

# Molecular Evolution of Mitochondrial 12S RNA and Cytochrome b Sequences in the Pantherine Lineage of Felidae

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DNA sequence comparisons of two mitochondrial DNA genes were used to infer phylogenetic relationships among 17 Felidae species, notably 15 in the previously described pantherine lineage. The polymerase chain reaction (PCR) was used to generate sequences of 358 base pairs of the mitochondrial 12S RNA gene and 289 base pairs of the cytochrome b protein coding gene. DNA sequences were compared within and between 17 felid and five nonfelid carnivore species. Evolutionary trees were constructed using phenetic, cladistic, and maximum likelihood algorithms. The combined results suggested several phylogenetic relationships including (1) the recognition of a recently evolved monophyletic genus *Panthera* consisting of *Panthera leo*, *P. pardus*, *P. onca*, *P. uncia*, *P. tigris*, and *Neofelis nebulosa*; (2) the recent common ancestry of *Acinonyx jubatus*, the African cheetah, and *Puma concolor*, the American puma; and (3) two golden cat species, *Profelis temmincki* and *Profelis aurata*, are not sister species, and the latter is strongly associated with *Caracal caracal*. These data add to the growing database of vertebrate mtDNA sequences and, given the relatively recent divergence among the felids represented here (1–10 Myr), allow 12S and cytochrome b sequence evolution to be addressed over a time scale different from those addressed in most work on vertebrate mtDNA.

## Introduction

In many habitats, the largest true carnivores are members of the Felidae. Currently, representatives of the genus *Panthera* dominate the extant carnivorous mammals in both size and prey utilization and are nearly cosmopolitan in their range. Felid predecessors, including the Rancho La Brea saber-toothed cat (*Smilodon fatalis*), the scimitar-toothed saber-toothed cat (*Homotherium serum*), and the Pleistocene lion (*Panthera atrox*), were also the dominant carnivores during their era (Van Valkenburgh 1991). Because of their prominent position atop the food chain, many aspects of felid biology have been well studied, including their behavior, ecology, morphology, evolution, and genetics (Guggisberg 1975; Kitchener 1991; Seidensticker and Lumpkin 1991). Furthermore, species conservation efforts have focused attention on the Felidae, since 36 of 37 recognized modern species are considered as either "endangered" or "threatened" by international conventions that list endangered species (USFWS 1973; IUCN 1986).

Key words: phylogeny, Felidae, mitochondrial DNA, Carnivora.

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In spite of the intensity and scope of cat research, there remains considerable uncertainty as to the evolutionary relationship among the 37 living cat species (Hemmer 1978; Leyhausen 1979; Neff 1982; Collier and O'Brien 1985; Kitchener 1991; Nowak 1991; Saito 1992). A major reason for this uncertainty is the rather recent radiation producing 37 distinct species within the last 10 Myr (Savage and Russell 1983; Nowak 1991; Wayne et al. 1991). Cats are typically very mobile and cosmopolitan, which for many species has led to considerable range overlap. For instance, the felids represented in our study (table 1) are found in Africa (lion, cheetah, caracal, serval, African golden cat), Asia (lion, snow leopard, marbled cat, clouded leopard, Temminck's golden cat), or Eurasia (tiger, leopard), and North and/or South America (jaguar, puma, cheetah [historically], lynxes, and ocelot). The domestic cat is currently cosmopolitan. The occurrence of multiple felid species in some regions (10 in Africa, 10 in South America), coupled with the relative recency of the felid radiation, suggests that speciation of cats may have been a swift response to differing environments encountered by these mobile creatures. For instance, the 10 cat species in South America have all either arisen in situ or entered after closure of the Panama land bridge about 2–3 MYA. Hence, it will be important to understand biogeography in the context of felid lineage separation and diversification.

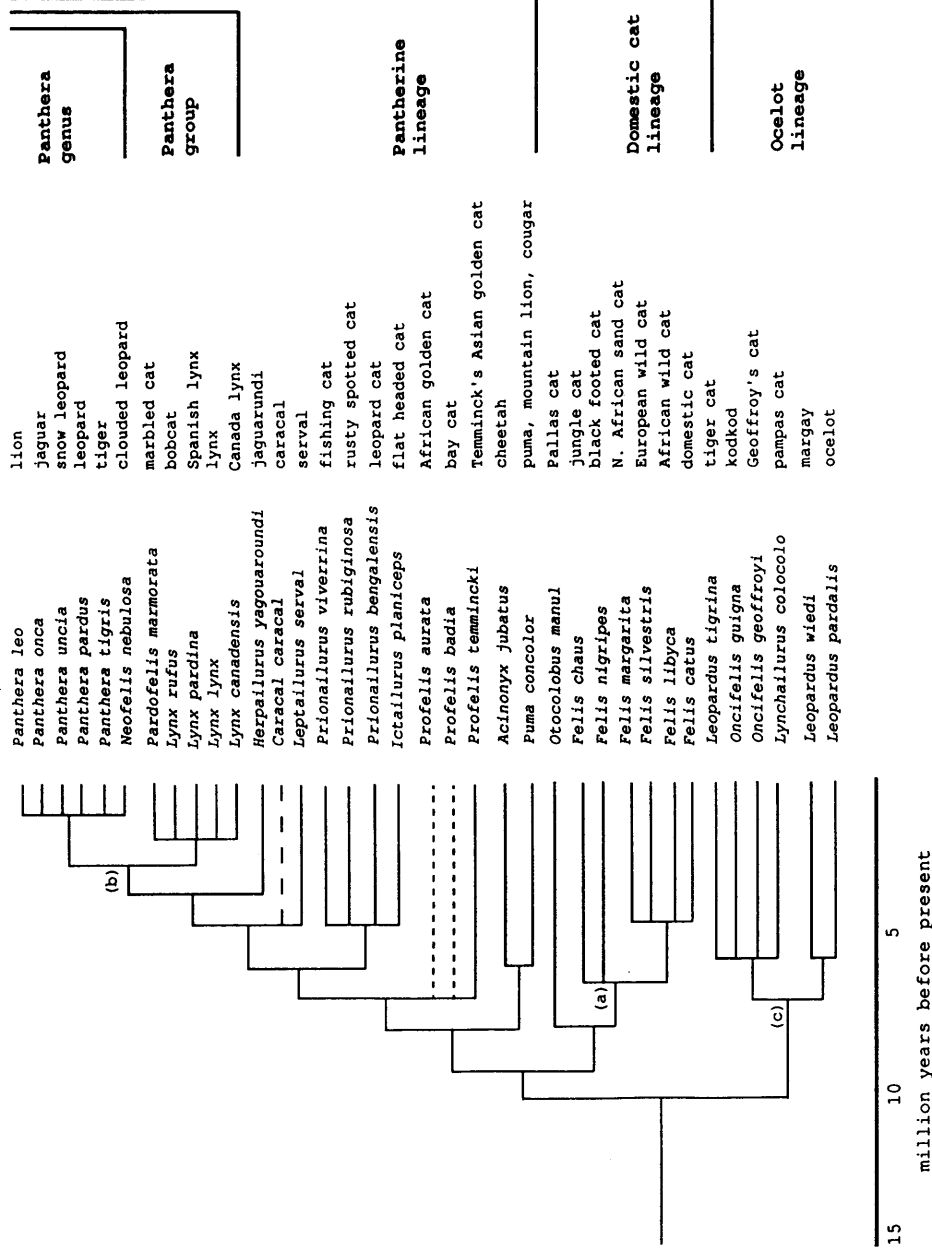


FIG. 1.—Phylogenetic relationships of Felidae based on prior molecular studies using immunological distance (Collier and O'Brien 1985), isozyme electrophoresis (O'Brien et al. 1987), karyology (Wurster-Hill and Gray 1975; Wurster-Hill and Centerwall 1982; Modi and O'Brien 1988), and endogenous retroviruses (Benveniste 1985). *a*, Entry of endogenous retroviral families feline leukemia virus and RD-114; *b*, members of *Panthera* group have identical karyotype; *c*, South American ocelot lineage; all have 36 chromosomes and share a metacentric chromosome C3. All other cat species have 2N = 38 chromosomes and lack C3. Dashed lines indicate tentative placement of species at position. Classification used as per Kitchener (1991) modified from O'Brien (1986). Estimated divergence times are from Wayne et al. (1991) and Savage and Russell (1983).

ation. The development of an accurate phylogenetic topology tracking the evolutionary history of the Felidae would offer a powerful adjunct to interpreting the evolutionary emergence of functional adaptations that characterize felid species. Substantial genetic differentiation within the Felidae below the species level can also be inferred from the large number of subspecies described in pumas and leopards. A comprehensive phylogenetic hierarchy would be highly useful in identifying and aid in ranking the units of conservation in the cat family.

The evolutionary relationships among members of the Felidae have been inferred using a variety of morphological and molecular techniques. Taxonomy, based on morphological and behavioral criteria, produced classifications that group the cats into as few as two, or

as many as 19, genera (Ewer 1973; Hemmer 1978; Leyhausen 1979; Neff 1982; Nowak 1991). In a recent study of 44 cranial characters in extant felid species, Salles (1992) resolved three primary clades corresponding roughly to *Leopardus* (ocelot), *Felis* (domestic cat), and the pantherine lineages, in agreement with earlier molecular data (Collier and O'Brien 1985; O'Brien 1986), although several of the pantherine cats (notably *Profelis temmincki* and *Pardofelis marmorata*) aligned outside the pantherine lineage.

O'Brien and collaborators (Collier and O'Brien 1985; O'Brien 1986; O'Brien et al. 1987; Wayne et al. 1988) used four molecular techniques (protein electrophoresis, microcomplement fixation, DNA-DNA hybridization, and G-banded karyology) to describe the phylogenetic relationships within the Felidae (fig. 1).

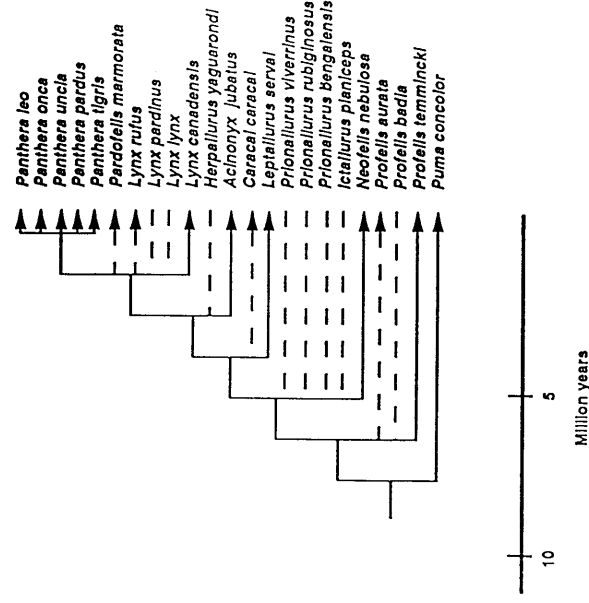


FIG. 2.—Phylogenetic tree of the pantherine lineage (Kitchener 1991; modified from O'Brien 1986) with estimated date of first appearance in fossil record. Species analyzed in present study are marked with an arrow. Dashed lines indicate uncertainty in the placement of the species within the evolution of the cats. Estimated divergence times are from Wayne et al. (1991) and Savage and Russell (1983).

Their results did not resolve all relationships but provided evidence for the occurrence of three primary radiations within the felids. Under the assumption of a molecular clock calibrated with paleontological fossil dates, their results suggested that the earliest separation occurred approximately 10 MYA when the ancestor to the South American ocelot lineage diverged. Seven to nine million years ago, the domestic cat lineage was formed, followed by a gradual divergence of the remaining large cats 1–8 MYA. This evolutionary scheme is supported by the presence of two different endogenous retroviral sequences, RD-114 and feline leukemia virus, found only in members of the domestic cat lineage (Benveniste 1985). Karyotypic evidence also supports these divisions; karyotypes are identical among members of the *Panthera* group, and members of the ocelot lineage have a distinctive karyotype of 36 chromosomes, including a Robertsonian fusion metacentric chromosome C3 (Wurster-Hill and Centerwall 1982; Modi and O'Brien 1988).

Members of genus *Panthera* or "great cats" include the lion, tiger, leopard, jaguar, and snow leopard. These species have been classified together because of the presence of a specialized jaw structure (flexible hyoid process) that consistently appears in most members of this group (Neff 1982; Salles 1992). However, species evolutionary relationships within *Panthera* are still unresolved. For instance, the earliest *Panthera* fossil from

lion	earliest fossil appearance million years	fossil date reference
jaguar	1-2	Neff, 1982
snow leopard	1.6	Kurten and Anderson, 1980
leopard	1.3	Turner, 1987
tiger	1.8	Neff, 1982
marbled cat		
bobcat	3-4	Werderlin, 1985
Spanish lynx		
lynx		
Canadian lynx	3-5	Turner, 1987; Ficcarelli, 1984
jaguarundi	3-5	Turner, 1987; Savage and Russell, 1983
cheetah		
caracal lynx		
serval		
fishing cat		
rusty-spotted cat		
leopard cat		
flat-headed cat		
clouded leopard		
African golden cat		
bay cat		
Temminck's Asian golden cat		
puma, mountain lion, cougar	3-3.5	Werderlin, 1985

the East African Pleistocene (2 Myr old) displays a lion- or tigerlike morphology, suggesting a close relationship between the two species. Yet, fossils of the North American lion, *Panthera atrox* (30,000 yr old), as well as fossils of a European Pleistocene cat *Panthera gombaszoegensis* (2 Myr old), display both primitive lion and jaguar features suggesting a closer relationship between these two species (Hemmer 1971; Neff 1982). Pocock (1907), however, suggested that the jaguar and leopard are closest based on morphological characteristics of extant representatives. Some controversy also exists on the inclusion of the snow leopard in this group because it lacks the flexibility in the hyoid process and because of morphological similarities to the cheetah, which also lacks this specialization.

The molecular-based "pantherine lineage" (fig. 2) is composed of the five *Panthera* species and several small and mid-sized felids: puma, marbled cat, bobcat, clouded leopard, cheetah, the golden cats, and jaguarundi (O'Brien 1986). As with *Panthera*, uncertainty exists over the relative placement of these species, particularly clouded leopard and cheetah. The cheetah is usually classified as a distinct monotypic genus due to its divergent morphological characteristics. However, its phylogenetic relationships to other members within the pantherine lineage is not well understood. A candidate relative of the cheetah, *Felis trumani* (20,000 yr old), reflects not only cheetah but

**Table 1**  
**Sample Sources**

Code	Species Name	Common Name(s)	Contact	Source
PleSER . . . . .	<i>Panthera leo leo</i>	Serengeti lion	Craig Packer	Serengeti Plains, Tanzania, Africa
PleNGC . . . . .	<i>Panthera leo leo</i>	Ngorongoro Crater lion	Craig Packer	Ngorongoro Crater, Tanzania, Africa
PleAT . . . . .	<i>Panthera leo leo</i>	Atlas lion	Mitchell Bush	National Zool. Park, Washington D.C.
PleSA . . . . .	<i>Panthera leo leo</i>	South African (zoo) lion	Mike Bleyman	CPT, Pittsboro, N.C.
PleAS . . . . .	<i>Panthera leo persica</i>	Asiatic lion	P. P. Rawal	Sakkarbaug Zoo, Junagadh, India
PtiAL . . . . .	<i>Panthera tigris altaica</i>	Siberian tiger	Bonnie Raphael	Stone Zoo, Stoneham, MA
PtiSU . . . . .	<i>Panthera tigris sumatrae</i>	Sumatran tiger	Don Jansen	Dallas Zoo, Dallas
PtiTI . . . . .	<i>Panthera tigris tigris</i>	Bengal tiger	Mike Fouraker	San Diego Zoo, San Diego
Ppa . . . . .	<i>Panthera pardus pardus</i>	Leopard	Don Jansen	Knoxville Zoo, Knoxville, Tenn.
PpaSL . . . . .	<i>Panthera pardus koiya</i>	Leopard—Sri Lanka	Mike Bleyman	San Diego Zoo, San Diego
Pun . . . . .	<i>Panthera uncia</i>	Snow leopard	Mitchell Bush	CPT, Pittsboro, N.C.
Pon . . . . .	<i>Panthera onca</i>	Jaguar	Sri Miththapala	National Zool. Park, Washington, D.C.
AjuJU . . . . .	<i>Acinonyx jubatus jubatus</i>	South African cheetah	Sue Mainka	MIN Zool. Garden, Apple Valley, Minn.
AjuRA . . . . .	<i>Acinonyx jubatus raineyi</i>	East African cheetah	Mike Bleyman	National Zoological Gardens, Dehiwela, Sri Lanka
PcoCO . . . . .	<i>Puma concolor coryi</i>	Puma, mountain lion	Don Jansen	Calgary Zoo, Canada
PcoOR . . . . .	<i>Puma concolor oregonensis</i>	Puma, mountain lion	Melody Roelke	CPT, Pittsboro, N.C.
Lru . . . . .	<i>Lynx rufus</i>	Bobcat, red lynx	Randall Junge	San Antonio Zoo
Lea . . . . .	<i>Lynx canadensis</i>	Canada lynx	Ann van Dyk	San Diego Zoo, San Diego
Pma . . . . .	<i>Pardofelis marmorata</i>	Marbled cat	Matthew George	Wildlife Safari, Winston, Ore.
Nne . . . . .	<i>Neofelis nebulosa</i>	Clouded leopard	Mitchell Bush	St. Louis Zoo, St. Louis
Cea . . . . .	<i>Caracal caracal</i>	Caracal lynx	Karl Ammann	DeWildt Center, South Africa
Lse . . . . .	<i>Leptailurus serval</i>	Serval	Wilber Amand	San Diego Zoo, San Diego
Pte . . . . .	<i>Profelis temmincki</i>	Temminck's golden cat	Roger Brannan	National Zool. Park, Washington, D.C.
Pau . . . . .	<i>Profelis aurata</i>	African golden cat	Melody Roelke	Kenya
Lpa . . . . .	<i>Leopardus pardalis</i>	Ocelot	Joe Maynard	Philadelphia Zoo
Fca . . . . .	<i>Felis catus</i>	Domestic cat	Mike Bleyman	Kansas City Zoo
Ccr . . . . .	<i>Crocuta crocuta</i>	Spotted hyena	Dirk van Damm	Everglades National Park, Fla.
Uth . . . . .	<i>Ursus americanus</i>	American black bear	Mitchell Bush	Bear Island, Big Cypress Swamp, Fla.
Uar . . . . .	<i>Ursus arctos yezonensis</i>	Brown bear	Mike Bleyman	Union Co., Oreg.
Cfa . . . . .	<i>Canis familiaris</i>	Domestic dog	Joe Maynard	Everglades National Park, Fla.
Cme . . . . .	<i>Canis mesomelas</i>	Black-backed jackal	Lyndsay Phillips	Rare Feline Breeding Colony, Calif.
			Ken Fletcher	CPT, Pittsboro, N.C.
			Lyndsay Phillips	CPT, Pittsboro, N.C.
			Dirk van Damm	Blijdorp Zoo, Rotterdam, Netherlands
			Lyndsay Phillips	National Zool. Park, Washington, D.C.
			Dianne Janczewski	CPT, Pittsboro, N.C.
			Lyndsay Phillips	CPT, Pittsboro, N.C.
			Ken Fletcher	Rare Feline Breeding Colony, Calif.
			Lyndsay Phillips	Blijdorp Zoo, Rotterdam, Netherlands
			Dianne Janczewski	Henry Doory Zoo, Omaha, Neb.
			Lynn Colly	FCRDC/NCI, Md.
			Dianne Janczewski	Henry Doory Zoo, Omaha, Neb.
			Lynn Colly	San Antonio Zoo
			Lyndsay Phillips	Shrine Circus
			Dianne Janczewski	FCRDC/NCI, Md.
			Lynn Colly	Johannesburg Zoo, South Africa

**Table 2**  
**Nucleic Acid Sequence Biological Specimens**

CODE	COMMON NAME(S)	DNA TISSUE SOURCE <sup>a</sup>	SPECIMEN NUMBER	NO. SEQUENCES OBTAINED <sup>b</sup>	
				12S Ribosomal	Cytochrome b
PleSER . . . .	Serengeti lion	WBC	Ple-S100	...	1
			Ple-S101	1 (1)	1
			Ple-S102	1	1
			Ple-S103	1	3 (2)
			Ple-S105	1	1*
	Total		5	9	
PleNGC . . .	Ngorongoro Crater lion	WBC	Ple-C2	1	...
			Ple-C4	1	1
			Ple-C6	1	...
			Ple-C8	1	1
			Ple-C11	1	1
	Total		5	3	
PleAT . . . . .	Atlas lion	WBC	Ple-7	2	1
			Ple-24	1	2
	Total		3	3	
PleSA . . . . .	South African (zoo) lion	TC	Ple-1	2	1
			Ple-23	1	...
			Ple-121	2	1
			Total	5	2
PleAS . . . . .	Asiatic lion	WBC	Ple-A5	2	2
			Ple-A6	1	1
			Ple-A9	1	1
			Ple-A13	1	1
			Ple-A20	1	1
	Total		8	5	
PtiAL . . . . .	Siberian tiger	Organ	Pti-66	2	1
			Pti-85	2	1
	Total		4	2	
PtiSU . . . . .	Sumatran tiger	WBC	Pti-83	1	1
			Pti-82	2	1
	Total		3	2	
PtiTI . . . . .	Bengal tiger	Organ	Pti-77	3 (2)	2 (2)
			Ppa-4	1	1
Ppa . . . . .	Leopard	Organ	Ppa-20a	2	2*
			Ppa-21a	1	1
			Ppa-31	1	1
			Total	5	4
Ppa-SL . . . . .	Leopard—Sri Lanka	WBC	Ppa-SL5	2	1
			Ppa-SL6	2	1
	Total		4	2	
Pun . . . . .	Snow leopard	WBC	Pun-9c	2	2*
			Pun-16	1	1
			Pun-11	4	2
	Total		7	5	
Pon . . . . .	Jaguar	TC	Pon-1	1	3
			Pon-9	1	2
	Total		2	5	
AjuJU . . . . .	South African cheetah	Organ	Aju-70	1 (5)	2 (3)
			Aju-72	2	...
			Aju-254	1	1
			Aju-31	...	1
			Aju-78	3	...
	Total		14	8	

Table 2 (Continued)

CODE	COMMON NAME(S)	DNA TISSUE SOURCE <sup>a</sup>	SPECIMEN NUMBER	NO. SEQUENCES OBTAINED <sup>b</sup>	
				12S Ribosomal	Cytochrome b
AjuRA	East African cheetah	Organ TC WBC WBC	Aju-227 Aju-196 Aju-231 Aju-232 Total	2 2*	...
PcoCO	Puma	WBC TC TC	Pco-71 Pco-156 Pco-120 Total	1 2 2*	1 1 3
PcoOR	Puma	Spleen Spleen	Pco-110 Pco-111 Total	2 1 3	1 1 2
Lru	Bobcat	Liver TC TC TC WBC	Lru-18 Lru-39 Lru-37 Lru-58 Lru-54 Total	2 (2) ...	2 (2) ...
Lca	Canada lynx	WBC	Lca-1	2	2
Pma	Marbled cat	WBC	Pma-5	2	2
Nne	Clouded leopard	Organ TC WBC	Nne-9 Nne-19E Nne-22 Total	1 1 2 4	1 1 2 2
Cca	Caracal lynx	TC	Cca-12	2	2
Lse	Serval	TC	Lse-1	2	2
Pte	Temminck's golden cat	WBC TC	Pte-6 Pte-8 Total	3 3 6	1 2 3
Pau	African golden cat	TC WBC	Pau-1 Pau-2 Total	2 2 4	2 2 4
Lpa	Ocelot	TC	Lpa-2	2	2
Fca	Domestic cat	TC WBC	Fca-553B Fca-65 Total	1 1 2	2 1 3
Cer	Spotted hyena	TC	Ccr-1	3	2
Uth	American black bear	Organ	Uth-1	2	2
Uar	Brown bear	TC	Uar-1	2	2
Cfa	Domestic dog	WBC	Cfa-9	2	...
Cme	Black-backed jackal	WBC	Cme-1	2	2

<sup>a</sup> TC-fibroblast tissue culture; WBC, white blood cells.

<sup>b</sup> The number of direct PCR sequences from each individual DNA sample is indicated under each gene. The number of M13 cloned sequences obtained from PCR products is in parentheses. Variant sequences within the indicated population or subspecies group are indicated by asterisks; the most common residue is used in analyzed sequences in fig. 4.

also snow leopard and puma characteristics (Orr 1969; Adams 1979).

In order to resolve some of these phylogenetic issues, two regions of the mitochondrial DNA were sequenced and analyzed. A 358-base-pair region of the 12S ribosomal RNA gene (12S) and a 289-bp region of

the cytochrome b protein coding gene (Cytb) were PCR amplified, sequenced, and compared from 75 individuals representing 17 felid and five nonfelid carnivore species (table 1). Multiple sequences were obtained, often from several individuals within each species, and PCR-derived sequences were compared to cloned sequences to verify

**Table 3**  
**PCR Primers (5'-3')**

	Size Bases	Human Map Position <sup>a</sup>	Strand <sup>b</sup>
<b>12S Ribosomal RNA:</b>			
12S-1	25	1067-1091	L
12S-2	20	1497-1478	H
12S-3	26	1254-1279	L
12S-4	26	1279-1254	H
<b>Cytochrome b:</b>			
Cytb-1	24	14700-14723	L
Cytb-2	25	15174-15150	H
Cytb-4	21	14938-14918	H

<sup>a</sup> Numbers refer to sequence position as in Anderson et al. (1981).

<sup>b</sup> Sequence is read from L (light) strand or H (heavy) strand.

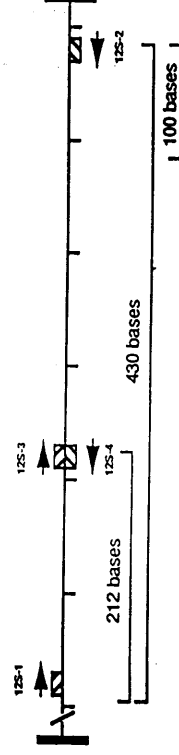
the PCR-derived sequences. Subspecies comparisons were made within tigers, pumas, cheetahs, leopards, and lions; population differences were also examined for the lion. Three methods of phylogenetic inference were employed—phenetic (distance based), maximum parsimony, and maximum likelihood—to decide which evolutionary relationships were suggested by the 12S RNA and Cytb data (Fitch and Margoliash 1967; Swofford 1989; Felsenstein 1990; Swofford and Olsen 1990). Trees were compared and a composite evolutionary scheme was determined based on relationships suggested by different gene sequences and by different phylogenetic algorithms. The inferred tree supported certain previously

established relationships and resolved several additional ancestral associations. Furthermore, the patterns of molecular evolution for the 12S and cytochrome b sequences were addressed with this phylogeny.

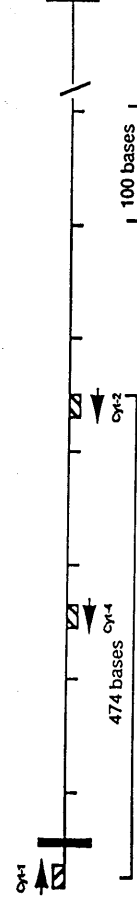
#### Material and Methods

Blood and skin biopsy samples were obtained from 75 individuals of feline and other carnivore species listed in table 1. DNA was obtained from frozen peripheral blood mononuclear cells or from primary fibroblast cultures. The number of sequences obtained from each specimen is presented in table 2. DNA extractions were performed using standard methods (Sambrook et al.

#### 12S RNA (human) 1023bp



#### Cytochrome b (human) 1141bp



**FIG. 3.**—Position and direction of PCR primers used for amplification of feline mtDNA 12S RNA and cytochrome b sequences. Primer sequences are in table 3.

1989); nucleotide sequences were obtained by PCR amplification of genomic DNA by methods of Saiki et al. (1985), with modification of Engelke et al. (1988). The PCR primers used are listed in table 3; their locations within the respective gene sequences are diagrammed in figure 3. A straight single-strand method of 40 cycles of PCR was performed on the 12S region in a programmable heat block (Perkin-Elmer Cetus DNA Thermal Cycler), denaturation at 92°C for 1 min., annealing at 50°C for 1 min. and extension at 72°C for 1 min. We prepared 100 µl reactions using 10 ng genomic DNA in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM each of dATP, dCTP, dGTP, and dTTP, 2.5 units *Thermus aquaticus* DNA polymerase, and a primer ratio of 100:1 (25 pmoles:0.25 pmoles) of primers 12S-1 and 12S-2, layered with mineral oil. The Cytb region was amplified using similar conditions, with primers Cytb-1 (25 pmoles) and Cytb-2 (25 pmoles) at an annealing temperature of 55°C. A second (single-stranded) amplification was subsequently performed using an unequal primer ratio (Cytb-1:Cytb-2) and 1 µl of the double-stranded product.

Single-stranded reaction products were resuspended in 2 ml dH<sub>2</sub>O and concentrated with Centricon 30 microconcentrators three times, dried in a vacuum centrifuge, and resuspended in 12 µl sterile dH<sub>2</sub>O. The dinucleotide chain-termination sequencing reaction was performed with the Sequenase kit (USB) as recommended by the manufacturer using primers 12S-2, 12S-3, Cytb-1, and Cytb-4. Fragments were separated in 6% polyacrylamide gel containing 1× TBE and 8 M urea for 2 h at 55 watts. Gels were dried onto Whatman 3M paper under a heated vacuum gel dryer and exposed to Kodak X-Omat film for 1–2 d.

Sequences obtained from cloned DNA were obtained from proteinase K (200 µg/ml) treatment of double-stranded products, incubated at 37°C for 30 min to digest polymerase; DNA was phenol-chloroform treated, ethanol precipitated, and resuspended in 16 µl 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TLE). Fragment ends were converted to blunt ends in 20 µl reactions at final concentrations of 1 U T<sub>4</sub> DNA polymerase B, 1.25 mM dNTPs, 1× React buffer #2 (USB), and incubated 20 min at room temperature. Fragments were isolated as above by electrophoresis and DEPH paper isolation and resuspended in 30 µl TLE; samples were ligated into *Sma*I digested M13mp19 in 10 µl reactions with 4 µl DNA, and final concentrations of 0.05 U/µl DNA ligase, 1× ligase buffer (USB), 0.25 µg/µl vector, and 3 µl dH<sub>2</sub>O, incubated at 14°C overnight. Volume was increased to 50 µl with dH<sub>2</sub>O. We added 2 µl DNA to 100 µl competent cells (*Escherichia coli* DH5a F'), incubated 30 min on ice, heat-shocked 15 min at 42°C,

and placed on ice for 2 min. We added 5 ml Top Agar (DIGENE), 80 µl 2% x-gal, and 40 µl 0.1 M IPTG to samples and plated onto L-broth plates containing 50 µg/µl ampicillin, incubated overnight at 37°C. Positive colonies (white) were excised and suspended in 3 ml Super Broth (DIGENE), incubated at 37°C overnight. We microfuged 1.5 ml of suspension 10 min; 1.2 ml supernatant was removed, and 300 µl 20% PEG/2.5 M NaCl was added and incubated at room temperature 10 min. Supernatant was aspirated off, pellet was resuspended in 200 µl TLE; 200 µl Phenol was added, sample vortexed, and microfuged 10 min. The top layer of sample was retained, EtOH precipitated, and resuspended in 30 µl dH<sub>2</sub>O. Sequencing was performed using USB sequenase kit as recommended by the manufacturer using the –40 primer.

Individual sequences were aligned using the PILEUP program of University of Wisconsin, GCG system package (Devereaux 1991). With 75 individuals sampled in our data set, there was ample opportunity to assess variation below the species level for several of the 21 species represented. In Tables 1 and 2, we have listed 31 “taxa”—note that we use the term taxa loosely here in that, in addition to true species and subspecies, we have included geographically isolated populations as well (tables 1 and 2). Perhaps surprisingly, there was very little variation among the sequences obtained within any of the taxa listed in table 2. Minor variants observed were: 12S—site 242 in AjuRA, site 67 in PcoCO; Cytb—site 85 in Ppa, site 142 in PleSER, site 161 in Pun, site 202 in Lru. In five of these six cases, the minor variants were the only variation seen at these sites across all taxa and in all six cases were restricted to a single sequence; hence by ignoring these minor variants we could use a single sequence for each gene for each taxon from table 2 (fig. 4). PILEUP alignments (default parameters: gap weight 5.0, gap length weight 0.3) were used to compute distance matrices (available on request). Although observed length variation was generally very minor, two distance matrices differing in their treatment of gaps were computed. Matrix 1 treated each gap, regardless of length, as a single difference, whereas matrix 2 simply excluded gaps entirely.

Evolutionary trees were constructed from the sequence data using random input of species order and also designated input order. Three sets of trees (12S, Cytb, and 12S/Cytb combined) were constructed using a designated outgroup for rooting. Phenetic trees were constructed via the Fitch-Margoliash algorithm of the PHYLIP program, which uses a distance matrix (Fitch and Margoliash 1967). Trees were also constructed using the maximum likelihood algorithm (DNAML in PHYLIP version 3.2; Felsenstein 1990). Maximum parsimony



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mony trees were obtained using the PAUP version 3 program (Swofford 1989) with heuristic search and random taxon addition and evaluated by bootstrap resampling analysis (100 iterations).

## Results

DNA sequence data were obtained from five non-felid carnivores and from 70 individuals belonging to 17 species of the Felidae including representatives of certain subspecies (lions, tigers, leopards, cheetah, and puma) and isolated populations (lions) (table 2; fig. 4). Most felid species were represented by two to five individuals; when only one individual was available, several sequences were compared, obtained from independent PCR amplifications (table 2). In no case did multiple amplifications of the same individual result in two different sequences. Furthermore, intraspecific variation was very limited. In addition to six polymorphic residues indicated in table 2 (by asterisk), 10 sites that differed between subspecies or isolated populations were identified. In the 12S sequence, four sites varied among lion populations and subspecies, one site among tiger subspecies, and one site between leopard subspecies; all were transitions. In the cytochrome b sequence, two sites differed among tiger subspecies and two additional sites varied between leopard subspecies; all but one (tiger) were transitions.

Likewise, insertion/deletion polymorphism (indels) was very restricted and not very informative. In the 12S sequence, one single bp deletion shared by clouded leopard (Nne) and marbled cat (Pma), and another unique to Temminck's golden cat (Pte), were observed among the Felidae. A few additional variable indel sites were observed when nonfelid carnivores were included (fig. 4A). Indel variation in the cytochrome b sequence was lion-specific—two deletions (18 bp and 3 bp) relative to the other carnivores (fig. 4B).

Three main modes of phylogenetic analysis (maximum parsimony, maximum likelihood, and phenetic) were performed for the 12S and cytochrome b sequences both as separate sequences and as a single combined sequence (fig. 5). Additionally, many subsets of the data (e.g., just felids, just transversions) were explored with PAUP (Swofford 1989) and MacClade (Maddison and Maddison 1992). All analyses agreed on the following

phylogenetic relationships (see fig. 5): (1) sister species status of lions (Ple) and leopards (Ppa); (2) clustering of five traditional *Panthera* species (Pti, Ple, Ppa, Pon, Pun) with the clouded leopard (Nne); (3) association of pumas (Pco) with cheetahs (Aju); (4) association of the African golden cat (Pau) with caracal (Cca) instead of the congeneric Asian (Temminck's) golden cat (Pte); (5) tigers (Pti) well separated from most other *Panthera*, although possibly a sister group to clouded leopards (Nne); and (6) ocelots (Lpa), hyenas (Ccr), and other nonfelid carnivores representing increasingly divergent outgroups with respect to the pantherine lineage felids. Furthermore, all conspecific sequences cluster together appropriately with generally little resolution among populations or subspecies.

Curiously, the placement of the lone representative of the domestic cat lineage (Fca) varies radically among our three principal analyses (fig. 5), ranging from within the non-*Panthera* pantherines to placement as an outgroup to all other felids, including ocelot (Lpa). The non-*Panthera* pantherines may be a sister clade to the *Panthera* (including Nne), although their monophyly will ultimately depend upon placement of the domestic cat (Fca) (see fig. 5).

The PAUP analysis of the combined sequences (omitting dog and treating gaps as missing data) resulted in eight trees, each with a minimum length of 535 steps. These trees differ primarily in their representation of the non-*Panthera* pantherine cats, which generally fall into three clades: pumas and cheetahs (Pco-Aju); serval, caracal, and African golden cat (Lse-Cca-Pau); and marbled cat and lynxes (Pma-Lru-Lca). One of these trees (fig. 5A) reduces the number of transversions required. Bootstrap analyses strongly support (>99%) the obvious associations: intraspecific (lions, leopards, tigers); congeneric (two bear species and two lynx species); and arctoid carnivores (canids and ursids) as the outgroup. Hyena (Ccr) was the outgroup to all felids in 87% of bootstrap analyses. Not-so-obvious associations corresponding to points 1–4 above had the following bootstrap support: (1) lions with leopard (58%); (2) *Panthera* includes clouded leopard (58%); (3) pumas with cheetahs (78%); and (4) African golden cat with caracal (95%). It should be noted that clustering snow leopard (Pun) and clouded leopard (Nne) with tigers (Pti) relative to

FIG. 4.—Nucleic acid sequences obtained and aligned from 31 carnivore "taxa" (including species, subspecies, and geographical populations) for the 12S (A) and Cytb (B) regions. Reference sequence used in all comparisons is the Serengeti lion (Pleser); dots and letters represent identical and different nucleotides, respectively, from Serengeti lion. Asterisks indicate deletion of base. PCR primer locations (see fig. 3) with reference to the sequences are as follows: 12S-1 adjacent to upstream end, 12S-2 31 bp downstream; Cytb-1 96 bp downstream, Cytb-2 37 bp upstream (referenced to human sequence; Anderson et al. 1981). The Cytb sequence is shown in reading frame with translation of the Serengeti lion sequence (Pleser) above.

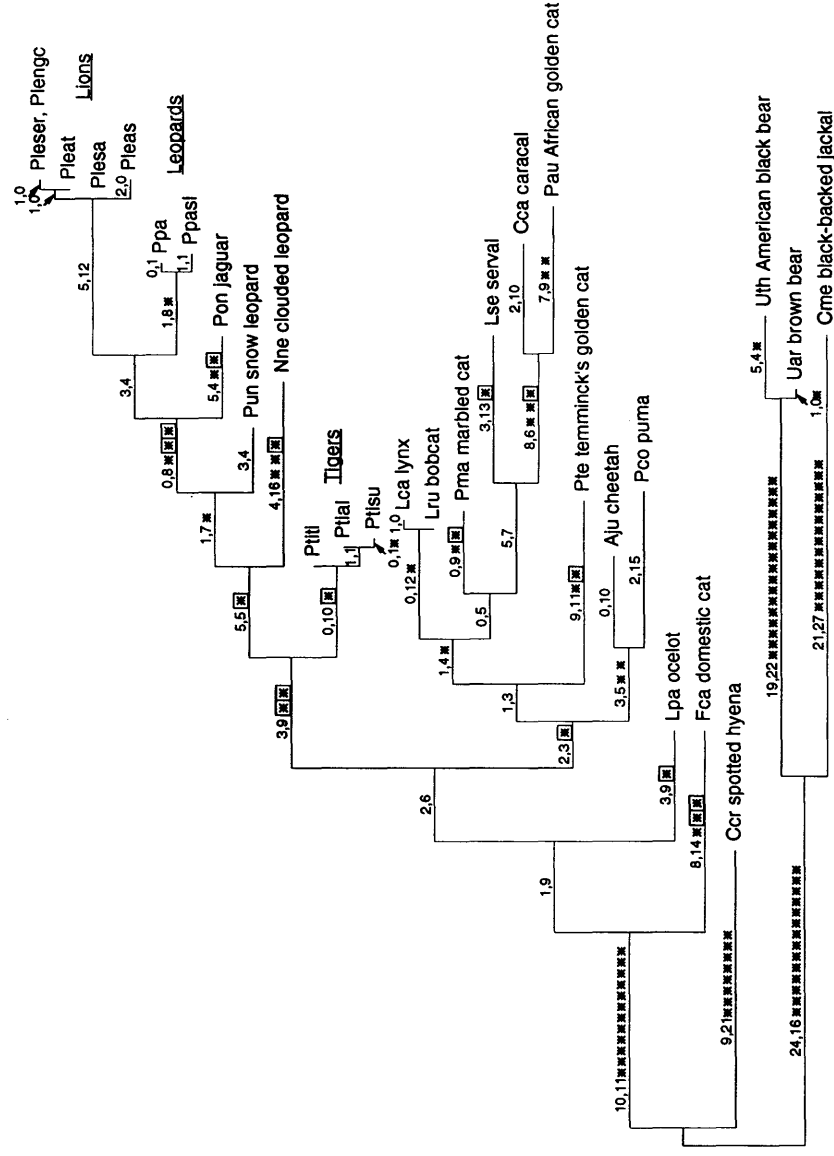


FIG. 5.—Inferred phylogenetic trees from analysis of combined 12S RNA and cytochrome b data from fig. 4. *A*. Maximum parsimony tree. This is one of eight minimum-length trees from 85,359 tested trees based on a heuristic search. In these heuristic searches, branch swapping (tree bisection and reconnection) and random taxon addition were used. Numbers given are site changes (12S, cytochrome b) for each branch. Asterisks indicate transversions and are boxed when homoplasy within felids is inferred. Ambiguous site changes were resolved to homogenize branch lengths when possible. Tree length is 535 with a consistency index (including all variable sites) of 0.51. *B*. Phenetic tree based on the least square method using the FITCH algorithm of PHYLIP (Fitch and Margoliash 1967); phenetic distance branch lengths indicated above each branch. This topology requires 545 changes, excluding indels. *C*. Maximum likelihood tree (Felsenstein 1990) (Ln likelihood = -3618.5; examined 1,882 trees) generated using empirical base frequencies and a transition/transversion ratio of 2.0. Limb lengths are the expected number of substitutions/site. This topology requires 540 changes, excluding indels.

the tree in figure 5*A* results in a tree one step longer overall but two transversions shorter.

## Discussion

Analysis of two mitochondrial DNA gene sequences, 12S RNA and cytochrome b, in samples from 17 cat species has provided new evidence that bears on the phylogenetic relationships among these species. The recency of the postulated divergence of species in the genus *Panthera* (1–2 Myr before the present) (Kurten and Anderson 1980; Neff 1982; Turner 1987; Wayne et al. 1988) makes this group a particular challenge for phylogenetic inference. In fact, the entire pantherine lineage divergence (fig. 2) may be no older than 5–10 Myr (Savage and Russell 1983; Wayne et al. 1991). If all divergence among the 17 sampled felid species took place between 2 and 10 MYA, we might expect to en-

counter difficulties similar to those seen in trying to resolve the human-chimp-gorilla trichotomy (e.g., Sibley and Ahlquist 1987). For this reason our laboratory has elected to apply several independent methods for determining the phylogeny of felids (O'Brien 1986; O'Brien et al. 1987) and to consider the concordance of the different methods as cumulative weight in support of association hypotheses (Nei 1987; Li and Graur 1991). We summarize these findings and inferred phylogenetic relationships in figure 6 and table 4.

Previous molecular data had identified three major lineages of the cat family (fig. 1) but failed to resolve the relationship within the lineages further (Collier and O'Brien 1985; O'Brien 1986). The pantherine lineage is the largest group, containing around 20 species of cats, including the genus *Panthera* and many mid-sized cats (e.g., cheetah, puma, lynx, golden cats). Immunological

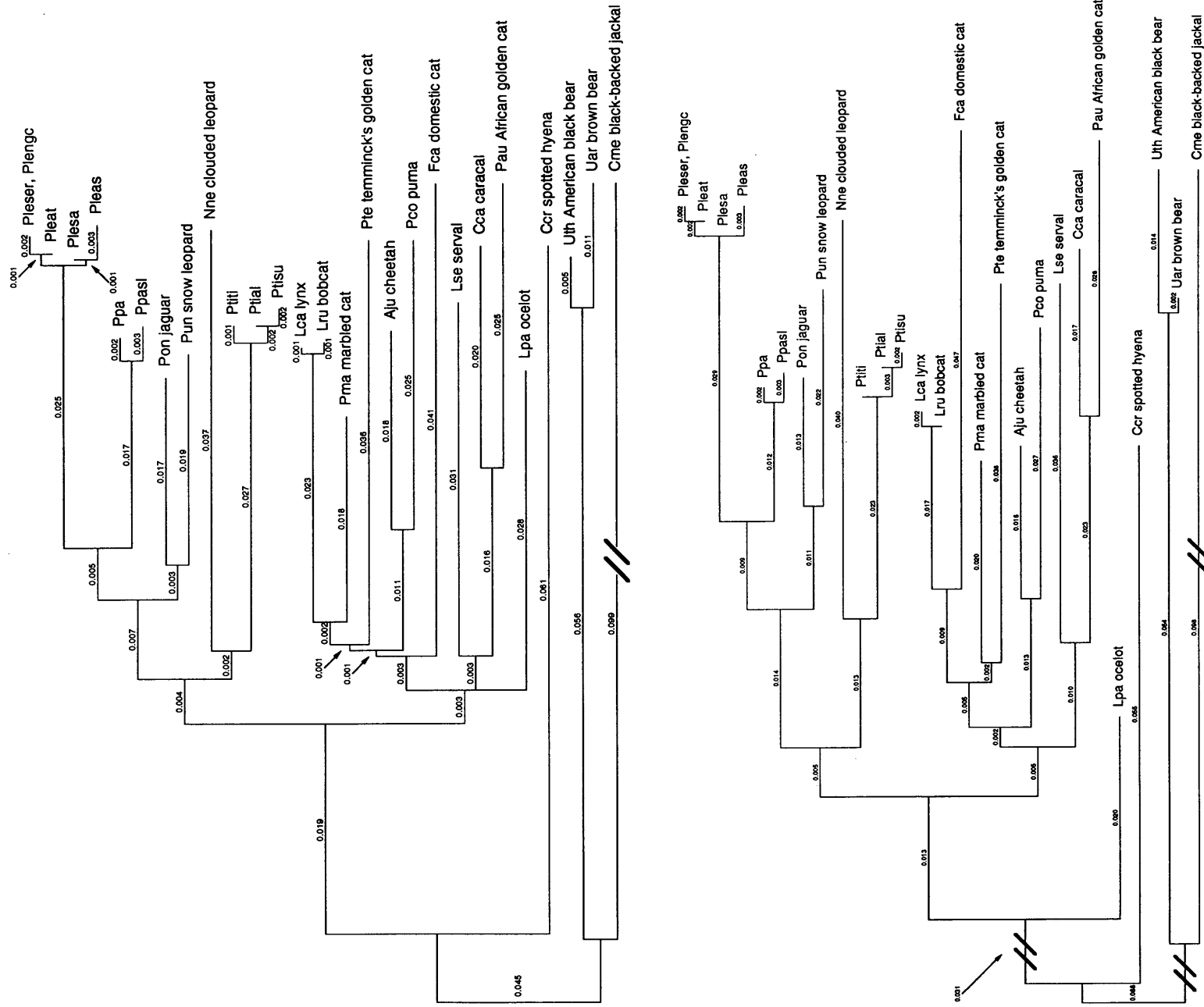
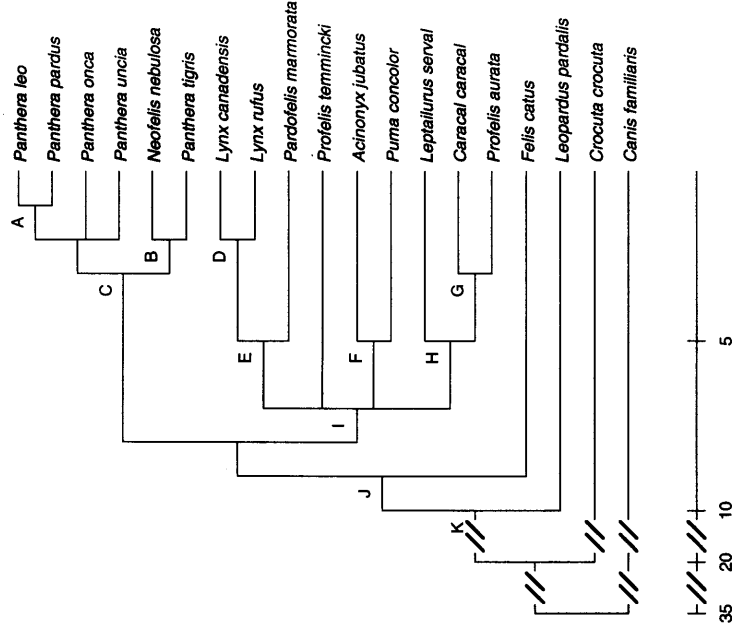


FIG. 5 (Continued)



Million Years Before Present

FIG. 6.—Phylogeny of the Felidae based on principle of majority rule from molecular data and calibrated using the divergence of the hyena at 20 MYA (Hunt 1989). Species associations shown here represent those observed in a majority of the trees, constructed from molecular genetic data (mtDNA sequencing, isozymes, albumin immunological distance, karyology, DNA-DNA hybridization, and endogenous retrovirus identification) and are supported by morphological data (Benveniste 1985; Collier and O'Brien 1985; O'Brien et al. 1987; Modi et al. 1988). Letters on divergence nodes represent association hypotheses that were evaluated by these methods as summarized in table 4.

distance and allozyme genetic distance data supported the monophyly of *Panthera* and suggested a close relationship of this genus to *Lynx* and *Pardofelis*. The *Lynx-Pardofelis-Panthera* association received additional support from karyological analysis since all members of all these genera had an identical karyotype (Modi and O'Brien 1988). Branching points for the remaining members of the pantherine lineage were suggested to be more ancient, but the precise relationships were left unresolved.

Phylogenetic relationships in the present study are deduced from both mitochondrial 12S and Cytb sequences (fig. 4) using several methods of analysis. These analyses did not resolve all relationships, but there were several concordant associations. These are

illustrated in figure 6 and include (1) confirmations of monophyly of the genus *Panthera* with a possible more recent association of Ple-Ppa; (2) inclusion of clouded leopard (Nne) as a sixth species within the genus *Panthera*, possibly as a sister group of tigers (Pti); (3) common ancestry of puma (Pco) and cheetah (Aju), strongly supported by all analyses; (4) placement of the ocelot lineage as an outgroup relative to the pantherine lineage; and (5) evidence for polyphyly in the ancestry of golden cats, Pau and Pte, and a recent association of Pau and Cca.

Each of these conclusions recapitulates earlier findings in the taxonomic literature of felid morphometrics and paleontology. For example Leyhausen (1979) suggested that tigers (Pti) and clouded leopard (Nne) should be aligned in a single genus, *Neofelis*. Our results would combine them as slightly more basal members of the monophyletic *Panthera* genus. An association between cheetah and puma has been suggested by the description of North American fossil specimens (*Felis trumani*), which are thought to be a link between the species (Orr 1969; Adams 1979). Although golden cats are considered sister taxa in the genus *Profelis* by Leyhausen (1979) and Ewer (1973), the two species are put into distinct genera by Hemmer (1978) and are well separated in the analyses by Salles (1992). Our data for both genes would support the latter convention. Finally, Leyhausen (1979) also aligns caracal (Cca) and the Asian golden cat (Pau), an association strongly supported by analyses of the 12S RNA and cytochrome b sequences.

Contrasting levels and patterns of variation were seen between the 12S and cytochrome b sequences. Of the 358 sites sequenced in the 12S gene, only 98 (27%) were variable; of these, only 52 were variable among felids, with 30 sites being phylogenetically informative. In contrast, 133 of the 289 (46%) sequenced cytochrome b sites were variable; of these 111 are variable among felids, with 73 sites being phylogenetically informative. Thus, not only are more cytochrome b sites variable in general, but the majority of these sites have sustained variation during the short time frame (<10 Myr) represented by the felid radiation.

The contrast in the overall level of variation between 12S and cytochrome b is time-dependent. Cytochrome b sequences appear to accumulate differences relatively rapidly and then stabilize, whereas 12S differences seem to accumulate more gradually but steadily. This contrast is apparent in figure 7, in which we have plotted pairwise nucleotide sequence differences for (1) intra-*Panthera* comparisons, including clouded leopard (Nne), estimated divergence within the last 3 Myr; (2) *Panthera* versus non-*Panthera* pantherine cats, diver-

**Table 4**  
**Summary of Phylogenetic Associations among Pantherine Lineage Species Supported by Molecular Genetic Data**

	AID	GD	MP	FM	COMBINED		KARYOLOGY
					12S RNA	AND Cytb	
A. PLE-PPA .....	ND	ND	+	+	+	+	+
B. NNE-PTI .....	-	-	+ <sup>a</sup>	+	+	+	+ <sup>b</sup>
C. Panthera Inc. NNE .....	-	+	+	+	+	+	+ <sup>b</sup>
D. LRU-LCA .....	+	ND	+	+	+	+	+
E. PMA-LRU-LCA .....	+	+	-	+	+	+	+
F. AJU-PCO .....	-	-	+	+	+	+	- <sup>c</sup>
G. CCA-PAU .....	ND	(+)	+	+	+	+	+ <sup>b</sup>
H. LSE-CCA-PAU .....	+	ND	+	+	+	+	+
I. Clade of non- <i>Panthera</i> pantherines .....	-	+	+	+	+	+	-
J. FCA lineage outside .....	+	ND	+	-	-	-	+
K. LPA lineage outside .....	+	ND	+	-	-	-	+

Previously proposed associations not depicted on figure 6:

PMA-LSE .....	-	ND	-	-	-	-	+ <sup>a</sup>
PAU-PTE .....	ND	ND	-	-	-	-	- <sup>b</sup>

NOTE.—+, supported; -, not supported and some contradiction; ND, not determined; methods as follows: AID, albumin immunological distance (Collier and O'Brien 1985); GD, allozyme genetic distance (O'Brien et al. 1987); combined 12S RNA and cytochrome b sequences using PAUP (maximum parsimony, MP), Fitch-Margolash (FM), and maximum likelihood (ML) algorithms, respectively (from fig. 5); and karyology, from Wurster-Hill and Centerwall (1982) and Modi and O'Brien (1988).

<sup>a</sup> Transversions given greater weight than transitions.

<sup>b</sup> One chromosome step.

<sup>c</sup> Two chromosome steps (Modi and O'Brien 1988).

gence 5–10 MYA; (3) all felids versus the spotted hyena (Ccr), divergence about 20 MYA; and (4) all felids versus the arctoid carnivores (canids and bears), divergence about 35 MYA.

The relative saturation of cytochrome b sites is also suggested by the distribution of the inferred number of changes per site (fig. 8) in the maximum parsimony tree of figure 5A. The mean number of changes per variable site in the 12S sequences (1.97) is markedly lower than that for cytochrome b sequences (2.96). The phylogenetic consequences of the discordancy between 12S and cytochrome b levels and patterns of sequence divergence are not completely obvious, but we can gain some insight from our analyses. For instance, figure 5A shows that the clustering of jaguar (Pon) with lions (Ple) and leopards (Ppa) is due solely to variation among the cytochrome b sequences. Similarly, sites defining distinct lineages for tigers (Pti), leopards (Ppa), lynxes (Lru and Lca), marbled cat (Pma), cheetahs (Aju), pumas (Pco), serval (Lse), and caracal (Cca) are found mostly or exclusively in cytochrome b. Thus, divergence of cytochrome b makes it a good candidate for phylogenetic studies of very recently diverged taxa, perhaps up to a

few million years ago. The principal drawback is that the majority of these changes are third-base transitions (71 of 99 sites varying among felids), which are notoriously prone to subsequent transitions (i.e., reversions) that generate homoplasy in phylogenetic analyses. In contrast, the 12S sequence was particularly important for moderate to long divergence times (5–35 Myr), differentiating all nonfelid outgroups, the domestic cat lineage (Fca), and several nodes among the pantherines (fig. 5A). The difference in the level of homoplasy is also shown by the consistency indices—0.64 for 12S sites and 0.44 for cytochrome b sites—for the tree in figure 5A.

Finally, it is worthwhile to consider the phylogenetic utility of the different classes of change (transition/transversion, position within codon for cytochrome b) within our data set. In table 5, we have categorized sites into transversions or transitions and excluded indels. Sites having both types of change were categorized as transversions. However, because many of the latter sites showed transversions between the nonfelid carnivores and felids but only transitions among felids, such sites were reclassified as transitions

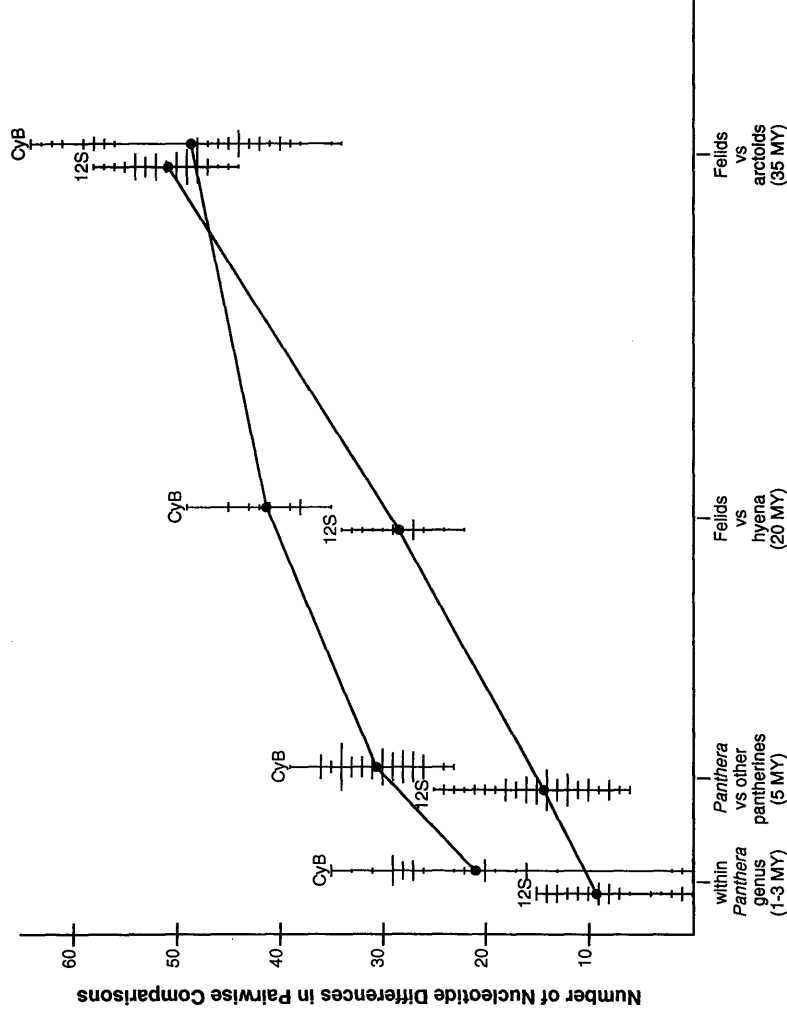


FIG. 7.—Comparison of nucleotide sequence divergence between 12S and cytochrome b. Number of differences are shown: (1) within *Panthera*, including clouded leopard (Nne), estimated divergence 1–3 MYA, 78 pairwise comparisons; (2) between *Panthera* and the non-*Panthera* pantherine cats, estimated divergence 5–10 MYA, 143 pairwise comparisons; (3) between felids and spotted hyena (Ccr), estimated divergence 20 MYA, 26 pairwise comparisons; and (4) between felids and arctoid carnivores (dogs and bears), estimated divergence 35 MYA, 104 pairwise comparisons for 12S and 78 for cytochrome b. Regions having indels longer than 1 bp were omitted for this analysis. Widths correspond to the number of pairwise comparisons having each level of divergence. Averages of the pairwise comparisons at time points 1 to 4 are shown and connected for each gene.

when we excluded the nonfelid carnivores. Because of the extreme transition bias seen with mitochondrial DNA, many authors (Irwin et al. 1991; Martin et al. 1992; Graybeal 1993, 1994; Adkins and Honeycutt 1994; Meyer 1994) have suggested that emphasis be given to transversions, especially when analyzing protein coding sequences. Unfortunately, our study only provided 24 transversions among felids, 16 of which were phylogenetically informative. If we take the maximum parsimony tree in figure 5A as the best topology for our combined data set, we see that transversions are generally more consistent (CI = 0.58 for cytochrome b, 0.67 for 12S) with the tree than are transitions (CI = 0.35 for cytochrome b, 0.62 for 12S).

If the cytochrome b sequence is largely saturated by about 20 Myr of divergence (fig. 7), one might hope that the level of homoplasy would decrease if we exclude the nonfelid carnivores, which diverged from felids 20–35 MYA (Wayne et al. 1991). Indeed,

maintaining the tree from figure 5A, we see (table 5) that most of the categories of change for cytochrome b show increases in the CI. Interestingly, because most of the cytochrome b changes within felids (71/99) are third-base transitions, the CI drops slightly compared to the CI when the outgroup carnivores were included. Similarly, the CI for 12S sites drops slightly when restricted to variation among felids, although in this case the CI for transitions also drops. Numerous authors (Irwin et al. 1991; Martin et al. 1992; Graybeal 1993; Adkins and Honeycutt 1994; Janke et al. 1994; Meyer 1994) have shown a dramatic difference between third-base transitions and other types of change for vertebrate cytochrome b. Our own results suggest essentially two time scales for vertebrate cytochrome b evolution: one for third-base transitions, so rapid that only divergences within a few million years are feasibly addressed, and one for most other types of change, spanning several million to 10s of millions of years. For

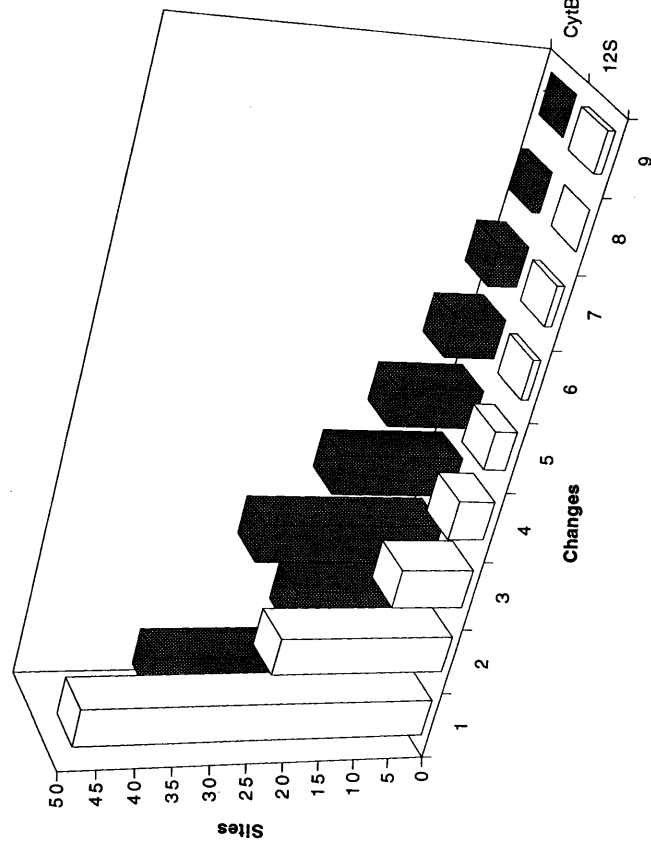


FIG. 8.—Distribution of the number of changes per variable site inferred from the maximum parsimony tree in fig. 5A. In 358 base pairs of 12S sequence there were 93 variable sites, and in 289 bp of cytochrome b sequence, there were 119 variable sites. The tree in fig. 5A calls for 183 changes in the 12S sequence and 352 changes in the cytochrome b sequence. The dog sequence and indel variants were excluded for this analysis.

even greater divergence times, it is possible that second-base transversions will provide the most reliable phylogenetic information, as these seem to accumulate very slowly.

**Table 5**  
**Consistency Indices (CI) as a Function of Class of Change, Using the Maximum Parsimony Tree of Figure 5A**

	OVERALL		WITHIN FELIDS	
	Sites	CI	Sites	CI
12S (358 bp):				
Transitions	62	0.62	42	0.57
Transversions	32	0.67	9	0.70
All sites	94	0.64	51	0.60
Cytochrome b (289 bp):				
Transitions:				
Codon position 1	12	0.44	10	0.48
Codon position 2	5	0.63	3	0.75
Codon position 3	60	0.32	71	0.38
All transitions	77	0.35	84	0.40
Transversions:				
Codon position 1	11	0.67	4	0.60
Codon position 2	2	1.00	0	...
Codon position 3	29	0.56	11	0.64
All transversions	42	0.58	15	0.63
All sites	119	0.44	99	0.43

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