Phylogenetic Reconstruction of South American Felids Defined by Protein Electrophoresis

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Abstract. Phylogenetic associations among six closely related South American felid species were defined by changes in protein-encoding gene loci. We analyzed proteins isolated from skin fibroblasts using two-dimensional electrophoresis and allozymes extracted from blood cells. Genotypes were determined for multiple individuals of ocelot, margay, tigrina, Geoffroy's cat, kodkod, and pampas cat at 548 loci resolved by two-dimensional electrophoresis and 44 allozyme loci. Phenograms were constructed using the methods of Fitch-Margoliash and neighbor-joining on a matrix of Nei's unbiased genetic distances for all pairs of species. Results of a relative-rate test indicate changes in two-dimensional electrophoresis data are constant among all South American felids with respect to a hyena outgroup. Allelic frequencies were transformed to discrete character states for maximum parsimony analysis. Phylogenetic reconstruction indicates a major split occurred approximately 5–6 million years ago, leading to three groups within the ocelot lineage. The earliest divergence led to *Leopardus tigrina*, followed by a split between an ancestor of an unresolved trichotomy of three species (*Oncifelis guigna, O. geoffroyi*, and *Lynchailuris colocolo*) and a recent common ancestor of *Leopardus pardalis* and *L. wiedii*. The results suggest that modern South American felids are monophyletic and evolved rapidly after the formation of the Panama land bridge between North and South America.

Introduction

Evolution of the cat family Felidae is characterized by an ancient ancestry combined with rapid, recent diversification. The ancestors of modern cats first appear in the fossil record about 35 million years ago (MYA) during the Oligocene (Savage and Russell 1983; Martin 1989). However, ancestral forms of extant felids first appear only 13–15 MYA during the Miocene (Savage and Russell 1983; Werdelin 1985; Collier and O'Brien 1985), and fossil evidence strongly suggests most modern species evolved more recently. For example, fossils indicate some of the earliest species to diverge from other felids—such as serval (*Leptailurus serval*) (Turner 1985), cheetah (*Acinonyx jubatus*) (Ficcarelli 1984; Turner 1987), caracal (*Caracal caracal*) (Savage and Russell 1983; Turner 1987), and puma (*Puma concolor*) (Kurten 1976; Van Valkenburgh et al. 1990)—evolved as recently 3–5 MYA. Other felids—such as jungle cat (*Felis chaus*) (Kurten 1968) and domestic cat (*Felis catus*) (Clutton-Brock 1987)—first appear in fossil deposits between 4,000 and 100,000 years old. At present, 37 extant species are distributed throughout the world in a variety of habitats (Nowak 1991). Due to its recent evolutionary history, phylogenetic representation of the Felidae offers insight into the maintenance and segregation of genetic polymorphisms among rapidly evolving species.

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Previous research using microcomplement fixation of serum albumin (Collier and O'Brien 1985), DNA hybridization (Benveniste 1985), allozymes (O'Brien et al. 1987), and sequence analysis of mitochondrial DNA (Janczewski 1992) has revealed three major groups within the Felidae. The earliest branch diverged approximately 12 MYA and led to the small, spotted, South American cats. The second group radiated approximately 8–10 MYA and is comprised of small cats (including the domestic cat) located in the Mediterranean region. The most recent group includes the *Panthera* genus and several species of intermediate-sized felids distributed world-wide.

The South American group, also named the ocelot lineage, is distinguished by an intriguing pattern of evolution. In contrast to its ancient ancestry, evidence suggests that extant species of South American felids are of very recent origin. An intermediate predecessor of the South American group (*F. lacustris* or *F. rexrodenosis*) first appears in the fossil record only 4–5 MYA (Werdelin 1985). Fossils of present-day *Leopardus* spp. found in South America are approximately 1.5–2.5 MY old (Berta 1983) and only 0.3–0.4 MY old in southern North America (Kurten and Anderson 1980). These species remain essentially endemic to South and Central America and consist of the ocelot (*Leopardus pardalis*), margay (*Leopardus wiedii*), tigrina (*Leopardus tigrina*), Geoffrey's cat (*Oncifelis geoffroyi*), kodkod (*Oncifelis guigna*), pampas cat (*Lynchailurus colocolo*), and Andean mountain cat (*Oreailurus jacobita*). Little is known of the elusive mountain cat; however, the common ancestry of the remaining six species is suggested by a shared karyology characterized by a reduction in chromosome number from 38 to 36 and possession of a metacentric C3 chromosome (Wurster-Hill and Centerwall 1982; Modi and O'Brien 1988).

Although the existence of the ocelot lineage is strongly supported, the phylogenetic relationships between the included species are not clear. For example, the number of genera proposed for the ocelot group varies from one to four. (See Nowak 1991; Leyhausen 1979; Hemmer 1978; Ewer 1973.) Consequently, phylogenetic analysis of the ocelot lineage would serve the dual purpose of estimating the extent and character of molecular genetic diversity maintained and segregated within closely related species and helping to clarify the Felidae classification.

Our approach in reconstructing the ocelot lineage phylogeny was to survey broad regions of the genome at one time by employing two techniques to detect changes in protein-encoding gene loci: two-dimensional protein electrophoresis (2DE) and allozyme electrophoresis (1DE). Phylogenetic reconstruction is possible because of the stochastic accumulation of changes within the genome since two species last shared a common ancestor. These changes can be expressed as an observable change in protein charge or molecular weight.

However, it is clear that rates of change vary among genes within the taxonomic group considered (Dayhoff 1978; Li et al. 1985). Consequently, using many loci provides an overall average measure of genetic differences among related species and improves the probability that the gene trees accurately depict species phylogeny (Nei et al. 1985; Pamilio and Nei 1988).

Two-dimensional protein electrophoresis has proven to be a powerful method for phylogenetic studies of other mammalian taxa such as bears (Goldman et al. 1989) and primates (Goldman et al. 1987; Janczewski et al. 1990). Conservative estimates indicate a sample of 400 protein-encoding gene loci isolated by 2DE is equivalent to surveying 100,000 nucleotides (Goldman and O'Brien 1993). We include an allozyme data analysis as an independent data set based on protein-encoding gene systems that have been even more informative in phylogenetic reconstruction (Nei 1987; Avise and Aquadro 1981; O'Brien et al. 1985).

Materials and Methods

Sample Preparation. Primary fibroblast cell lines were cultured from skin punch biopsies of the species listed in Table 1. Outgroup species consisted of a felid from the pantherine lineage, jaguar, *Panthera onca* (PON), and spotted hyena, *Crocuta crocuta* (CCR), as a nonfelid representative from within the carnivore order. Preparation of proteins for two-dimensional electrophoresis followed the methods of Janczewski et al. (1990) with the exception of a change in the incubation time for radiolabeling with ^{35}S-methionine from 4 h to 18 h. Lysates from each individual were stored at −70°C.

Two-Dimensional Electrophoresis. Proteins were separated by 2DE using the protocol developed for the investigator 2-D Electrophoresis System (Millipore Corp 1991). This system offered technological advances which improved resolution and precision. In the first stage of isoelectric focusing, we employed analytical-tube gels (18 cm × 1 mm inner diameter) manufactured with an inert thread running the length of the gel. Approximately 10–15 μl of cell lysate (150,000 cpm/μl) was loaded onto the gel. Separation of proteins occurred at 100 μamps/gel over an 18 volt-hour (V-h) interval.

Prior to separation by SDS-PAGE, tube gels were removed from the chamber, chilled, extruded from the tube into equilibration buffer, and soaked for 2 min. Gels were then applied to 14% Duracryl acrylimide slab gel for separation by molecular weight. Optimal resolution was achieved by increasing the concentration of the running buffer in the upper chamber to 4× instead of 2× as specified in the Millipore protocol. Gels were run at a maximum voltage of 500 V and dried, and protein spots of differing intensity were visualized using sequential autoradiography of 2, 7, and 21 days.

Allozyme Analysis. Allozyme data were generated using samples of erythrocyte, leukocyte, and fibroblast proteins isolated from individual species. All three tissue extracts from multiple individuals of each species were applied to starch gels and stained for specific protein-encoding gene loci listed in Table 5 following the methods of O'Brien et al. (1983, 1985). Confirmation of allozyme shifts between different tissue extracts was required for acceptance of a mobility difference in order to exclude epigenetic tissue isozyme variation.

Data Analysis. Allelic frequencies for each locus were estimated from individual genotypes and used to calculate Nei's unbiased ge-

Table 1. Species list

Species (code)	Common name	No. of individuals	2DE	1DE	Sources
Crocuta crocuta (CCR)	Spotted hyena	2	X	—	Henry Doorly Zoo, Omaha, Nebraska
Leopardus pardalis (LPA)	Ocelot	3	X	X	Octagon Wildlife Park, Fort Meyers, Florida; Cheyenne Mountain Zoo, Colorado Springs, Colorado
Leopardus tigrina (LTI)	Tigrina	3	X	X	Blijdorp Zoo, Rotterdam, The Netherlands; Cincinnati Zoo, Cincinnati, Ohio
Leopardus wiedii (LWI)	Margay	1	X	X	Blijdorp Zoo, Rotterdam, The Netherlands
Lynchailurus colocolo (LCO)	Pampas cat	3	X	X	Blijdorp Zoo, Rotterdam, The Netherlands; Cincinnati Zoo, Cincinnati, Ohio
Oncifelis geoffroyi (OGE)	Geoffroy's cat	3	X	X	Washington State University, Pullman, Washington; Blijdorp Zoo, Rotterdam, The Netherlands
Oncifelis guigna (OGU)	Kodkod	2	X	X	Zoologica National de Chile, Santiago Chile
Panthera onca (PON)	Jaguar	2	X	X	National Zoological Park, Washington, DC; Johannesburg Zoological Garden, Johannesburg, South Africa

netic distance (Nei 1978) between all pairs of species examined using Biosys-1 (Swofford and Selander 1981). Phenograms describing the associations among the species were constructed from the distance matrix using Fitch-Margoliash and neighbor-joining algorithms from Phylip version 3.4 (Felsenstein 1991). Character data were generated for each species by transforming allelic frequencies into discrete states. These data were subsequently analyzed by maximum parsimony using the program PAUP version 3.0 (Swofford 1991).

Results

Phenetic Analysis

Two-Dimensional Protein Electrophoresis Data

Over 800 protein spots were resolved by two-dimensional electrophoresis. Based on optimal resolution in all individuals examined, we selected 548 protein-encoding gene loci for our analysis (Fig. 1). Protein polymorphisms were visualized as either a vertical shift in molecular weight or as a horizontal displacement of a spot due to change in charge (Fig. 2). In this analysis, 46 of the 548 presumptive loci varied between species with invariant proteins serving as positional markers for variable proteins. Most (42 of 46) of the variable proteins were single-step shifts in charge (Table 2). Distributions of electromorphs at each variant locus among the species examined are presented in Table 3.

Values for Nei's unbiased genetic distance were estimated for all pairwise combinations of species (Table 4). Each species was approximately equidistant (D = 0.0206 ± 0.002; N = 548 loci) from the outgroup species of *Crocuta crocuta* (CCR). Using a fossil date of approximately 35 MYA since the felids and *C. crocuta* last shared a common ancestor (Martin 1989), we estimate rate of change for the 2DE metric to be 0.028% per million years.

Phenograms were constructed using Fitch-Margoliash least squares and neighbor-joining algorithms on variable loci (N = 46). Since the remaining 502 loci were invariant, there were no differences in overall tree topology between phenograms based on 548 compared with 46 variable loci. Three major groups were resolved within the ocelot lineage (Fig. 3). The earliest species to diverge from an ancestral species of the five South American cats was the tigrina, *L. tigrina.* The other five species assorted into two groups: the first a pairing of *L. pardalis* and *L. wiedii* and the second a trichotomy of *O. guigna, O. geoffroyi,* and *L. colocolo* (Fig. 3A,B). Using the KITSCH algorithm, which uses the Fitch-Margoliash method under the presumption of molecular clock (with contemporaneous species tips), the order of the trichotomy changes slightly, but the approximate time of ocelot lineage divergence (12 MYA) and *C. crocuta*–felid split (35 MYA) are both supported (Fig. 3C).

Allozyme Analysis

Forty protein-encoding gene loci were resolved using starch gel electrophoresis (1DE). Of these, 22 allozyme loci were variable among the species examined (Table 5). Genetic distance varied between 0.143 and 0.277 within the ocelot lineage (Table 6). Phenetic analysis of 1DE data lent (Fig. 4) support to the overall topology and three groups by 2DE. The slightly earlier divergence of *L. tigrina* is observed followed by the three-species group of *O. guigna, O. geoffroyi,* and *L. colocolo* and a more recent pairing of *L. wiedii* and *L. pardalis.* The presumption of a molecular clock using
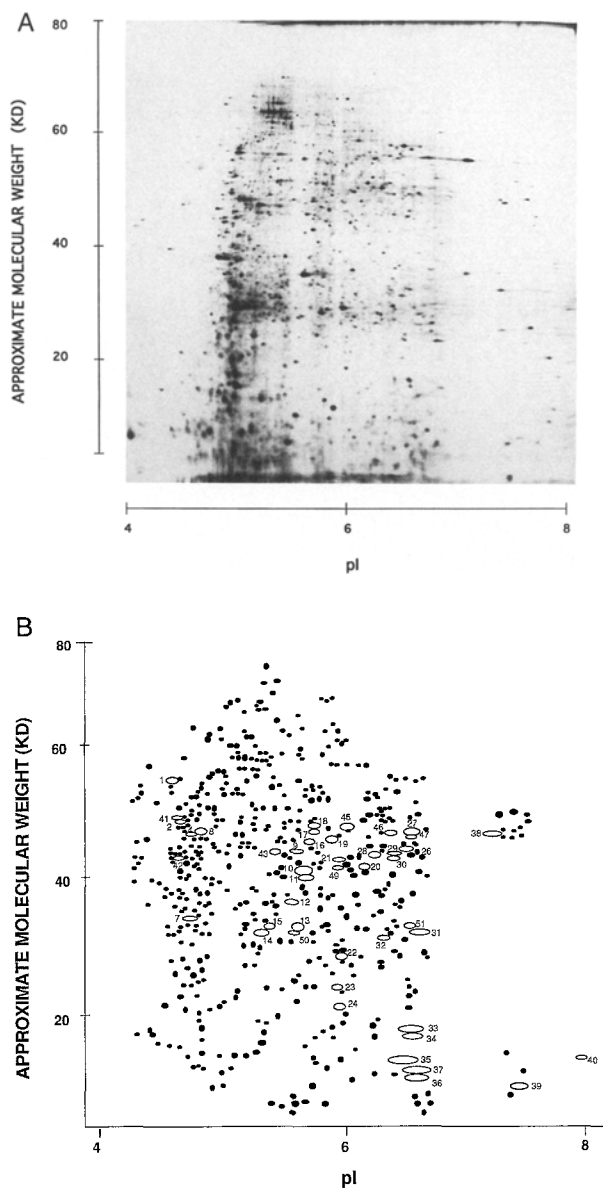
Fig. 1. A *Leopardus tigrina.* Autoradiogram of two-dimensional gel of skin cell fibroblast proteins labeled with ^{35}S-methionine. **B** Schematic illustration of 548 loci used in the analysis. All variant loci were characterized by alleles with differing isoelectric points (horizontal shifts in position). Invariant loci are closed ellipses and variant loci are open ellipses.

Fig. 2. An example of interspecific variation at a homologous locus (see Fig. 1B), between *Leopardus tigrina* (**left**), heterozygous with alleles A and B and *Leopardus pardalis* (**right**), homozygous for allele B. Allele B is positioned directly right of allele A (*arrow*) in both species.

Table 2. Categories of protein variation across 548 loci resolved by 2DE

Category	No. of loci	Fraction of total
Invariant	502	0.916
Variant	46	0.083
Acidic and basic	41	0.893
Missing	2	0.042
Missing and other	3	0.063
Two-step variant	3	0.063

maximum parsimony analysis using PAUP (Swofford 1991). A heuristic search for the most parsimonious trees produced seven equally parsimonious trees of 113 steps. However, no consistent tree topology was determined with consensus algorithm of Adams (1972), strict (Sokal and Rohlf 1981), and majority rule (Margush and McMorris 1981).

We repeated the heuristic search using a bootstrap resampling of the data set. After 100 iterations, the majority-rule consensus tree had poor support for all nodes (22–45%). An input tree identical to the tree derived from the distance matrices (Figs. 3A, 4A) had a length of 117 (four steps longer than the shortest tree) and is illustrated for limb length, homoplasy per limb, and consistency index in Fig. 5A. We conclude that the parsimony analysis of 2DE data does not provide adequate support for resolving this group, although neither does it conflict with the conclusions of the phenetic analysis.

Allozyme Data

Transformation of genotypes into 97 character states for analysis by maximum parsimony also failed to resolve the associations among the six species. A heuristic search yielded one tree of 58 steps. After 100 iterations of a bootstrap analysis, low values for nodes within the tree gave little support for species associations. However, as seen with 2DE data, an increase in tree

the phenetic distance matrix causes rearrangements of some of these species, emphasizing the sensitivity of the topology to differing rates of evolution.

Character State Analysis

Two-Dimensional Protein Electrophoresis
Transformation of the 2DE data set presented in Table 3 to a presence-absence code produced 108 characters that were analyzed as cladistic characters in a
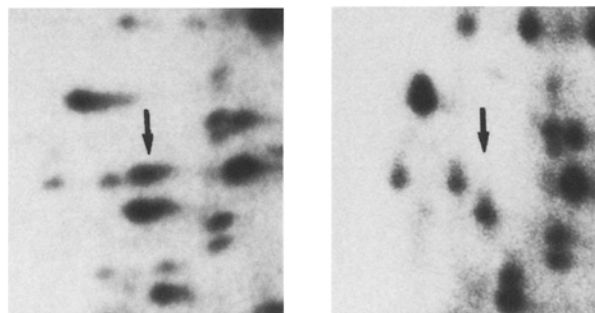
Table 3. Distribution of protein phenotypes pooled across individuals within each of seven felid species and one hyena for 46 variable loci resolved by 2DE (Fig. 1b)[a]

Protein	Species							
	CCR	LCO	LPA	LTI	LWI	OGE	OGU	PON
1	A	AB	AB	AB	AB	AB	A	A
2	B	A	A	A	AB	A	AB	AB
4	AB	AB	A	A	A	A	AB	A
6	AB	AB	AB	AB	AB	AB	AB	AB
7	AB	AB	AB	B	B	AB	B	AB
8	A	AB	AB	B	B	AB	B	AB
9	BC	B	B	AB	BC	AB	AB	B
10	AB	B	AB	A	B	B	B	A
11	A	AB	B	AB	B	AB	AB	B
12	B	B	B	B	B	B	AB	B
13	AB	B	AB	B	AB	B	B	M
14	AB	AB	A	AB	A	AB	AB	AB
15	AB	A	A	AB	A	A	A	A
16	AB	B	AB	AB	B	B	AB	B
17	B	B	AB	AB	B	B	AB	B
18	B	AB	AB	AB	B	AB	AB	AB
19	B	A	A	AB	A	A	AB	AB
20	B	AB	AB	AB	AB	AB	AB	AB
21	AB	A	A	AB	B	AB	A	B
22	AB	B	B	A	B	B	B	B
23	AB	A	AB	AB	A	AB	A	AB
24	A	A	AB	AB	A	AB	AB	AB
26	B	B	B	AB	B	B	B	B
27	A	AB	A	A	A	A	AB	AB
28	B	AB	B	AB	B	B	B	B
29	AB	AB	AB	A	A	AB	AB	AB
30	B	AB	AB	AB	AB	AB	AB	AB
31	C	AB	AB	AB	A	A	AB	AB
32	B	AB	B	B	B	B	AB	AB
33	A	AB	AB	AB	A	B	B	AB
34	A	AB	B	B	AB	AB	A	A
35	AB	AB	AB	AB	A	AB	AB	AB
36	A	A	AB	AB	A	AB	B	AB
37	AB	AB	AB	B	B	AB	AB	B
38	B	AB	B	B	B	AB	B	AB
39	AB	AB	B	AB	B	AB	AB	AB
40	AB	AB	AB	AB	A	B	AB	AB
41	A	A	AB	AB	A	A	AB	A
42	B	B	B	B	B	B	AB	B
43	N	N	AN	AN	A	N	N	N
45	B	A	A	A	A	A	A	A
46	AB	B	AB	B	B	B	B	B
47	N	B	BN	BN	B	BN	BN	N
49	BN	B	AB	B	B	B	AB	B
50	N	A	A	A	A	A	A	A
51	N	B	B	B	B	B	B	B

[a] A = acidic, B = basic, C = two steps basic, N = missing. Allele M = unscorable for technical reasons. Species code see Table 1

length of four steps derived the identical tree determined by phenetic methods (Fig. 5B).

Comparison of Genetic Distance Values

We compared the genetic distance values of both 1DE and 2DE data with previous research on closely related mammalian species performed in our laboratory (Table 7). The comparison indicated 2DE values for the South American lineage were lower than expected. Values from the present study reflected an overall trend that previously unresolved loci, detected by increased sensitivity in 2DE methods, were disproportionally invariant. In contrast, 1DE data were fairly consistent within the Felidae. Minimum genetic-distance values for the ocelot lineage were approximately four times those es-

Table 4. Nei's unbiased genetic distance for all 548 loci (above diagonal) and for 46 variable loci (below diagonal) resolved by 2DE

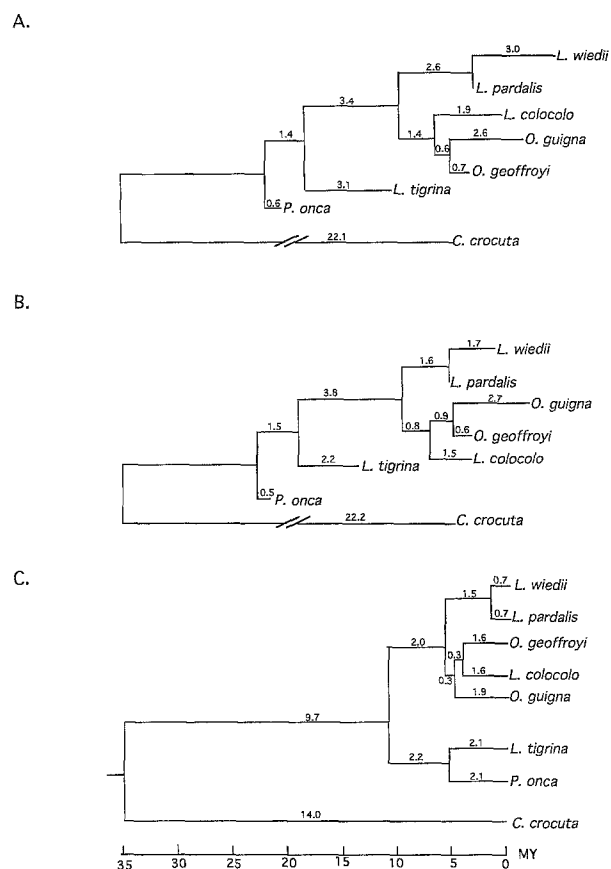|	CCR	LCO	LPA	LTI	LWI	OGE	OGU	PON
CCR	*****	0.019	0.022	0.019	0.022	0.021	0.021	0.016
LCO	0.274	*****	0.003	0.009	0.007	0.003	0.004	0.008
LPA	0.312	0.032	*****	0.005	0.001	0.003	0.005	0.007
LTI	0.277	0.123	0.061	*****	0.009	0.006	0.008	0.003
LWI	0.319	0.087	0.015	0.120	*****	0.007	0.008	0.008
OGE	0.308	0.033	0.037	0.084	0.091	*****	0.003	0.006
OGU	0.305	0.050	0.066	0.115	0.107	0.034	*****	0.006
PON	0.228	0.105	0.096	0.043	0.099	0.075	0.086	*****

Fig. 3. Phenetic analysis of two-dimensional electrophoretic data (N = 46 variable protein loci) based on a distance matrix of Nei (1978) unbiased minimum genetic distance (Table 4). All trees generated using Phylip 3.4 computer package. Trees derived from (**A**) neighbor-joining and (**B**) Fitch-Margoliash least squares (average percent standard deviation = 17.3) methods were drawn using branch-length values representing estimated % genetic distance. (**C**) Tree derived under the molecular clock hypothesis, assuming equal rates of change among all lineages, using Fitch-Margoliash least-squares method with contemporary tips (average percent standard deviation = 25.7).

timated for the most recently evolved felid group, the *Panthera* genus.

Discussion

The phylogenetic analysis of six species of small South American cats of the ocelot lineage based upon 548

proteins resolved by 2DE plus 40 allozyme loci affirmed the close monophyletic relationship of the species and provided support for their resolution into three distinct groupings. The earliest divergence led to *L. tigrina*, followed by an ambiguous trichotomy of three species, *O. guigna*, *O. geoffroyi*, and *L. colocolo*, and lastly the more recent association of *L. pardalis* and *L. wiedii*. This scheme was supported by phenetic analyses of both 2DE and allozyme data sets (Figs. 3, 4).

Because of the recency of divergence combined with the slow rate of change for the tested protein loci, the parsimony analysis was not particularly informative as all the trees had high degrees of homoplasy due to transmission of shared ancestral characters (symplesiomorphies). This situation can overwhelm phylogenetically informative character divergence (synapomorphies or shared derived characters) and obscure the actual evolutionary relationship (Rogers 1984; Menotti-Raymond and O'Brien 1993). Nevertheless, a maximum parsimony analysis of the present data did not contradict the phenetic inference (Fig. 5) derived from genetic distance matrices based on allele frequency differences.

The association of *L. pardalis* and *L. wiedii* is consistent with the karyotypic data which link these species due to their sharing of a unique deletion of chromosome D2 (Modi and O'Brien 1988). Furthermore a multivariant analysis of skull morphology grouped these two taxa together, but suggested an association with *L. tigrina* as well (Glass and Martin 1978). This morphological association with *L. tigrina* is inconsistent with the present molecular data, which might be explained by loss of shared primitive morphological features in other species of this linkage.

In an attempt to resolve the topology of South American lineage, we performed jackknife analysis using Asian golden cat (*Profelis temmicki*), leopard cat (*Prionailurus bengalensis*), domestic cat (*Felis catus*), jaguarundi (*Herpailurus yagouaroundi*), and the puma (*Puma concolor*) as alternative felid outgroups. Although jackknife analysis offers no statistical test of significance in phylogenetic analysis, it allowed us to estimate the relative influence of using different species as the outgroup for the overall topology. Using both Fitch-Margoliash and neighbor-joining algorithms, 2DE data split into the same three groups irrespective of the

Table 5. Distribution of alleles at polymorphic loci defined by 1DE (1 = most common allele; 0 = unscorable for technical reasons)[a]

	LCO	LPA	LTI	LWI	OGE	OGU	PON
AK-1	1	1	1	1	0.8	1	1
ALB	1	1	1	0.8/1	1	1	1
CPK-B	1	1	2	1	1	1	1
DIA-A	0.5/1	1	1	1	1	1	0
DIA-C	0.3	0.4	1	0.8	1	1	2
ESU-1	1/2	1	1	1	1	1	1
CA-2	1	1	1	1	1	1	0.5
G6PD	1	0.8	1	0.8	1	1	1
GPT	1/2	1	2	1	2	1	2
GSR	1	1/2	1	1	1	1	2
IDH-1	1	1	0	0.8	1	1	1
MDH-1	1	1	1/2	1/2	1	1	1
ME-1	1	0.8/1	1	1	1	0.8	1
MPI	0.8	1/2	1	2	1	1	1
NP	1	1	1	1	1	1	1.5
PEP-C	1	1/2	1	1	1.5/2	1.5/2	0.9
PGD	1	1/2	1	1	1	1/2	1
PGM-1	1	0	1	1	0.5/1	1	0.5/1
PGM-3	0.8	0	1	0.8	1	0	0.5/1
PK	0	1	0	1	1	1	1.5
SOD-2	1	1	1	1	1	1	0.9
TF	1	0.3	2	1	1	1	2

[a] Invariant loci included: ACP-1, CAT, ESα, GLO, GOT-1, GOT-2, GPI, GUS-B, HBB, HK-1, IDH-2, LDH-A, LDH-B, MDH-2, PEP-B, PGAM, PP, TPI. Allele names represent relative electrophoretic mobility relative to an arbitrary type = 1.0. Gene abbreviations follow human nomenclature (McAlpine et al. 1985)

Table 6. Matrix of Nei's unbiased genetic distance based on allelic frequencies at 40 allozyme loci

	LCO	LPA	LTI	LWI	OGE	OGU	PON
LCO	*****						
LPA	0.211	*****					
LTI	0.193	0.245	*****				
LWI	0.185	0.168	0.252	*****			
OGE	0.168	0.210	0.153	0.277	*****		
OGU	0.202	0.143	0.219	0.210	0.144	*****	
PON	0.404	0.391	0.326	0.543	0.425	0.442	*****

felid outgroup. Allozyme data were less robust but consistently paired the *L. pardalis* with the margay, *L. wiedii*.

Originally proposed by Zuckerkandl and Pauling (1962), the concept of a molecular clock can be used to estimate divergence times when the rate of change for each metric is linear and constant among all lineages within the phylogeny considered. Previous studies with primates and ursids reveal that 2DE estimates show a proportionality with evolutionary time (Wayne et al. 1991). Similarly, our study reveals an equivalent rate of change among all felid species with respect to the outgroup of *C. crocuta*. Assuming a constant metric, represented by the Fitch-Margoliash algorithm modified to accommodate contemporary tips (Fig. 3), we estimate that the two major groups of South American felids diverged between 5 and 6 MYA. The subsequent radiation within the ocelot lineage was estimated to have oc-

curred 2–5 MYA. This interval coincides with the estimated time of formation of a land bridge between North and South America between the late Miocene (Martin 1989) and the late Pliocene (Wayne et al. 1991). Prior to this period, no mammalian carnivores were present in South America (Patterson and Pascual 1972).

Recent radiation of extant species of South American felids is consistent with high rates of extinction and reevolution of adaptive forms observed in carnivores. Typically, this basic pattern consists of total extinction of the lineage followed by the reevolution of the same adaptive type (Martin 1989). High rates of speciation over a short interval result in a significant amount of random segregation of ancestral genetic polymorphisms among extant species within a monophyletic lineage (Wu 1991). Therefore, our inability to completely resolve the ocelot lineage using a broad screening survey of the genome is probably attributable to the short time since the six species last shared a common gene pool.

Clearly, the number of changes in protein-encoding loci detected between each pair of species using 2DE is very few relative to the abundance of ancestral polymorphisms. This is particularly obvious in the parsimony analysis. Even though we employed twice the number of loci (using higher-resolution technology) previously reported (Goldman et al. 1987, 1989; Janzcewski et al. 1990), the proportion of variable loci declined. Consequently, estimated rate of change for the 2DE metric in cats was 34 times less than the value de-
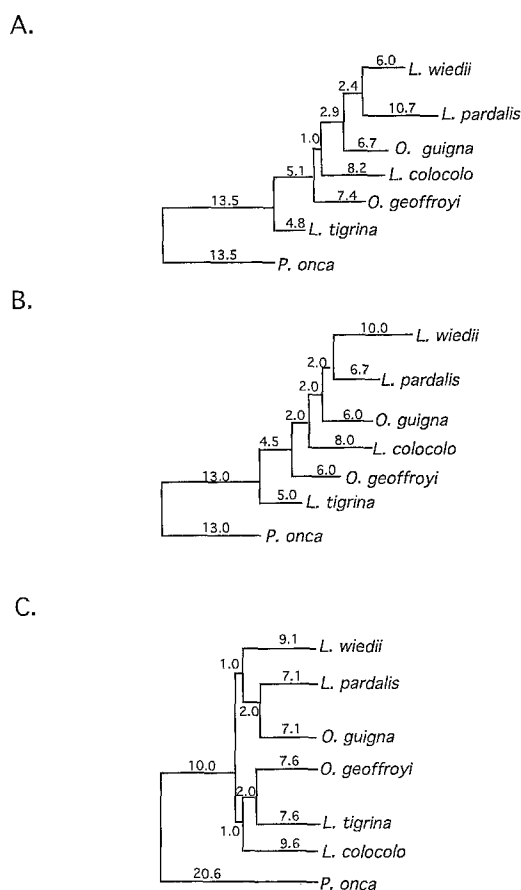
A.

B.

C.

Fig. 4. Phenetic analysis of allozyme electrophoresis (N = 40 protein-encoding gene loci) based on a distance matrix of Nei (1978) unbiased minimum genetic distance (Table 6). All trees generated using Phylip 3.4 computer package. Trees derived from (A) neighbor-joining and (B) Fitch-Margoliash least-squares (average percent standard deviation = 10.8) methods were drawn using branch-length values representing estimated % genetic distance. (C) Tree derived under the molecular clock hypothesis, assuming equal rates of change among all lineages, using Fitch-Margoliash least-squares method with contemporary tips (average percent standard deviation = 15.7).
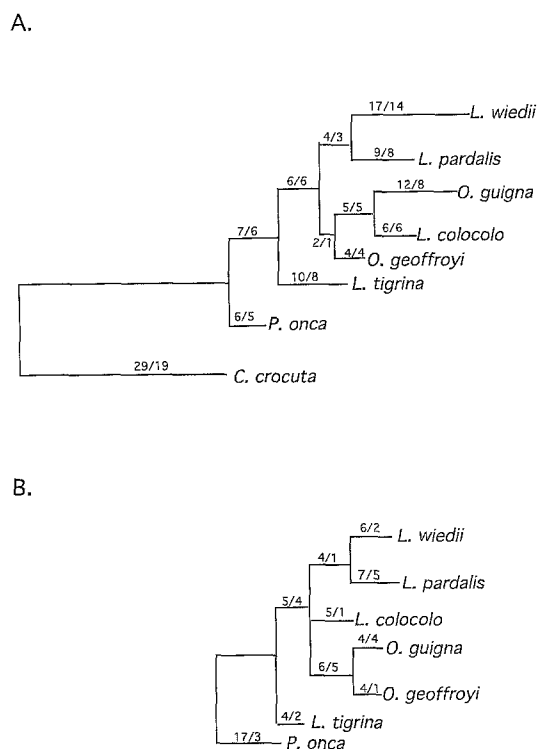
A.

B.

Fig. 5. Maximum parsimony analysis of allele frequencies coded into presence and absence data. Numbers on each limb correspond to No. steps/No. homoplasies required to construct tree. Both trees constructed using a user-defined tree based on the topology estimated by phenetic analysis (Figs. 3, 4). In both data sets, an increase of four steps in total tree length recapitulated the corresponding phenetic tree. A Two-dimensional electrophoresis data: N = 108 character states, tree length = 117 steps, CI = 0.583. B Allozyme data: N = 97 character states, tree length = 62 steps, CI = 0.774.

Table 7. Interspecies genetic distance estimates using 2DE and allozyme data sets

Genus[a]	2DE	1DE	Reference
Apes[a] (7 spp.)	0.014–0.159	0.072–0.548	Janczewski et al. (1990)
Ursus (5 spp.)	0.028–0.060	0.018–0.108	O'Brien et al. (1985); Goldman et al. (1989)
Canis (3 spp.)		0.036–0.240	Wayne and O'Brien (1987); Wayne et al. (1989)
Mustela (4 spp.)		0.081–0.320	O'Brien et al. (1989)
Panthera (5 spp.)	0.003–0.015[b]	0.034–0.229	O'Brien et al. (1987)
Ocelot lineage	0.001–0.009	0.143–0.277	Present study

[a] Except for the apes, all groups of species are within a single genus. For the apes, the values include human, chimpanzees, gorilla, orangutan, and gibbons (Janczewski et al. 1990)

[b] (JPS and SJO, unpublished observation)

termined for primates (Goldman et al. 1987). This discrepancy emphasizes the dependence of 2DE protein evolutionary rates on the geologic time interval. Both primates and bears evolved gradually over the past 25 MY compared with the shorter interval of 10 MYA for the Felidae. We suggest further confirmation of the phylogenetic relationships among the cats of the ocelot lineage using a gene, or genes, with a more rapid mutation rate.

specific Federal Fish and Wildlife permits (Convention on International Trade in Endangered Species of Wild Fauna and Flora [CITES]: Endangered and Threatened Species, Captive Bred) issued to the National Cancer Institute, National Institutes of Health (principal officer S.J. O'Brien) by the U.S. Fish and Wildlife Services of the Department of the Interior.

References

Adams EN III (1972) Consensus techniques and the comparison of taxonomic trees. Syst Zool 21:390–397

Avise JC, Aquadro CF (1981) A comparative summary of genetic distance in vertebrates. Evol Biol 14:114–126

Benveniste RE (1985) The contributions of retroviruses to the study of mammalian evolution. In: MacIntyre RJ (ed) Molecular evolutionary genetics. Plenum Press, New York, pp 359–417

Berta A (1983) A new species of small cat (Felidae) from the late Pliocene-early Pleistocene Uquian of Argentina. J Mammal 64: 720–725

Clutton-Brock J (1987) A natural history of domesticated animals. Cambridge University Press, Cambridge, England

Collier GE, O'Brien SJ (1985) A molecular phylogeny of the Felidae: immunological distance. Evolution 39:473–487

Dayhoff MO (1978) Survey of new data and computer methods of analysis. In: Dayhoff MO (ed) Atlas of protein sequence and structure, vol 5. Natl Biomed Res Found, Silver Springs, MD, pp 41–45

Ewer RF (1973) The carnivores. Cornell University Press, New York

Felsenstein J (1991) PHYLIP—phylogeny inference package (version 3.4). University of Washington, Seattle, WA

Ficcarelli G (1984) The Villafranchian cheetahs from Tuscany and remarks on the dispersal and evolution of the genus Acinonyx. Palaeontographia Italica 73:94–103

Glass GE, Martin LD (1978) A multivariate comparison of some extant and fossil Felidae, Carnivora. Carnivore 1:80–88

Goldman D, Giri PR, O'Brien SJ (1987) A molecular phylogeny of the hominoid primates as indicated by two-dimensional protein electrophoresis. Proc Natl Acad Sci USA 84:3307–3311

Goldman D, Giri PR, O'Brien SJ (1989) Molecular genetic-distance estimates among the Ursidae as indicated by one- and two-dimensional protein electrophoresis. Evolution 43:282–295

Goldman D, O'Brien SJ (1993) Two-dimensional protein electrophoresis in phylogenetic studies. In: Zimmer EA, White TJ, Cann RL, Wilson AC (eds) Molecular evolution: producing the biochemical data. Methods in enzymology, vol 224:113–121

Hemmer H (1978) The evolutionary systematics of living Felidae: present status and current problems. Carnivore 1:71–79

Janczewski DN (1992) Phylogenetic relationships of the great cats based on mitochondrial DNA sequence analysis. PhD dissertation, Graduate School of the University of Maryland, College Park, MD

Janczewski DN, Goldman D, O'Brien SJ (1990) Molecular divergence and variation of orangutan (Pongo pygmaeus) based on isozyme and two-dimensional electrophoresis. J Hered 81:375–387

Kurten B (1976) Fossil puma (Mammalia: Felidae) in North America. Neth J Zool 26:502–534

Kurten B (1968) Pleistocene mammals of Europe. Aldine, Chicago

Kurten B, Anderson E (1980) Pleistocene mammals of North America. Columbia University Press, New York

Leyhausen P (1979) Cat behavior. Garland Press, New York

Li W-H, Wu C-I, Luo C-C (1985) A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. Mol Biol Evol 2:150–174

McAlpine PJ, Shows TB, Miller RL, Pakstis AJ (1985) The 1985 cat-

alog of mapped genes and report of the nomenclature committee. Eighth International Workshop on Human Gene Mapping. Cytogenet Cell Genet 40:8–66

Margush T, McMorris FR (1981) Consensus n-trees. Bull Math Biol 43:239–244

Martin LD (1989) Fossil history of the terrestrial Carnivora. In: Gittleman JL (ed) Carnivore behavior, ecology and evolution. Cornell University Press, Ithaca, NY

Menotti-Raymond M, O'Brien SJ (1993) Dating the genetic bottleneck of the African cheetah. Proc Natl Acad Sci USA 90:3172–3176

Millipore Corp (1991) Investigator 2 D electrophoresis system. Publication 17957.

Modi W, O'Brien SJ (1988) Quantitative cladistic analysis of chromosomal banding among species in three orders of mammals: hominoid primates, felids and arvicolid rodents. In: Gustafson JP, Appels R (eds) Chromosome structure and function. Plenum Press, New York, pp 215–242

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–90

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Nei M, Stephens JC, Saitou N (1985) Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. Mol Biol Evol 2:66–85

Nowak RM (1991) Walker's Mammals of the world, 5th ed. Johns Hopkins University Press, Baltimore, MD

O'Brien SJ, Collier GE, Benveniste RE, Nash WG, Newman AK, Simonson JM, Eichelburger MA, Seal US, Bush M, Wildt DE (1987) Setting the molecular clock in the Felidae: the great cats, panthera. In: Tilson RL (ed) Tigers of the world. Noyes Publications, Park Ridge, NJ, pp 10–27

O'Brien SJ, Martenson JS, Eichelberger MA, Thorne ET, Wright FW (1989) Genetic variation and molecular systematics of the black-footed ferret. In: Seal US, Thorne ET, Bogan MA, Anderson SH (eds) Conservation biology and the black-footed ferret. Yale University Press, New Haven, pp 21–23

O'Brien SJ, Nash WG, Wildt DE, Bush M, Benveniste RE (1985) A molecular solution to the riddle of the giant panda's phylogeny. Nature 317:140–144

O'Brien SJ, Wildt DE, Goldman D, Merril CR, Bush M (1983) The cheetah is depauperate in genetic variation. Science 221:459–462

Pamilio P, Nei M (1988) Relationships between gene trees and species trees. Mol Biol Evol 5:568–583

Patterson B, Pascual R (1972) The fossil mammal fauna of South America. In: Keast A, Erk C, Glass B (eds) Evolution, mammals and southern continents. State University of New York Press, Albany, NY pp 247–309

Rogers JS (1984) Deriving phylogenetic trees from allele frequencies. Syst Zool 33:52–63

Savage DE, Russell DE (1983) Mammalian paleofaunas of the world. Addison-Wesley, London

Sokal RR, Rohlf FJ (1981) Taxonomic congruence in the Leptopodomorpha reexamined. Syst Zool 30:309–325

Swofford DL (1991) PAUP, phylogenetic analysis using parsimony, version 3.0. Illinois Natural History Survey, Champaign, IL

Swofford DL, Selander RK (1981) Biosys-1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72:281–283

Turner A (1985) Extinction, speciation, and dispersal in African larger Carnivores from the late Miocene to recent. S Afr J Sci 81:256–257

Turner A (1987) New fossil carnivore remains from the Strekfontein hominid site (Mammalia: Carnivora). Ann Transvaal Museum 34:319–347

Van Valkenburgh B, Grady F, Kurten B (1990) The Plio-Pleistocene cheetah-like cat Miracinonyx inexpectatus of North America. J Vert Paleontol 10:434–454

Wayne RK, Benveniste RE, Janczewski DN, O'Brien SJ (1989) Molecular and biochemical evolution of the Carnivora. In Gittleman JL (ed) Carnivore behavior, ecology and evolution. Cornell University Press, Ithaca, NY, pp 465–494

Wayne RK, O'Brien SJ (1987) Allozyme divergence within the Canidae. Sys Zool 36:339–355

Wayne RK, Van Valkenburgh B, O'Brien SJ (1991) Molecular distance and divergence time in carnivores and primates. Mol Biol Evol 8:297–319

Werdelin L (1985) Small Pleistocene felines of North America. J Vert Paleontol 5:194–210

Wu C-I (1991) Inferences of species phylogeny in relation to segregation of ancient polymorphisms. Genetics 127:429–435

Wurster-Hill DH, Centerwall WR (1982) The interrelationships of chromosome banding pattern in canids, mustelids, hyena and felids. Cytogenet Cell Genet 34:178–192

Zuckerkandl E, Pauling L (1962) Molecular disease, evolution and genic heterogeneity. In Kasha M, Pullman B (eds) Horizons in biochemistry. Academic Press, NY, pp 189–225