

The Complete Nucleotide Sequence of the Domestic Dog (*Canis familiaris*) Mitochondrial Genome

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The complete nucleotide sequence of the mitochondrial genome of the domestic dog, *Canis familiaris*, was determined. The length of the sequence was 16,728 bp; however, the length was not absolute due to the variation (heteroplasmy) caused by differing numbers of the repetitive motif, 5'-GTACACGT(A/G)C-3', in the control region. The genome organization, gene contents, and codon usage conformed to those of other mammalian mitochondrial genomes. Although its features were unknown, the "CTAGA" duplication event which followed the translational stop codon of the COII gene was not observed in other mammalian mitochondrial genomes. In order to determine the possible differences between mtDNAs in carnivores, two rRNA and 13 protein-coding genes from the cat, dog, and seal were compared. The combined molecular differences, in two rRNA genes as well as in the inferred amino acid sequences of the mitochondrial 13 protein-coding genes, suggested that there is a closer relationship between the dog and the seal than there is between either of these species and the cat. Based on the molecular differences of the mtDNA, the evolutionary divergence between the cat, the dog, and the seal was dated to approximately 50 ± 4 million years ago. The degree of difference between carnivore mtDNAs varied according to the individual protein-coding gene applied, showing that the evolutionary relationships of distantly related species should be presented in an extended study based on ample sequence data like complete mtDNA molecules. © 1998 Academic Press

Key Words: mitochondrial genome; complete nucleotide sequence; evolutionary relationships; Carnivora; dog; seal; cat.

INTRODUCTION

The size (16 ~ 17.5 kb), gene constituents (22 tRNA, 2 rRNA, and 13 protein-coding genes), and genomic

organization of mammalian mitochondrial DNA (mtDNA) are becoming well characterized. In all mammalian species the mitochondrial genomes reveal an overall conservation of gene order and a very compact organization of genetic information (Anderson *et al.*, 1981; Attardi, 1985; Janke *et al.*, 1994). The mtDNA of most animals is maternally inherited without recombination and evolves rapidly (Roy *et al.*, 1994). The rate of nucleotide substitution in mitochondrial genomes is 10 times greater than that in nuclear genomes, which is presumably due to a reduced fidelity of mtDNA replication and DNA repair, and due to the high levels of oxygen radicals in the electron transport process. The relatively high rate of evolution of mitochondrial genes makes mtDNA sequence comparisons especially useful for estimating the dates of relatively recent evolutionary events as well as the evolution of distantly related taxa.

To date, the complete nucleotide sequences of mitochondrial genomes of 19 mammalian species have been published (human, mouse, cow, rat, fin whale, blue whale, harbor seal, grey seal, horse, American opossum, hedgehog, gorilla, common and pygmy chimpanzee, Bornean orangutan, Sumatran orangutan, Indian rhinoceros, donkey, platypus, guinea pig, cat, white rhinoceros, wallaroo, and armadillo) (Anderson *et al.*, 1981, 1982; Bibb *et al.*, 1981; Gadaleta *et al.*, 1989; Arnason *et al.*, 1991, 1993; Arnason and Johnsson, 1992; Arnason and Gullberg, 1993; Xu and Arnason, 1994; Janke *et al.*, 1994; Krettek *et al.*, 1995; Horai *et al.*, 1995; Xu and Arnason, 1996; Xu *et al.*, 1996a,b; Janke *et al.*, 1996; D'Erchia *et al.*, 1996; Lopez *et al.*, 1996; Xu and Arnason, 1997; Janke *et al.*, 1997; Arnason *et al.*, 1997). These mammalian mtDNA data have contributed to the study of molecular dynamics, the evolution of genomes, mammalian phylogeny, and comparative genetics (Kocher *et al.*, 1989; Li and Graur, 1991; Charlesworth *et al.*, 1994; O'Brien, 1994a,b; Pecon *et al.*, 1994; Avise, 1994; Morin *et al.*, 1994; D'Erchia *et al.*, 1996; Penny and Hasegawa, 1997). The partial comparisons of mtDNA at the population and species level have been used to estimate the evolutionary divergence times of the human, primate, white

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sturgeon, feline, and canine species (Aquadro and Greenberg, 1983; Ramharack and Deeley, 1987; Ruvulo *et al.*, 1993; Brown *et al.*, 1993; Janczewski *et al.*, 1995; Vila *et al.*, 1997). Nevertheless, the use of complete mtDNAs is essential for comparison because it has been shown that individual mtDNA genes may provide different answers to phylogenetic questions (Arnason and Johnsson, 1992; Cao *et al.*, 1994; Xu *et al.*, 1996a; Xu and Arnason, 1996). In recent years, phylogenetic analyses based on a concatenated sequence of the inferred amino acid sequence of protein-coding mtDNA genes (Cao *et al.*, 1994; Krettek *et al.*, 1995; D'Erchia *et al.*, 1996; Xu *et al.*, 1996b; Janke *et al.*, 1996, 1997; Penny and Hasegawa, 1997; Arnason *et al.*, 1997) made it possible to establish phylogenetic relationships among seven mammalian orders: Primates (human, chimpanzee, gorilla, orangutan, and gibbon), Artiodactyla (cow), Cetacea (whale), Carnivora (seal and cat), Rodentia (mouse and rat), Perissodactyla (horse and rhinoceros), and Marsupialia (opossum and wallaroo). The overall evidence of the above phylogenetic relationship suggested that Marsupialia and Monotremata are an outgroup to the eutherians (Janke *et al.*, 1996, 1997). The insectivores represent an earlier branch among eutherian orders (Krettek *et al.*, 1995) and rodents are consistently found basal to primates and ferungulates. Carnivora are joined with Perissodactyla (Xu *et al.*, 1996b), and Ferungulata contains all these orders including now the Cetacea (Janke *et al.*, 1994, 1997) as a sistergroup to Artiodactyla. As such, the complete sequence comparisons of mammalian mtDNAs have contributed to the documentation of a reliable phylogenetic relationship among mammals. However, although the phylogenetic relationship among mammalian orders seems to be firmly established, there remains an uncertainty concerning the relationships within the order, especially between carnivores (cat, dog, and seal). The main split within Carnivora is that between Feliodea (cats) and Canoidea (dogs and seals) as has been proposed by morphological data. However, it was difficult to demonstrate this basic split by molecular data. The comparisons within Carnivora at the level of the entire mtDNA molecule have so far been limited by paucity of data. Moreover, the phylogenetic position of the Canidae relative to other mammalian orders is still unsettled. Until now the complete mtDNA sequences of only three carnivore species (*Phoca vitulina*, *Halichoerus grypus*, and *Felis catus*) have been reported (Arnason and Johnsson, 1992; Arnason *et al.*, 1993; Lopez *et al.*, 1996).

In the present study we report on the first complete nucleotide sequence of the mitochondrial genome of the domestic dog, *Canis familiaris*, and compare it with the mtDNAs of other carnivore species (the cat and two species of seal). We also propose a dating of the evolutionary divergence between the cat, the dog, and the seal from the comparison of the mtDNAs. The

addition of the dog mtDNA to previously reported carnivore mtDNAs will contribute to a further understanding of the evolutionary relationships within this group.

MATERIALS AND METHODS

MtDNA was isolated from the liver of a Korean native dog, Sapsaree, according to Zimmerman's method (1988) and purified by cesium chloride ultracentrifugation (Lansman *et al.*, 1981). The entire mtDNA was recovered in four *Apa*I fragments (7.0, 4.8, 3.7, and 1.2 kbp) and cloned into the plasmid pGEM vector. Each cloned fragment was further digested with *Bam*HI, *Hind*III, *Eco*RI, or *Xba*I and subcloned into plasmids pGEM5Zf or pGEM7Zf. The entire mtDNA molecule was covered by natural (not PCR) clones. The mtDNA sequence was determined manually by polyacrylamide gel electrophoresis using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with [³⁵S]dATP. Sequencing was carried out using both universal and numerous synthetic oligonucleotide primers. For complete sequencing, deletion mutants were constructed with intervals of about 200 bp according to the manufacturer's protocol (Erase-a-Base system, Promega). The entire DNA sequences were determined from both strands. Sequences that contained any ambiguous or unreadable nucleotides were sequenced again with synthetic internal primers.

The mtDNA sequences, inferred amino acid sequences, and secondary structure of the ribosomal and transfer RNAs were analyzed using the PC/GENE software program (Bairoch, 1992). The pairwise comparisons of two rRNA and 13 protein-coding genes from the carnivore species were performed with the previously determined mtDNAs retrieved from the GenBank database (Accession No. U20753 for the cat, X72004 for the grey seal, and X63726 for the harbor seal). In all comparisons, the rRNA genes or protein-coding genes of the cat and dog were compared with both the harbor seal and the grey seal and then the values were averaged.

The complete mtDNA sequence of the dog, *C. familiaris*, was deposited in the GenBank database under Accession No. U96639.

RESULTS

Composition and Organization of Dog mtDNA

The length of the mtDNA molecule of the dog, *C. familiaris*, was 16,728 bp, having a control region 1270 bp long including 30 tandem repeats of a 10-nt motif between conserved sequence block (CSB) I and II. Complete sequencing relied on the universal primers supplemented with 20 specific internal primers, 17 subclones, and 42 deletion mutants. The organization

and direction of 13 open reading frames, 22 tRNA genes, two rRNA genes (12S and 16S rRNA), and the regulatory control region were the same as those in other mammalian species studied previously. The total base compositions of the L-strand were: A, 31.6%; C, 25.5%; G, 14.1%; and T, 28.8%. This composition of dog mtDNA exhibited a higher dT content than that of the seal and cat mtDNAs (Arnason and Johnsson, 1992; Lopez *et al.*, 1996). Also, similar to the seal and cat mtDNAs, the dog mitochondrial genome exhibited a higher overall dG content among mammals showing that dA or dC have converged into dG or dT. Characteristics of the dog mitochondrial genome are shown in Table 1. The numbering system for the dog mtDNA

followed the mouse and horse conventions (Bibb *et al.*, 1981; Xu and Arnason, 1994), which allocated position one of those completed mtDNA sequences to the 5' terminus of the tRNA-Phe encoding gene. The limits and nomenclature of each gene were determined by comparison with other known mammalian mtDNAs. The site of origin of light strand replication (OLR) was located within a cluster of five tRNA genes (Table 1). Although the 37-bp length of the dog OLR was one base longer than that of other mammals, its features were highly conserved with other mammals and had a stable stem-loop structure (free energy: -17.4 kcal/mol), consisting of an 11-bp stem region with complete intra-strand complementarity and a 14-base loop region as in

TABLE 1
Characteristics of the Domestic Dog Mitochondrial Genome

Gene	From 5' to 3'	Length ^a	Start codon	Stop codon	5' Intervening spacer
tRNA-Phe	1-70	70			
12S rRNA	71-1,024	954			
tRNA-Val	1,025-1,091	67			
16S rRNA	1,092-2,673	1582			
tRNA-Leu (UUR)	2,674-2,747	74			
NADH dehydrogenase subunit 1 (ND1)	2,750-3,706	957	ATG	TAA	AA
tRNA-Ile	3,706-3,774	69			
tRNA-Gln	3,845-3,771	75 L			
tRNA-Met	3,847-3,916	70			C
NADH dehydrogenase subunit 2 (ND2)	3,917-4,960	1044	ATA	TAG	
tRNA-Trp	4,959-5,026	68			ATCAAATCACCTC ^b
tRNA-Ala	5,108-5,040	69 L			C
tRNA-Asn	5,181-5,110	72 L			
Origin of L-strand replication (OLR)	5,182-5,218	37			
tRNA-Cys	5,282-5,215	68 L			
tRNA-Tyr	5,350-5,283	68 L			
Cytochrome <i>c</i> oxidase subunit I (CO I)	5,352-6,896	1545	ATG	TAA	T
tRNA-Ser (UCN)	6,964-6,894	71 L			
tRNA-Asp	6,969-7,036	68			TCAG
Cytochrome <i>c</i> oxidase subunit II (CO II)	7,037-7,720	684	ATG	TAA	
tRNA-Lys	7,738-7,804	67			CCTAGACTAGATCTACT
ATPase 8	7,806-8,009	204	ATG	TAA	A
ATPase 6	7,967-8,647	681	ATG	TAA	
Cytochrome <i>c</i> oxidase subunit III (CO III)	8,647-9,430	784	ATG	Taa ^c	
tRNA-Gly	9,431-9,498	68			
NADH dehydrogenase subunit 3 (ND3)	9,499-9,845	347	ATA	Taa ^c	
tRNA-Arg	9,845-9,912	68			
NADH dehydrogenase subunit 4L (ND4L)	9,913-10,209	297	ATG	TAA	
NADH dehydrogenase subunit 4 (ND4)	10,203-11,580	1378	ATG	Taa ^c	
tRNA-His	11,579-11,649	71			
tRNA-Ser (AGY)	11,650-11,709	60			
tRNA-Leu (CUN)	11,710-11,779	70			
NADH dehydrogenase subunit 5 (ND5)	11,780-13,600	1821	ATA	TAA	
NADH dehydrogenase subunit 6 (ND6)	14,111-13,584	528 L	ATG	TAA	
tRNA-Glu	14,179-14,110	70 L			
Cytochrome <i>b</i>	14,184-15,323	1140	ATG	AGA	ATTA
tRNA-Thr	15,324-15,393	70			
tRNA-Pro	15,458-15,393	66 L			
Control region	15,459-16,728	1270			

Note. Positions include the 5' and 3' nt of each gene. The numbering of positions starts with the 5' position of tRNA-Phe.

^a L denotes light strand (sense).

^b Denotes the 3' intervening spacer of tRNA-Trp.

^c Signifies an incomplete termination codon, and the lower case denotes a predicted codon after posttranscriptional polyadenylation.

the OLR of the harbor seal. In this structure, the stem overlapped with tRNA-Cys gene by 4 nucleotides.

Protein-Coding Genes and Codon Usage

The total number of the inferred amino acids from the 13 protein-coding genes of the dog mtDNA was 3792, showing that this number was the same as that of the cat mtDNA but shorter than that of the seal mtDNA by 3 amino acids in the nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 5 gene. As in other mammals, all protein coding genes except for the NADH6 gene are encoded on the H-strand so that the L-strand gives the sense reading frames. All protein-coding genes were oriented in the same direction, like their homologous counterparts found in other mammalian mtDNA. The pattern of codon usage in dog mtDNA followed the preference patterns reported in other mammalian mtDNA sequences (Table 2). However, compared to the cat and seal codon usage, the canine pattern shows an increase in CTT at the expense of CTA for Leu, an increase of TCT or TCG at the expense of TCA for Ser, and an increase of GCT or GCG at the

expense of GCC for Ala. These differences can partially account for the higher content of dG and dT in the overall nucleotide composition of canine mtDNA. Initiation codons (ATG or ATA) were the same as those of most other mammals. Regarding termination codons, the 11 genes usually used TAA. Of these, three genes had incomplete termination codons, which were presumably completed as TAA by posttranscriptional polyadenylation (Anderson *et al.*, 1981). The NADH2 and cytochrome *b* genes used TAG and AGA codons, respectively, as termination codons as in some other mammals. Also similar to other mammals, the coding sequences of the ATPase 8 and ATPase 6, the NADH4 and NADH4L, and the NADH5 and NADH6 genes overlapped each other by 43, 7, and 17 nucleotides, respectively.

tRNA and rRNA Genes

The limits and directions of the dog tRNA genes were determined by alignment with those of other mammalian tRNA genes (Table 1). The combined length of the tRNA genes of the dog was 1519 nt, compared to 1507

TABLE 2
Total Codon Usage in the Dog Mitochondrial Genome

Phe	TTT	107 (108 ^a , 68 ^b)	Ser	TCT	68 (33, 35)	Tyr	TAT	69 (66, 50)	Cys	TGT	5 (11, 8)
	TTC	128 (117, 163)		TCC	75 (73, 74)		TAC	74 (76, 84)		TGC	22 (14, 16)
Leu	TTA	118 (108, 68)		TCA	78 (112, 106)	Ter	TAA ^c	11 (11, 11)	Trp	TGA	93 (92, 91)
	TTG	28 (17, 20)		TCG	13 (8, 10)		TAG	1 (1, 1)		TGG	10 (12, 13)
Leu	CTT	96 (59, 57)	Pro	CCT	64 (57, 42)	His	CAT	37 (31, 36)	Arg	CGT	6 (6, 5)
	CTC	90 (97, 95)		CCC	62 (59, 81)		CAC	57 (63, 64)		CGC	10 (9, 13)
	CTA	230 (273, 297)		CCA	60 (72, 68)	Gln	CAA	68 (84, 72)		CGA	45 (45, 43)
	CTG	33 (42, 58)		CCG	7 (7, 7)		CAG	21 (7, 14)		CGG	4 (7, 4)
Ile	ATT	175 (151, 131)	Thr	ACT	75 (72, 51)	Asn	AAT	66 (56, 40)	Ser	AGT	18 (18, 17)
	ATC	167 (179, 203)		ACC	89 (101, 107)		AAC	91 (94, 107)		AGC	32 (34, 42)
Met	ATA	202 (182, 192)		ACA	120 (124, 137)	Lys	AAA	80 (89, 89)		AGA	1 (1, 1)
	ATG	47 (68, 57)		ACG	17 (14, 22)		AAG	18 (14, 12)		AGG	0 (0, 0)
Val	GTT	50 (33, 56)	Ala	GCT	63 (51, 46)	Asp	GAT	28 (29, 26)	Gly	GGT	45 (33, 26)
	GTC	27 (38, 44)		GCC	86 (116, 98)		GAC	41 (39, 46)		GGC	51 (57, 48)
	GTA	84 (95, 90)		GCA	88 (84, 100)	Glu	GAA	72 (76, 74)		GGA	95 (97, 109)
	GTG	24 (21, 28)		GCG	14 (8, 8)		GAG	21 (23, 25)		GGG	28 (30, 22)

^a Indicates number of the total codon usage of the cat mitochondrial genome (Lopez *et al.*, 1996).

^b Indicates number of the total codon usage of the Harbor seal mitochondrial genome (Arnason and Johnsson, 1992).

^c Termination codons for COIII, ND3, and ND4 genes were included as TAA under the assumption that the posttranscriptional polyadenylation of the canine mRNAs for these genes was similar to that in other mammals.

nt in the cat and 1510 nt in the seal. As in other mammals, 22 tRNA genes were found in the dog mtDNA, and had a similar organization. The small number of tRNA genes in the mammalian mtDNA is thought to be the result of strong pressure for the economization of the genome size (Anderson *et al.*, 1981, 1982; Bibb *et al.*, 1981; Clary and Wolstenholme, 1985; Wolstenholme *et al.*, 1987; Gadaleta *et al.*, 1989; Desjardins and Morais, 1990; Okimoto *et al.*, 1992). The structural features of the dog mitochondrial tRNA molecules, such as the anticodon (AC) stem and loops, T-Ψ-C, and amino acid-acceptor (A-A) arms, showed an L-shaped tertiary structure identical to those found in other mammalian mitochondrial tRNA molecules (Cedegren *et al.*, 1981; Kumazawa and Nishida, 1993). The tRNA-Ser-AGY, which was smallest in size, lacked the entire dihydrouridine (DHU) arm as in other mammalian tRNAs (Arcari and Brownlee, 1980; de Bruijn *et al.*, 1980; Udea *et al.*, 1985).

The 12S and 16S rRNA genes were separated by the tRNA-Val gene and were bound on their other sides by the genes for tRNA-Phe and tRNA-Leu (UUR), respectively. The sequences around the 3' end of the mammalian rRNA genes form a distinctive stem and loop structure (Anderson *et al.*, 1981, 1982; Bibb *et al.*, 1981; Gadaleta *et al.*, 1989). Asakawa *et al.* (1995) suggested that such a structure in the 12S rRNA and 16S rRNA could play an essential role in the protein synthesis and transcriptional regulation in mitochondria, respectively. The 3' contiguous region of the 12S rRNA of dog mtDNA was also inferred to have a stable secondary structure, showing free energy of -7.9 kcal/mol. Although the 3' contiguous region of the 16S rRNA genes showed some sequence variations among mammals, it could also be folded into stable stem and loop structure (free energy: -7.7 kcal/mol in the dog). The 3' regions of both rRNAs of dog mtDNA showed the most stable secondary structure in comparison with other mammals.

Control Region (D-Loop Region)

The control region of dog mtDNA was 1270 bp long. However, the length of the control region was not absolute due to pronounced heteroplasmy caused by variable numbers of tandem repeats of 5'-GTACAC-GT(G/A)C-3' between CSB I and CSB II. The existence of repeat motifs in the control region and the heteroplasmy caused by variable repeats were characteristic of several mammal mtDNAs: seal, cat, pig, horse, rabbit, donkey, hedgehog, platypus, rhinoceros, and opossum. The sequence of repeat motif in dog mtDNA was similar to that of pig mtDNA (Ghivizzani, 1993), except for an A/G alteration at the 9th position of the 10-bp unit. In most mammals, the repetitive portions are located in the 3' portion of the control region or between CSB I and CSB II (Walberg and Clayton, 1981; Clayton, 1991). However, in feline mtDNA (Lopez *et al.*,

1996) two repetitive sequences exist, that is, RS3 between CSB I and CSB II and RS2 between tRNA-Pro and the conserved core. Also, in the control region of rabbit mtDNA (Mignotte *et al.*, 1990), the short repeats are located between CSB I and CSB II, and the long repeats are located between tRNA-Phe and CSB III. In spite of the diversity of nucleotide sequences in the control region, a characteristic pattern of a secondary structure for the possible signaling of the start and termination of the D-loop strand has been proposed for mammals containing repetitive portions (Mignotte *et al.*, 1990). In the control region of dog mtDNA, the 30 repeat motifs between CSB I and CSB II could also be folded into a regular, compact, and stable secondary structure with free energy of -157.0 kcal/mol for the possible functioning in D-loop replication. Sequence similarities between repeats and the formation of secondary structures are consistent with a model of concerted evolution due to unidirectional replication slippage or unequal crossing over (Ohno, 1970; Buroker *et al.*, 1990; Hillis *et al.*, 1991; Wilkinson and Chapman, 1991).

Untranslated Sequences

The untranslated spacer nucleotides between the dog mitochondrial genes totaled 44 bp, 12 bp longer than those of cat. In the intervening space between the cytochrome *c* oxidase subunit II (COII) gene and the tRNA-Lys gene was the canine-specific "CTAGA" duplication, which was not observed in other mammals. When nucleotide sequences of the 3'-untranslated region among mammals were compared, the mRNAs of other mammalian COII genes except for those of canine and primates had only very short 3'-untranslated regions. Ramharack and Deeley (1987) suggested that the 3'-untranslated regions of primate COII genes have evolved through duplication events occurring within 2–3 nucleotides following the COII translational stop codon. Also, they proposed that the 3' contiguous region of primate COII mRNAs could form stable stem and loop structures immediately preceding the duplication site, thereby facilitating the mutational events involved. However, our data showed that the free energy (-2.4 kcal/mol) of the 3' contiguous region of the dog COII mRNA with a 17-nt-untranslated region did not show any discrepancy with that of other mammals which had minor untranslated regions. In particular the free energy (-10.3 kcal/mol) of the 3' contiguous region of the seal COII mRNA, which has only one 3'-untranslated nucleotide, was higher than those (free energy: -6.0 kcal/mol in human and -4.4 kcal/mol in chimpanzee) of primate COII mRNAs. Accordingly, whether or not the presence of untranslated regions in both dog and primate sequences can be correctly attributed to the same mutational event is difficult to assess at present.

TABLE 3
Percentage Differences of rRNAs from Pairwise Comparisons of Carnivores
(Ti, Transitions; Tv, Transversions)^a

	Dog/seals ^b					Dog/cat					Cat/seals ^b				
	Gap	Ti	Tv	Ti/Tv	Total	Gap	Ti	Tv	Ti/Tv	Total	Gap	Ti	Tv	Ti/Tv	Total
12S rRNA	2.2	6.8	4.8	1.5	13.8	1.7	6.7	5.5	1.2	13.9	2.8	7.2	4.9	1.5	14.9
16S rRNA	1.4	6.1	7.5	0.8	15.0	2.1	6.5	6.5	1.0	15.1	1.5	6.4	6.2	1.1	14
Mean value	1.8	6.5	6.2	1.2	14.4	1.9	6.6	6.0	1.1	14.5	2.2	6.9	5.6	1.3	14.5

^a Each gap was counted as a single difference.

^b The rRNA genes of the dog (and cat) were compared with both the harbor seal and the grey seal and then the values were averaged.

Comparison with the mtDNA of the Cat and Seal

Besides providing details of the genome analysis of dog mtDNA, the purpose of this study is to document the evolutionary relationship with previously reported carnivores (the cat, the harbor seal, and the grey seal). In order to determine the relationship between the cat, the dog, and the seal, we examined the possible differences between the mtDNAs of these groups by comparing 2 rRNA and 13 protein-coding genes. The percentage differences of the two rRNA genes with respect to total difference, transition difference, and transversion difference were studied for each species-pair of dog/seals, dog/cat, and cat/seals (see Table 3). In the combined sequence of the two rRNA genes, the mean of the nucleotide differences for each species-pair was 14.4, 14.5, and 14.5%, respectively, showing that Transition/Transversion (Ti/Tv) ratios ranged from 1.1 to 1.3% with a mean value of 1.2%. Interestingly, the dog/seals showed a higher similarity than the cat/seals in the 12S rRNA gene, and vice versa in the case of the 16S rRNA gene, indicating that the phylogeny provided by only an individual rRNA gene may obscure the evolutionary relationships among carnivore species. Table 4 shows the differences and rank (in parentheses) of the total nucleotide and the inferred amino acid sequences in the 13 protein-coding genes within each species-pair, in order of the increasing differences in each pairwise comparison. Among the three species-pairs, the range of the total nucleotide differences was 17.7–25.5% and that of total amino acid differences was 1.6–33.6%. Unlike other genes, the amino acid differences (31.3%) in the ATPase8 gene exhibited a higher mean value than the nucleotide differences (25.2%), showing that a considerable portion of the nucleotide differences of this gene was related to replacement substitutions in all the species-pairs.

Among the three species-pairs, the mean values of the nucleotide differences of the 13 protein-coding genes were 21.1, 21.2, and 20.3%, respectively, whereas the mean differences in the inferred amino acid sequences were 11.5, 11.7, and 11.9%, revealing a noticeable discrepancy between the two modes of compari-

sons. Compared with the total nucleotide differences, the amino acid differences in the protein-coding genes show a relatively good consistency for the same gene as well as being relatively insensitive to molecular saturation (Xu *et al.*, 1996a,b). Therefore, when arranged according to the combined molecular differences for both the two rRNA genes and the inferred amino acid sequences, the order of the three species-pairs was dog/seal < dog/cat < cat/seal.

This study also showed that the degree of difference between carnivore mtDNAs varied according to the individual protein-coding gene applied, suggesting that the evolutionary rate of the same protein-coding gene may differ considerably between the divergences of the carnivore species. As shown in Table 4, the individual

TABLE 4
Percentage Differences between the Mitochondrial Protein-Coding Genes of Carnivores^a

Gene	Total nucleotide differences/amino acid differences		
	Dog/seals ^b	Dog/cat	Cat/seals ^b
NADH1	(2)19.0/5.7(3)	(1)17.9/6.3(4)	(1)15.8/7.7(5)
NADH2	(11/12)23.6/18.9(7)	(12)23.7/22.2(12)	(11)22.8/23.1(12)
CO I	(6)20.1/2.5(1)	(3)19.5/2.5(1)	(3)18.3/1.6(1)
CO II	(1)17.7/4.2(2)	(4/5)19.7/4.0(2)	(4)18.6/3.8(2)
ATPase8	(13)25.5/33.6(13)	(13)25.0/31.3(13)	(13)25.0/29.1(13)
ATPase6	(5)20.0/8.0(5)	(8/9)21.6/9.7(6)	(7/8)20.3/8.0(6)
CO III	(8)20.8/7.5(4)	(2)19.2/5.0(3)	(2)17.9/6.3(3)
NADH3	(7)20.6/12.2(11)	(8/9)21.6/11.3(8)	(6)20.2/11.8(8)
NADH4L	(10)22.8/8.2(6)	(7)21.2/8.2(5)	(9)20.9/6.15(4)
NADH4	(3)19.7/9.5(8)	(11)23.3/11.5(9)	(10)21.7/12.0(9)
NADH5	(11/12)23.6/16.7(12)	(10)22.1/16.7(11)	(12)23.9/18.4(11)
NADH6	(9)20.8/11.4(10)	(4/5)19.7/12.6(10)	(7/8)20.3/15.7(10)
Cytochrome <i>b</i>	(4)19.8/11.2(9)	(6)21.1/10.3(7)	(5)19.4/10.5(7)
Mean value	21.1/11.5	21.2/11.7	20.3/11.9

^a Figures in parentheses show the position with respect to the increasing difference in each mode of comparison.

^b The protein-coding genes of the dog (and cat) were compared with both the harbor seal and the grey seal and then the values were averaged.

genes exhibited a somewhat inconsistent ranking position both in modes of comparison as well as in different species-pairs; in particular, the positions of the COIII and NADH4 genes showed a conspicuous inconsistency in the total nucleotide difference within different species-pairs. This inconsistency for the same gene, as discussed by Xu *et al.* (1996a), was more pronounced in comparisons based on the total nucleotide differences than on the amino acid differences.

The mitochondrial cytochrome *b* gene has been generally used for the documentation of evolutionary relationships between several mammals (Irwin *et al.*, 1991; Arnason and Gullberg, 1996). The conservative nucleotide substitutions of the cytochrome *b* gene, as reviewed by Arnason and Gullberg (1996), have provided results that are consistent with accepted phylogenetic relationships spanning a wide temporal range. The comparison of the cytochrome *b* gene among the three carnivore species-pairs is summarized in Table 5. The mean of the conservative differences between the three carnivore species was 11%. With respect to the conservative differences of the cytochrome *b* gene, a closer relationship between the dog and the seal was also notable. As is evident in Table 5, the Ti/Tv ratios of the third codon position within all species-pairs showed a very low value (mean value of 1.1) in comparison to those (mean value of 13.0) within the closely related species-pairs previously studied (Xu *et al.*, 1996a), suggesting that transitional saturation was pronounced among carnivore species. Therefore, as mentioned earlier, total nucleotide differences may cause considerable error in the estimation of the evolutionary separation among distantly related species.

In our data the 12S rRNA differences were in line with the amino acid differences, whereas the 16S rRNA differences conformed to the total nucleotide differences of the protein-coding genes. The total percentage differences between the tRNA genes within each species-pair of dog/seals, dog/cat, and cat/seals were 13.4, 12.5, and 13.8%, respectively, showing lower difference val-

ues than those of the rRNA genes in all the species-pairs.

The Phylogenetic Position of the Dog among Ferungulate Taxa

As mentioned in the Introduction, the phylogenetic relationships among mammalian orders based on the combined sequences of mtDNA protein-coding genes are well established. Phylogenetic analyses have grouped Carnivora, Perissodactyla, and Artiodactyla (+Cetacea) into a superordinal clade (as a group Ferungulata). In the present study the phylogenetic position of the dog relative to seven other mammalian species (six Ferungulate taxa and one rodent as an outgroup) was addressed based on the concatenated sequences of 12 protein-coding genes encoded by their mitochondrial genome. The NADH6 gene was not included as it is encoded on a different strand relative to the remaining 12 protein-coding genes. For the tree reconstruction, the maximum likelihood analysis of PUZZLE program (Strimmer and Haeseler, 1996) was accomplished. In this analysis, as a model of amino acid substitution the mtREV24 (Adachi and Hasegawa, 1996) was used, and uniform rate over all sites was assumed. The phylogenetic tree based on a maximum likelihood analysis is shown in Fig. 1. The reliability value of quartet puzzling for the internal branches of the tree topology is shown as a percentage and the Carnivora branches are designated with lowercase letters (a-c). A clade (branch a) comprising the Carnivora group (cat, dog, and seals) is supported by a reliability value of 100%. The phylogenetic analysis showed the clade (branch b) separating the dog and the seals from the cat, supporting our data based on the molecular studies of mtDNA between the cat, the dog, and the seal. Also, the phylogenetic analysis supported a sister group relationship between Carnivora and Perissodactyla. By supplementing the carnivore group with an additional dog sequence, the present maximum likelihood topology resolves the relationship of the carnivore species as well as recapitulating the results of the previous phylogenetic relationship (Xu *et al.*, 1996b; Penny and Hasegawa, 1997; Arnason *et al.*, 1997).

The Dating of the Evolutionary Divergence within the Carnivore Species

Among the carnivore species, evolutionary divergence based on the molecular study of mtDNA is relatively unknown, although several molecular techniques, such as protein electrophoresis, DNA hybridization, and microcomplement fixation, have been used to reconstruct phylogenetic relationships and to estimate divergence time (Wayne *et al.*, 1991).

Recently, phylogenetic analyses based on a concatenated sequence of the inferred amino acid of protein-coding mtDNA genes have contributed to establish reliable phylogenetic relationships between mamma-

TABLE 5

Comparison of the Cytochrome *b* Gene in Three Carnivore Species-Pairs^a

	Dog/seals	Dog/cat	Cat/seals
Percentage nt difference	19.8	21.1	19.4
Percentage cons. nt difference	10.5	11.3	11.2
Percentage aa difference	11.2	10.3	10.5
Ratio nt substitution	2.8/1.0/9.6	2.7/1.0/9.6	2.7/1.0/12.1
Ratio cons. nt substitution	1.8/1.0/4.3	1.7/1.0/4.5	2/1.0/6.2
Ratio 3rd codon pos. Ti/Tv	1.26	1.14	0.95

^a Cons. (conservative) difference defined as all nonsynonymous nucleotide substitutions in the first codon position, all substitutions in the second codon position, and transversions in the third codon position. nt, nucleotide; aa, amino acid.

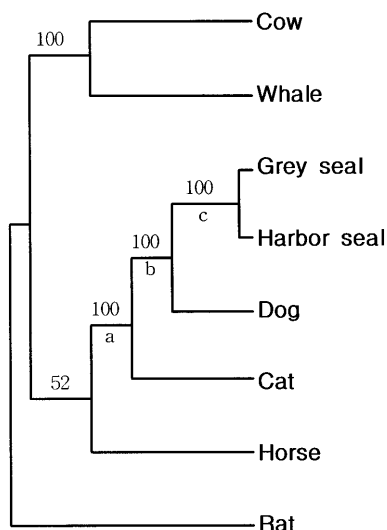


FIG. 1. Phylogenetic position of the dog relative to seven other mammalian species (six ferungulate taxa and one rodent), based on 12 proteins encoded by their mitochondrial genome. All mitochondrial genomes are available in the GenBank database: cat (U20753), harbor seal (X63726), grey seal (X72004), cow (J01394), fin whale (X61145), horse (X79547), and rat (X14848). The maximum likelihood method of PUZZLE Version 4.0 (Strimmer and Haeseler, 1996) was used to reconstruct the phylogenetic tree from the concatenated amino acid sequences of protein-coding genes, except for NADH 6 which is encoded on a different strand relative to the remaining 12 protein coding genes. As a model of amino acid substitution the mtREV24 (Adachi and Hasegawa, 1996) was used, and as a model of rate heterogeneity uniform rate over all sites was taken into account. For robustness of the tree, the analysis was accomplished using 1000 puzzling steps. The reliability value of quartet puzzling for the internal branches of the tree topology is shown as a percentage.

lian orders. Also, as discussed earlier, the analyses on the basis of the conservative nucleotide differences of the cytochrome *b* gene have generally been in good agreement with accepted phylogeny. Accordingly, the differences of a concatenated amino acid sequence of protein-coding genes and the differences of the conservative nucleotide of the cytochrome *b* gene have made it

possible to propose a date for the evolutionary separation between the cat, the dog, and the seal.

The genetic distances between the eight mammalian species based on the concatenated amino acid sequence of 12 protein-coding mtDNA genes are given in Table 6. By applying, as a reference, a divergence time of 60 million years ago (MYA) for artiodactyls and cetaceans (Arnason and Gullberg, 1996), the values in Table 6 suggest that the evolutionary divergences within each species-pair of dog/seal, dog/cat, and cat/seal were dated to 44, 46, and 47 MYA, respectively. In addition, based on the differences of the conservative nucleotide of the cytochrome *b* gene (Table 5), the evolutionary divergences within above carnivore species were dated to approximately 50 ~ 54 MYA.

In this study, the differences of the concatenated amino acid sequences as well as the differences of the conservative nucleotide of the cytochrome *b* gene showed similar values within each species-pair, showing mean values of 11.7% and 11.0%, respectively. Also, the phylogenetic topology showed that the carnivore species formed a clade, sharing a common ancestor. Therefore, there is no reason to attempt to discriminate absolutely the divergence time of the cat/dog/seal. In the present study, the molecular differences of the mtDNA indicate that the cat, the dog, and the seal had a common ancestor 50 ± 4 MYA.

DISCUSSION

The genome organization, gene contents, and codon usage of the dog mitochondrial genome conformed to those of other mammalian mitochondrial genomes, except for the "CTAGA" duplication events following the translational stop codon of the COII gene. As mentioned in the Introduction, the evolutionary relationships between carnivore species, based on the comparison of complete mtDNA, have so far been limited due to the paucity of data. The present inclusion of the complete sequences of dog mtDNA have

TABLE 6

Genetic Distances Based on Amino Acid Differences between Eight Mammalian Species^a

	Cow	Whale	Seal (G)	Seal (H)	Dog	Cat	Horse	Rat
Cow	0.00000							
Whale	0.15462	0.00000						
Seal (G)	0.15912	0.19541	0.00000					
Seal (H)	0.15451	0.19371	0.01589	0.00000				
Dog	0.15623	0.20172	0.11399	0.11318	0.00000			
Cat	0.14930	0.19725	0.12109	0.12020	0.11790	0.00000		
Horse	0.13409	0.17155	0.14517	0.14486	0.13839	0.13336	0.00000	
Rat	0.29314	0.31965	0.30257	0.30092	0.29510	0.28529	0.28538	0.00000

^a Order: Artiodactyla (cow), Cetacea (fin whale), Carnivora (Grey (G) seal, Harbor (H) seal, dog, cat), Perissodactyla (horse), Rodentia (rat). Distance values were obtained from the maximum likelihood distances of the PUZZLE program (Strimmer and Haeseler, 1996) based on a comparison of the concatenated amino acid sequences (3627 aa) of 12 protein-coding mtDNA genes.

made it possible to address the relationships between carnivore species. The combined molecular differences, in two rRNA genes as well as in the inferred amino acid sequences of the mitochondrial 13 protein-coding genes, would suggest that there is a closer relationship between the dog and the seal than there is between either of these species and the cat. Our data support the previous morphological data that indicate a main split between Feliodea (cats) and Canoidea (dogs/seals). This study also showed that the evolutionary rates provided by an individual mtDNA gene may differ quite considerably, suggesting that the evolutionary relationship of distantly related species should be examined with caution unless supported with sufficient sequence data and/or a comprehensive representation of taxa. The different patterns characterizing the individual protein-coding gene in a pairwise comparison of carnivore species support the previous reports (Arnason *et al.*, 1993; Cao *et al.*, 1994; Xu *et al.*, 1996a) that the same protein-coding gene may show different evolutionary rates in different pairwise comparisons, making it difficult to establish a general evolutionary relationship from each gene on its own.

The availability of mtDNA molecules of the dog and other carnivore species (cat and seal) made it possible to propose the dating of the evolutionary separation of these groups on the basis of complete mtDNA data. The proposed dating of the divergence between the cat, the dog, and the seal, at 50 ± 4 MYA, conformed to the estimates of Wayne *et al.* (1991) who placed the maximum divergence date of the Carnivora species at around 55 million years based on a protein electrophoresis and DNA hybridization. Also, the dating was in line with the divergence time (approximately 50 ± 5 MYA) based on the transversion rates of the 12S rRNA gene which has been found to correlate with time (Arnason and Johnsson, 1992). However, the dating was not consistent with divergence dates from fossil records. The fossil records which estimate the divergence time of the Carnivora species are relatively plentiful and have been well studied. Most paleontological evidence suggests that the divergence time of the Carnivora species is no older than 40 MYA. The paleontological record of Canidae was traceable to 32–35 MYA (Savage and Russell, 1983), and the earliest divergence of Felidae in fossil records was estimated at 35–40 MYA (Radinsky, 1975; Hunt, 1989). Also, the fossil records placed the divergence time of Pinnipedia at 27–30 MYA (Berta *et al.*, 1989). However, generally paleontological records grossly underestimate the age of evolutionary divergences. As discussed by Xu *et al.* (1996b), although fossil records are reasonably complete and correctly estimated, the time of evolutionary separation should be estimated before the development of morphological traits that can be recognized among fossil finds. The present molecular analysis suggests that the age of Carnivora fossils falls well within the dating of the

carnivore separation. The dog, *C. familiaris*, is one of the oldest domesticated animals and one of the most familiar to man. In recent times, dogs have been a common focus for research concerning their evolution and origin (Roy *et al.*, 1994; Vila *et al.*, 1997). Accordingly, the full sequence of dog mtDNA will be useful for the further study of the evolution and genetics of the canine species as well as for the documentation of the phylogenetic position of the dog among mammalian orders.

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