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Phylogeographic Patterns and Evolution of the Mitochondrial DNA Control Region in Two Neotropical Cats (Mammalia, Felidae)

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Abstract. The ocelot (*Leopardus pardalis*) and margay (*L. wiedii*) are sister-species of Neotropical cats which evolved from a lineage that migrated into South America during the formation of the Panamanian land bridge 3–5 million years ago. Patterns of population genetic divergence of each species were studied by phylogenetic analyses of mitochondrial DNA (mtDNA) control region sequences in individuals sampled across the distribution of these taxa. Abundant genetic diversity and remarkably concordant phylogeographic partitions for both species were observed, identifying parallel geographic regions which likely reflect historical faunal barriers. Inferred aspects of phylogeography, population genetic structure, and demographic history were used to formulate conservation recommendations for these species. In addition, observed patterns of sequence variation provided insight into the molecular evolution of the mtDNA control region in closely related felids.

Key words: *Leopardus pardalis — Leopardus wiedii* — Mitochondrial DNA control region — Phylogeography — Felid evolution

Introduction

The Neotropical Region has been inhabited by 10 extant species of wild felids since Plio-Pleistocene times, 1–5 million years ago (Berta 1983; Webb 1985). Seven of these species comprise a monophyletic group called the ocelot lineage (Collier and O'Brien 1985) and probably radiated from a common ancestor during the formation of the Panamanian isthmus (Pecon-Slattery et al. 1994; Johnson et al. 1996; Johnson and O'Brien 1997), 3–5 MYA (Webb 1985; Collins et al. 1996). Within this lineage, morphological and molecular genetic data consistently indicate that the ocelot (*Leopardus pardalis*) and the margay (*L. wiedii*) are sister-species (Hemmer 1978; Salles 1992; Pecon-Slattery et al. 1994; Johnson and O'Brien 1997). These two species are sympatric throughout most of their ranges, occurring from southern United States (ocelot) or northern Mexico (margay) to southern Brazil and northern Argentina. The ocelot is a medium-sized cat (7–15.8 kg) which occupies a wide variety of habitats from moist forests to drier scrubland. The margay is smaller (2.5–4 kg) and is believed to prefer forested environments (Nowak 1991; Redford and Eisenberg 1992; Oliveira 1994).

Currently both ocelots and margays are threatened by habitat loss and fragmentation, hunting, and human persecution. To develop adequate conservation strategies for these felid species, it is essential to understand their geo-

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Table 1. Samples analyzed in the present study

Geographic origin	Samples[a]	Institution/ contact
N Mexico	Lwi49	Sonoran Ecological Center
C Mexico	Lpa116	Idaho State University/Jon Landre
S Mexico	Lpa35, Lpa37, Lwi42, Lwi52	Parque Zool. De Leon; Balam; Zoomat
Guatemala	Lpa29, Lpa31, Lpa32, Lwi35, Lwi36, Lwi39, Lwi40	Autosafari Chapin
Nicaragua	Lpa25, Lwi30, Lwi31	Managua Zoo; Juigalpa Zoo
Costa Rica	Lwi22, Lwi24	Las Pumas
Panama	Lpa17, Lpa18, Lwi20	Summit Zoo
Venezuela	Lpa83; Lpa85	Leslie Pantin, private collection; Valencia Aquarium
Trinidad	Lpa92, Lpa93, Lpa94	Emperor Valley Zoo
French Guiana	Lpa115, Lpa125, Lpa151, Lwi104	Prog. Faune Sauvage
Amazonas, N Brazil (north of the Amazon)	Lpa10, Lpa11, Lpa169, Lwi18	CENAP/IBAMA; Cent. Inst. Guerra na Selva (CIGS)
E Pará, N Brazil (south of the Amazon)	Lpa180, Lpa181, Lpa165, Lpa166, Lpa182, Lwi62, Lwi63	PZB Carajas/F. Lima; São Paulo Zoo/F. Simon; Sorocaba Zoo/A. Nunes
C Brazil	Lpa66, Lwi65	Goiânia Zoo
E Brazil	Lpa171	Uberaba Zoo/Jussara Tebet
SW Brazil	Lpa183, Lwi64	Curitiba Zoo/R.N. Morais; Campinas Zoo
S Brazil	Lpa53, Lpa56, Lpa57, Lpa58, Lpa59, Lpa60, Lpa73, Lpa140, Lpa170, Lwi69, Lwi70, Lwi130, Oge54	CENAP/IBAMA; Itaipu Zoo, Brazil/W. Moraes; Maison Forestier Zoo; UFRGS/Ivan Gonçalves; Cachoeira do Sul Zoo
Bolivia	Lpa99, Lpa100, Lwi82	Santa Cruz Zoo
Paraguay	Lwi66, Lwi67	Itaipu Zoo, Paraguay

[a] Lpa, code for *Leopardus pardalis;* Lwi, code for *L. wiedii;* Oge, code for *Oncifelis geoffroyi*

graphic patterns of population subdivision, so that they can be managed on the basis of historical demographic partitions and current genetic differentiation (Ryder 1986; Avise et al. 1987; Moritz 1994; Wayne 1996). Current conservation efforts have generally been based on subspecies designations, which have recognized about 11 trinomials for each of these species (e.g., Pocock 1941; Cabrera 1961; CBSG 1995). However, these subspecific classifications were generally based on very few specimens, restricted geographic sampling, and morphological characters which undergo extensive individual variation (Ximenez 1974), and so are often not reliable indicators of historical population subdivisions.

Molecular markers provide important measures of population structure and geographic differentiation and have been employed to assess questions in evolutionary and conservation biology (e.g., Nei 1987; Avise 1994; Avise and Hamrick 1996). Mitochondrial DNA (mtDNA) analyses, particularly focusing on fast-evolving segments of the noncoding control region, have been extensively employed to infer population structure and/or history of a wide variety of organisms (e.g., Stanley et al. 1996; Bonatto and Salzano 1997; Vilà et al. 1997). Characterized by high substitution rates, matrilinear mode of inheritance, and haploid, nonrecombining gene content, mtDNA is an effective marker for evolutionary studies at recent levels of divergence (Avise et al. 1987; Moritz et al. 1987; review by Avise 1994).

In the Felidae family, mtDNA polymorphisms have been used to infer phylogenetic relationships and evolutionary history (e.g., Menotti-Raymond and O'Brien 1993; Janczeswski et al. 1995; Masuda et al. 1996; Johnson et al. 1996, 1998; Johnson and O'Brien 1997) and patterns of intraspecific molecular variation in leopards (*Panthera pardus*) (Miththapala et al. 1996) and tigers (*P. tigris*) (Wentzel et al. 1997).

The objective of the present study was to characterize the molecular genetic variation in ocelot and margay populations on a broad geographic scale using mtDNA control region sequences. Observed phylogeographic partitions and estimated population genetic parameters were employed to define major subdivisions and to draw inferences on the population history of these species. The usefulness of the first hypervariable segment (HVS-I) of the mtDNA control region for evolutionary studies in felids and the implication of our results for ocelot and margay conservation efforts were addressed.

Materials and Methods

Sample Collection and Laboratory Techniques

Biological material (blood, skin biopsies, and tissue samples) of ocelots and margays was obtained from captive animals of known origin, wild animals captured for ecological studies, or road-killed individuals, from all major geographic regions across the species' ranges (Table 1, Fig. 1).

Genomic DNA was extracted from blood and tissue samples following standard protocols (Sambrook et al. 1989; Lahiri and Nurnberger 1991; Laird et al. 1991). The 5′ portion of the mtDNA control region, which contains the HVS-I, was amplified by PCR (Saiki et al. 1985) using the human primers L15996 and H16498 (Ward et al. 1991)

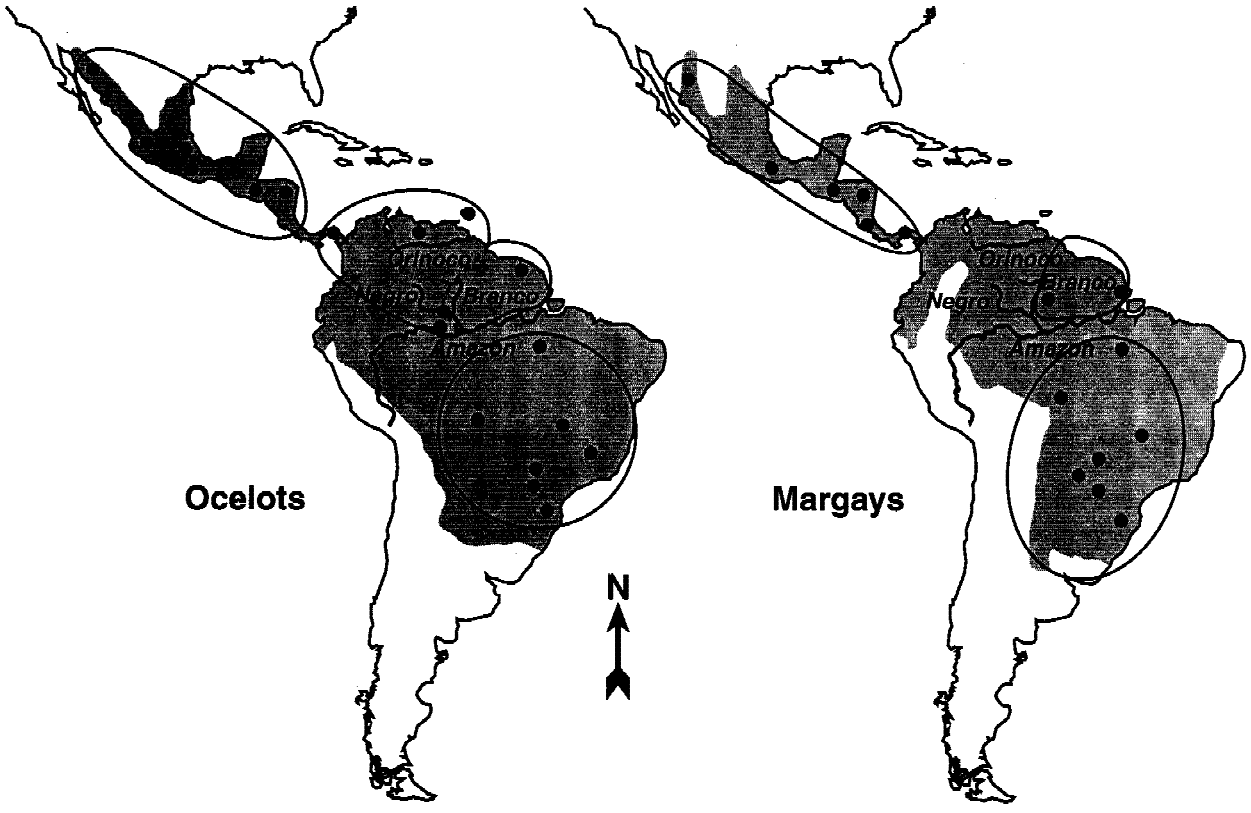

Fig. 1. Map showing the current distribution [*shaded area* (adapted from Oliveira 1994)] of ocelots (*Leopardus pardalis*) and margays (*L. wiedii*). *Circles* represent approximate collection areas of samples. *Solid lines* show major phylogeographic groups identified in the present study in each species; *dashed lines* indicate uncertainty as to current boundaries between such groups. Major rivers which may have influenced phylogeographic partitions identified in this study are shown.

(Fig. 2). PCR was performed in a MJ Research thermocycler following 35–43 cycles of 45 s at 94°C, 45 s at 52°C, and 1 min 30 s at 72°C. Solid-state sequencing of PCR products was performed using Dynabeads (Dynal) (Hultman et al. 1989) and a T7 Sequencing Kit (Pharmacia); alternatively, products were purified and sequenced with a Sequenase PCR Product Sequencing Kit (United States Biochemical).

Sequencing was performed by the chain-termination method (Sanger et al. 1977) with each of the primers used in the PCR amplification and α-^{35} S-dATP. Sequencing reaction products were separated in 5% acrylamide denaturing gels, which were then fixed, dried onto the glass plate, and placed against an X-ray film (Kodak) for 2–5 days.

This part of the mtDNA of felids contains a repetitive region (Hoelzel et al. 1994) which generates high levels of heteroplasmy and is flanked on both sides by extremely variable segments (unpublished data), so it was not possible to sequence across it or to design primers which could adequately bypass this array. For this reason, only one strand of each fragment was sequenced. To minimize potential errors, two or more independent readings, gel runs, and PCR and/or sequencing reactions were performed for several individuals, and in every case the result obtained was consistent for all replicates. Sites which could not be unambiguously scored after these attempts were treated as missing information and eliminated from the analyses in a pairwise fashion. Fragment A, sequenced with primer L15996, included an imperfect repeat (~30 bp long) located on one side of the tandem array. Fragment B, sequenced with primer H16498, included the first (3′ side) repeat (82 bp long) of the tandem array. Proposed molecular models (e.g., Buroker et al. 1990; Wilkinson and Chapman 1991) and our own results (unpublished data) indicate that the first and last (imperfect) repeats were not subjected to concerted evolution and could thus be phylogenetically informative. Further, our analyses with and without the repeats yielded similar results.

Due to the presence of indels, sequences were aligned by eye. A highly variable segment with a large number of indels among the different sequences was eliminated from the analyses. Sequences have been deposited in GenBank (accession numbers AF085366–AF085411), and the alignment used for the present study is available at http://rex.nci.nih.gov/RESEARCH/basic/lgd/front_page.htm.

Sequence Analysis

Nucleotide composition in the analyzed segments was assessed with MEGA (Kumar et al. 1993); transition/transversion ratios were estimated with the program PAMP [included in the package PAML (Yang 1995)] from expected substitution frequencies in a neighbor-joining (Saitou and Nei 1987) tree. The presence of phylogenetic signal in the data sets was tested using the approach described by Hillis (1991) and Hillis and Huelsenbeck (1992) and implemented in test version 4.0d59 of PAUP* (written by D.L. Swofford, Smithsonian Institution). This was done by computing the g_1 statistic (Hillis 1991), derived from the skewness of the distribution of parsimony-generated tree lengths, for an exhaustive search in a subset of eight or nine mtDNA lineages (herein referring to individual sequences or haplotypes) which were representative of each major phylogenetic group and, also, by repeating this procedure in three independent sets of 1000 random trees.

Phylogenetic analysis was performed using combined A + B fragments and three approaches: maximum parsimony, minimum evolution, and maximum likelihood. (1) Maximum-parsimony (MP) trees were estimated with test version 4.0d59 of PAUP*, using 10 independent heuristic searches with random taxon addition. Reliability of the inferred clades was assessed by performing 100 bootstrap replications (Felsenstein 1985; Hillis and Bull 1993) with heuristic searches. (2) Minimum evolution (ME) trees were estimated with test version 4.0d59

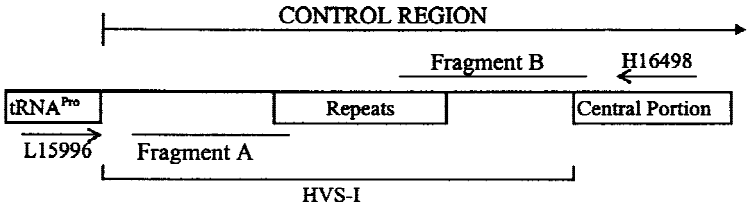

Fig. 2. Structure of the 5′ portion of the mitochondrial DNA control region of felids showing the first hypervariable segment (HVS-I), the annealing positions of human primers L15996 and H16498, and the location of the fragments analyzed in the present study (A, 226 bp; B, 218 bp). L and H in primer names refer to the light and heavy strands of the mtDNA, and numbers are the position of the 3′ nucleotide of the primer in the human mtDNA reference sequence (Anderson et al. 1981).

of PAUP*, using the neighbor-joining algorithm followed by a branch-swapping procedure (heuristic search option), and Tamura–Nei (1993) distances corrected with the γ distribution of rate variation among sites (Yang 1996). The α parameter of the γ distribution was specifically estimated for each data set with Yang and Kumar's (1996) method and the program PAMP. Reliability of these trees was assessed using 100 bootstrap replications. (3) Maximum-likelihood trees were produced with the program DNAML contained in the package PHYLIP 3.5 (Felsenstein 1993), using the estimated transition/transversion ratio and empirical base frequencies.

Trees of ocelot mtDNA lineages were rooted using multiple margay sequences as outgroups, and margay trees were rooted using ocelot sequences. For combined trees, a sample from another ocelot lineage species, the Geoffroy's cat (*Oncifelis geoffroyi*), was used as the outgroup. To assess the occurrence of saturation of substitutions in our data set at the interspecific level of divergence, we inspected the branch lengths and pairwise distances in outgroup–ingroup and within-ingroup comparisons and analyzed plots of total substitutions vs. transversions at different levels of divergence. Since the latter are known to accumulate much more slowly than transitions in the mtDNA (Brown 1985), they are less likely to be saturated and, so, could provide a relative ''time scale'' for measuring saturation in total substitutions.

Gene diversity (the probability that two randomly chosen mtDNA lineages are different in the sample) and nucleotide diversity (π per nucleotide site, i.e., the probability that two randomly chosen homologous nucleotides are different in the sample) (Nei 1987) were estimated from the data using the package ARLEQUIN (Schneider et al. 1997). Analyses were performed for broad populations defined on the basis of geographic criteria and, also, for more restricted groups defined by phylogeographic patterns observed in the mtDNA lineages. As measures of differentiation among populations we estimated fixation indices (F_{st}), using an analysis of molecular variance (AMOVA) approach (Excoffier et al. 1992), and the mean number of migrating females between populations per generation (N_m) (Slatkin 1987), both as implemented in ARLEQUIN.

Results

Sequencing Results

PCR products from the mtDNA control region HVS-I were sequenced for 39 ocelots, 24 margays, and a Geoffroy's cat, *Oncifelis geoffroyi* (Table 1). PCR products varied in size roughly from 600 to 1000 bp, due mainly to the presence of a variable number of units (80–82 bp each) in the tandem array, and exhibited high levels of heteroplasmy. We sequenced the HVS-I in two fragments, A and B. Fragment A contained 226 bp [positions 16,340–16,537 in the domestic cat mtDNA reference sequence (Lopez et al. 1996)], and fragment B spanned 218 bp (positions 16,698–16,911 in the reference sequence). For five margays (Lwi20, 22, 31, 62, 63), only sequences of fragment B were obtained. ''Indel hotspots'' were observed in both fragment A and fragment B; 21 bp of the former had to be removed from the data set because of possible alignment ambiguities, so that a total of 423 bp was used for the analyses.

Twenty-four unique sequences were identified for ocelots and 21 for margays, identified by 70 and 81 variable sites, respectively (Table 2). Both species had high levels of diversity, and most individuals had unique sequences. Sequence identity was observed only among individuals from nearby geographic locations (see Figs. 3 and 4), and similar mtDNA lineages invariably belonged to individuals from the same geographic region. For instance, all three ocelots from French Guiana (Lpa115, 125, and 151) had the same sequence, as did all ocelots from Trinidad (Lpa92, 93, and 94) and one from Venezuela (Lpa83). Similarly, three margays from Guatemala (Lwi35, 36, and 39) shared a mtDNA lineage, as did two margays from the same region of southern Brazil (Lwi69 and 70).

Phylogenetic and Geographic Relationships Among Sequences

Assessment of phylogenetic signal (g_1 statistic) in the data sets produced statistically significant results ($P < 0.01$) and confirmed the presence of useful historical information in the data. Rate variation among sites was high, as indicated by low estimates of the γ distribution α parameter (0.1253 to 0.2736). Average nucleotide composition was 31% A, 28.6% T, 26.4% C, and 14.1% G. Estimated transition/transversion ratios were very high, averaging 21.46 among ocelots and 70.8 among margays. All trees produced with different phylogenetic methods generated consistent results, in which the distinction of major clusters was visible in both species, with minor topological and branch-length differences between trees constructed with different algorithms.

Clear geographic partitions were observed in the trees of both species. Ocelots (Fig. 3) presented four main phylogeographic groups: (1) southern South America (all mtDNA lineages sampled south of the Amazon River, including Bolivia and several regions of Brazil); (2) north–northwestern (NNW) South America (all Venezuela and Trinidad samples, Brazilian individuals sampled on the northern side of the Amazon River, and two sequences from Panama); (3) north–northeastern (NNE)

Table 2. mtDNA diversity estimates in ocelot and margay phylogeographic groups

	N	mtDNA lineages	Variable sites	No. pairwise differences	% pairwise divergence[a]	Gene diversity[b]	Nucleotide diversity[a,b]
Ocelots	39	24	70	1–28	0.26–14.73	0.962 ± 0.015	0.068 ± 0.034
S. South America	19	10	20	1–11	0.26–3.71	0.895 ± 0.048	0.022 ± 0.012
N. South America	13	8	49	5–25	1.40–12.16	0.885 ± 0.067	0.052 ± 0.028
NNW S. America[c]	9	6	26	5–12	1.40–4.43	0.833 ± 0.127	0.023 ± 0.013
NNE S. America[d]	4	2	11	11	4.74	0.5 ± 0.265	0.029 ± 0.020
Central America	7	6	16	1–14	0.27–4.79	0.952 ± 0.096	0.020 ± 0.012
Margays	24	21	81	1–40	0.36–23.33	0.985 ± 0.018	0.183 ± 0.092
S. South America	10	9	47	2–23	0.60–13.30	0.978 ± 0.054	0.089 ± 0.049
N. South America	2	2	11	11	4.36	1.0 ± 0.5	0.065 ± 0.066
Central America	12	10	35	1–18	0.36–14.73	0.955 ± 0.057	0.100 ± 0.054
C. America 1[e]	5	5	17	2–15	0.98–10.67	1.0 ± 0.127	0.093 ± 0.058
C. America 2[f]	7	5	18	1–13	0.36–5.93	0.857 ± 0.137	0.049 ± 0.029

[a] Calculated using γ-corrected Tamura–Nei distances (α = 0.1508 for ocelots and 0.2736 for margays)
[b] Estimated using only sites which had less than 5% missing data (0.05 allowed missing level per site in ARLEQUIN)
[c] Ocelot samples from Venezuela, Trinidad, Panama, and northern Brazil
[d] Ocelot samples from French Guiana and northern Brazil
[e] Margay samples from Panama, Costa Rica, and Nicaragua
[f] Margay samples from Guatemala and Mexico

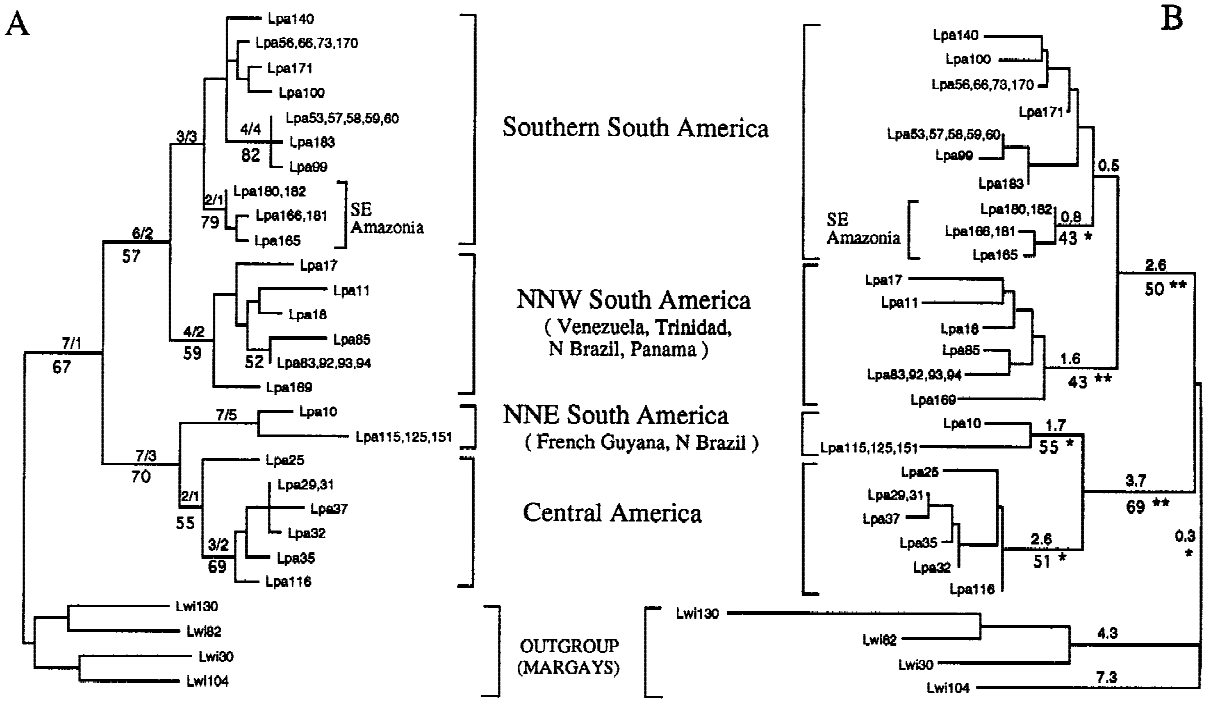

Fig. 3. Phylogenetic relationships among mitochondrial DNA lineages of ocelots based on 423 base pairs of the control region. Labels are sample identification numbers; main phylogeographic groups are indicated by *brackets* and identified in the center. *Numbers above branches* indicate number of steps/number of homoplasies **(A)** or branch lengths (×100) **(B);** *numbers below branches* are bootstrap support values. (A) Majority-rule consensus of 47 maximum-parsimony trees (length = 162, CI = 0.586), generated with PAUP*, using the Accelerated Transformation option for character-state optimization. Differences among the trees were limited to minor variation in branch lengths and in topology within groups; the only main group not present in all the trees was NNE South America (51%). (B) Minimum-evolution tree generated with PAUP*, using Tamura–Nei distances corrected with the γ distribution of rate variation among sites (α = 0.1253). Groups supported by the maximum-likelihood analysis (ln likelihood, −1437; 2084 trees examined) are shown: *branch significantly positive ($P < 0.01$), confidence interval overlaps zero; **branch significantly positive ($P < 0.01$), confidence interval does not overlap zero.

South America (one mtDNA lineage from French Guiana and one from northern Brazil); and (4) Central America. These groups were consistently identified by most phylogenetic approaches as reciprocally monophyletic clades, except for the NNE South American cluster, which was depicted in some analyses as paraphyletic with regard to Central America. The Central American group was distinct and had a diagnostic 7-bp deletion in fragment A. NNE South America and Central America groups were closely associated, as were southern South

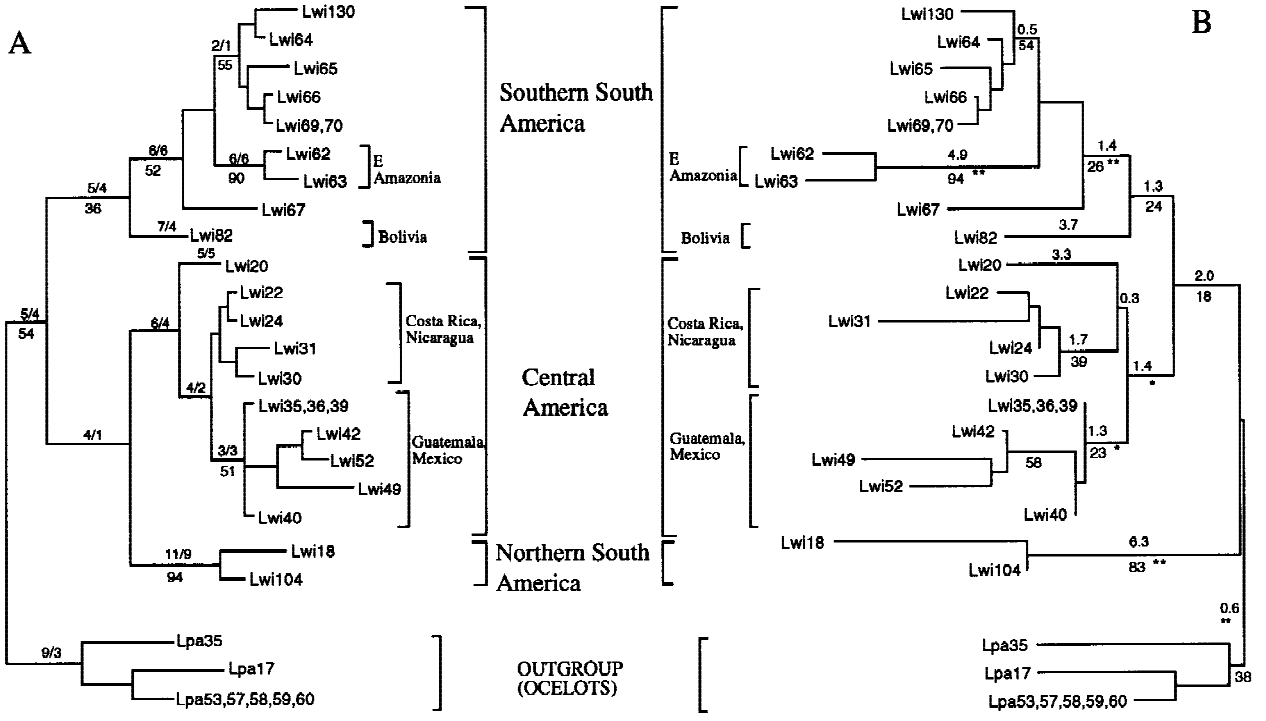

Fig. 4. Phylogenetic relationships among mitochondrial DNA lineages of margays. Segment length, method details, labels, symbols, and comments are the same as presented in Fig. 3. **(A)** Majority-rule consensus of 24 maximum-parsimony trees (length = 186; CI = 0.527); the southern South American group was present in 75% of the trees, due to changes in the position of the basal sequence Lwi82; the association of the northern South American and Central American groups was present in 63% of the trees. **(B)** Minimum-evolution tree generated with corrected Tamura–Nei distances ($\alpha = 0.2151$). Groups supported by the maximum-likelihood analysis (ln likelihood, −1505; 946 trees examined) are shown: *branch significantly positive ($P < 0.01$), confidence interval overlaps zero; **branch significantly positive ($P < 0.01$), confidence interval does not overlap zero.

America and NNW South America. This clustering into two major phylogenetic groups (Fig. 3) was supported by bootstrap values of 57 and 70% in the MP tree and 50 and 69% in the ME tree. Geographic substructure was visible within the ocelot mtDNA phylogenetic groups. Within southern South America sequences from the southeastern Amazon region (Lpa165, 166, 180, 181 and 182) formed a distinct clade (with 79% MP bootstrap support) apart from other samples from south of the Amazon River. Within NNW South America, the sequence from Trinidad/Venezuela was consistently associated with the other Venezuelan sequence.

Margays (Fig. 4) presented three major mtDNA phylogenetic groups: one formed by all South American sequences from south of the Amazon River, another with all Central American sequences, and a third with the two sequences from northern South America. Within southern South America, a sequence from Bolivia was consistently basal in this clade. The remainder, from several regions of Brazil and Paraguay, formed a single cluster. Within this group, two samples from the eastern Amazon region (Lwi62 and 63) were closely related to each other (bootstrap values of 90% MP, 94% ME) and distinct from the rest. In the Central American major group, two clusters were observed, one containing sequences from Costa Rica and Nicaragua, and the other composed of individuals from Guatemala and Mexico. An individual from Panama (Lwi20) appeared basal in this major clade and, in some analyses, weakly associated with the Costa Rica/Nicaragua subgroup. The third major group was composed of the two northern South American mtDNA lineages, which clustered together with high bootstrap support in all analyses (94% MP, 83% ME) and were consistently different from both other major groups. The relationship of this group to the other two is not clear; in most analyses it seemed to be more divergent from both of them than they are from each other, but at this point their initial divergence should be regarded as trichotomy.

Population Structure and Genetic Diversity

Broad populations defined by geographic criteria for both species were (1) southern South America, (2) northern South America, and (3) Central America. The point of separation between northern and southern South America was considered to be the Amazon River, based on results from the phylogenetic analyses of mtDNA lineages described above. Analyses performed with phylogeographic subgroups recognized two clusters of oce-

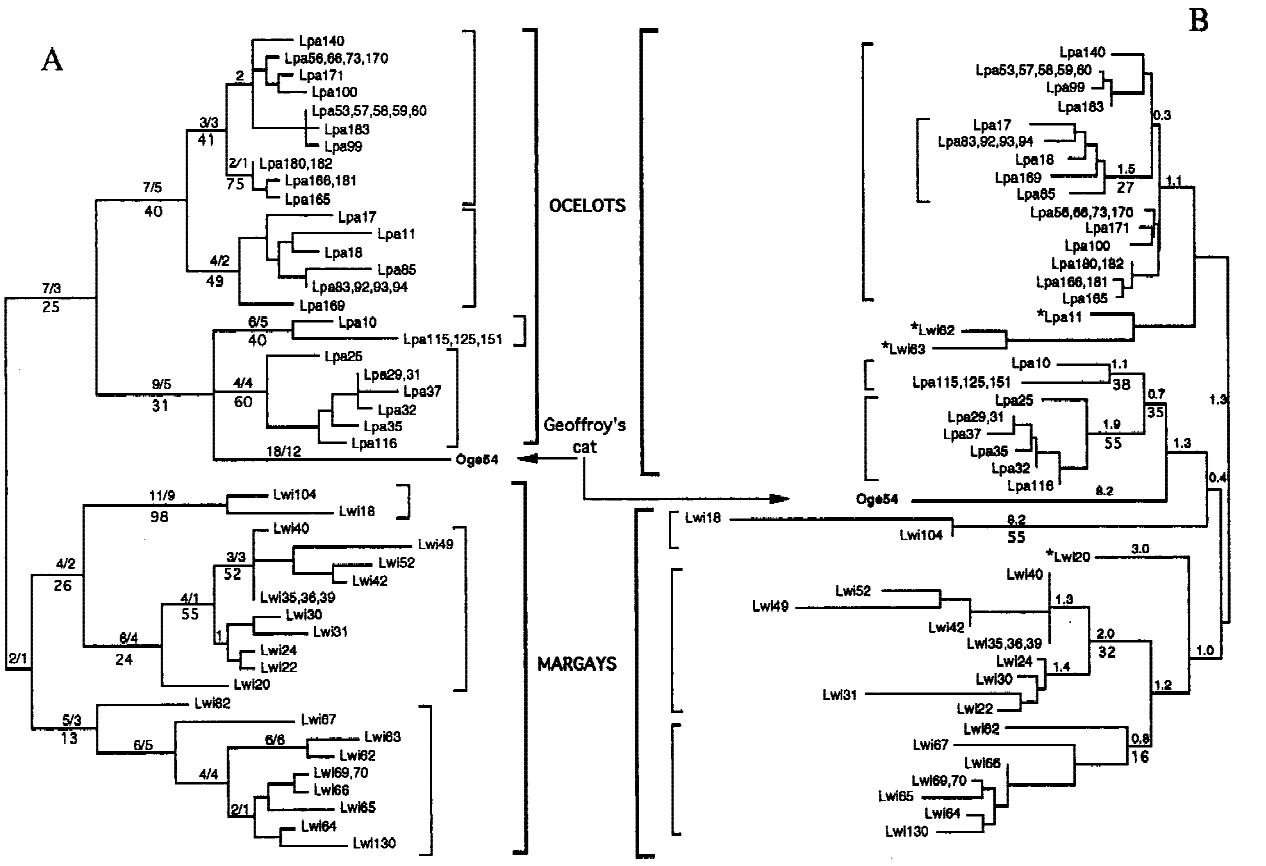

Fig. 5. Phylogenetic relationships among mtDNA lineages of ocelots, margays, and Geoffroy's cat. Major phylogeographic groups in each species are indicated by *brackets.* Segment length, labels, method details, and symbols are the same as presented in Figs. 3 and 4, unless stated otherwise. **(A)** Majority-rule consensus of 1069 maximum-parsimony trees (length = 278; CI = 0.450), generated by a heuristic search with simple taxon addition. *Numbers below branches* are support values generated by 100 bootstrap replications constrained to generate a maximum of 1000 trees. **(B)** Minimum-evolution tree generated using corrected Tamura–Nei distances ($\alpha = 0.1885$). *Asterisks* indicate individuals misplaced from their usual position in intraspecific trees.

lot lineages in northern South America and two Central American groups of margays. High levels of mtDNA control region sequence diversity were observed in all groups of both species (Table 2). Genetic diversity indices were similar in all geographic groups of each species and tended to be higher in margays than in ocelots. F_{st} values were 0.602 for ocelots and 0.622 for margays when only the broadest geographic groups (three major regions mentioned above) were considered, showing that more than 60% of the mtDNA variability in our samples was distributed among populations. When the two groups of northern South American ocelots and the two groups of Central American margays, were considered separate populations, F_{st} values were 0.748 for the former and 0.634 for the latter. The estimated N_m was less than 1 in all comparisons, indicating that very low levels of mtDNA gene flow has occurred recently among these geographic regions.

Interspecific Comparisons and Outgroup Placement

Phylogenetic analyses that included both ocelot and margay sequences affirmed the geographic groupings of each species but generally gave weak support for the monophyly of these species. Branches separating species were generally short (often shorter than several within-species branches) and had weak statistical support (Figs. 3–5). This correlates with the observation that a single site (position 25 in fragment A) is diagnostic for the two species. Trees including ocelot and margay mtDNA sequences showed a trend for branch lengths of margay lineages to be longer than those of ocelots (Fig. 5B), which is related to the higher diversity and divergence estimates for the latter species (Table 2). Certain ocelot sequences were similar to some of the margays', leading to unstable rooting of intraspecific trees when these sequences were included as outgroups (not shown, but see Fig. 5). Similarly, the Geoffroy's cat sequence appeared phylogenetically unstable; it failed to connect at the root between the ocelot and the margay lineages and clustered, instead, to internal groups within one or the other species, such as the NNE South American/Central American ocelot group (Fig. 5). Graphic comparisons of total pairwise differences vs. transversions within and between species indicated that although minimum ob-

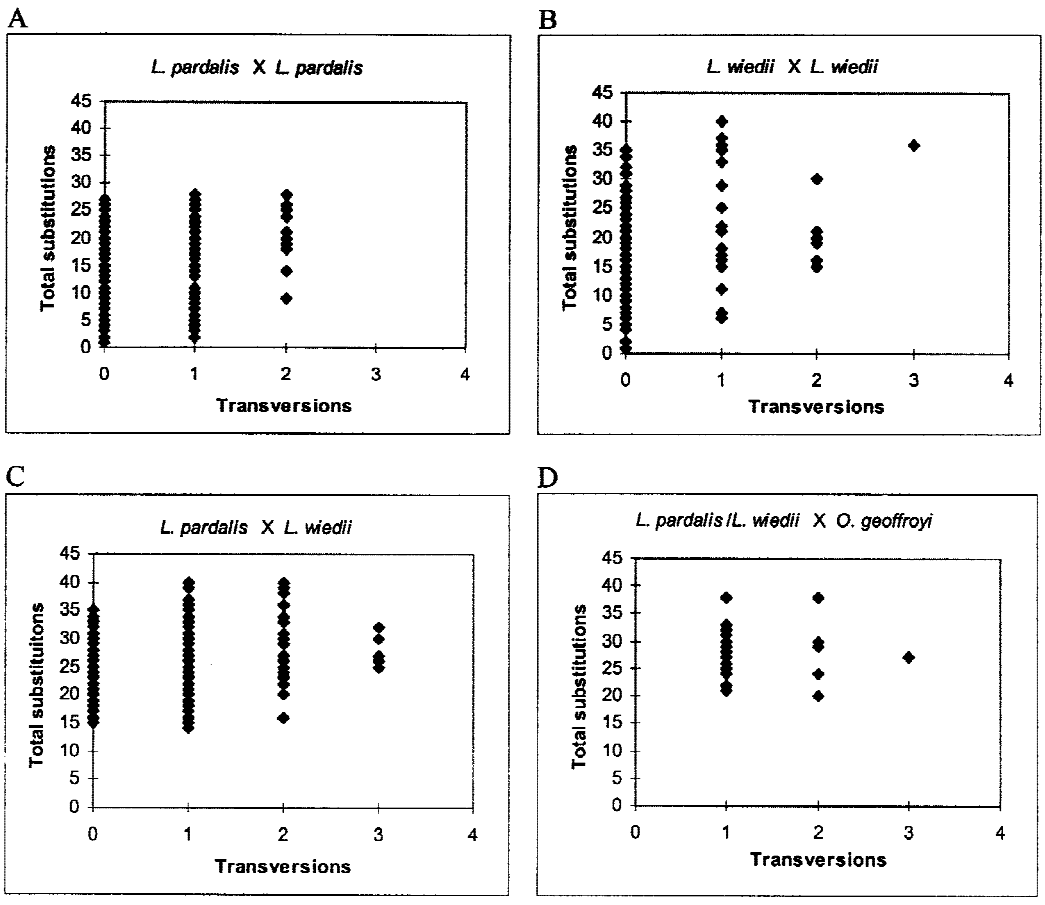

Fig. 6. Graphs comparing the increase in total substitutions vs. the increase in transversions in A + B fragments. **A** Pairwise comparisons among ocelot mtDNA lineages. **B** Pairwise comparisons among margay lineages. **C** Pairwise comparisons involving ocelot vs. margay lineages. **D** Pairwise comparisons involving ocelot and margay mtDNA lineages vs. the Geoffroy's cat lineage.

served differences clearly increased as more divergent taxa were compared, total substitutions tended to plateau in relation to transversion increments (Fig. 6).

Discussion

Genetic Diversity and Control Region Evolution

Ocelots and margays present among the highest levels of mtDNA control region diversity so far observed in any organism (e.g., Bickham et al. 1996; Stanley et al. 1996; McMillan and Palumbi 1997; Parker and Kornfield 1997; Vilà et al. 1997; Wooding and Ward 1997), suggesting either very fast substitution rates in this segment or a long evolutionary history with no significant population bottlenecks in the recent past. The latter seems more likely and agrees with results obtained for sequence variation in other mtDNA regions and in a hypervariable segment of the major histocompatibility complex (unpublished data).

Margays presented slightly higher levels of control region diversity than ocelots, at both species- and population-level comparisons (Table 2, Fig. 5). If accurate, this relatively greater margay control region diversity might suggest that the evolutionary rate of the HVS-I is faster in margays than in ocelots, possibly due to different molecular or demographic processes in these species (Mindell and Thacker 1996). An alternative possibility is that the rate of sequence evolution is similar in both species and that margay lineages are older than ocelots'. Further study on the molecular evolution of this mtDNA region in felids, as well as correlation of different life history traits with phylogeographic patterns and population genetic parameters, should help clarify this issue.

The short branches and weak support observed in the basal ocelot/margay split might be interpreted as being caused by the incomplete sorting of ancestral polymorphisms in recently derived sister species (Avise 1994). However, analyses of other mtDNA segments (Johnson and O'Brien 1997, unpublished data) support a clear separation between ocelot and margay mtDNA lineages at about 3 MYA. Thus we favor the view that the observed patterns of mtDNA control region variation probably result from saturation at informative sites in comparisons between these two species. This phenomenon would be more apparent as more divergent groups are compared. The unstable placement and comparatively

short branch length of the Geoffroy's cat in the phylogenetic reconstructions support this hypothesis. The observed tendency of total substitutions (predominantly transitions) to form a plateau when plotted against transversions, especially in interspecific comparisons (Fig. 6), also indicates that total changes are saturated after that limit. Two factors leading to the rapid saturation of informative sites may be suggested: first, the observed high variation in substitution rate among sites, evidenced by very low values of the α parameter of the γ distribution, should mean that most variability is restricted to a few nucleotide positions (Yang 1996); and second, the observed transition/transversion ratio, which is usually high in animal mitochondrial DNA (Brown 1985), and in our data sets reaches up to 70:1, leads to an increased probability of character-state reversion. Similar observations have recently been reported in other organisms (e.g., McMillan and Palumbi 1997).

These findings suggest that this control region segment should be applied chiefly for intraspecific studies, for which it has shown to be highly informative, and also in comparisons among very recently separated felid species. Because saturation at informative sites may be extensive in interspecific comparisons, problems can also arise in the rooting of intraspecific phylogenies based on this mtDNA segment, similar to what probably happens with humans and chimpanzees (Maddison et al. 1992; Stoneking et al. 1992).

Comparative Phylogeographic Patterns and Population History

Observed patterns of phylogeographic partitions in ocelot mtDNA lineages (Figs. 1A and 3) indicate that in the past this species was divided for a significant period of time into two major populations. These two ancestral groups possibly inhabited different parts of northern South America, since this is the only region where both major groups are currently present. Only lineages descending from one such group were identified presently in Central America, indicating that this region was colonized solely or predominantly by that ancestral population. The phylogenetic pattern of this group indicates that a finer geographic substructure currently exists in this region and lends support to a south–north direction in the colonization of Central America by this species, as expected if ocelots originated in South America. The existence of a distinct geographic cluster currently inhabiting NNE South America (French Guiana and northern Brazil, north/northeast of the Amazon River) is also suggested, although support for its monophyly is weak in some analyses. The patterns observed in the other major group of ocelot mtDNA lineages suggest that a second important episode of population division happened in South America. A plausible cause for such division is the Amazon River, since each phylogenetic group defined by this separation seems to occur only on one side of the river. Current gene flow between the two northern South American ocelot groups is uncertain at this point, and more detailed sampling in this region is required to clarify this issue. Our results suggest that they represent two distinct historical groups, isolated in the past and possibly still kept at low gene flow levels by geographic barriers such as the Rio Negro. Rivers have been historically implicated as faunal barriers in the Amazon region. Although recent work indicates that their influence is not always as critical and direct as thought previously (Patton et al. 1994, 1996), further study in this area is required to understand better the interaction between the past dynamics of major watercourses and the phylogeographic partitions of taxa with different life history and dispersal characteristics. Among southern South American ocelots, the distinction between lineages from the southeastern part of the Amazon region from the rest of Brazil and Bolivia may reflect historical isolation by distance.

Margays present phylogeographic patterns (Figs. 1B and 4) concordant in several aspects to those of ocelots. A clear division between Central and South American lineages exists, perhaps caused by the same historical process that produced the major split in ocelots. In addition, a third major group seems to have been formed in northern South America. The phylogenetic patterns observed among margay Central American lineages are similar to those of ocelots; there is evidence for geographic substructure, in which Panama, Nicaragua + Costa Rica, and Guatemala + Mexico present consistent differentiation from each other. A south–north direction in the colonization of Central America by margays is also implied by the observed structure, though a more comprehensive sampling would be required to test this hypothesis. As in ocelots, the pattern observed among southern South American margay lineages exhibits a distinction between the eastern Amazonian region (south of the Amazon river) from the rest of Brazil, indicative of isolation by distance or recent regional barriers to gene flow. However, the pattern for margays differs from that of ocelots in that the only sequence from Bolivia (Lwi82) seems to be differentiated from those of Brazil and Paraguay, consistently occupying a basal position in this clade. In the case that this geographic distinction is corroborated by more comprehensive sampling, it would indicate more limited historical gene flow in margays than in ocelots in this region, possibly associated with stronger barriers to the former imposed by dryer environments.

Major splits between the observed mtDNA lineage clades indicate long periods of geographic isolation between populations (Avise 1994). Within each major group in both species, levels of geographic substructure suggest recent population division or more likely, historically limited gene flow (Avise et al. 1987). This

would be in agreement with the fact that most of the long-distance dispersion in felid populations is usually performed by males (Oliveira 1994). Females, through which mtDNA is transmitted, are largely philopatric, which should contribute to enhancing the differentiation among populations in regard to mtDNA phylogenies.

Population Structure and Genetic Diversity

Central American and southern South American populations of both species, and also northern South American margay lineages, form monophyletic groups in the mtDNA lineage trees, which completely agree with broad present-day geographic partitions. However, northern South American ocelot populations are more complex, presenting two distinct clusters of lineages descending from both populations formed at the first major split. Ocelot nucleotide diversity estimates support this observation (Table 2), showing similar levels of diversity in Central America, southern South America, and each northern South American group, but a twofold higher value when northern South America is considered as a single population. In margays, genetic diversity estimates are similar for all geographic groups; it is possible that a subdivision pattern in northern South America similar to the ocelots' would emerge with a more comprehensive sampling of this region. F_{st} values for both species indicate that a significant portion of the mtDNA diversity in our sample is structured between defined geographic populations (Excoffier et al. 1992), thus supporting the occurrence of very strong regional subdivision. Estimation of the expected number of migrant females between populations per generation (N_m) indicates that mtDNA gene flow among these populations is very low, which leads to their differentiation over time (Slatkin 1987).

Implications for Conservation

Results presented here have implications for the conservation and management of these species in the wild and in captivity. Central American and southern South American groups of each species can certainly be regarded as ''evolutionarily significant units'' [ESU (Ryder 1986)] since they fulfill the major requirement of reciprocal monophyly of mtDNA lineages (Moritz 1994). As such, they should be conserved and managed as separate entities. Northern South American margays, and the two groups of northern South American ocelots, could be provisionally regarded as independent ESUs, but further studies are required to improve our knowledge on population structure in this region for both species. The geographic substructure observed in the mtDNA phylogenies should also be considered for conservation purposes. Margay populations from Panama, Costa Rica + Nicaragua, Guatemala + Mexico, Bolivia, and eastern Amazonia should be viewed at least as independent ''management units'' (Moritz 1994), since their degree of mtDNA differentiation indicates that their demographic connection to other regions is low. The same would apply to ocelot populations from southeastern Amazonia, Nicaragua and Mexico + Guatemala. The relationships among the presently identified geographic groups, and the existence of other demographic units in both species, should be investigated with a more comprehensive geographic sample and other approaches, including the use of nuclear molecular markers.

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