Tracking the Evolution of the Elusive Andean Mountain Cat (*Oreailurus jacobita*) From Mitochondrial DNA

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Rarely observed in the wild, the existence of the Andean mountain cat (*Oreailurus jacobita*) has been established based on only 3 skulls and 14 museum skins. The Andean mountain cat's evolutionary relationship to other felids based on morphological characters is largely contradictory, with evidence aligning it with South American small spotted cats (ocelot lineage) or alternatively with pantherine lineage felids. Here we describe the phylogenetic distinctiveness and placement of the Andean mountain cat using DNA extracted from pieces of nine independent pelt specimens, including one confiscated from a trapper in 1995. A phylogenetic analysis of DNA sequences from three rapidly evolving mitochondrial genes (16S rRNA, NADH-5, and ATP-8) indicate that the Andean mountain cat is a distinct species belonging to the ocelot lineage. Our findings suggest that the Andean mountain cat diverged from a common ancestor with the ocelot (*Leopardus pardalis*) and margay (*L. wiedii*) and exhibits moderate levels of genetic variation.

Although the majority of living carnivore species are well described, there are exceptions such as two olingo species (*Bassaricyon pauli* and *B. lasius*), the Colombian weasel (*Mustela felipi*), and two cat species, Bornean bay cat (*Pardofelis badia*) and Andean mountain cat (*Oreailurus jacobita*) (Wozencraft 1993). In the scientific literature, only 3 skulls (Kuhn 1973; Pearson 1957; Philippi 1873) and 14 skins (Burmeister 1879; Cornalia 1865; Greer 1965; Matschie 1912; Pearson 1957; Philippi 1870; Pocock 1941; Schwangart 1941; Scrocchi and Halloy 1986; Yepes 1929) have been discussed, although an additional 20 skins are mentioned as present in the museum in Buenos Aires (Cabrera 1961; Scrocchi and Halloy 1986). Sightings of live Andean mountain cats have been rare, with only two documented cases (Scrocchi and Halloy 1986; Zeisler 1992). Most specimens in museum collections were collected over 60 years ago (Grimwood 1969; Scrocchi and Halloy 1986; Zeisler 1992). Virtually nothing is known about Andean mountain cat behavior and biology. They appear to be restricted to arid and semiarid zones above tree line, at 3,000–4,500 m (altiplano), in remote portions of the Andes of Chile, Argentina, Bolivia, and Peru (Figure 1) (Scrocchi and Halloy 1986).

Within South America there are 10 recognized cat species, including the Andean mountain cat (Wozencraft 1993). These species appeared in South America after the Panamanian land bridge formed in the late Pliocene, or 2–4 million years ago (Patterson and Pascual 1972). South American felids belong to three evolutionarily distinct groups, which have been distinguished using a variety of molecular genetic techniques (Collier and O'Brien 1985; Johnson and O'Brien 1997; Masuda et al. 1996; O'Brien et al. 1987; Pecon Slattery et al. 1994). The first group, which is comprised of puma (*Puma concolor*) and jaguarundi (*Herpailurus yagouaroundi*), along with the cheetah (*Acinonyx jubatus*) (currently restricted to Africa and Saudi Arabia), apparently diverged in North America from a common ancestor with other felid species 5–8 million years ago (Johnson and O'Brien 1997). The second group, the *Panthera* genus, currently has only one American member, the jaguar (*Panthera onca*) (Johnson et al. 1996; Johnson and O'Brien 1997). Cat species of this group, which include the lion (*P. leo*), leopard (*P. pardus*), and tiger (*P. tigris*), generally have broad geographic distributions in a wide variety of habitats. The third group, the ocelot lineage, is composed of species restricted to Central and South America: ocelot (*Leopardus pardalis*), margay (*L. wiedii*), tigrina (*L. tigrina*), Geoffroy's cat (*Oncifelis geoffroyi*), kodkod (*O. guigna*), and pampas cat (*Lynchailurus*

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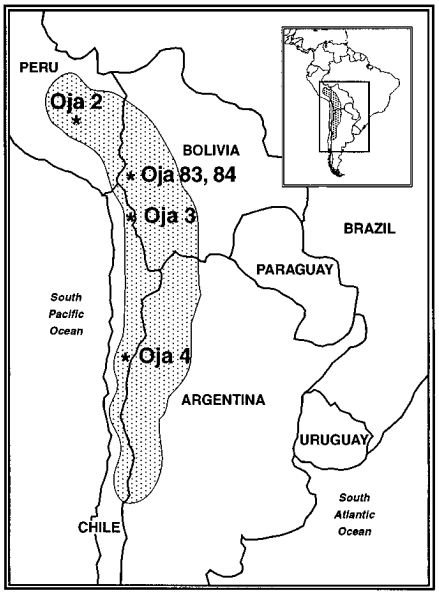

Figure 1. Map of the distribution of Andean mountain cat (modified from Nowell and Jackson 1996) and approximate collection sites of the specific Andean mountain cat samples.

Table 2. Primer sets used in gene amplifications and approximate length of product

1. 16S gene (Hoelzel and Green 1992)	
16S-1F: GTGCAAAGGTAGCATAATCA	
16S-4R: TGTCCTGATCCAACATCGAG	(ca. 376 bp)
Heminested set a	
16S-1F: (from above)	
16S-4NR: AACCTAAATT(G/A)TCGGCCCAT	(ca. 200 bp)
Heminested set b	
16S-1NF: ATGGGCCGA(C/T)AATTTAGGTT	
16S-4R: (from above)	(ca. 175 bp)
2. NADH-5 gene	
ND5-1F: GTGCAACTCCAAATAAAAG	
ND5-2R: GGGTCTGAGTTTATATATC	(315 bp)
Heminested set a	
ND5-1F: (from above)	
ND5-2NR: TA(G/A)GAGA(C/T)TGT(G/A)GTTTTTAC	(119 bp)
Heminested set b	
ND5-1NF: TATGTAAAAAC(C/T)ACA(G/A)TCTC	
ND5-2R: (from above)	(196 bp)
3. ATP-8 gene	
AP8-1F: GCATTAACCTTTTAAGTTAAAG	
AP8-2R: GGCGAATAGATTTTCGTTCA	(ca. 162 bp)

All primers are listed in 5′ to 3′ orientation and F refers to sense and R to antisense.

colocolo). Further phylogenetic resolution among ocelot-lineage cats has been achieved with five distinct categories of molecular markers (Johnson et al. 1996; Johnson and O'Brien 1997; Masuda et al. 1996; Pecon Slattery et al. 1994; Wurster-Hill and Centerwall 1982) and generally recognizes three subgroups: one composed of ocelot and margay, another consisting of Geoffroy's cat and kodkod, and a third genetically diverse subgroup made up of tigrina and pampas cat.

The Andean mountain cat's evolutionary relationship to other felids based on morphological characters is largely contradictory. The classification of the Andean mountain cat as a unique genus or subgenus (*Oreailurus*) originally was advocated based on a distinctive auditory bulla, with a larger anterior than posterior chamber (Cabrera 1940), and this has been independently suggested by others (Pocock 1941; Schwangart 1941). These and other morphological characters alternatively align Andean mountain cat with pampas cat of the ocelot lineage or with

Table 1. Identification code, museum identification code, geographic origin, and source for each sample

Species	ID	Museum ID	Origin	Contact	Source
Oreailurus jacobita	Oja 1	BM 23.11.18.2	Unknown	Richard Sabin	The Natural History Museum, London
	Oja 2	MVZ 116.317	Peru	James Patton	Museum of Vertebrate Zoology, Berkeley
	Oja 3		Chile	Piwonka Z.	Personal collection
	Oja 4		Chile		Chile Natural History Museum, Santiago
	Oja 7	MACN 29.200	Argentina	Martha Piantanida	Museo de Historia Natural Bernardino Rivadavia, Buenos Aires
	Oja 10	MACN 37.32	Argentina	Martha Piantanida	Museo de Historia Natural Bernardino Rivadavia
	Oja 11	MACN 42.113	Argentina	Martha Piantanida	Museo de Historia Natural Bernardino Rivadavia
	Oja 83	UG 2337	Bolivia	Hans-Jurg Kuhn	Universitat Gottingen, Gottingen
	Oja 84	ZSM 1984/12	Bolivia	Richard Kraft	Zoologische Staatssammulung, Munich
Leopardus pardalis	Lpa 11		Costa Rica	Lilly & Werner Hagnauer	Las Pumas, Costa Rica
	Lpa 31		Guatemala		Auto Safari Chapin, Guatemala
Leopardus wiedii	Lwi 22		Costa Rica	Lilly & Werner Hagnauer	Las Pumas, Costa Rica
	Lwi 41		Guatemala		Auto Safari Chapin, Guatemala
Leopardus tigrina	Lti 8		Colombia	Pat Quillen	SOS Care, California, USA
	Lti 11		Brazil	Pat Quillen	SOS Care, California, USA
	Lti 13		Costa Rica	Lilly & Werner Hagnauer	Las Pumas, Costa Rica
Lynchailurus colocolo	Lco 9		Uruguay		Parque Zoológico de Mercedes, Uruguay
	Lco 11		Chile		Zoológico de Quilpeu, Chile
Oncifelis geoffroyi	Oge 12				Washington State University
	Oge 21		Argentina		Zoológico de la Plata, Argentina
	Oge 32				
Oncifelis guigna	Ogu 2		Chile	Victor Riveros	Zoológico Nacional de Chile
	Ogu 3		Chile	Victor Riveros	Zoológico Nacional de Chile
Felis catus	Fca 117			Martin Kriete	NIH Animal Center, Maryland, USA
Herpailurus yagouaroundi	Hya 16		Argentina	Juan Romero	Parque Zool. de Buenos Aires, Argentina

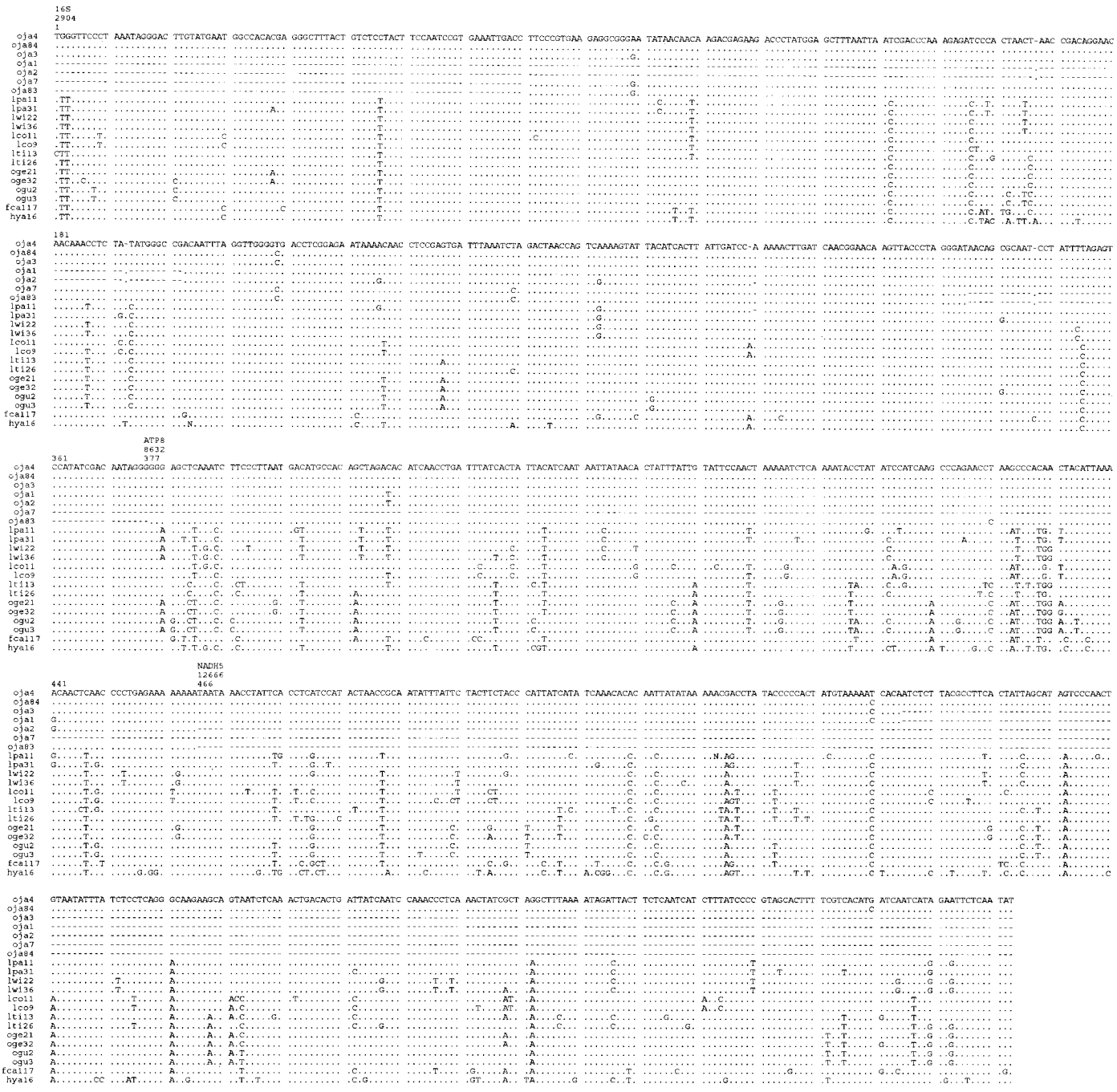

Figure 2. Sequences from portions of 16S, NADH-5, and ATP-8 mitochondrial genes. Reference numbers for each gene correspond to the domestic cat mitochondrial DNA sequence (Lopez et al. 1996).

rusty-spotted cat (*Prionailurus rubiginosa*) (Salles 1992), a member of the Pantherine lineage (Collier and O'Brien 1985) which is currently restricted to a small portion of India and Sri Lanka.

Here we employ a molecular genetic approach to discern the evolutionary associations of Andean mountain cat. We sequenced segments from three mitochondrial genes (16S ribosomal RNA, NADH dehydrogenase subunit 5, and ATPase-8) believed to be evolving at a moderate to rapid rate in carnivores (Lopez et al. 1997) and therefore informative in distinguishing associations among recently derived species. In addition to being of systematic interest, these molecular genetic data will aid in setting conservation and research priorities (Nowell and Jackson 1996) and will provide genetic markers useful in distinguishing Andean mountain cats from other species, such as the pampas cat, with which they are often confused.

Materials and Methods

Sample Collections

Because no Andean mountain cat individuals are currently held in captivity, samples were obtained from historic material (hides) in museums or private ownership. Nine samples of Andean mountain cat pelts, collected from the wild between 1929 and 1995, were used for this analysis (Table 1, Figure 1). Samples from other South American cat species were obtained

Table 3. Number of base-pair differences among 21 felid samples (above diagonal) and Kimura's two-parameter genetic distances with transition/transversion ratios of 11.0 (below diagonal)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. oja4	—	2	3	3	4	3	4	54	53	54	53	64	64	69	60	63	68	69	70	74	105
2. oja84	0.2	—	1	4	5	1	2	56	55	56	55	66	66	71	62	65	70	71	72	76	107
3. oja3	0.4	0.2	—	3	5	1	2	45	44	41	39	48	49	52	45	48	52	56	57	57	76
4. oja1	0.6	0.8	0.6	—	2	2	5	30	28	30	28	37	35	39	37	39	41	44	45	42	59
5. oja2	1.1	1.4	1.4	0.6	—	4	7	17	20	22	21	26	22	28	24	31	32	37	37	25	35
6. oja7	1.2	0.4	0.4	1.5	3.1	—	0	13	12	10	10	10	11	11	7	10	10	13	13	17	17
7. oja83	0.9	0.5	0.5	1.6	2.2	0.0	—	33	33	31	30	31	30	34	24	33	33	41	41	37	45
8. lpa11	6.5	6.8	7.1	6.6	4.9	5.4	0.8	—	29	32	35	67	61	65	60	56	61	60	61	72	100
9. lpa31	6.4	6.7	6.9	6.2	5.8	5.0	0.8	0.3	—	39	40	67	63	62	57	61	64	63	64	74	98
10. lwi22	6.5	6.8	6.4	6.6	6.5	4.1	7.5	3.8	4.6	—	7	68	66	65	58	57	62	63	64	77	103
11. lwi36	6.4	6.6	6.1	6.2	6.1	4.1	7.2	4.1	4.8	0.8	—	67	65	60	57	54	59	62	63	78	98
12. lco11	7.8	8.1	7.6	8.3	7.7	4.1	7.6	8.2	8.2	8.4	8.2	—	16	67	65	62	67	70	71	78	106
13. lco9	7.8	8.1	7.8	7.8	6.5	4.6	7.3	7.4	7.7	8.1	8.0	1.8	—	63	63	60	65	68	69	76	104
14. lti13	8.5	8.7	8.2	8.8	8.4	4.5	8.3	7.9	7.6	7.9	7.3	8.2	7.7	—	39	46	49	51	52	81	100
15. lti26	7.3	7.5	7.0	8.3	7.1	2.8	5.7	7.3	6.9	7.0	6.9	7.9	7.7	4.6	—	43	48	48	49	81	95
16. oge21	7.7	8.0	7.6	8.8	9.3	4.1	8.0	6.8	7.4	6.9	6.5	7.6	7.3	5.5	5.1	—	7	25	26	79	102
17. oge32	8.4	8.6	8.3	9.3	9.7	4.1	8.0	7.5	7.8	7.6	7.2	8.2	8.0	5.9	5.8	0.8	—	29	30	82	106
18. ogu2	8.5	8.7	8.9	10.0	11.3	5.4	10.2	7.3	7.7	7.7	7.5	8.6	8.4	6.1	5.7	2.9	3.4	—	1	83	107
19. ogu3	8.6	8.9	9.1	10.3	11.3	5.4	10.2	7.4	7.8	7.8	7.7	8.7	8.5	6.2	5.9	3.0	3.5	0.1	—	84	108
20. fca117	9.2	9.4	9.2	9.6	7.4	7.2	9.2	8.9	9.2	9.6	9.7	9.7	9.4	10.1	10.1	9.8	10.3	10.4	10.5	—	88
21. hya16	13.6	13.9	12.7	14.0	10.7	7.4	11.4	12.9	12.6	13.3	12.6	13.8	13.5	12.9	12.3	13.1	13.8	13.9	14.0	11.2	—

during visits to international institutions (Table 1). For outgroup species we sequenced gene segments from jaguarundi (*H. yagouaroundi*) and domestic cat (*Felis catus*).

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted from nine pelts using a silica/guanidium thiocyanate technique (Boom et al. 1990; Pääbo et al. 1988). No detectable DNA (as visualized on agarose gel) or PCR product (see below) was obtained from seven additional pelt samples or any of the negative controls. Sample preparation and DNA extraction from pelts were conducted in a laminar flow hood in an area isolated from other felid samples to provide minimal opportunity for contamination. For the other felid samples, total genomic DNA was extracted from frozen leukocytes or primary fibroblast cultures from skin biopsies following the standard phenol/chloroform methods described in Modi et al. (1987) and Sambrook et al. (1989).

Nucleotide sequences were obtained by PCR amplification of genomic DNA (Engelke et al. 1988; Saiki et al. 1985) using oligonucleotide primers which amplified portions of 16S, NADH-5, and ATP-8 mitochondrial genes (Table 2); each primer included universal-tailed sequencing primers (Applied Biosystems Inc.). Samples which did not amplify well using primers for the NADH-5 and 16S gene segments were sequenced using two hemi-nested primer sets which together covered the same sequence. Methods and conditions were similar for each primer set. Thirty cycles of PCR were performed in a programmable heat block (Perkin-Elmer Cetus DNA Thermal Cycler); each cycle with a 1 min denaturation step at 92°C, 1 min annealing at 48°C, and a 1 min extension step at 72°C. Fifty or 75 μl reactions were prepared using 7.5 ng genomic DNA in 10 mM Tris-HCL, pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP, 2.5 units *Thermus aquaticus* DNA polymerase, a primer volume ratio of 1:1 and an upper layer of mineral oil. Bovine serum albumin (2 μg/mg) was added to pelt-extracted PCR reactions to overcome inhibition.

Reaction products were resuspended in 2 ml dH_2O, concentrated with Centricon 100 microconcentrators, and sequenced in both forward and reverse directions with a prism dye primer kit and an ABI 373A automated DNA sequencer (Applied Biosystems Inc.). A consensus of the forward and reverse sequences was determined using the Sequencher computer program (Gene Codes Corporation). Sequence data were deposited in GenBank.

Phylogenetic Analyses

Alignments of the sequences were obtained using the algorithm of Needleman and Wunsch (1970) with the PILEUP program of the Genetics Computer Group (University of Wisconsin) computer package and were visually confirmed. Percentage differences among samples were estimated using Kimura's two-parameter model (Kimura 1980) using the DNADIST program of PHYLIP (version 3.5; Felsenstein 1993). Transition/transversion ratios were estimated using computer program MEGA (version 1.01; Kumar et al. 1993).

Phylogenetic trees were constructed using three methods: (1) the minimum evolution method estimated by the neighbor-joining algorithm (Saitou and Nei 1987) with Kimura distances followed by bootstrapping with 100 resamplings using PHYLIP (version 3.5; Felsenstein 1993); (2) the maximum parsimony method using the random addition of taxa and the branch-and-bound search options of PAUP (version 3.1.1; Swofford 1993) followed by the bootstrapping option with 100 resamplings; and (3) the maximum likelihood method (Felsenstein 1981) using the PHYLIP computer package (version 3.5; Felsenstein 1993). These methods are based on different evolutionary assumptions and may produce different phylogenetic trees (Felsenstein 1993; Huelsenbeck and Hillis 1994). Bootstrap proportions of greater than 70% were considered to reflect reliable support for each node (Hillis and Bull 1993).

We combined the sequences of each of the genes in a "total evidence" analysis because results from the three mitochondrial genes were largely similar and because mitochondrial genes should be evolving as a single inherited unit (Kluge 1989; Huelsenbeck et al. 1996).

Results and Discussion

Nucleotide sequences (883 bp) were obtained by PCR amplification of genomic DNA using oligonucleotide primers which amplified portions of 16S, NADH-5, and ATP-8 mitochondrial genes (Table 2). Par-

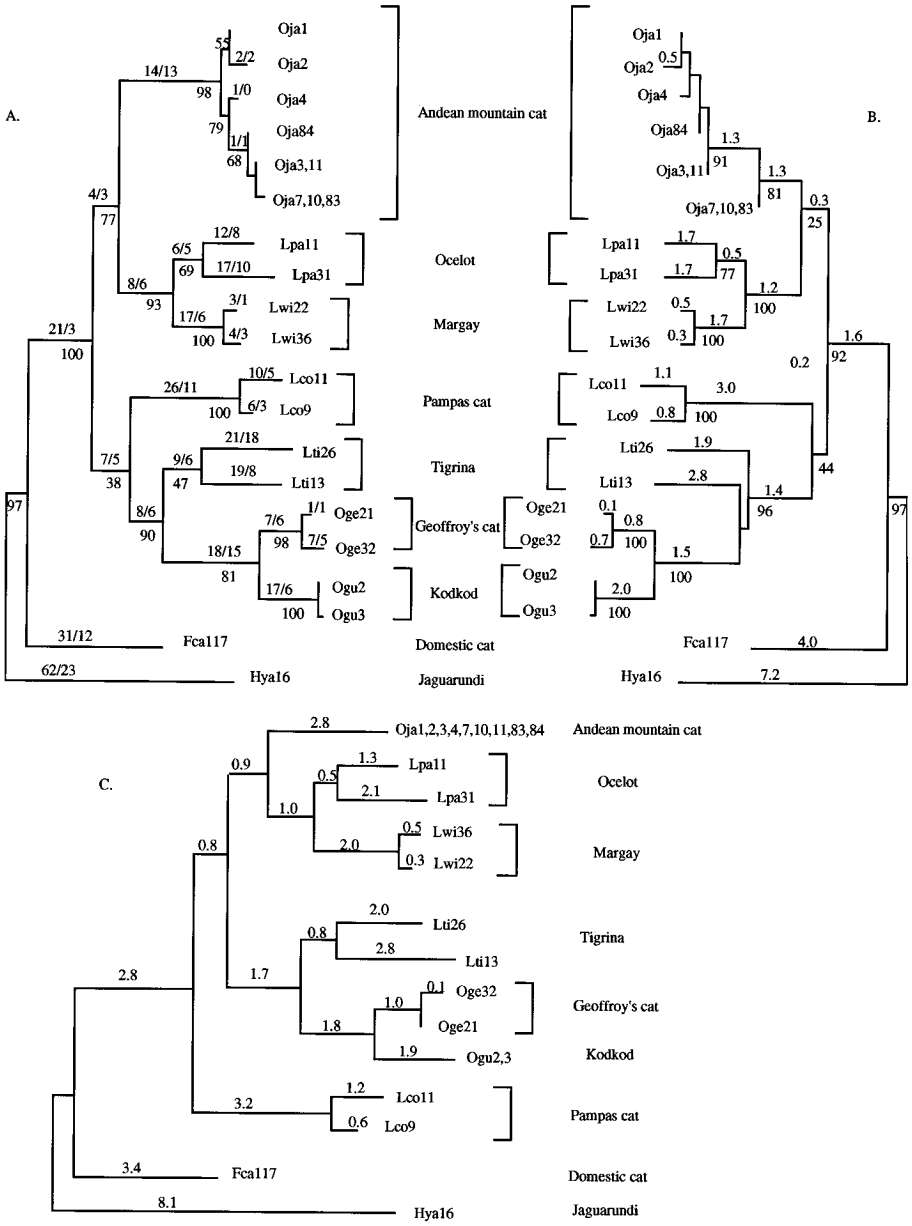

Figure 3. Phylogenetic relationships among multiple individuals of Andean mountain cat and other ocelot lineage felid species. **(A)** Phylogenetic tree constructed with maximum parsimony method estimated with PAUP with the branch-and-bound search option and a transition/transversion ratio of 11.0. There were three most-parsimonious trees (tree length = 747, CI = 0.686) which varied in their relative position of the haplotypes of Oja84, Oja3, and Oja7. Number of base pair changes (unweighted) followed by number of homoplasies are above branches. Percentage bootstrap support (100 iterations) for each node are below branches. **(B)** Phylogenetic tree constructed with minimum evolution method estimated with neighbor-joining algorithm with Kimura distances (tr/tv = 11.0). Percentage differences are indicated above branches and percent bootstrap support are shown below the branches. **(C)** Phylogenetic tree constructed with maximum likelihood method (1,561 trees examined, Ln likelihood = −3,121, tr/tv = 11.0); nodes with confidence intervals overlapping zero were reduced to polytomies; all nodes depicted in phylogram had significant positive support ($P < .01$).

tial sequences were obtained for Oja1 (482 bp), Oja2 (363 bp), Oja3 (684 bp), Oja7 (133 bp), Oja10 (133 bp), Oja11 (684 bp), and Oja 83 (433 bp) (Figure 2, Table 3). Sequences of Oja7, Oja10, and Oja83 were identical and Oja11 was identical with Oja3, resulting in 6 haplotypes.

Phylogenetic analyses using the three phylogenetic algorithms (minimum evolution, maximum parsimony, and maximum likelihood) of the combined sequences definitively placed Andean mountain cat within the monophyletic ocelot lineage (Figure 3). The three different phylogenetic analyses uniformly corroborated the unique identity of Andean mountain cat by clustering the six genotypes into a single group (81–97% bootstrap support).

However, the phylogenetic algorithms differed slightly in their support for a few of the interspecific relationships (Figure 3). For example, maximum parsimony and maximum likelihood analyses provided some evidence that Andean mountain cat formed a distinct cluster with ocelot and margay (77% maximum parsimony bootstrap support) separate from tigrina, pampas cat, Geoffroy's cat, and kodkod samples. However, in the minimum evolution analysis using the neighbor-joining algorithm, the link between the Andean mountain cat and the clade of ocelot and margay was very weak (25% bootstrap support). When we constrained the Andean mountain cat to cluster with the pampas cat, as has been proposed by some researchers, the shortest tree estimated from the maximum parsimony analysis was 758 steps long, compared with a 746-step unconstrained tree.

The three phylogenetic algorithms also differed in placement of ocelot lineage species relative to the outgroups. Minimum evolution and maximum parsimony analyses suggested that ocelot lineage species formed two groups, one composed of the Andean mountain cat, ocelot, and margay, and the other composed of the pampas cat, tigrina, Geoffroy's cat, and kodkod. In contrast, maximum likelihood analysis rooted the ocelot lineage slightly differently by making the pampas cat the most basal group. Results from each gene segment analyzed separately were consistent with the patterns presented above, but bootstrap and maximum likelihood support for among-species-level relationships were weaker due to a reduction in phylogenetic signal.

Estimating the divergence date of Andean mountain cat from other South American species is complicated by a sparse and poorly understood fossil record. A possible ancestor of South American small cats of the ocelot lineage (*F. lacustris* or *F. rexroadensis*) first appears in the fossil record in North America 4–5 million years ago (Werdelin 1985). The earliest fossils of modern-day *Leopardus* spp. in South America are 1.5–2.5 million years old (Berta 1983) and are only 0.3–0.4 million years old in southern North America (Kurtén and Anderson 1980). Estimates of when the ocelot lineage species last shared a common ancestor range from 5–6 million years ago (Johnson et al. 1996; Johnson and O'Brien 1997; Pecon Slattery et al. 1994). These dates are within the range of estimates for the formation of the Panamanian land bridge linking North and South America between the late Miocene and the late Pliocene (Patterson and Pas-

cual 1972). This chronology of evolutionary events suggests that Andean mountain cats could have differentiated in either North or South America, perhaps by adapting to the drier alpine ecosystems along the Andes while the progenitor to ocelot/margay adapted to more mesic and forested regions in Central America and western South America. Further characterization of the relationships and identification of ancient lineages among extant ocelot and margay populations may help elucidate this evolutionary history.

Low prey densities in Andean habitats and a paucity of visual sightings of Andean mountain cat suggest that they are relatively rare. However, pairwise Kimura distances among Andean mountain cat samples ranged from 0.4–1.5%, suggesting that their populations have not suffered severe reductions in numbers or bottlenecks, which might have reduced genetic variability. These values are comparable to intraspecific estimates for Geoffroy's cat (one from Uruguay and the other from central Argentina) and kodkod (0.8 and 0.1%, respectively). Detailed information on Andean mountain cat populations, which may be reduced and widely separated in the Andean mountains, are needed to further refine our understanding of the rare cat's genetic patterns.

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