Genomic Ancestry of the American Puma (*Puma concolor*)

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Puma concolor, **a large American cat species, occupies the most extensive range of any New World terrestrial mammal, spanning 110 degrees of latitude from the Canadian Yukon to the Straits of Magellan. Until the recent Holocene, pumas coexisted with a diverse array of carnivores including the American lion (*Panthera atrox*), the North American cheetah (*Miracynonyx trumani*), and the saber toothed tiger (*Smilodon fatalis*). Genomic DNA specimens from 315 pumas of specified geographic origin (261 contemporary and 54 museum specimens) were collected for molecular genetic and phylogenetic analyses of three mitochondrial gene sequences (*16S rRNA, ATPase-8,* and *NADH-5*) plus composite microsatellite genotypes (10 feline loci). Six phylogeographic groupings or subspecies were resolved, and the entire North American population (186 individuals from 15 previously named subspecies) was genetically homogeneous in overall variation relative to central and South American populations. The marked uniformity of mtDNA and a reduction in microsatellite allele size expansion indicates that North American pumas derive from a recent (late Pleistocene circa 10,000 years ago) replacement and recolonization by a small number of founders who themselves originated from a centrum of puma genetic diversity in eastern South America 200,000–300,000 years ago. The recolonization of North American pumas was coincident with a massive late Pleistocene extinction event that eliminated 80% of large vertebrates in North America and may have extirpated pumas from that continent as well.**

Pumas (also called mountain lions, or cougars) occupy a vast range of ecological zones (Figure 1A) as diverse as desert, savannah, tropical rain forest, and alpine steppes (Anderson 1983; Hansen 1992). Adult size varies from 50 to 70 kg at the equator to twice that size in the extreme reaches of the Canadian Yukon and Patagonian pampas. The puma fossil record is less than half a million years old, but both molecular and morphologic studies suggest that the puma's origin dates to the late Miocene [5–8 million years before the present (MYBP)], when pumas evolved from a common ancestor with the African cheetah (*Acinonyx jubatus*) and American jaguarundi (*Herpailurus yaguaroundi*) (Janczewski et al. 1995; Johnson and O'Brien 1997; Pecon-Slattery and O'Brien 1998; Van Valkenburgh et al. 1990). Pumas most certainly arrived in South America 2–4 MYBP during the Great American Interchange, when eutherian carnivores first migrated south from North America with the geologic joining of the Panamanian land bridge (Marshall et al. 1982; Stehli and Webb 1985; Webb 1978; Webb and Marshall 1981; Webb and Rancy 1996). Although the event would suggest that modern pumas originated from a North American ancestry, the puma fossil record in South America is so poor that a more recent neotropical origin and northward dispersal cannot be ruled out (Kurten 1976; Werdelin 1989).

The peopling of North and South America clearly diminished puma populations in recent centuries, reducing the range by two-thirds in North America as a consequence of habitat destruction and human depredation (Figure 1A). Except for the small endangered population of Florida panther (*Puma concolor coryi*) living in cypress swamps of south Florida, pumas are extinct east of the Mississippi (Hansen 1992; Maehr 1998; Roelke et al. 1993). Nonetheless, elimination of bounties and recent legislative protection has led to an increase in puma numbers in many areas since 1900. Some 32 separate geographic subspecies of puma have been described based on geographic and morphometric criteria (Figure 1A) (Neff 1983; Young and Goldman 1946). The eastern cougar (*P. c.*

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couguar) and the Wisconsin puma (*P. c. schorgeri*) are presumed extinct, and three subspecies (*P. c. coryi, P. c. costaricensis,* and *P. c. brownii*) are classified as endangered or threatened by the U.S. Fish and Wildlife Service (Hansen 1992).

Here we examine the extent of genetic diversity using three groups of genetic markers (mtDNA coding genes, Y-chromosome *Zfy* intron sequence, and 10 nuclear feline microsatellite loci) among 315 individual pumas: 261 contemporary animals and 54 museum specimens, each of known geographic origin representing 31 of 32 named subspecies. The pattern of variation within and among subspecies was employed to verify phylogeographic subdivision and to interpret the natural history of the species. The cumulative data support the recognition of six operational taxonomic units (OTUs) or subspecies based on their sharing of a "unique geographic range or habitat, a group of phylogenetically concordant" phenotypic characters and "a unique natural history" (Avise and Ball 1990; O'Brien and Mayr 1991). Molecular markers show that individuals from the North American continent comprise a large panmictic population and display reduced genetic variation relative to South American pumas, an indication of an historic founder effect in the North American puma ancestry. Four of the six recognized puma OTUs reflect geographic (potential faunal) boundaries, while the other two are likely hybrid (or intergrade) zones from geographically adjacent subspecies. Consistency of the genetic/phylogenetic results with each category of genetic markers offers confidence in subspecies classification and has implications for natural history and present conservation management of the puma.

Materials and Methods

Samples

Biological samples were collected from 315 pumas and were obtained from the wild (n = 148), captive facilities (n = 113), and museums (n = 54) (Figure 1A). Samples included 1–35 animals from each of 32 subspecies. Captive animals were wild born and of known geographic origin. Samples were assigned to the nearest subspecies based on geographic location (Cabrera 1963; Jackson 1955; Neff 1983; Young and Goldman 1946). Population samples were selected to exclude known related individuals, or in the case of Florida, known hybrids (O'Brien et al. 1990). Samples of cheetah, jaguarundi, Geoffroy's cat (*Oncifelis geoffroyi*), and domestic cat (*Felis catus*) were included as outgroup species.

DNA was extracted from white blood cells, whole blood, primary fibroblast cultures from skin biopsies, or tissues following a phenol-chloroform protocol (Sambrook et al. 1989). DNA from hide, hair, and bone samples was extracted using a silica based, guanidium extraction method (Höss and Pääbo 1993; Johnson et al. 1998; Pääbo et al. 1988).

mtDNA Markers

Products for both single-strand conformation polymorphism (SSCP) and sequencing of mtDNA regions were obtained by PCR amplification of genomic DNA (Saiki et al. 1985) using primers that amplify portions of the *16S rRNA* (*16S*), *NADH-5* (*ND5*), and *ATPase-8* (*ATP8*) genes (Johnson et al. 1998; Johnson and O'Brien 1997). PCR amplification of museum samples was performed with two pairs of hemi-nested primers for *16S* and *ND5* (Johnson et al. 1998) except 16S-3F-5′GAGACCCATTAATTTCAACCG-3′ is substituted for 16S-1NF. PCR reactions were performed using 20 ng of genomic DNA in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.16 mg/ml BSA, 1 μM of each primer, and 1 unit *Taq* polymerase enzyme in a volume of 10 μl (2.5 units in 25 μl for museum specimens). Thermocycling conditions consisted of 0.5 min denaturation at 94°C, 1.5 min annealing at 49°C, and 1 min extension at 72°C for 30 cycles (35 or 40 cycles for museum samples). Resulting products were visualized on a 2% agarose gel in TBE buffer.

SSCP analyses were performed on mtDNA amplification products from contemporary samples (Orita et al. 1989a;b; Poduslo et al. 1991). PCR was performed as described above with the addition of 1 μCi of dCTP[α-^{32}P]. The PCR product was denatured at 95°C and electrophoresed on a 4.5% nondenaturing polyacrylamide gel with 10% glycerol at 60 W for 6 h. Several individuals with identical banding patterns were sequenced to confirm that each had the same allele. PCR products from museum specimens were directly sequenced. The mtDNA PCR products were sequenced in both forward and reverse directions using a Prism Dye Primer kit and were analyzed by an ABI 373A automated DNA sequencer (Applied Biosystems Inc., Foster City, CA). Sequences were deposited in GenBank (accession numbers AF241812–AF241820).

Phylogenetic Analyses of mtDNA Haplotypes

Phylogenetic analyses of mtDNA variation were conducted for samples which successfully amplified for all three gene segments (n = 286). Sequences for each gene were combined for a total evidence approach (Huelsenbeck et al. 1996) covering 891 bp. The combined data were edited and aligned using the program SEQUENCHER (version 3.0, Gene Codes Corp., Ann Arbor, MI) and verified visually. Genetic distances were estimated both by absolute number of base pair differences among haplotypes (p distance) and by the Tajima-Nei model of substitution (Tajima and Nei 1984) as implemented in the program PAUP* (used with permission of D. Swofford). Phylogenetic relationships among the haplotypes were estimated using three major methods—minimum evolution estimated by neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML)—using the program PAUP*. Concordance among the resultant topologies was considered to be strong support for the observed phylogeny.

NJ trees were generated with the Tajima-Nei distance estimates and a general heuristic search with tree bisection-reconnection branch swapping. The MP analysis was conducted by a general heuristic search using simple sequence addition of sequences and tree bisection-reconnection branch swapping. ML trees were generated using the HKY or F84 model (Hasegawa et al. 1985). The starting tree was obtained from the NJ analysis, the shape parameter ($\propto$) for the gamma distribution of rate heterogeneity among sites and the transition:transversion (Ts:Tv) ratio were set at default values, and base frequencies were empirically derived. An iterative process whereby each successive ML tree incorporated parameters estimated from the preceeding ML analysis continued until an optimal tree was consistently derived. Relationships among mtDNA haplotypes were estimated using a minimum spanning network, computed from the absolute number of differences between haplotypes using the program MINSPNET (Excoffier et al. 1992).

Zfy Intron Sequence

The terminal intron of *Zfy*, located on the Y chromosome, was examined in 14 representative male pumas (and one female for a negative control). PCR was per-

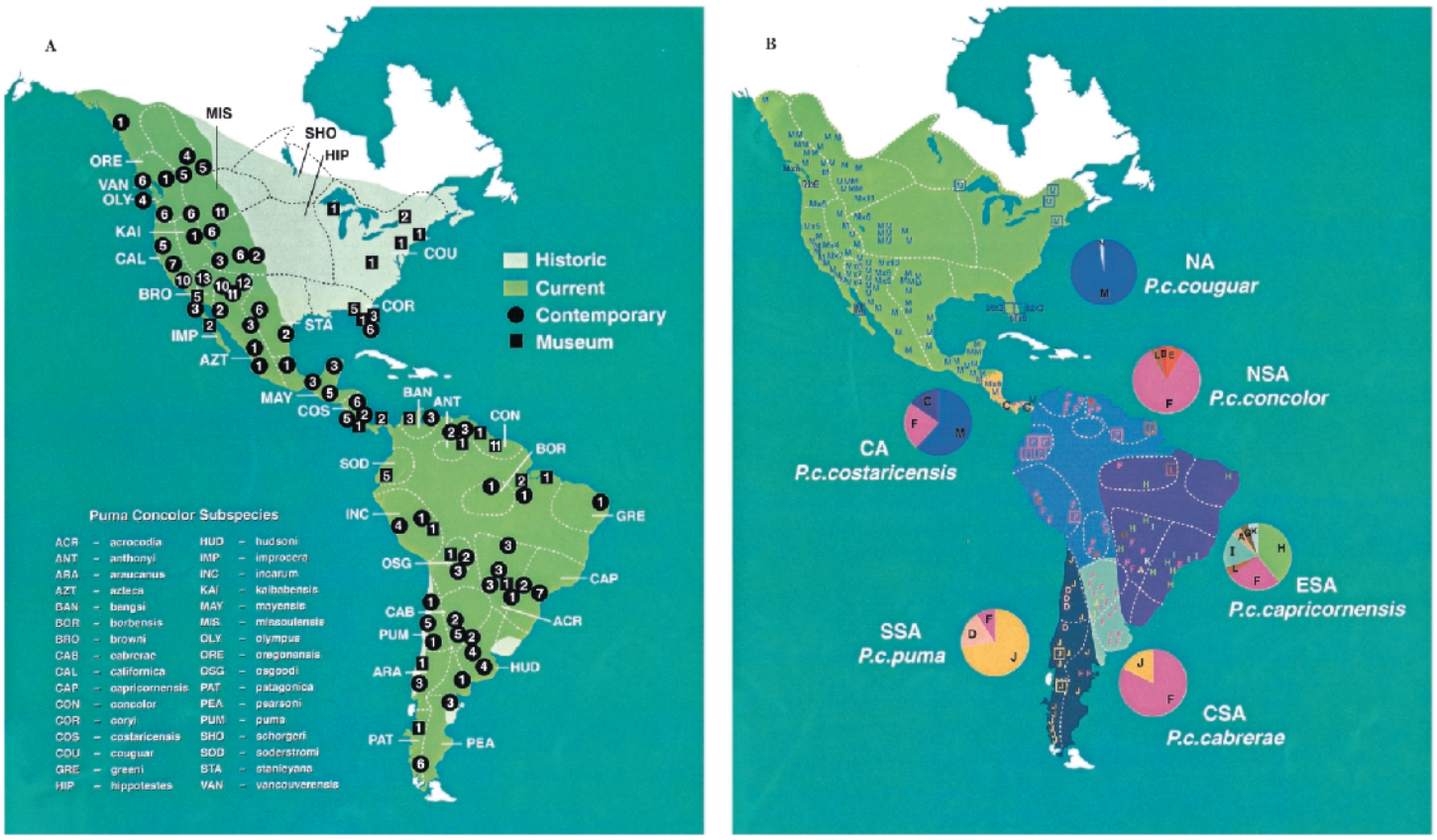

Figure 1. **(A)** Geographic ranges of 32 currently recognized subspecies of puma (*Puma concolor*) (Cabrera 1963; Jackson 1955; Neff 1983; Young and Goldman 1946). The subspecies are labeled with a three-letter code, and the total number of samples examined here are indicated. Collaborators and institutions that supplied puma specimens are (listed alphabetically by institution): Alberto Paras, Africam Safari, Mexico; Agustin Iriarte, Agriculture Service SAG, Chile; Charles Land, Alaska Department of Fish and Game; Ernesto Boede, Aquarium J.V. Seijas, Venezuela; Don Buckley, Arizona; Lisa Haynes, Stan Cunningham, Matt Peirce, and Harley Shaw, Arizona Game and Fish Department; Troy Best, and Ned Gentz, Auburn University, Alabama; Lorenza Calvo, Aurora National Zoo, Guatemala; Nini N. 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Junge, St. Louis Zoological Park, Missouri; Anabel Herrera, Summit Zoo, Panama; Jan Englund and Olavi Gronwald, Swedish Museum of Natural History, Stockholm, Sweden; Bill Franklin, Torres del Paine National Park, Chile; Mike Syvanen, University of California, Davis; Frank Iwen and Adrian Wydeven, University of Wisconsin Zoological Museum, Madison, Wisconsin; Ken Elowe, Utah Division of Wildlife Resources; Esmeralda Mujica, Valencia Aquarium, Venezuela; Melody Roelke, Vancouver Game Farm, Canada; Briggs Hall, Don Magoon, Jim Byse, and Willie Nation, Washington Department of Fish and Game; Scott Citino, White Oak Plantation, Florida; Melody Roelke, Laurie Marker, and Mike Briggs, Wildlife Safari, Oregon; Melody Roelke, Yosemite National Park, California; Dr. Riveros, Zoologico Nacional de Chile; Miguel Alvarez, Zoomat, Mexico. **(B)** Distribution of mtDNA haplotypes across the puma's ranges. Letters and pie charts refer to mtDNA haplotypes specified by fifteen single nucleotide polymorphisms defined in pumas (Table 1). The geographic ranges of six revised subspecies of pumas as defined here by mtDNA and microsatellite analysis are indicated. Dotted lines demarcate former subspecies range (Neff 1983; Young and Goldman 1946). Nx4 and Mx3,4,5 etc., indicates the number of individuals in the defined location that had the N or M haplotype, respectively. Squares around the haplotype letter indicates a museum sample.

formed using first-round primers ZF2F and ZF1R, and second round "nested" primers ZFY2F and ZFY1R (Pecon-Slattery and O'Brien 1998). PCR products of 350 bp from the *Zfy* intron were sequenced in both forward and reverse directions using the Dye Terminator Prism sequencing kit and analyzed by an ABI 373A automated DNA sequencer [Applied Biosystems Inc. (ABI), Foster City, CA]. *Zfy* sequences were edited and aligned using the program SEQUENCHER (version 3.0, Gene Codes Corporation, Ann Arbor, MI). The sequence was deposited in GenBank using accession number AF241870.

Table 1. Geographic location and ancestral inferences of 14 mtDNA haplotypes resulting from 15 polymorphic sites in combined analyses of the 16S (382 bp), ATP8 (191 bp), and ND5 (318 bp) genes, in 286 pumas[a]

	16S		ATP8					ND5										
Haplotype	3063[b]	3094	8630	8681	8700	8725	8756	12723	12751	12809	12819	12834	12840	12908	12909	ANC[c]	*N*[d]	Geographic location
A	T	G	C	C	T	T	C	T	A	A	C	C	G	T	A	9	1	Paraguay
B	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	9	1	Venezuela
C	.	.	T	.	.	.	T	.	.	.	.	.	.	.	G	8	2	Costa Rica, Panama
D	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	7	4	Chile
E	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	7	2	Bolivia, Peru
F	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	8	46	nA, Bo, Br, CR, E, G, Pa, Pe, V[e]
G	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	8	1	Brazil
H	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	**10**	9	Brazil, Paraguay
I	.	.	.	.	.	.	T	.	.	.	.	.	A	.	G	**10**	4	Brazil
J	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	9	19	Argentina, Chile
K	C	.	.	.	.	C	.	.	.	.	T	.	.	.	.	8	1	Brazil
L	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	9	2	Fr. Guyana, Brazil
M	.	A	.	T	.	.	T	.	G	.	.	.	.	.	.	9	190	North America, CR, Panama
N	.	A	.	T	.	.	T	.	G	.	.	.	.	C	.	8	4	Washington, USA
Hya	C	A	C	C	T	T	C	T	A	A	T	C	G	T	A		286	total samples
Aju	T	A	C	C	T	T	T	C	A	A	C	C	A	T	A			
Oge	T	A	C	C	T	T	T	T	A	A	C	C	A	T	A			
Fca[b]	A	A	C	C	T	T	T	C	A	A	T	C	A	T	A			
ancestral state[c]		A	C	C	T	T	T		A	A		C	A	T	A			
codon position	n/a	n/a	n/a	3	1	2	3	3	1	2	3	3	3	2	3			
aa change	n/a	n/a	n/a	—	S > P	V > A	—	—	I > V	Q > R	M > I	—	—	V > A	—			

[a] Base pairs that are identical to haplotype A are indicated by a period.
[b] From the complete *Felis catus* mtDNA sequence (Lopez et al. 1996).
[c] The number of sites with inferred ancestral state based on occurrence in three of four outgroup species' (Hya, Aju, Oge and Fca) mtDNA homologue.
[d] Number of individuals with each haplotype.
[e] Abbreviations: nA, northern Argentina; Bo, Bolivia; Br, Brazil; CR, Costa Rica; E, Ecuador; G, Guyana; Pa, Paraguay; Pe, Peru; V, Venezuela; (Aju) cheetah, (Hya) jaguarundi, (Oge) Geoffroyi's cat, and (Fca) domestic cat.

Microsatellite PCR and Length Determination

The 10 most polymorphic microsatellite markers were selected (FCA008, FCA035, FCA043, FCA082, FCA090, FCA096, FCA117, FCA166, FCA249, FCA262) from 43 loci examined which were initially characterized in the domestic cat (Menotti-Raymond et al. 1997; Menotti-Raymond et al. 1999). Of the 10 loci, 7 were unlinked on a genetic recombination map, one (FCA166) was unmapped and two (FCA043 and FCA117) were 20 cM apart (Menotti-Raymond et al. 1999). PCR amplification was performed as described (Menotti-Raymond et al. 1997), except for FCA116 which amplified using the following primers: FCA166F 5′-AGGTATTCTTCATCCCTAGGCA-3′ and FCA166R 5′-TGTGCTGACAGCACCGAG-3′ (Culver 1999). Products were electrophoresed on a 6% polyacrylamide gel using an Applied Biosystems 373A automated sequencer. Microsatellite allele sizes were estimated by comparison with a GS350 TAMRA (ABI) internal size standard, and two pumas were used as allele size controls on each gel. Data were collected and analyzed using the programs GENESCAN (version 1.2.2-1) and GENOTYPER software (version 1.1) (ABI).

Phylogenetic Analyses of Microsatellite Data

Pairwise genetic distances among individuals, among subspecies, and among groups of pumas were estimated using the kinship coefficient (Dkf) and proportion shared alleles (Dps) algorithms (Bowcock et al. 1994) as implemented in the program MICROSAT (version 1.5, Minch et al.). These distance estimators, which are based on shared alleles, are hypothesized to be more appropriate for comparisons among populations (Driscoll 1998; Goldstein and Pollock 1997). Phylogenetic relationships were determined among individuals that amplified for eight or more loci (n = 262) and among subspecies incorporating all samples that amplified for at least one microsatellite locus (n = 277). Phylogenetic trees were constructed from the Dkf and Dps distance matrixes using the NEIGHBOR option of the program PHYLIP (version 3.572) (Felsenstein 1993). Trees were drawn using the program TREEVIEW (version 1.5) (Page 1996).

Genetic Diversity and Population Structure Analysis

Measures of genetic diversity were estimated for individuals, as well as within and between subspecies. Nucleotide diversity (π) (Nei and Li 1979) was estimated from mtDNA haplotype data, using the program SENDBS (version 2, National Institute of Genetics, Shizuoka, Japan). Average heterozygosity (H_0), average variance in number of repeats, and average range of number of repeats were estimated from microsatellite data using the program MICROSAT (version 1.5, Minch et al.). Deviations from Hardy–Weinberg equilibrium (Guo and Thompson 1992) were tested for each microsatellite locus using the program ARLEQUIN (version 1.1) (Schneider et al. 1997).

Population subdivision was estimated by F_{ST} (Reynolds et al. 1983) and number of migrants (Slatkin 1994) for both mtDNA sequence data and microsatellite length variation data, using only the contemporary samples (excluding museum specimens). Estimates of F_{ST} among mtDNA haplotypes were obtained by analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using Tajima-Nei (1984) distances as implemented in the program ARLEQUIN. With microsatellite data, F_{ST} estimates were generated by AMOVA with the "number of different alleles" distance method using ARLEQUIN. The significance levels of F_{ST} were assessed after employing a Bonferroni adjustment (Weir 1996) for multiple comparisons. Alternative ways of subdividing the individuals into groups were compared to determine which partitions best accounted for the genetic variance. An AMOVA was also performed among subspecies within groups (Excoffier et al. 1992).

Divergence Time Estimates

Divergence dates among mtDNA haplotypes and outgroup species were estimat-

Table 2. Measures of genetic variation across combined analyses of three mtDNA gene segments 16S (382 bp), ATP8 (191 bp), and ND5 (318 bp), and 10 microsatellite loci in pumas grouped by subspecies

Subspecies, continent, or group[a]	Number of individual mtDNA/ μsat[b]	mtDNA Haplotype							Microsatellite loci: Total number of alleles[c]	% P	Average H_o (SE)[e] %	Average number of alleles per locus	Average variance[d]	Average range[d]	Maximum range
North American subspecies:															
P. c. missoulensis	23/22	**M**							34	100	48 (0.40)	3.4	5.1	5.5	9
P. c. oregonensis	16/15	**M**							32	100	35 (0.24)	3.2	4.0	5.2	9
P. c. vancouverensis	6	**M**							18	50	7 (0.35)	1.8	1.1	1.9	5
P. c. olympus	4			N					18	50	31 (0.18)	1.8	3.0	2.5	9
P. c. californica	25/24	**M**							32	90	27 (0.03)	3.2	2.9	5.1	9
P. c. kaibabensis	5	**M**							32	90	53 (0.35)	3.2	4.4	4.9	9
P. c. hippolestes	14	**M**							32	90	43 (0.44)	3.2	3.4	4.5	9
P. c. couguar + schorgeri (ancient)	3 + 1	**M**							11	20	22 (0.23)	1.2	2.1	0.8	6
P. c. browni	15	**M**							39	100	54 (0.45)	3.9	3.8	5.4	9
P. c. browni (ancient)	4	**M**							13 (3)	80	38 (0.53)	1.6	9.2	4.1	14
P. c. azteca	35	**M**							46	100	50 (0.27)	4.6	4.5	6.4	13
P. c. stanleyana	10	**M**							38	80	47 (0.18)	3.8	4.1	4.5	9
P. c. coryi	6	**M**							12	20	5 (0.0)	1.2	0.3	0.5	6
P. c. coryi (ancient)	6/4	**M**							9 (6)	50	42 (0.38)	2.3	9.6	4.8	10
P. c. improcera (ancient)	1/0	**M**													
P. c. mayensis	12	**M**							49	100	60 (0.0)	4.9	7.2	7.8	17
P. c. costaricensis	13	**M**		**F**		C			54	100	63 (0.11)	5.4	11.1	8.4	20
South American subspecies:															
P. c. borbensis	2			**F**		**H**			25	100	72 (0.24)	2.8	10.2	5.6	12
P. c. borbensis (ancient)	1/0		**L**												
P. c. greeni	1					**H**			15	50	50 (0.0)	1.5	9.5	2.6	11
P. c. acrocodia	11	A		**F**	G	**H**	**I**	K	72	100	68 (0.27)	7.2	6.9	8.4	19
P. c. capricornensis	8			**F**		**H**	**I**		49	100	77 (0.04)	4.9	8.5	7.9	19
P. c. bangsi	5			**F**					43	100	81 (0.18)	4.3	6.9	6.2	18
P. c. concolor	3			**F**		B			37	100	80 (0.0)	3.7	6.5	5.8	12
P. c. concolor (ancient)	2		**L**	**F**					19 (11)	90	69 (0.27)	2.4	5.7	3.3	5
P. c. soderstromi (ancient)	4			**F**					19	90	52 (0.15)	1.9	8.9	3.2	12
P. c. incarum	5			**F**		**E**			55	100	78 (0.58)	5.5	8.7	5.5	15
P. c. incarum (ancient)	1			**F**					2 (0)[1]	100	na	na	na	na	7
P. c. osgoodi	5			**F**		**E**			49	100	81 (0.0)	4.9	10.3	7.0	18
P. c. cabrerae	10	**J**		**F**					57	100	77 (3.61)	5.7	9.6	8.4	21
P. c. hudsoni	7			**F**					51	100	74 (2.90)	5.1	8.5	8.1	19
P. c. puma	6	**J**				**D**			39	100	69 (0.35)	3.9	8.0	5.8	11
P. c. araucanus	4	**J**				**D**			30	100	43 (0.53)	3.0	6.5	5.1	10
P. c. araucanus (ancient)	2	**J**							18 (5)	100	83 (0.35)	2.0	5.3	3.0	6
P. c. patagonica	6	**J**							32	100	63 (0.35)	3.2	9.4	6.2	20
P. c. pearsoni	4	**J**		**F**					34	100	78 (0.24)	3.8	7.3	6.0	10

[a] Subspecies ordered in a geographical cline from north to south, ancient samples came from museums.
[b] Number of individuals typed for mtDNA/microsatellite analysis; one value indicates the same number for both.
[c] Alleles found in museum but not in modern samples of the same population are in parentheses.
[d] Variance and range in the number of repeats averaged across all loci.
[e] Average observed heterozygosity across all loci and standard error.

ed by averaging all pairwise (p) distances between haplotypes. Feline-specific mtDNA divergence rates of 1.39% (*ATP8*), 1.22% (*ND5*), and 0.97% (*16S*) per million years (MY) were developed previously (Lopez et al. 1997). A weighted average, based on the number of base pairs used for each gene, was used to obtain the composite divergence rate of 1.15%/MY. A divergence time was also estimated from *Zfy* sequence data using a rate for felids of 0.11%/MY (Pecon-Slattery and O'Brien 1998). From this rate, the estimated time to acquire one mutation, in the 350 bp of intronic sequence, was approximately 2.5 MY.

Results

DNA was extracted and amplified by polymerase chain reaction (PCR) from a total of 315 pumas, collected from throughout their range (Figure 1A). The sequences of three mitochondrial genes [382 bp of *16S rRNA* (*16S*), 191 bp of *ATPase*-8 (ATP8) and 318 bp of (ND5) *NADH-5*] were determined from pumas using specific primers designed from an alignment of available mammalian mtDNA sequences (Lopez et al. 1996). Sequence analysis revealed two single nucleotide polymorphisms in the *16S* gene, five in the *ATP8* gene, and eight in the *ND5* gene (Table 1). All polymorphisms were transitions and 6 of 13 mutations in protein coding genes were nonsynonymous. RNA secondary structure estimated by FoldRNA (GCG, Version 8 1994) indicated that the two 16S rRNA variants were noncompensatory.

SSCP assays (Orita et al. 1989a;b; Podulso et al. 1991) were developed (Table 1) and used to screen pumas in Figure 1A. The 15 polymorphic sites defined 14 mtDNA haplotypes (Tables 1 and 2, Figure 1B). All pumas north of Nicaragua (N = 186), except four from the Pacific Northwest, shared the M haplotype, including historic museum specimens from two possibly extinct North American subspecies (*P. c. couguar* and *P. c. schorgeri*). Three haplotypes were present in Central American (C,F,M) pumas and 11 (A,B,D-L) were found in South American pumas. Haplotypes M and F had wide distributions throughout North and South America, respectively, while the other haplotypes were geographically restricted (Table 2, Figure 1B). Haplotype L was unique to museum samples, one from French Guyana and a second from Brazil (located at opposite sides of the Amazon River, Figure

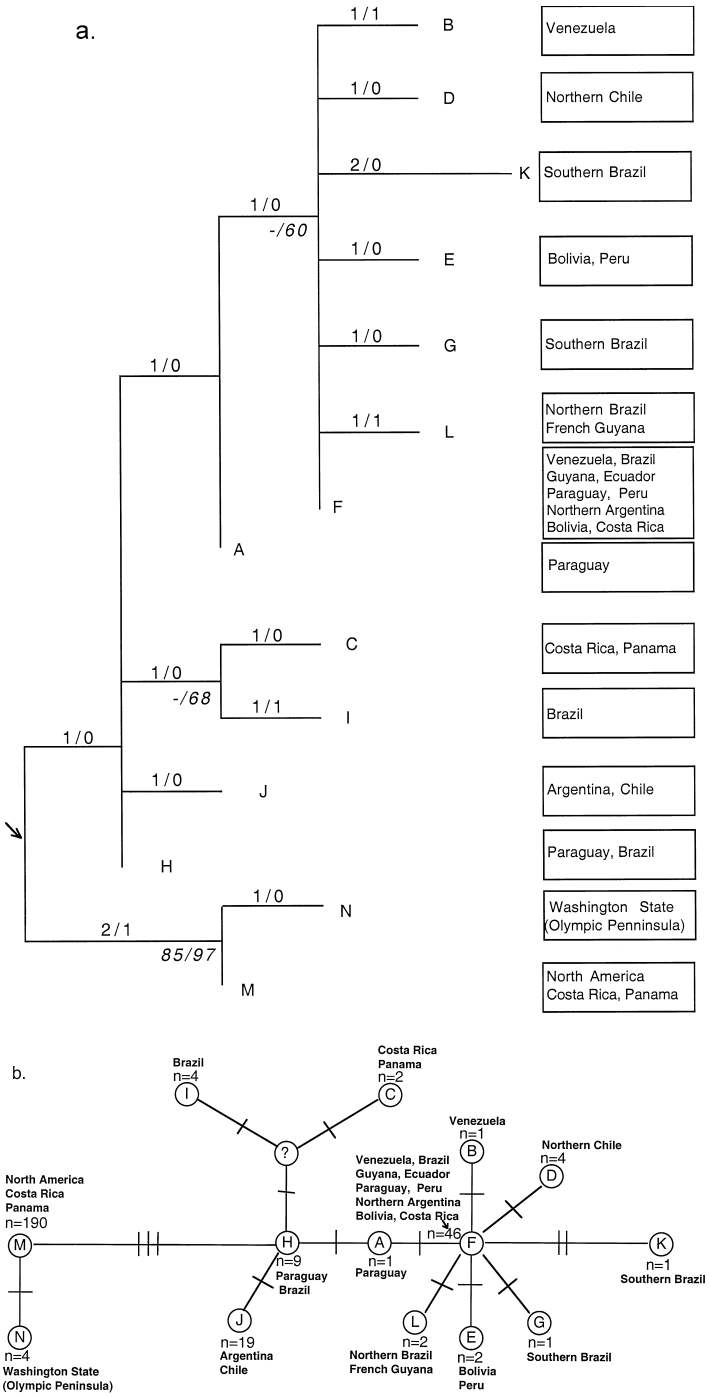


Figure 2. **(A)** Maximum parsimony topology of the 14 haplotypes from the combined 16S (382 bp), ATP8 (191 bp), and ND4 (318 bp) mitochondrial genes (Table 1). The single MP tree (CI = 0.882, total tree length = 17) rooted with the NA clade is depicted. Trees derived from MP, NJ, and ML analyses had identical topologies. Numbers above branches represent the number of steps/number of homoplasies. Numbers below branches are bootstrap values (from 100 replicates) from NJ analysis/MP analysis; dashes indicate a bootstrap value of less than 50%. The NJ analysis generated a single tree (ME = 0.0194). All nodes of the ML tree were significant (a consensus of 100 trees, -ln likelihood = 1311.0909). Rooting by outgroup taxa of jaguarundi, cheetah, and Geoffroyi's cat was uninformative due to prohibitively large interspecific genetic distances (0.09–0.12) relative to intraspecific values. Thus trees are unrooted but the arrow indicates position of the midpoint. **(B)** Minimum spanning network of the 14 mitochondrial haplotypes (Table 1), consisting of *16S, ATP8,* and *ND5* genes using MINSPNET (Orita et al. 1989a) and the number of individuals with each haplotype. Cross marks on branches define the number of substitutions between connected haplotypes. A hypothetical ancestor (?) likely connects I, C, and H as indicated.

1B). Overall mtDNA diversity among all haplotypes (π) was 0.318%.

The phylogenetic relationship among the 14 mtDNA haplotypes was examined using maximum parsimony (MP), minimum evolution (ME), and maximum likelihood (ML) algorithms. Each method produced an identical topology which is illustrated by a maximum parsimony tree (Figure 2A). Beyond continental distinctions (M,N versus others; bootstrap proportion 85% MP, 97% NJ) there was little mtDNA defined phylogeographic structure among populations from different regions of the continents. The average amount of mtDNA sequence differences among haplotypes was low among all pumas: 0.1–0.9% as compared to 9.0–11.0% between homologous segments from the cat species listed in Table 1 (http://lgd.nci.nih.gov).

To explore the ancestry of these haplotypes, nucleotide differences among pumas were compared to homologous residues from four outgroup species (cheetah, jaguarundi, Geoffroy's cat, and domestic cat; Table 1). A nucleotide site polymorphism in pumas was considered ancestral if at least three of four outgroup taxa shared the same nucleotide. Using this criterion, ancestral nucleotide states were indicated at 12 of 15 variable sites. The two haplotypes with the most ancestral states (10 of 12), H and I (Table 1), were restricted to Brazil and Paraguay (Figure 1B). The minimum spanning network (Figure 2B) indicates that all haplotypes are one to four mutational steps from the ancestral central H and F haplotypes.

The mammalian *Zfx* and *Zfy* genes on the respective X and Y chromosomes include introns that have been shown to be powerful nuclear indicators of phylogenetic divergence in cats and other species (Bianchi et al. 1992; Pecon-Slattery and O'Brien 1998; Shimmin et al. 1993). DNA sequence of the more rapidly evolving *Zfy* intron was determined in 14 puma specimens selected from a wide geographic distribution. Among seven North American and seven South American male pumas, no DNA sequence variation was observed in a 350 bp region of the intron.

In contrast to limited variation in mtDNA coding genes and Y chromosome *Zfy* intron sequences, appreciable allelic diversity was apparent in a genotypic screen of 10 unlinked nuclear microsatellite loci. Ten loci selected from a group of 253 markers that have been isolated from and mapped in domestic cat were typed in 277 pumas (258 contemporary and 19

Table 3. Measures of genetic variation across 10 microsatellite loci and mtDNA gene segments, in pumas grouped by phylogeographic group

Continent or group	Number of individual mtDNA/ μsat	mtDNA Haplotype								π ($\times 10^2$)	Microsatellites Average H_o(SE)[a] %	Total number of alleles	Number of unique alleles	Average number of alleles per locus (SE)	Average variance	Average range	Maximum range
Puma concolor	286/277				A–N					0.32	52 ( )[b]	121	—	12.1	6.9	12.5	21
North America-NA (15 subsp)	186/78	**M**	N							0.02	42 (0.16)	64	5	6.4[c]	4.8[c]	9.8	18
Central America-CA (1 subsp)	13/13	**M**	**F**	C						0.40	63 (0.11)	54	12	5.4	11.1	8.4	20
South America-SA	87/86	A		B	D–L					0.30	71 (0.33)	110	41	11.1	9.3	10.8	21
ESA (4 subsp)	23/22	A	**F**	H	G	I	K	**L**		0.22	71 (0.09)	86	5	8.6	8.0	9.3	19
NSA (5 subsp)	25/25	B	**F**	E				**L**		0.04	75 (0.52)	91	5	9.1	8.2	10.0	19
CSA (2 subsp)	17/17		**F**		**J**					0.10	75 (0.46)	67	1	6.7	9.6	9.2	21
SSA (4 subsp)	22/22		**F**	D	**J**					0.19	64 (1.16)	60	1	6.0	8.3	8.6	20

NA, North America; CA, Central America; ESA, eastern South America; NSA, northern South America; CSA, central South America; SSA, southern South America, continent (NA, North America; SA, South America), and species.

[a] Average observed percent heterozygosity across all loci and standard error.

[b] Significantly different from South American subspecies ($P < .05$).

[c] Significantly different from South American subspecies ($P < .01$).

museum specimens). A summary of the quantity and pattern of microsatellite variability observed in each of 30 previously named trinomial subspecies is presented in Table 2. The results indicate that all South American subspecies display appreciable microsatellite diversity, with 90–100% of loci polymorphic and an average heterozygosity of 43–83%. Most North American subspecies were also variable, although four subspecies (*P. c. coryi, P. c. couguar, P. c. olympus,* and *P. c. vancouverensis*) showed reduced levels of microsatellite variation compared to other North American subspecies. This reduction may reflect evidence for historic inbreeding as has been reported for *P. c. coryi* (Roelke et al. 1993), but could also result from small sample size for these subspecies which were represented by few (two to six) individuals.

A comparison of both mtDNA and microsatellite allele variation revealed a marked difference between pumas from North versus South America (summarized in Table 3). South American subspecies displayed greater overall variation than the North American population in several measures including the number of mtDNA haplotypes (11 versus 2, respectively); mtDNA genetic diversity (π = 0.3 versus 0.02, respectively); number of microsatellite alleles (110 versus 64, respectively); number of unique or continent specific alleles (41 versus 5); average microsatellite heterozygosity (71% versus 42%) ($P <$ .05); and average number of alleles per locus (11.1 versus 6.4) ($P < .01$). In addition, the molecular size distribution or variance in allele size for microsatellite loci (i.e., the average number of repeat motifs between alleles) differed appreciably between North (4.8) and South America (9.3) ($P < .01$). The lower variance likely reflects the disjunct pattern of microsatellite allele distribution of North American pumas compared to a more continuous distribution among two South American locales illustrated in Figure 3. In North American subspecies, one to three microsatellite alleles predominate for 7 of 10 loci and for these loci, a single allele occurred at ≥40% frequency (all except FCA-043, -082, -096) (Figure 3). By contrast, South American populations' allele size class distributions were more continuous in that no predominant alleles (≥40%) were present in any of the 10 loci.

The relationships among free-ranging puma samples were examined in a phylogenetic analysis of composite microsatellite genotypes of 262 individuals (Figure 4A,B). Minimum evolution topologies (estimated by the NJ algorithm) using two genetic distance measures: percent allele sharing, Dps, and mean kinship, Dkf, previously determined to be applicable for closely related populations (Driscoll et al. in preparation; Goldstein and Pollock 1997) clustered puma individuals from the same geographic locales, although the statistical bootstrap support was low. Continental concordance had few exceptions, although no phylogeographic structure was apparent within North American (NA) populations. With a few exceptions (20 of 262 genotypes), the genetic distance-based trees indicate a consistent parti-

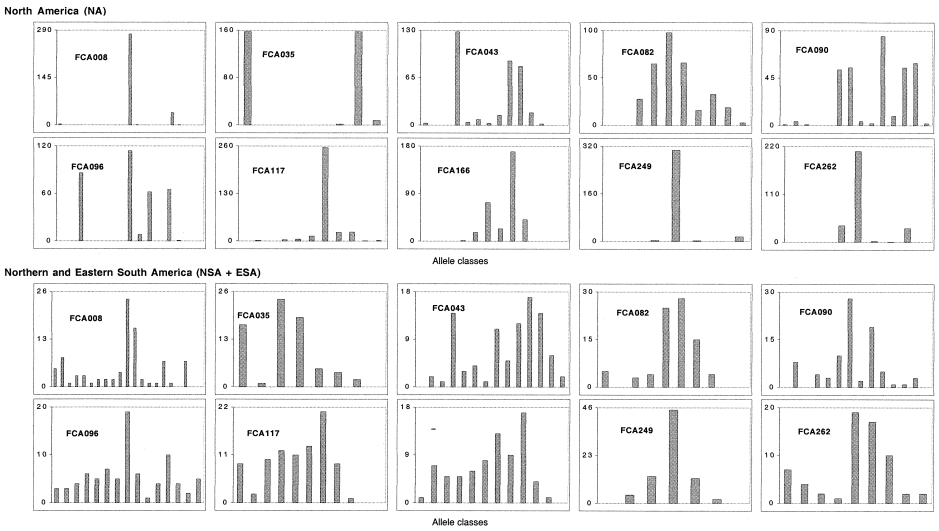


Figure 3. Frequency distribution of alleles at 10 microsatellite loci among combined North American subspecies (N = 186 individuals from 15 named subspecies, Figure 1A) and two combined phylogeographic groups: northern South America (NSA) (N = 25 pumas) and eastern South America (ESA) (N = 22 pumas). These groups were selected as a subset of all South American populations likely to reflect ancestral geographic region based on mtDNA analysis (see text). Horizontal axis includes 2 bp (CA repeat) increments on either size of the most common allele in the geographic population. Vertical axis is the count of number of alleles.

Table 4. Population pairwise F_{ST} microsatellite estimates above the diagonal and mitochondrial genes below the diagonal

	NA	CA	ESA	NSA	CSA	SSA
NA	—	0.110*	0.103*	0.059*	0.186*	0.367*
CA	0.784*	—	0.179*	0.126*	0.126*	0.228*
ESA	0.815*	0.057	—	0.031*	0.094*	0.330*
NSA	0.958*	0.492*	0.384	—	0.077*	0.316*
CSA	0.935*	0.233	0.177*	0.107*	—	0.172*
SSA	0.835*	0.240	0.186*	0.526*	0.281*	—

Below diagonal F_{ST} for the combined mitochondrial genes 16S (382 bp), ATP8 (191 bp), ND5 (318 bp), and Tajima–Nei distance method (Tajima and Nei 1984) in the six groups, NA (North America), CA (Central America), ESA (eastern South America), NSA (northern South America), CSA (central South America), and SSA (southern South America).

Museum samples were excluded. To equalize sample size among groups, each North American subspecies was limited to only three or fewer randomly chosen contemporary samples ($n = 129$).

* $P < .0033$ (after Bonferroni adjustment), based on 100 permutations.

tioning among South and Central American pumas, which correspond to five geographic areas (Figures 1B and 4A,B): southern South America (SSA) including Chile and southwestern Argentina (Patagonian/Andean region); eastern South America (ESA) including Brazil (south of Amazon River) and Paraguay; northern South America (NSA) including Venezuela, Ecuador, Bolivia, Guyana, and Colombia; Central South America (CSA) including northeast Argentina; and Central America (CA) including Costa Rica, Panama, and Nicaragua.

Phylogenetic analyses of individual genotypes constrained in accordance with previously named trinomial subspecies reinforced the phylogeographic conclusions (Figure 4C,D). North American subspecies clustered with strong bootstrap support (73% Dkf and 60% Dps). Three subspecies had unusually long branch lengths in the Dkf tree (Figure 4d) due to a small sample size (*P. c. couguar, P. c. greeni,* and *P. c. borbensis*), while others with long branch lengths proved to be homozygous at five to eight loci (*P. c. olympus, P. c. vancouverensis,* and *P. c. coryi*).

Population distinctiveness among the six puma groups was examined by Wright's F_{ST} values for both mtDNA haplotypes and for composite microsatellite genotypes (Table 4). Significant differences were obtained for all pairwise group comparisons with microsatellites and for 11 of 15 mtDNA comparisons. Similar subdivision was affirmed by calculating M, migrant number (Slatkin 1994), and AMOVA analysis (Excoffier et al. 1992; Schneider et al. 1997). The combined phylogeographic analyses of microsatellite genotypes (Figure 4) and mtDNA coding gene haplotypes (Figures 1B and 2) form the basis for recognition of the six taxonomic units or subspecies of modern pumas (Figure 1B). Other geographic partitions were used to test F_{ST} and M; however, the six groups described here best explained the observed genetic variation. Boundaries between some of these groups were somewhat arbitrary due to either incomplete sampling in the area or to slight differences between results of the mtDNA and microsatellite analyses. The CSA group was recognized because individuals from this area shared affinities with the other South American groups, but could not be readily assigned to either of these groups. Boundaries of the CSA group were defined as the region between Rio Negro and Rio Paraná and included the *P. c. cabrerae* and *P. c. hudsoni* subspecies.

A summary of the distribution of mtDNA and microsatellite variation among pumas considered as a species, as continental populations, and as the six phylogeographic groups is presented in Table 3. The most diverse group based on mtDNA haplotypes was ESA (7 haplotypes; $\pi = 0.02$), while NA had the fewest (2 haplotypes; $\pi = 0.22$). For microsatellites the groups with the highest diversity (ESA and NSA) were central to the pumas range and, of interest, were the focus of the most ancestral mtDNA haplotypes, H and I (Figure 1B). Pumas from ESA or NSA had the greatest number of alleles, the most unique alleles, and the highest heterozygosity (Table 3). A broad continuous distribution of allele size classes was found among pumas from ESA and NSA (Figure 3), in contrast with other groups whose allele sizes were largely a subset of the ESA/NSA size classes.

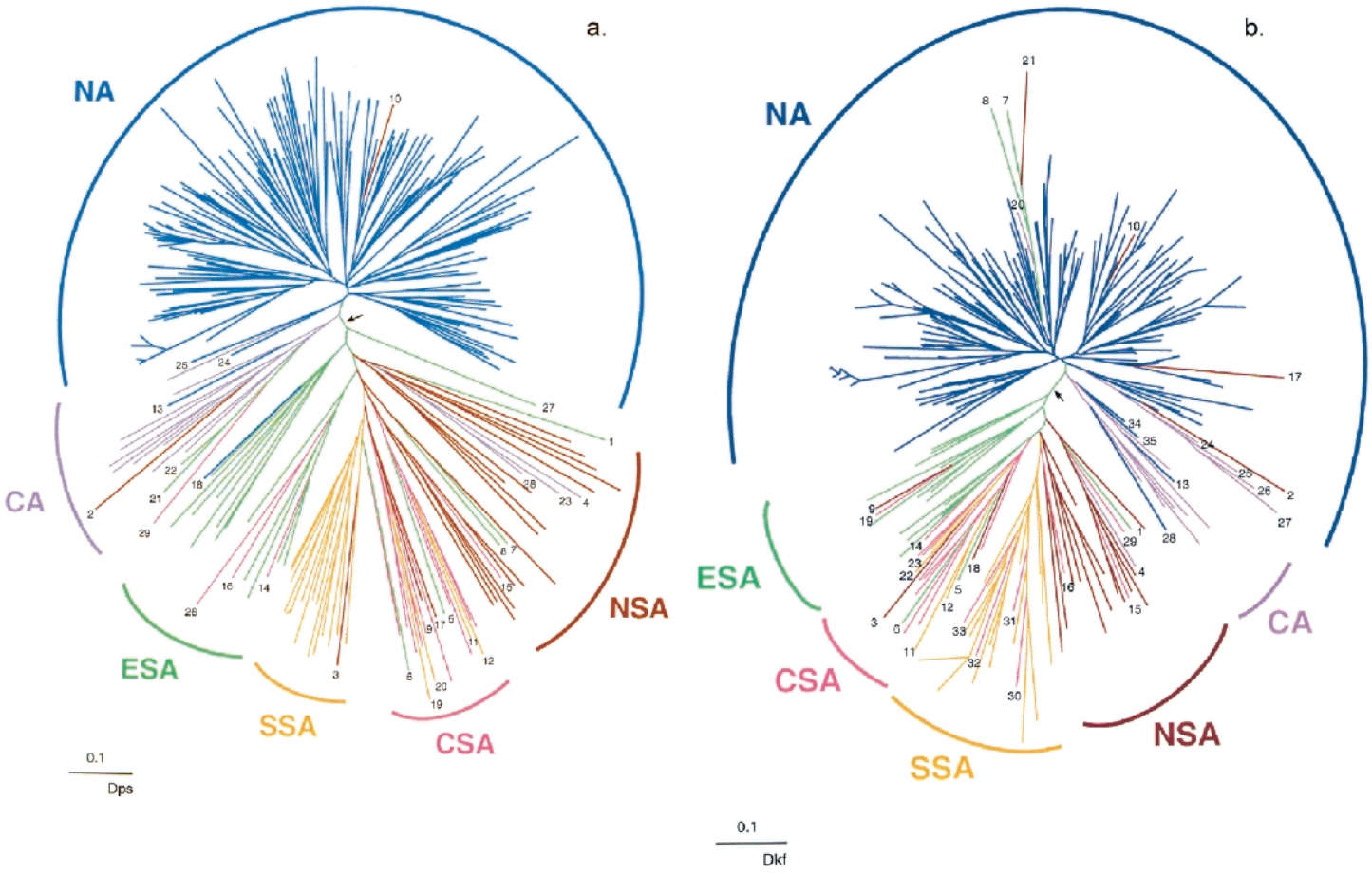
When the six puma groups were examined phylogenetically using microsatellite genotypes (Figure 4E), we observed strong support for group definition, but some inconsistency in the hierarchical relationships among the groups, particularly in South America. CA and NA clustered together (bootstrap = 98%; 100% for Dkf and Dps, respectively) as did CSA and SSA (bootstrap = 98% and 91% for Dkf and DPs, respectively), but the relationship of ESA and NSA differed depending on the genetic distance measure employed (Figure 4E). Five of the groups (all except CSA) had at least one unique population specific mtDNA haplotype (Table 3) and each had a distinct frequency distribution of mtDNA haplotype frequencies (Figure 1B).

Using previously calibrated divergence rates for the combined three feline mtDNA genes (Lopez et al. 1997)—the composite mutation rate for this combined region of mtDNA was estimated as 1.15%/MY. Using this divergence rate, puma and jaguarundi last shared a common ancestor 4.2 MYBP, while extant puma lineages diverged approximately 390,000 years ago and North American pumas shared a common ancestor less than 20,000 years ago. The *Zfy* intronic sequence divergence rate in felids has been estimated at one mutation every 2.5 MY (Pecon-Slattery and O'Brien 1998). From this rate, extant puma lineages are probably less than 2.5 MY old, since no variation was detected among pumas in our sample.

Discussion

Distinguished by a hemispheric distribution from the Arctic tundra of the north to Patagonia in the south, pumas (*P. concolor*) exhibit both broad and fine-scale patterns of genetic differentiation. Concordance between microsatellite and mitochondrial DNA sequence variation revealed an evolutionary history marked by ancient ancestry and a period of recent North American repopulation. Previous classification into 32 subspecies (Neff 1983; Young and Goldman 1946) was not affirmed; rather modern populations assorted by molecular genetic criteria into six broad phylogeographic regions (Figure 1B) with evidence of historic inbreeding in a few isolated localities and admixture in others.

The six regions (Figure 1B) were defined based on phylogenetic partitions as indicated by composite individual and population genotypes at 10 unlinked feline microsatellite loci (Figure 4), by the distribution and frequency of mtDNA haplotypes of 15 polymorphic nucleotide sites within three genes (*16S, ATP8, ND5*; Table 1) and by the pattern of diversity indicated by these loci. The puma groups roughly correlate with geographic barriers that could restrict migration and gene flow. North American pumas (NA) are made up


a.
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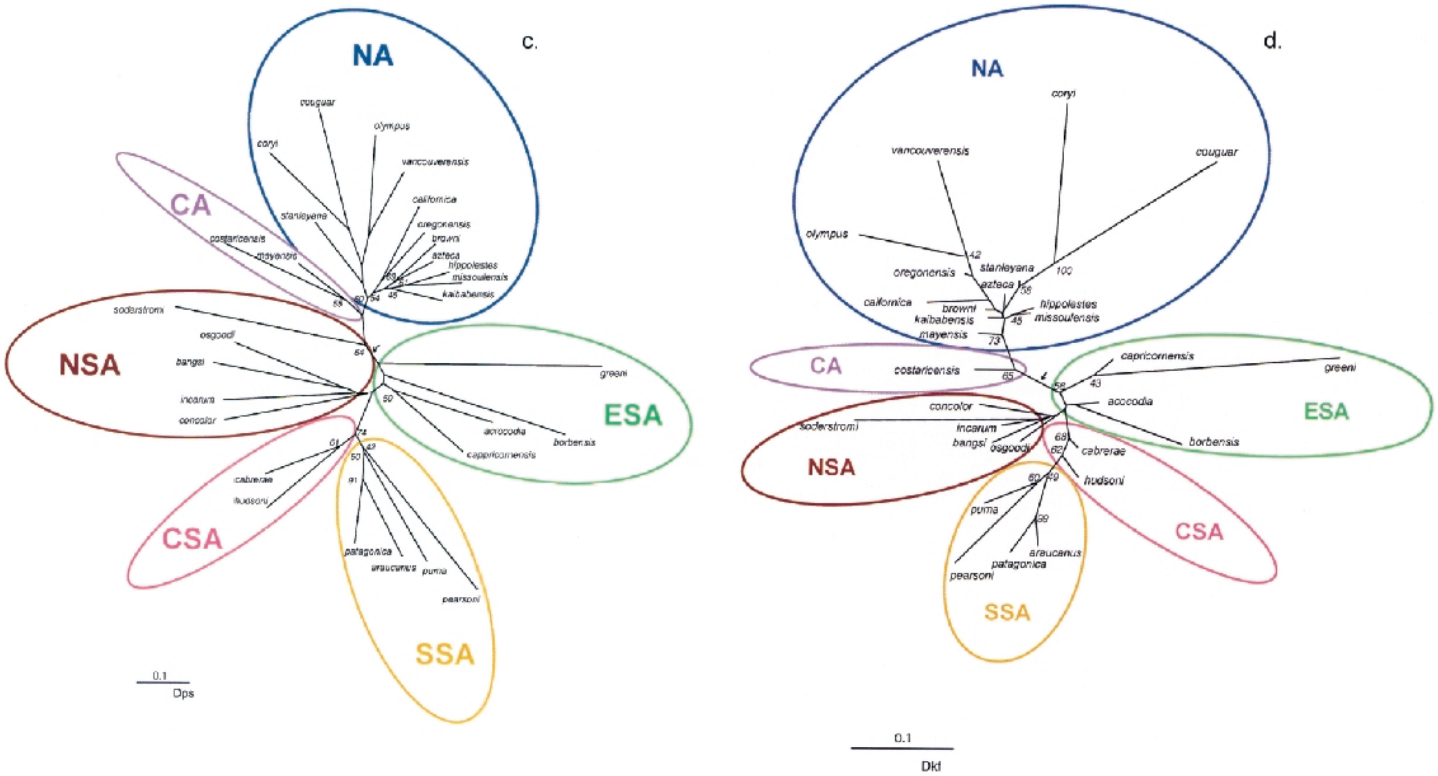
c.
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azteca
hippolestes
mssoulensis
kaibabensis
costaricensis
mayensis
sodorstromi
osgoodi
bangsi
incarum
concolor
greeni
acrocodia
borbensis
capricornensis
cabrerae
hudsoni
patagonica
araucanus
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couguar
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azteca
californica
brownt
kaibabensis
hippolestes
mssoulensis
mayensis
costaricensis
capricornensis
greeni
concolor
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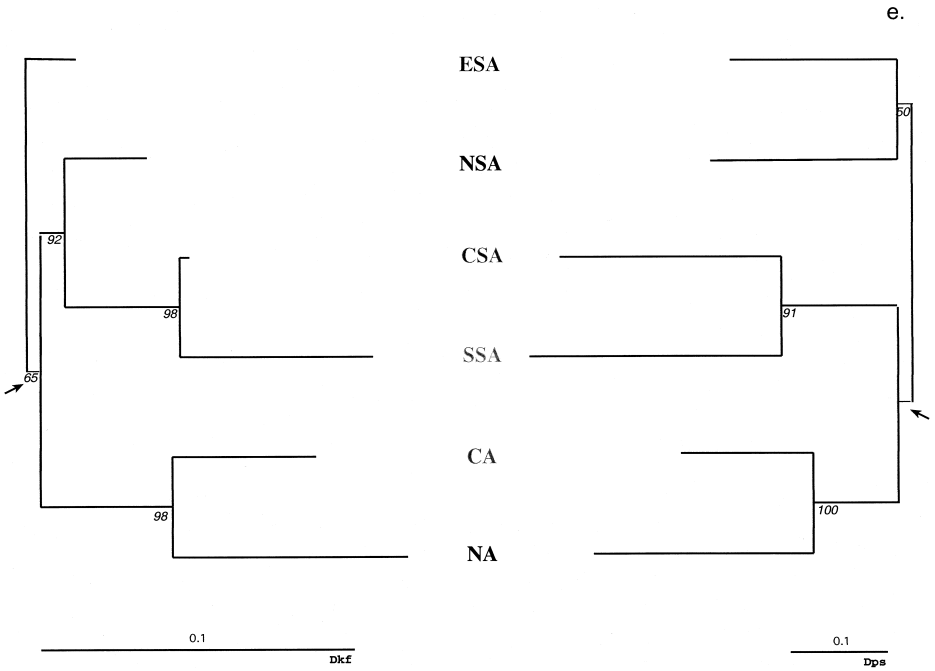

Figure 4. Continued. **(E)** ME-NJ tree using D_{kf} and D_{ps} genetic distance among the six phylogeographic subspecies defined by this study. Numbers on nodes are bootstrap percentages.

of 15 previously named subspecies north of Nicaragua. Central American pumas (CA) inhabit mostly tropical rainforest. Bounded by two major rivers, Rio Negro and Rio Paraná, central South American (CSA) pumas include two previously named subspecies from the pampas desert. Eastern South America (ESA) pumas inhabit several biogeographic zones bordered by the Amazon, Rio Paraná, and the Paraguay rivers and include four previous subspecies. Demarcated by the Amazon and Paraguay rivers, the northern South American (NSA) region includes six named subspecies distributed across multiple habitat types. Southern South America (SSA) includes four subspecies of puma from Patagonia and the diverse mountainous habitats of Argentina and Chile.

Patterns of mtDNA and microsatellite variation revealed differences in relative genetic diversity (Table 3) that can be interpreted in the context of geography and puma natural history. As ESA is the population distinguished by the most ancestral (Table 1) and central (Figure 2B) mtDNA haplotypes, modern puma genomic diversity likely traces its origin to near or within the ESA locale. This inference is supported by the observation that ESA has the highest number of mtDNA haplotypes (n = 7), near maximal microsatellite diversity, and the broadest distribution of allele size classes. In contrast, NA had the lowest variation both in mtDNA and in microsatellite measures. NA pumas are predominately one mitochondrial haplotype (M) with an infrequent second haplotype (N) restricted to the Olympic peninsula. The entire NA puma sample (n = 191) possessed less microsatellite diversity than any of the other phylogeographic groups with nearly all alleles being a subset of those found in Central and South America. Considered together, the reduction in mtDNA and microsatellite variation is indicative of either an historic founder effect or a population bottleneck that effectively reduced overall genomic diversity (Maruyama and Fuerst 1985; Mayr 1970; Nei et al. 1975).

The date for the postulated bottleneck in the history of North American pumas was estimated by comparing the variance in microsatellite allele to that in other populations that have also experienced historic demographic reductions (Driscoll 1998; Menotti-Raymond and O'Brien 1993; O'Brien et al. 1985; O'Brien et al. 1987). Microsatellite allele variance is a measure of the breadth of time-dependent mutational expansion that microsatellites show in a population (Driscoll 1998; Goldstein et al. 1995; Reich and Goldstein 1998). Because allele variance takes longer for reconstitution after a bottleneck than overall heterozygosity or allele number (Driscoll 1998; Goldstein et al. 1995; Goldstein and Pollock 1997; Reich and Goldstein 1998) it is a valuable chronometer for founder events that are older than the period required for reconstitution of heterozygosity.

The average allele variance among the entire North American population was 4.8, compared to 8.2–11.1 for other puma subspecies (Table 3). The observed NA allele variance is equivalent to the restricted allele variance observed in African cheetahs (based on a larger screen of 88 microsatellite loci) (Driscoll 1998), a species documented by several measures to have experienced a severe population bottleneck around 10,000–12,000 years ago, near the end of the Pleistocene (Menotti-Raymond and O'Brien 1993; O'Brien et al. 1985; O'Brien et al. 1987). If microsatellite allele variance reconstitution following demographic homogenization occurred at the same rate in cheetahs and pumas, the results imply that NA pumas are descended

←

Figure 4. Phylogenetic relationships among 262 individual pumas constructed from 10 microsatellite loci. Unrooted NJ trees estimated using **(A)** proportion of shared alleles, Dps; and **(B)** mean kinship, Dkf, genetic distances with a 1-ps transformation. Individuals, subspecies, or groups are color coded as indicated by geographic area abbreviations defined in text, Figure 1B, and Table 3. Numbered taxa indicate exception to phylogeographic clusters and correspond to 1, Brazil (Pco-194); 2, Venezuela (Pco-295); 3, Peru (Pco-409); 4, Panama (Pco-542); 5, Brazil (Pco-696); 6, Paraguay (Pco-699); 7, Paraguay (Pco-701); 8, Paraguay (Pco-714); 9, Guyana (Pco-856); 10, Ecuador (Pco-867); 11, Chile (Pco-215); 12, Chile (Pco-212); 13, Belize (Pco-271); 14, Argentina (Pco-580); 15, Argentina (Pco-574); 16, Argentina (Pco-562). Numbers 16–29 on the Dps tree **(A)** correspond to 17, Bolivia (Pco-707); 18, Mexico (Pco-757); 19, Argentina (Pco-566); 20, Argentina (Pco-564); 21, Brazil (Pco-195); 22, Brazil (Pco-196); 23, Costa Rica (Pco-547); 24, Arizona (Pco-604); 25, Mexico (Pco-592); 26, Arizona (Pco-561); 27, Brazil (Pco-697); 28, Costa Rica (Pco-548); 29, Argentina (Pco-576). Numbers 16–35 on the Dkf tree (5B) correspond to 17, Peru (Pco-406); 18, Peru (Pco-407); 19, Argentina (Pco-571); 20, Nicaragua (Pco-551); 21, Paraguay (Pco-710); 22, Argentina (Pco-565); 23, Chile (Pco-597); 24, Costa Rica (Pco-545); 25, Nicaragua (Pco-554); 26, Nicaragua (Pco-549); 27, Nicaragua (Pco-550); 28, Mexico (Pco-593); 29, Argentina (Pco-576); 30, Argentina (Pco-560); 31, Argentina (Pco-568); 32, Argentina (Pco-579); 33, Argentina (Pco-573); 34, Arizona (Pco-73); 35, Arizona (Pco-163). Exceptional individuals (11% of total) in some cases can be explained by size homoplasy of microsatellite alleles, demonstrated to occur for some loci based on sequence analysis of 85 microsatellite homozygotes within pumas (Culver et al., in preparation). **(C)** NJ-ME analysis using combined population genotypes based on Dps distance between the 29 named subspecies (Neff 1983; Young and Goldman 1946) (Figure 1A). *P. c. shorgeri* and *P. c. couguar* were combined. Bootstraps values over 40% are indicated on divergence nodes. **(D)** NJ-ME analysis of combined subspecies genotypes using the D_{kf} genetic distance as in **(C)**.

Table 5. Trinomial subspecies designation and diagnostic characters for the six phylogeographic groups

Group	Subspecies (citation)[a]	mtDNA sites[b]	mtDNA haplotypes[c]	Microsatellite alleles	Citation
NA	*P. c. couguar*	12908	N	*FCA043-104; FCA082-252; FCA090-95, -122; FCA117-152*	*Kerr 1792*
CA	*P. c. costaricensis*	8630	C	*FCA082-236; FCA 166-223*	*Merriam 1901*
ESA	*P. c. capricornensis*	sites 3063, 12723, and 12819	A, H, G, I, K	*FCA008-140, -146, -150, -164; FCA043-120*	*Merriam 1901*
NSA	*P. c. concolor*	12834 and 12840	B, E	*FCA008–162; FCA035-124; FCA043-142; FCA166-221; FCA249-251*	*Linneaus 1771*
CSA	*P. c. cabrerae*	None	None	*FCA008-176*	*Pocock 1904*
SSA	*P. c. puma*	12809	D	*FCA249-239*	*Molina 1782*

[a] Citation for subspecies designation with the earliest description of those describing subspecies in the geographic locale.
[b] Sites listed relative to domestic cat mtDNA sequence (Lopez et al. 1996).
[c] See Table 1 for list of haplotypes, Table 3 for unique haplotypes.

from a founder event of approximately the same age as the cheetah's (i.e., ~10,000 years ago). Since North American puma fossils date to more than 300,000 years ago (Kurten 1976; Savage and Russell 1983; Turner 1997), the interpretation would suggest a recent replacement of North American pumas by a small number of ancestors of modern NA pumas. Toward the end of the late Pleistocene, a massive extinction event abruptly eliminated 80% of large mammal species (N = 35–40 species including cheetahs, lions, and saber-toothed Smilodon) from North America (Martin 1989; Martin and Wright 1967; Pielou 1991). Perhaps NA pumas were eliminated at about the same time by the same event, followed by repopulation and dispersal of a handful of SA migrants who had survived the cataclysm.

Despite apparent phylogenetic uniqueness (Figure 4) and moderate F_{ST} distinctiveness (Table 4), two phylogeographic regions (CA and CSA) resemble hybrid zones. CA pumas retain three mtDNA haplotypes: the predominant North American haplotype (M), the ubiquitous South American haplotype (F), and a unique CA haplotype (C) (Figure 1B). CA pumas exhibit the largest average variance in microsatellite repeat number (11.1) among the six phylogeographic zones. These patterns suggest recent gene flow to CA from both NA and NSA. The CSA group also shares mtDNA haplotypes (J and F, Figure 1B) with neighboring subspecies plus phylogenetic affiliation of microsatellite genotypes (Figure 4), observations consistent with gene flow to CSA populations from adjoining biogeographic regions.

Within modern North American pumas, several smaller populations have unique features (Table 2). The number of polymorphic microsatellite loci and amount of genetic variation were lower in three subspecies (*P. c. coryi, P. c. vancouverensis,* and *P. c. olympus*). In contrast to outbred puma populations in northern Yellowstone, southern Idaho, and the San Andres Mountains, with 9–10 polymorphic microsatellite loci and heterozygosity values of 0.42–0.52 (Culver et al., in preparation), populations on Vancouver Island, the Olympic Peninsula, and Florida's Big Cypress Swamp contain only 2–5 polymorphic microsatellite loci and heterozygosity values from 0.05 to 0.31. The Florida population is highly inbred (eight fixed loci), which is consistent with its demographic history (Belden 1986; Maehr 1998; Roelke et al. 1993). Museum samples from the Florida population dating to the turn of the 19th century show much higher heterozygosity levels (0.42 versus 0.05), variance in number of repeats (9.6 versus 0.3), and range in number of repeats (4.8 versus 0.5), consistent with a 20th century reduction of genetic diversity in Florida pumas (Table 2).

Conclusions

A molecular genetic analysis of mitochondrial and nuclear genomic variability across intercontinental puma populations has revealed consistent patterns that can be interpreted in the context of demographic and migratory history. Six phylogeographic groups were resolved with no evidence for additional within-group subdivision. We detect little support for the retention of 32 previously named subspecies (Neff 1983; Young and Goldman 1946) and suggest that subspecific taxonomy of the puma be revised to designate six phylogeographic subspecies. This recommendation is based on previous criteria (Avise and Ball 1990; O'Brien and Mayr 1991) which designate that subspecies share a unique range, a group of phylogenetic concordant characters, and a unique natural history relative to other subdivision of the species. We list in Table 5 the proposed Latin trinomial names of the six subspecies and the molecular genetic phenotypic characters specific to each.

Until recently, one subspecies, *P. c. couguar*—NA, occupied the entire North American continent. In spite of its wide distribution, the subspecies shows markedly reduced mtDNA and microsatellite variability relative to the southern subspecies. Most likely modern North American pumas descended from a founder event involving a small number of individuals who migrated "out of South America" approximately 10,000–12,000 years before the present and subsequent to the abrupt Pleistocene extinction of large North American mammal species. The cause of this near global extinction event is still uncertain but may have eliminated not only North American lions, cheetahs, and saber-toothed cats, but also the North American pumas (Martin 1989; Martin and Wright 1967; Pielou 1991). Supplemental tables can be inspected on the LGD web site (http://www.lgd.nci.nih.gov).

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