20753 and U20754, respectively.

RESULTS

Composition of Feline Cytoplasmic mtDNA

The *F. catus* mitochondrial genome is composed of 13 structural open reading frames (ORFs), 22 tRNA genes, both large and small subunit rRNA genes, and a regulatory control region (Fig. 1). The feline mtDNA sequence possesses several features observed in other mammalian species. First, all of the ORFs are oriented in the same direction as homologous ORFs found in other mammalian mitochondrial genomes, with no major rearrangements (Fig. 2A). Second, ND1, ND2, COIII, ND3, and ND4 genes lack complete termination codons (Table 1). However, complete stop codons may be read within the tRNA genes directly downstream of both the ND1 and the ND2 genes. Presuming that polyadenylation of processed transcripts occurs in feline mtDNA according to the model prescribed for humans (Anderson *et al.*, 1981), most stop codons in feline mtDNA appear to be TAA. Third, disregarding the ORFs that have stop codons in their downstream tRNA genes (e.g., ND1 and ND2), coding sequences overlap between the ATPase 8 and ATPase 6, ND4 and ND4L, and ND5 and ND6 genes. Fourth, the cat light (L)-strand origin of replication (ORL) is less d(C-G)-rich (53%) than the harbor seal (*P. vitulina*) sequence, but feline ORL can still fold into a stable stem-loop structure (Fig. 2B). Neither of the two rRNA genes nor any of the major structural genes show large length differences relative to other mammalian mtDNAs.

The total length of the cat mtDNA sequence is 17,009 bp, with a total base composition of 32.5% A, 26.2% C, 14.2% G, and 27.1% T. Similar to seal mtDNAs, the cat mitochondrial genome exhibits a higher overall dG content among mammals but retains the strong bias against dG at the third codon position in structural genes. The pattern of codon usage in feline mtDNA (Table 2) follows the preference patterns observed in other mammalian mtDNA sequences, with the possible exception of the Ile and Phe codons. Compared to harbor seal codon usage, feline mtDNA shows an increase in TTT, probably at the expense of TTC codons. Also, the cat initiates the ND2 gene with a non-AUG codon, ATC (Ile), a result also observed in ND3 and ND5 genes in mouse and horse (Bibb *et al.*, 1981; Arnason, 1994) (Table 1). In contrast to other mammalian genomes, the ATPase 8 gene is extended by the duplication of one Q residue at the 3′ end, which may have resulted from slippage during DNA replication. Finally, a total of 34 bp constitute the untranslated spacer nucleotides between mitochondrial genes.

Analysis of tRNA Genes

The canonical secondary structure features common to most vertebrate mitochondrial tRNA molecules (Cedergren *et al.*, 1981; Kumazawa and Nishida, 1993), such as the anticodon (AC) stem loops and T-Φ-C and amino acid-acceptor (A-A) arms, are also observed in some feline mitochondrial tRNAs (Fig. 2C). Compared with cow or harbor seal tRNA sequences, most differences in the cat occur in the T-Φ-C arm, the dihydrouridine loop, or the "variable" loop region between the AC stem and the T-Φ-C arm, and many substitutions in one stem strand were compensated in the corresponding stem strand. Insertion or deletion mutations (indels) are observed in the cat dihydrouridine loops of His, Gln, Phe, Pro, Tyr, Leu, and Asp tRNAs relative to bovine mtDNA genes. For example, the tRNA-Phe gene is 3 bp longer in cat than in cow mtDNA. The AC loop region is the most conserved tRNA region among all comparisons. Furthermore, the cat tRNA-Leu (CUN) gene is longer relative to the harbor seal sequence, which is best explained by an arbitrary placement of the ND5 5′ gene boundary in *P. vitulina* mtDNA, causing it to be three residues (M-K-V) longer and discordant with all other mammalian ND5 sequences. Feline tRNA sequences for Ala, Val, and Met contain a large number of mutations in the AC stem compared with that in bovine mtDNA, although most changes are compensated.

Mitochondrial DNA Control Region of the Cat

The feline CR spans about 1560 bp (Fig. 3A). An unusual characteristic of the cat mtDNA CR is the presence of two distinct sites of repetitive sequences (RS2 and RS3) on opposite sides of the highly conserved core of the control region, which together extend the CR 447 bp longer than the human sequence. The locations of these repeats appear to be highly conserved compared to other mammalian CR repeats (Wilkinson and Chapman, 1991; Ghivizzani *et al.*, 1993; Arnason and Johnsson, 1992). RS3, a 294-bp d(C-A)-rich repeat, which resembles nuclear microsatellites (Love *et al.*, 1990) as well as other carnivore CR repeats (Hoelzel *et al.*, 1994), is found at the L strand 3′ end (beginning at nt pos. 270) of feline mtDNA (Fig. 1). RS3 contains a 6- to 8-bp core unit, ACACACGT, imperfectly repeated 37 times in the mtDNA sequence. The RS2 element at the 5′ (left) end of the CR L strand consists of three complete 80- to 82-bp monomers (a–c), which are highly conserved with each other (91–98% similarity) (Fig. 3B). The cat RS2 repeats also show 72–75 and 67–74% sequence similarity to homologous mtDNA CR repeats observed in the evening bat and masked shrew, respectively (Wilkinson and Chapman, 1991; Stewart and Baker, 1994). The 3′-most cat repeat (RS2c) showed the greatest divergence at its own 3′ end, while the most 5′ repeat unit (pos. 16504), possessing 94% similarity and one deletion relative to the consensus, is truncated after only 34 bp in feline mtDNA. The cat RS2 sequences contain several palindromic motifs (5′ TACAT----ATGTA 3′) beginning at pos. 16507 that could potentially form secondary structures and possibly function as terminal-associated sequences (TAS) involved in D-loop replication (Brown, 1986; Foran *et al.*, 1988; Saccone *et al.*, 1991; Madsen *et al.*, 1993). Array secondary structures appeared more stable with two or three RS2 repeats versus one repeat unit (Fig. 3C). These secondary structures may have facilitated the expansion/contraction of repeat numbers following the model of Buroker *et al.* (1990). The nonintegral repeat number may reflect mutational decay or misaligned slippage during rounds of duplication. Preliminary data indicate that the RS2 region is hypermutable and highly heteroplasmic within individuals of other species of Felidae (M. Culver, unpublished observations).

Comparison of Cytoplasmic mtDNA and Numt Homologous Regions

Homologous *Numt* DNA sequences extend 7946 bp and were derived from a single monomer of the tandemly repeated chromosomal array estimated to range from 38 to 76 copies (Lopez *et al.*, 1994; Lopez, 1995). The overlapping homologous region between cytoplasmic mtDNA and *Numt* begins at cytoplasmic nt pos. 529 within the RS3 repeat and includes about 80% of the COII gene to nucleotide 8454 (Fig. 1). The last 304 bp of the *Numt* COII gene in Fig. 1 occur upstream of the CR RS3 repeat sequences in the original pNumt.1 clone, corroborating the observations of an *in situ* excision, circularization, nuclear integration, and tandem amplification at the chromosomal *Numt* locus (Lopez *et al.*, 1994).

A total uncorrected nucleotide sequence difference

FIG. 1. The complete nucleotide sequences of domestic cat (*F. catus*) cytoplasmic mtDNA (top) and one aligned *Numt* DNA repeat unit (bottom). The L-strand sequence is shown. Alignment of both sequences in the 7946-bp region of homology was performed with GAP (GCG, 1994), using a gap weight of 5.0 and a gap length weight of 0.3. Indels are marked by dots and highlighted with gray shading. The translated amino acid sequences for each structural gene are given above the nucleotide sequences using standard IUPAC nomenclature and translated with the mitochondrial genetic code. Repetitive regions in the control region are marked as either RS2 or RS3 following the nomenclature of Hoelzel (1993). At RS2, boundaries of each monomer are marked (▼), and the total region is delineated (▽). RS2 palindromes are marked by arrows below the sequence, with the 5′ → 3′ arrows showing strong conservation to TAS consensus sequences (Foran *et al.*, 1988). All nucleotide numbering in the text refers to the cytoplasmic mtDNA designations of the cat and follows homologous human mtDNA conventions (Anderson *et al.*, 1982). Abbreviations for all mitochondrial genes were based on human nomenclature (Wallace, 1992), except for the tRNA genes, which followed Arnason and Johnsson (1992). Due to typeset editing, sequence line 33 beginning with nucleotide 3177 is 1 bp short, and line 7 beginning with 601 is 1 bp longer than normal due to an insertion.

FIGURE 1

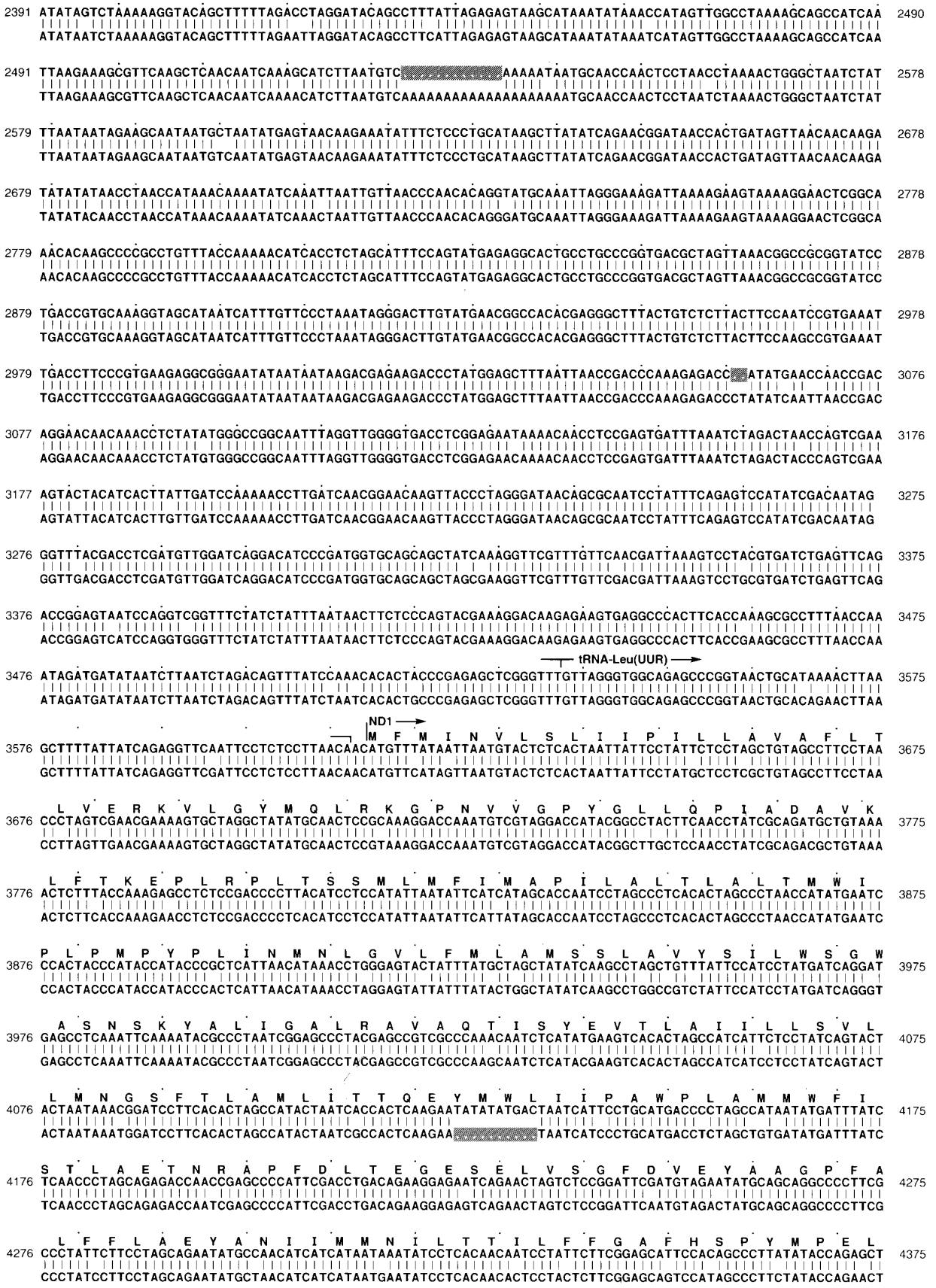


FIG. 1—*Continued*

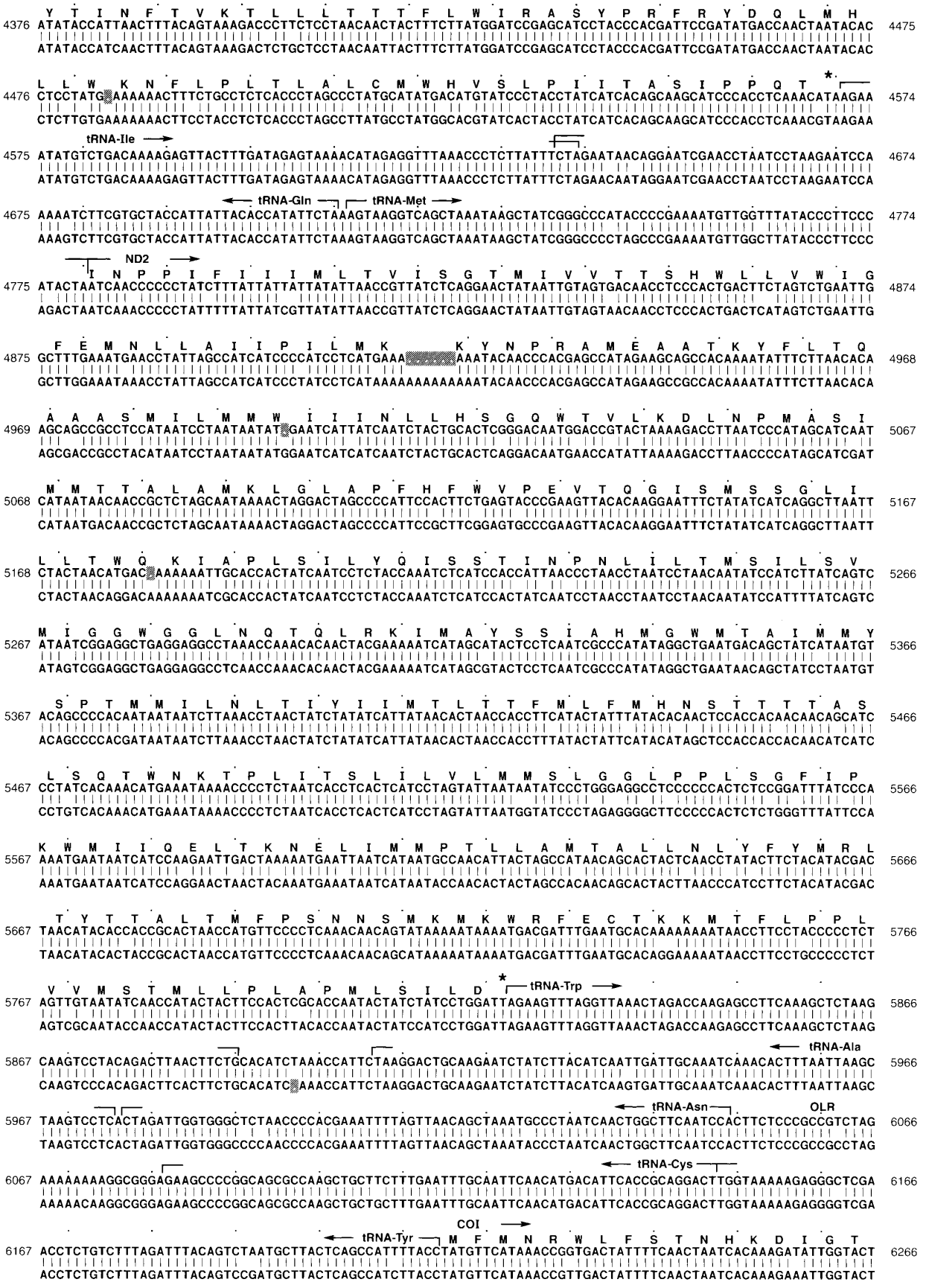


FIG. 1—*Continued*

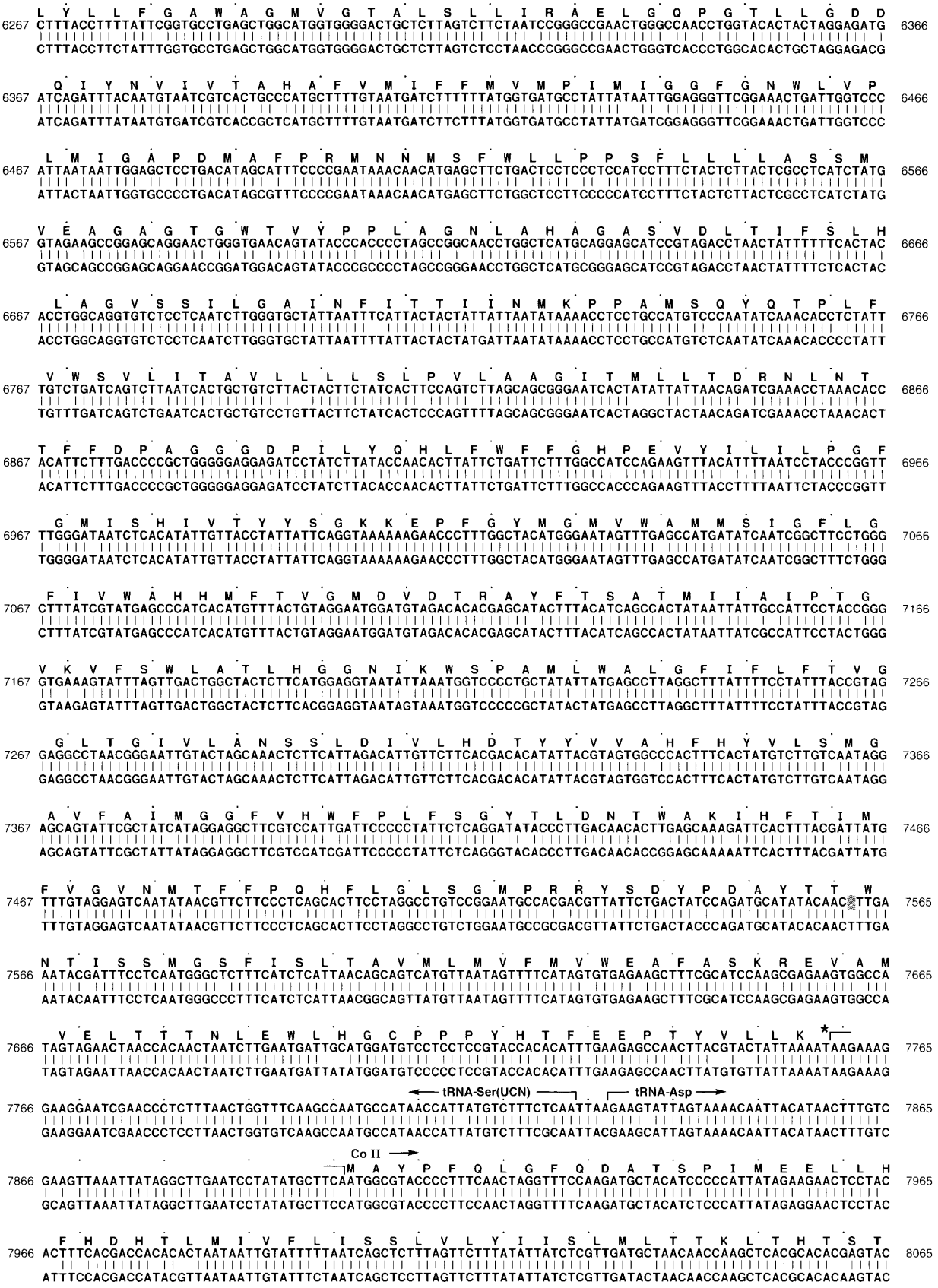


FIG. 1—*Continued*

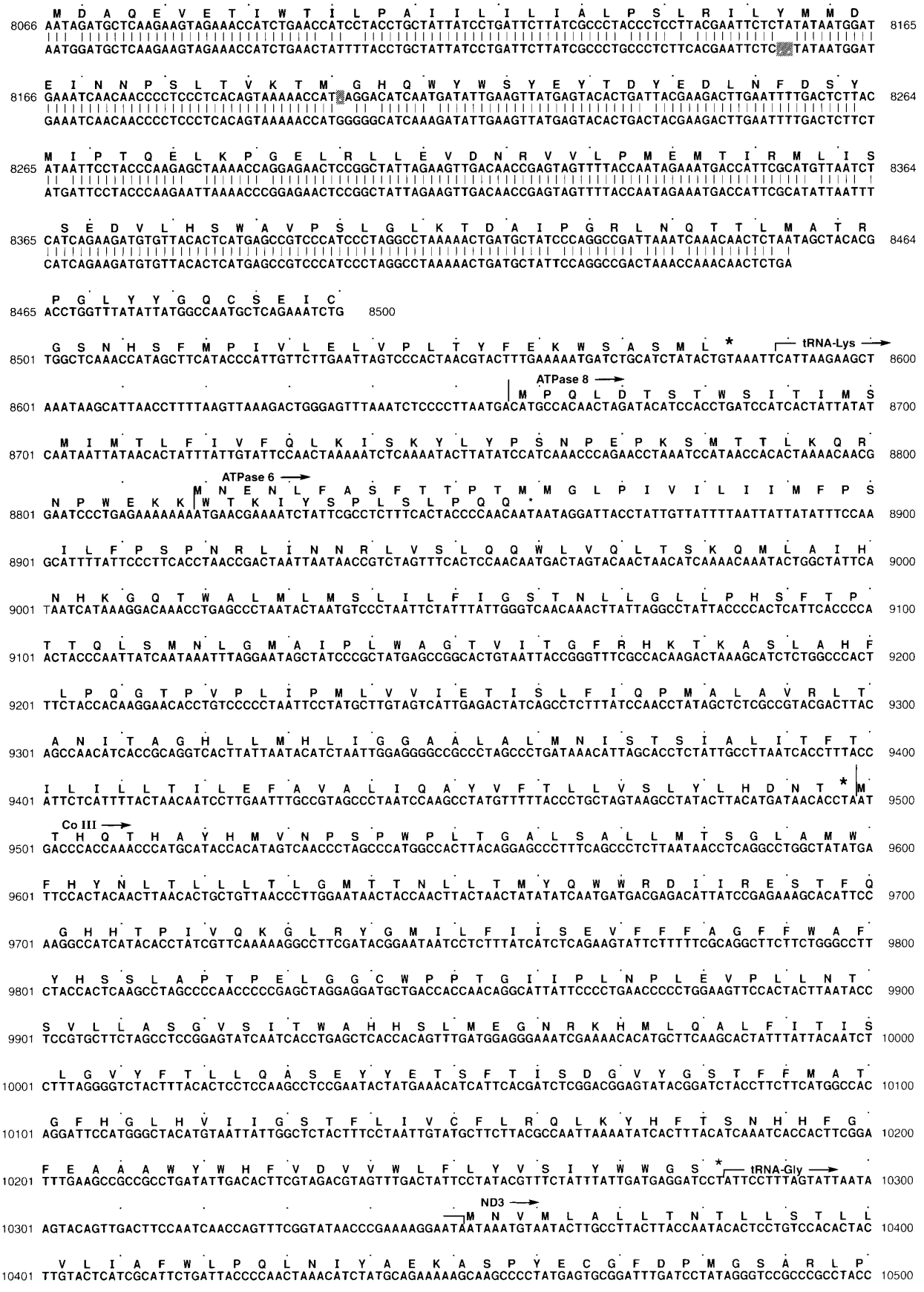


FIG. 1—*Continued*

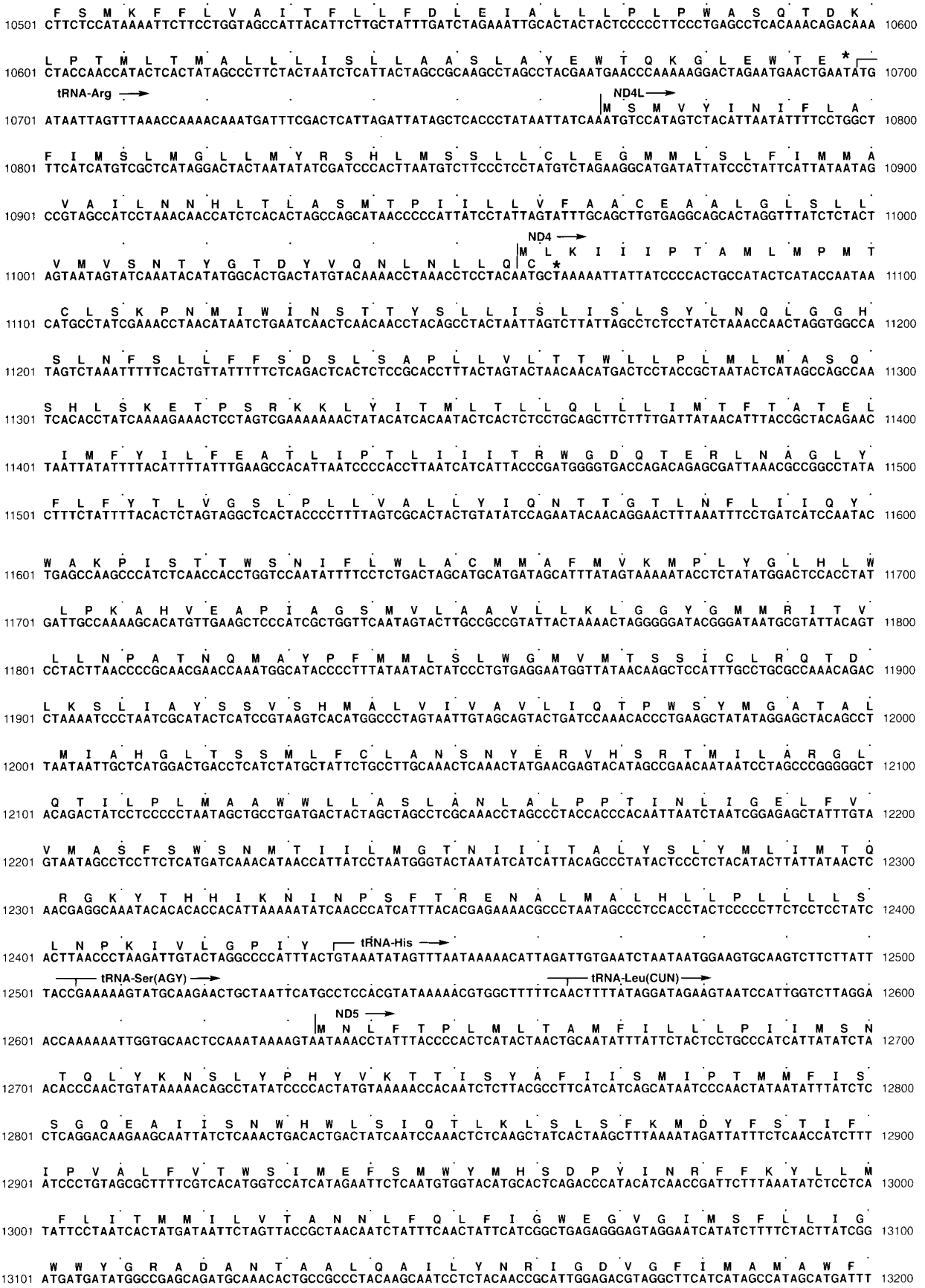


FIG. 1—*Continued*

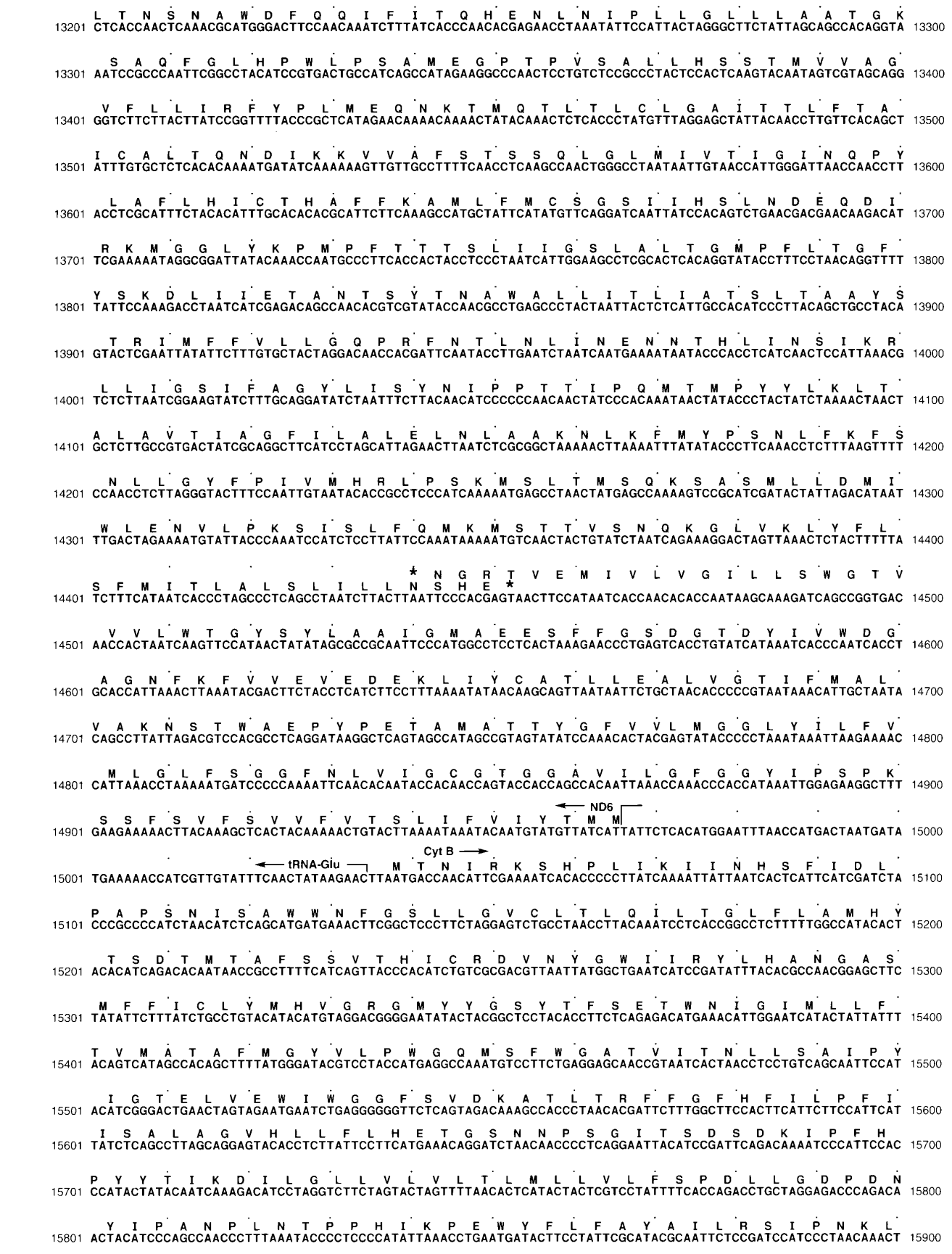
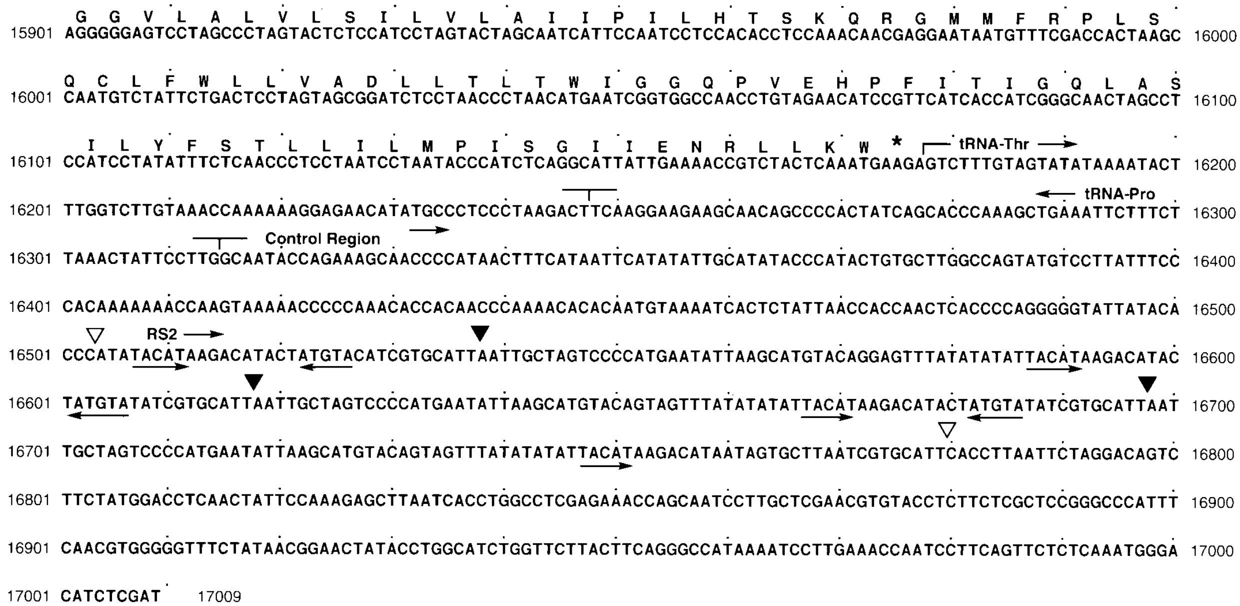


FIG. 1—*Continued*

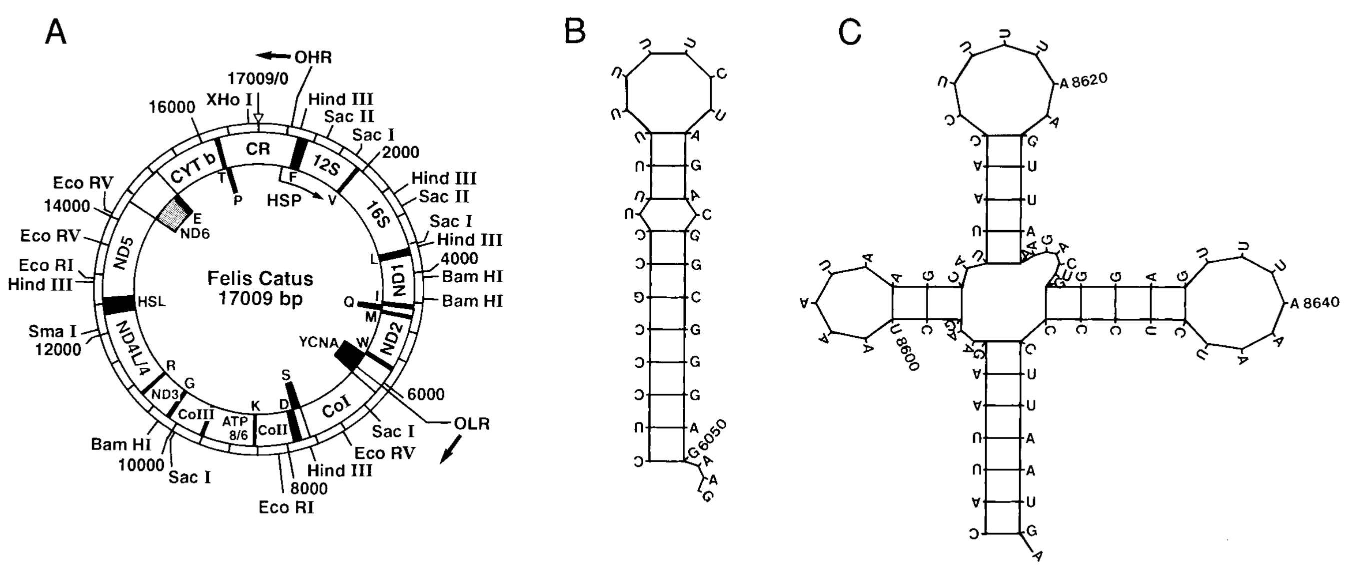


G G V L A L V L S I L V L A I I P I L H T S K Q R G M M F R P L S
15901 AGGGGAGTCCTAGCCCTAGTACTCTCCATCCTAGTACTAGCAATCATTCCAATCCTCCACACCTCCAAACAACGAGGAATAATGTTTCGACCACTAAGC 16000
Q C L F W L L V A D L L T L T W I G G Q P V E H P F I T I G Q L A S
16001 CAATGTCTATTCTGACTCCTAGTAGCGGATCTCCTAACCCTAACATGAATCGGTGGCCAACCTGTAGAACATCCGTTCATCACCATCGGGCAACTAGCCT 16100
I L Y F S T L L I L M P I S G I I E N R L L K W * tRNA-Thr
16101 CCATCCTATATTTCTCAACCCTCCTAATCCTAATACCCATCTCAGGCATTATTGAAAACCGTCTACTCAAATGAAGAGTCTTTGTAGTATATAAAATACT 16200
tRNA-Pro
16201 TTGGTCTTGTAAACCAAAAAAGGAGAACATATGCCCTCCCTAAGACTTCAAGGAAGAAGCAACAGCCCCACTATCAGCACCCAAAGCTGAAATTCTTTCT 16300
Control Region
16301 TAAACTATTCCTTGGCAATACCAGAAAGCAACCCCATAACTTTCATAATTCATATATTGCATATACCCATACTGTGCTTGGCCAGTATGTCCTTATTTCC 16400
16401 CACAAAAAAACCAAGTAAAAACCCCCAAACACCACAACCCAAAACACACAATGTAAAATCACTCTATTAACCACCAACTCACCCCAGGGGGTATTATACA 16500
RS2
16501 CCCATATACATAAGACATACTATGTACATCGTGCATTAATTGCTAGTCCCCATGAATATTAAGCATGTACAGGAGTTTATATATATTACATAAGACATAC 16600
16601 TATGTATATCGTGCATTAATTGCTAGTCCCCATGAATATTAAGCATGTACAGTAGTTTATATATATTACATAAGACATACTATGTATATCGTGCATTAAT 16700
16701 TGCTAGTCCCCATGAATATTAAGCATGTACAGTAGTTTATATATATTACATAAGACATAATAGTGCTTAATCGTGCATTCACCTTAATTCTAGGACAGTC 16800
16801 TTCTATGGACCTCAACTATTCCAAAGAGCTTAATCACCTGGCCTCGAGAAACCAGCAATCCTTGCTCGAACGTGTACCTCTTCTCGCTCCGGGCCCATTT 16900
16901 CAACGTGGGGGTTTCTATAACGGAACTATACCTGGCATCTGGTTCTTACTTCAGGGCCATAAAATCCTTGAAACCAATCCTTCAGTTCTCTCAAATGGGA 17000
17001 CATCTCGAT 17009

FIG. 1—*Continued*

of about 5.1% exists between the two feline mtDNA homologues. The overall base composition of *Numt* and cytoplasmic mtDNA are very similar: cytoplasmic, 33.3% A, 26.4% T, 24.8% C, and 15.4% G vs *Numt*, 32.6% A, 25.8% T, 25.2% C, and 16.3% G. When changes in homologous coding gene regions (ND1, ND2, CO1, and CO2) were compared according to codon position, 72% of the base substitutions occur at the third position, and 23/51 (45%) first position substitutions are at synonymous Leu codons. These data suggest that the majority of mutational differences are synonymous, indicating mutational drift principally in the cytoplasmic organelle constrained by selective pressures.

A comparison of the pattern of mutational divergence between feline cytoplasmic mtDNA and *Numt*, relative to mtDNA divergence between related species from other mammalian families (e.g., fin/blue whales, harbor/grey seals), is presented in Table 3. All three comparisons involve recently diverged mtDNA sequences (seals, 2–2.5 MYA; whales, <5.0 MYA; and *Numt*/cyto-



A
OHR
17009/0
16000
XHo I
Hind III
Sac II
Sac I
2000
Hind III
Sac II
Sac I
Hind III
4000
Bam HI
Bam HI
6000
OLR
Sac I
Eco RV
Hind III
8000
Eco RI
Sac I
10000
Bam HI
Sma I
12000
Eco RI
Hind III
Eco RV
14000
Eco RV
CR
12S
16S
ND1
ND2
CoI
CoII
ATP 8/6
CoIII
ND3
ND4L/4
ND5
ND6
CYT b
HSP
HSL
YCNA
Felis Catus
17009 bp
B
A 6050
C
A 8620
A 8640
U 8600

FIG. 2. (A) Physical map of coding genes and major restriction sites within the cat cytoplasmic mtDNA. Genes on the inner circle are transcribed from the L strand. Locations of the tRNA genes (shaded boxes) conform to the canonical placental mammalian arrangement and have been previously drawn (Lopez *et al.*, 1994). Listed enzymes recognize 6-bp sites and cut less than four times, except *Hin*dIII, which has five sites. The following abbreviations were used: HSP, putative heavy-strand promoter; OHR, origin of heavy-strand replication; OLR, origin of light-strand replication. (B) Predicted secondary structure of the OLR (L-strand origin of replication) (energy = −14.0). Sequences are shown in H-strand orientation. (C) Representative tRNA cloverleaf secondary structure of tRNA-Lys in feline mtDNA. Diagram was produced with the FOLD program of Zuker and Steigler (1981) in GCG (energy = −11.0).

TABLE 1

Characteristics of the Domestic Cat Cytoplasmic Mitochondrial Genome

Gene	From	To	Size[a]	Start codon	Stop codon	5′ intervening spacer
tRNA-Phe	866	935	70			
12S rRNA	936	1895	960			
tRNA-Val	1896	1963	68			
16S rRNA	1964	3537	1574			
tRNA-Leu (UUR)	3538	3612	75			
NADH dehydrogenase subunit 1 (ND1)	3615	4571	957	ATG	TAa[b]	AC
tRNA-Ile	4571	4639	69			
tRNA-Gln	4637	4710	74 L			
tRNA-Met	4712	4780	69			A
NADH dehydrogenase subunit 2 (ND2)	4781	5822	1042	ATC	Taa[b]	
tRNA-Trp	5823	5890	68			
tRNA-Ala	5907	5975	69 L			CACATCTAAACCATTC
tRNA-Asn	5977	6049	73 L			A
Origin of L-strand replication (OLR)	6050	6081	32			
tRNA-Cys	6082	6147	66 L			
tRNA-Tyr	6148	6214	67 L			
Cytochrome c oxidase subunit I (COI)	6216	7760	1545	ATG	TAA	T
tRNA-Ser (UCN)	7759	7828	70 L			
tRNA-Asp	7833	7901	69			TTAA
Cytochrome c oxidase subunit II (COII)	7902	8585	684	ATG	TAA	
tRNA-Lys	8589	8656	68			ATT
ATPase 8	8658	8861	204	ATG	TAA	C
ATPase 6	8819	9499	681	ATG	TAA	
Cytochrome c oxidase subunit III (COIII)	9499	10282	784	ATG	Taa[b]	
tRNA-Gly	10283	10351	69			
NADH dehydrogenase subunit 3 (ND3)	10352	10698	347	ATA	TAa[b]	
tRNA-Arg	10699	10767	69			
NADH dehydrogenase subunit 4L (ND4L)	10768	11064	297	ATG	TAA	
NADH dehydrogenase subunit 4 (ND4)	11058	12435	1378	ATG	Taa[b]	
tRNA-His	12436	12504	69			
tRNA-Ser (AGY)	12505	12563	59			
tRNA-Leu (CUN)	12564	12633	70			
NADH dehydrogenase subunit 5 (ND5)	12634	14454	1821	ATA	TAA	
NADH dehydrogenase subunit 6 (ND6)	14438	14965	528 L	ATG	TAA	
tRNA-Glu	14966	15034	69 L			
Cytochrome B (Cyt B)	15038	16177	1140	ATG	AGA	TTA
tRNA-Thr	16178	16247	70			TT
tRNA-Pro	16248	16314	67 L			
Control region (CR)	16315	865	1559			

Note. In the comparison with Numt: (a) there is a 335-bp overlap of control region sequences with Numt; (b) The 16-bp spacer region between tRNA-Trp and tRNA-Ala contains 1 gap; and (c) a total of two mutations occur in other spacer regions. L, light-strand transcript.

[a] ORFs end at the last base of putative stop codon.

[b] Signifies an incomplete termination codon as shown in Fig. 1; lowercase denotes predicted codon after polyadenylation.

plasmic, ca. 2.0 MYA) (Arnason *et al.*, 1993; Arnason and Gullberg, 1993; Lopez *et al.*, 1994). The percentage similarity values for each gene appear comparable among the three datasets and likely reflect gene-specific evolutionary rates. Thus, CO subunit and rRNA genes are the most conserved, followed by the ND1 and ND2 genes in all three comparisons. Moreover, the varying Ti:Tv ratios observed between the different gene classes (protein, tRNA, and rRNA) probably reflect the different selective constraints acting on each gene class. For all three groups, rRNA genes consistently show the lowest Ti:Tv ratios. Despite having the longest estimated divergence times among the three data sets, the whale data set retains a high overall Ti:Tv ratio of 9:1, similar to the seal ratio of 11:1. In contrast, overall (3.8:1) and individual feline gene Ti:Tv ratios greatly deviate from both seal and whale data sets, while corresponding DNA sequence similarities remain relatively uniform (Table 3). Furthermore, between the two seals, only 5% of third codon position changes were transversions, while twice as many transversions accrue at the same position in cat mtDNAs. The greatest flux of cat Ti:Tv values appear in the tRNA class, which also had the lowest ratios (1.6:1). Last, examination of the mutational spectra from three genes—16S rRNA, ND1, and ND2—indicates that the most prominent differences between datasets involve the number of A ↔ C and G ↔ T transversions, which are increased about 4- to 10-fold in feline mtDNA sequences relative to that in either seal or whale comparisons.

The feline homologous mtDNA region contains a to-

TABLE 2

Total Codon Usage in the Cat Mitochondrial Genome

First position	Second codon position								Third position
	T		C		A		G		
T	TTT (Phe)	108 (68)	TCT (Ser)	33 (35)	TAT (Tyr)	66 (50)	TGT (Cys)	11 (8)	T
	TTC	117 (163)	TCC	73 (74)	TAC	76 (84)	TGC	14 (16)	C
	TTA (Leu)	108 (68)	TCA	112 (106)	TAA (Stop)	8 (7)	TGA (Trp)	92 (91)	A
	TTG	17 (20)	TCG	8 (10)	TAG (Stop)	1 (0)	TGG	12 (13)	G
C	CTT (Leu)	59 (57)	CCT (Pro)	57 (81)	CAT (His)	31 (36)	CGT (Arg)	6 (5)	T
	CTC	97 (95)	CCC	59 (42)	CAC	63 (64)	CGC	9 (13)	C
	CTA	273 (297)	CCA	72 (68)	CAA (Gln)	84 (72)	CGA	45 (43)	A
	CTG	42 (58)	CCG	7 (7)	CAG	7 (14)	CGG	7 (4)	G
A	ATT (Ile)	151 (131)	ACT (Thr)	72 (51)	AAT (Asn)	56 (40)	AGT (Ser)	18 (17)	T
	ATC	179 (203)	ACC	101 (107)	AAC	94 (107)	AGC	34 (42)	C
	ATA	182 (192)	ACA	124 (137)	AAA (Lys)	89 (89)	AGA (Stop)	1 (1)	A
	ATG (Met)	68 (57)	ACG	14 (22)	AAG	14 (12)	AGG (Stop)	0 (0)	G
G	GTT (Val)	33 (26)	GCT (Ala)	51 (46)	GAT (Asp)	29 (26)	GGT (Gly)	33 (48)	T
	GTC	38 (44)	GCC	116 (98)	GAC	39 (46)	GGC	57 (36)	C
	GTA	95 (90)	GCA	84 (100)	GAA (Glu)	76 (74)	GGA	97 (109)	A
	GTG	21 (28)	GCT	8 (8)	GAG	23 (25)	GGG	30 (22)	G

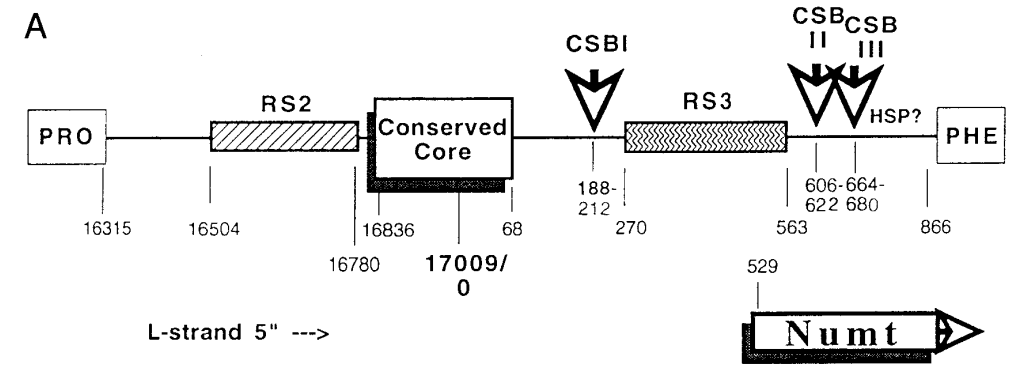
Note. Numbers in parentheses indicate total codon usage in the harbor seal mtDNA sequence for comparison (Arnason *et al.*,1991). Codons were counted with the CODONFREQUENCY option in GCG (1994). Due to the assumption of posttranscriptional polyadenylation of feline mRNAs similar to other mammals (Clayton, 1991), termination codons for COIII, ND3, and ND4 genes were not included in the present tally (see Table 1). The potential TAA and TAG termination codons for ND1 and ND2 are counted here but have not been empirically verified.

tal of 21 gaps, representing indels that ranged from 1 to 10 bp (Figs. 1A and 1B); of these, 12 were single nucleotide indels. Fourteen of the gaps (66%) are insertions in the *Numt* sequence, which lengthen *Numt* by at least 20 bp over the homologous cytoplasmic mtDNA. Five gaps occur in the CR, 7 in the rRNA genes, 5 in the ND subunits, and only 3 in the CO genes. A large proportion (25%) of gap mutations are found in the variable 3′ terminus of the CR, reconfirming the relaxed mutational constraints in this region. These mutations may derive from DNA polymerase slippage during DNA replication, since at least 8 indels occurred at sites that are "simple," homopolymeric, or with one alternating nucleotide motif (Tautz *et al.*, 1986; Newfeld *et al.*, 1994). For example, two gaps involving >1 bp occur at sites with alternating residues or direct repeats (nt pos. 1848 and 4124). Other long (12 and 6 bp) insertions of poly-(A) sequences occur in the 16S rRNA gene (pos. 2533) and in the ND2 gene (pos. 4918), respectively. These observations plus preliminary measures of cryptic simplicity suggest the influence of stochastic DNA turnover mechanisms with respect to indel mutations and other sequence changes (Dover, 1982; Tautz *et al.*, 1986; Hoelzel *et al.*, 1993). Because many of the indels would disrupt ORFs for mitochondrial structural proteins, the lineage of these mutations likely derive from the *Numt* sequence.

Phylogenetic analysis with feline mtDNA and *Numt* 16S rRNA sequences was conducted to show the relationship of feline mtDNA with other mammalian mtDNAs (Fig. 4). A maximum parsimony topology extends the conclusion of Janke *et al.* (1994), with additional cat sequences, and recapitulates phylogenetic relationships produced with other algorithms (e.g., neighbor-joining, maximum likelihood) (Felsenstein, 1993). We used the 16S rRNA gene, since frameshift mutations in *Numt* usually obliterated most amino acid identity (but not DNA homology) after alignment. Nevertheless, other conserved mitochondrial genes, such as COI and COIII, produced branching hierarchies similar to the 16S rRNA results, which show that the closest affinities of the two feline mtDNAs are with each other and with the seal sequences, as well as the recapitulation of an artiodactyl-carnivore grouping (Li *et al.*, 1990).

DISCUSSION

Feline mtDNA is distinguished from other mammalian mtDNA sequences by its possession of a large, 7.9-kb tandemly repeated homologue in the nuclear genome, termed *Numt* (Lopez *et al.*, 1994). In the cytoplasmic mitochondrial genome of the cat, the control region is longer than average (1559 bp) due to two repetitive motifs, RS2 and RS3 (Fig. 3), at opposite ends, but its length does not exceed the 1838-bp CR of lagomorphs (Mignotte *et al.*, 1990; Biju-Duval *et al.*, 1991). The compact vertebrate mitochondrial genome structure as defined by Attardi (1985) is probably maintained by selective pressures and therefore may limit the accrual of novel features such as CR simple repeats (Wallace, 1992; Hoelzel, 1993; Hoelzel *et al.*, 1994; Ghivizzani *et al.*, 1993; Buroker *et al.*, 1990; Rand, 1993).



B

```
                                20                  40                  60                  80
Cat- Rs2a   AATTGCTAGTCCCCATGAAT ATTAAGCATGTACAGGAGTT TATATATATTACATAAGACA TACTA**TGTATATCGTGCA TT
Cat- Rs2b   .................... .............T.... .................... .................... ..
Cat- Rs2c   .................... .............T.... .................... ..A..GTGC.TA........ ..
Bat         ..ACTA..T...A..... .....A........TA.A.A ...TA.............. ..TA.TG.A..*..T..A.. ..
Sorexa      ..CATG..T...A....C.. .*...........TACCA. .T.TA...G.....T.T... .C..............A.. ..
```

C

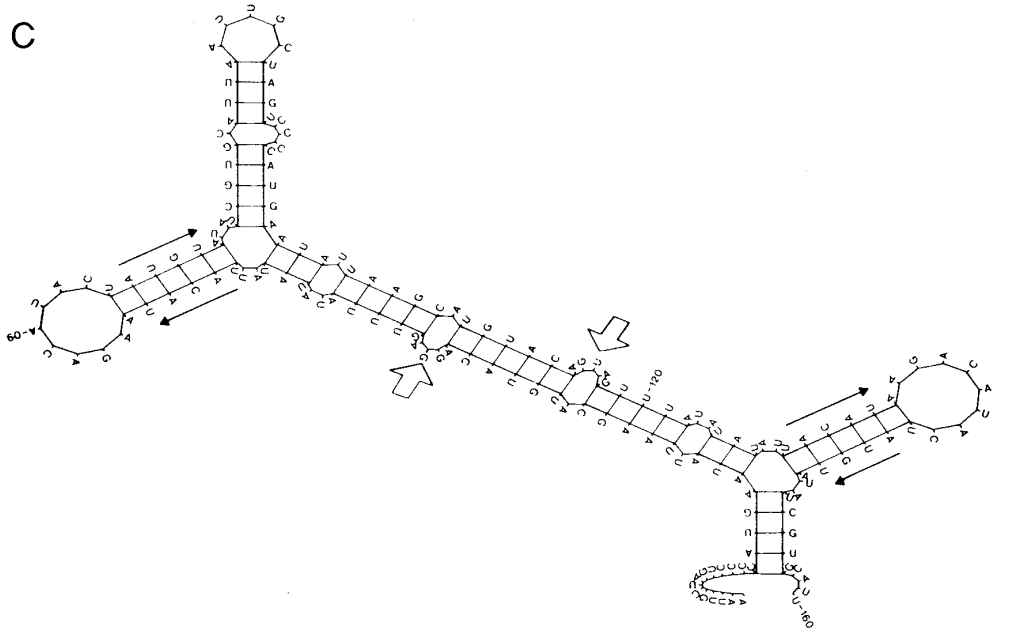


FIG. 3. (A) Schematic diagram of the feline control region (drawn to scale). Numbers correspond to cytoplasmic mtDNA nucleotide positions shown in Fig. 1. Hatched boxes represent repetitive sequence sites, RS2 or RS3, following the terminology of Hoelzel (1993). CSB I–CSBIII designate closest matches to previously identified "conserved sequence blocks" (Saccone *et al.*, 1991; Lopez *et al.*, 1994). (B) Multiple alignment of three complete RS2-type repeats (80, 80, and 82 bp, respectively) in the cat cytoplasmic CR. The RS2 region spans nt positions 16504 to 16779 in feline mtDNA. Evening bat and masked shrew (Sorexa) sequences are also listed for comparison. (C) Secondary structure of two of three 80-bp repeats at the RS2 site (pos. 16538) in the CR (energy = −25.3) produced by FOLD. Black arrows mark the location of palindromic sequences shown in Fig. 1A, and white arrows indicate the substitution observed at pos. 36 in two repeats.

With respect to codon usage, base composition, size, and order of mitochondrial genes, the *F. catus* cytoplasmic mtDNA conforms to most placental mammalian mtDNA genomes (Pepe *et al.*, 1983; Gadeleta *et al.*, 1989; Anderson *et al.*, 1982; Wolstenholme, 1992; Kumazawa and Nishida, 1993).

Analysis of mutation patterns between the two feline mtDNA sequences revealed several findings. For example, the comparison of nucleotide substitution patterns among the three closely related mammalian pairs indicated a lower bias against transversions and no significant net increases in dA and dT content in *Numt* relative to felid cytoplasmic mtDNA (Table 3), which challenges expectations for pseudogenes and noncoding sequence evolution (Gojobori *et al.*, 1982; Li *et al.*, 1984, 1985). The ratio of transitions to transversions has been shown to exceed 20:1 in recently diverged mtDNA sequences (Brown *et al.*, 1982; Ruvulo *et al.*, 1993),

TABLE 3

Nucleotide Substitution Patterns from Pairwise Comparisons of Closely Related Mammalian Taxa

	Numt/Domestic cat						Harbor seal/grey seal						Fin whale/blue whale					
Gene	Substitutions	Gap	Ti	Tv	Ti:Tv	% Similar	Substitutions	Gaps	Ti	Tv	Ti:Tv	% Similar	Substitutions	Gaps	Ti	Tv	Ti:Tv	% Similar
Control region[b]	21	5	16	5	3:1	92	11	1	10	1	10:1	97	12	1	7	5	1.4:1	96
t-PHE	1	0	1	0	1:0	98	1	0	1	0	1:0	99	8	0	7	1	7:1	89
12S rRNA	32	2	22	10	2:1	96	20	1	17	2	3:1	98	44	6	36	8	5:1	95
t-VAL	3	0	2	1	2:1	96	2	0	2	0	2:0	97	2	0	1	1	1:1	97
16S rRNA	42	5	30	12	2:1	97	35	6	28	7	4:1	97	80	3	67	13	5:1	95
t-Leu (UUR)	3	0	3	0	3:0	95	0	0	0	0		100	2	2	2	0	2:0	95
ND 1	61	2	52	9	6:1	93	29	0	29	0	29:0	97	72	0	70	2	35:1	92
t-ILE	0	0	0	0		100	1	2	0	1	0:1	97	1	0	1	0	0	98
t-GLN	3	0	3	0	3:0	96	1	1	1	0	1:0	99	0	0	0	0		100
t-MET	4	0	1	3	1:3	94	1	1	1	0	1:0	98	1	0	1	0	1:0	98
ND 2	77	3	62	15	4:1	93	46	0	43	3	14:1	96	101	0	89	12	7:1	90
t-TRP	2	0	1	1	1:1	97	1	1	1	0	1:0	99	1	0	1	0	1:0	98
t-ALA	1	0	0	1	0:1	99	0	0	0	0		100	2	0	2	0	2:0	97
t-ASN	3	0	3	0	3:0	96	0	0	0	0		100	0	0	0	0		100
OLR	2	0	1	1	1:1	94	0	0	0	0		100	1	0	1	0	1:0	97
t-CYS	1	0	0	1	0:1	98	0	1	0	0		100	2	0	2	0	2:0	97
t-TYR	4	0	3	1	3:1	94	5	0	5	0	5:0	93	0	0	0	0		100
CO I	86	1	73	13	6:1	94	56	0	52	4	13:1	96	115	0	107	8	13:1	92
t-SER (UCN)	3	0	1	2	1:2	96	1	0	1	0	1:0	99	4	0	4	0	4:0	94
t-ASP	3	0	1	2	1:2	96	1	0	1	0	1:0	99	0	0	0	0		100
CO II[a]	36	2	33	3	11:1	94	14	0	14	0	14:1	94	21	0	19	2	10:1	92
Totals	388	20	308	80	3.8:1	95	225	14	206	18	11:1	97	469	12	417	52	9:1	94
Gene Class Totals																		
tRNA	31	0	19	12	1.6:1	97	14	6	13	1	13:1	98	23	2	21	2	10:1	97
rRNA	74	7	52	22	2.4:1	97	55	7	45	9	5:1	98	124	9	103	21	5:1	95
Protein	260	8	220	40	6:1	93	145	0	138	7	20:1	96	309	0	285	24	12:1	92

Note. Mean similarities in structural genes were calculated with a penalty of 1 substitution for each gap. Ti:Tv ratios were not listed for genes with 100% similarity. Boxed values designate overall Ti:Tv ratios for each mammalian group. DNA sequences and estimated divergence times for the seal (2.0–2.5 MYA) and whale (5–7 MYA) pairs were derived from Arnason and Johnsson (1992), Arnason *et al.* (1991), and Arnason and Gullberg (1993). The 2.0-MYA divergence time for the two cat mtDNAs was based on their divergence and reference mutation rates for nuclear pseudogenes (Lopez *et al.*, 1994) and also conforms with previously estimated divergence times for other species of genus *Felis* (Collier and O'Brien, 1985) known to carry nuclear mtDNA.

[a] Comparison involves only the first 250 bp that are homologous between cat mtDNA sequences.

[b] Comparisons encompass the extreme 3′ end of the CR: 336 bp in the cat (see Fig. 1), 346 bp in the seals, and 350 bp in whales.

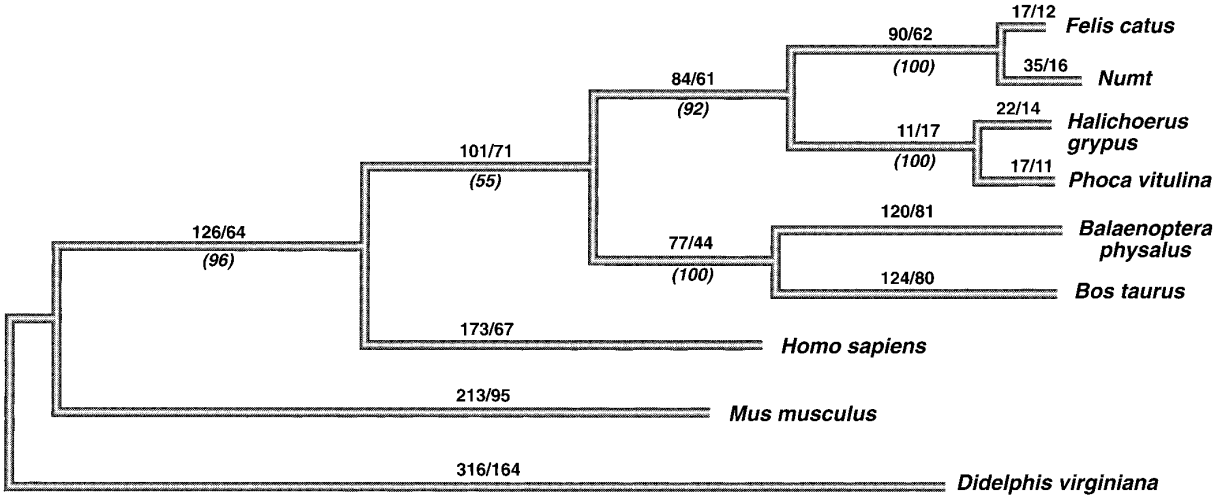


FIG. 4. Phylogenetic reconstruction with total 16S rDNA gene sequences. The 50% majority-rule consensus tree was created with PAUP 3.1.1., employing unweighted maximum parsimony criteria and branch-swapping options (Swofford, 1993). Alignment of the complete gene (ca. 1600 bp) from the respective taxa was performed with default parameters (gap weight = 3.0) of PILEUP in GCG. The tree length equals 1615 steps, with a consistency index of 0.755. Numbers above the branches designate the number of total changes/homoplasies. Bootstrap percentages in support of each node from 100 replications are shown in italics for each node.

while transversions cause more amino acid replacements and accumulate with increasing divergence time (Aquadro and Greenberg, 1983; Jukes, 1987). As one plausible explanation for the lower felid Ti:Tv ratios, elevated Ti:Tv ratios are usually more skewed in mtDNA rather than nuclear DNA comparisons (DeSalle *et al.*, 1987).

Disparities in mutational spectra probably also relate to the dissimilar cellular environments between mitochondria and the nucleus, which encompass differences in the degree of oxidative damage to DNA, the presence or absence of different enzymes and DNA polymerases involved in repair mechanisms, and the physical structure of the double helix *in vivo* (Clayton, 1991; Miquel, 1992; Martin *et al.*, 1995; Boulikas, 1992; Wallace, 1992). For example, a relationship has been observed between hypermutable nucleotide hot spots induced by reactive oxygen species and the "pausing" of mammalian β-polymerase at specific DNA secondary structures during DNA replication (McBride *et al.*, 1991; Feig and Loeb, 1993). The increase in C ↔ A and G ↔ T transversions in several genes between the two feline sequences parallels this altered mutation spectra. Various molecular architectures likely differ between nuclear and cytoplasmic compartments, since mtDNA does not wrap around histone proteins within mitochondria. Perhaps most influential on mutational spectra, however, are the greater levels of modified DNA (8-hydroxydeoxyguanosine, formamidopyrimidines, alkylated residues) found in the mitochondrial organelle compared to that in the nucleus, caused by more encounters with various reactive oxygen species (H_2O_2, O_2^-, hydroxyl radicals and singlet oxygen molecules) (Richter *et al.*, 1988; Miquel, 1992). DNA mismatch repair of the nuclear genes is likely influenced by the occurrence of methylated residues (Hare and Taylor, 1985), which may be distinct in newly integrated *Numt*. However, the paucity of quantitative data on mtDNA methylation (Pollack *et al.*, 1984) limit conclusions about its effects on general mutation patterns.

In sum, the structure and gene content of the domestic cat mitochondrial genome resembles the mtDNA of other placental mammals, except for an elongated control region attributable to two separate stretches of repetitive sequences. Simple repetitive DNA motifs are associated with several indel sites identified in cat homologous mtDNA sequences, which most likely originate in the nucleus due to the consequent disruption of ORFs in the functional genome. The nuclear mtDNA homologue, *Numt*, resembles a nuclear pseudogene sequence that, by comparison with cytoplasmic mtDNA, offers an unusual opportunity for directly analyzing intracellular (paralogous) duplication events (Goodman, 1981; Hardison and Gelinas, 1986; Fukuda *et al.*, 1985; Smith *et al.*, 1991; Zullo *et al.*, 1991; Lopez, 1995) as well as the differences in mutational constraint of the same genes in different cellular organelles.

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