



Symposium Article

Population Structure of mtDNA Variation due to Pleistocene Fluctuations in the South American Maned Wolf (*Chrysocyon brachyurus*, Illiger, 1815): Management Units for Conservation

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Abstract

The maned wolf (*Chrysocyon brachyurus*) is one of the largest South American canids, and conservation across this charismatic carnivore's large range is presently hampered by a lack of knowledge about possible natural subdivisions which could influence the population's viability. To elucidate the phylogeographic patterns and demographic history of the species, we used 2 mtDNA markers (D-loop and cytochrome *b*) from 87 individuals collected throughout their range, in Argentina, Bolivia, Brazil, and Uruguay. We found moderate levels of haplotype and nucleotide diversity, and the 14 D-loop haplotypes were closely related. Genetic structure results revealed 4 groups, and when coupled with model inferences from a coalescent analysis, suggested that maned wolves have undergone demographic fluctuations due to changes in climate and habitat during the Pleistocene glaciation period approximately 24000 years before present (YBP). This genetic signature points to an event that occurred within the timing estimated for the start of the contraction of the *Cerrado* around 50000 YBP. Our results reveal a genetic signature of population size expansion followed by contraction during Pleistocene interglaciations, which had similar impacts on other South American mammals. The 4 groups should for now be considered management units, within which future monitoring efforts should be conducted independently.

Resumen

El aguará guazú (*Chrysocyon brachyurus*) es uno de los cánidos más grandes de Sudamérica y en la actualidad la conservación de este carnívoro carismático se ve obstaculizada por la falta de conocimiento acerca de las posibles subdivisiones naturales que podrían influir en la viabilidad de las poblaciones. Para dilucidar los patrones filogeográficos y la historia demográfica, hemos utilizado dos marcadores del ADN mitocondrial (*D-loop* y el *citocromo b*) en 87 individuos colectados en toda su área de distribución geográfica, en la Argentina, Bolivia, Brasil y Uruguay. Encontramos niveles moderados de diversidad haplotípica y nucleotídica estando los 14 haplotipos del *D loop* estrechamente relacionados. Los resultados en combinación con el análisis coalescente revelaron estructuración genética en cuatro grupos, y sugirieron que la especie ha sufrido fluctuaciones demográficas debido a los cambios en el clima y hábitat en el período de la glaciación Pleistocénica aproximadamente 24.000 AAP. Esta marca genética apunta a que el evento ocurrió alrededor 50.000 AAP en el inicio de la contracción del Cerrado. Nuestros resultados revelan una marca genética ocurrida durante la expansión del tamaño de la población, seguido de la contracción durante las interglaciaciones en el Pleistoceno, efecto que ha tenido impactos similares en otros mamíferos sudamericanos. Los cuatro grupos deben ser considerados por el momento unidades de manejo en las cuales deben focalizarse esfuerzos de monitoreo y manejarse de forma independiente.

Subject areas: Molecular systematics and phylogenetics; Population structure and phylogeography

Key words: climate change, conservation genetic units, gene flow, landscape fragmentation, maned wolf

Expanding knowledge, technologies, and applications of genetic techniques represent some of the most rapidly evolving and exciting tools for canid conservation (Boitani *et al.* 2004; Estes *et al.* 2011). In several instances, successful management of large canids in the northern hemisphere has been based on genetic characterization using molecular markers (Vilà *et al.* 1999; Hoban *et al.* 2013; Cronin *et al.* 2015). In South America, the maned wolf (*Chrysocyon brachyurus*, Illiger 1815) is the largest canid (an average male weighs about 23 kg with a mean total length of 147 cm), and it inhabits a largely transformed and fragmented landscape (Dietz 1985). More than half of the *Cerrado*'s 2 million km² has been converted into pasture, cropland, and other uses (Klink and Machado 2005; Vynne 2014). Until recently, maned wolves occurred over a wide geographic range of 5 million km² (Dietz 1985; Queirolo *et al.* 2011). Over the last 100 years, their range has been dramatically reduced and fragmented, but small populations are still found, as far west to the Peruvian pampas, and south through the Chaco of Paraguay, Argentina, and Uruguay (Figure 1; Dietz 1985; Beccacesi 1990, 1992, 1993; Mones and Olazarri 1990; Miatello and Cobos 2008; Queirolo *et al.* 2011). Only 5–10% of the 22 known remaining maned wolf populations are located in protected areas (Queirolo *et al.* 2011; Muir and Emmons 2012). The IUCN Red List categorizes them as “Near Threatened” because their current global population is estimated at about 13 000–15 000 mature individuals, and they are likely to experience a continuing decline of nearly 10% in the next decade (Muir and Emmons 2012). The species is also listed in CITES Appendix II and in the United States Endangered Species list and protected by law in many parts of its range, but enforcement is frequently problematic (Bernardes *et al.* 1990).

Maned wolves are monogamous and have 1 mate per year, litter average size 4, and are found in low densities on large home ranges of approximately 80 km² (Jácomo *et al.* 2009). The available field data show that a female daughter can remain as a presumed helper that inherits the maternal territory on death of her mother, but that male offspring and the mates of females that die all disperse. This

suggests that there is a tendency for them to establish local matrilineal territories (Emmons 2012, 2014).

Previous studies of regional maned wolf genetic variability in the Brazilian *Cerrado* reported moderately low levels of genetic diversity and no geographic structure, a result in contrast with other Neotropical Canidae such as the crab eating fox (*Cerdocyon thous*) and the pampas fox (*Lycalopex gymnocercus*) (Prates Júnior 2008; Lion *et al.* 2011; Fontoura-Rodrigues and Eizirik 2014). These studies were based on small sample sizes from few locations, because wolves are difficult to capture. However, recently developed non-invasive genetic technologies have made it possible to obtain DNA samples without capturing or harming animals and from places where the animals have only been detected by the presence of their feces (Mannise *et al.* 2012).

In particular, it is crucial to uncover the presence of demographic partitions that could impact gene flow patterns and result in a hierarchical distribution of the genetic diversity among populations throughout the geographic range of the species (Moritz 1994, 1995; Crandall *et al.* 2000). Moritz (1999) proposed that 2 types of conservation genetic units could be recognized with molecular data: evolutionarily significant units (ESUs), defined as historically isolated and independently evolving sets of populations, without regard to the current distribution of phenotypic variation and management units (MUs), defined as populations that do not show reciprocal monophyly for mtDNA alleles, yet have diverged in allele frequency and are significant for conservation in that they represent populations connected by such low levels of gene flow that they are functionally independent.

Our aim was to elucidate the species phylogeographic patterns and demographic history with the objective to design effective conservation and management guidelines. We analyzed representative samples from throughout the species range, including samples from Argentina, Bolivia, Brazil, and Uruguay. We predicted that we would detect low or no genetic subdivision between locations because, until the last century, large parts of their historical range were interconnected by grassland/open *Cerrado*. We also sought to investigate the

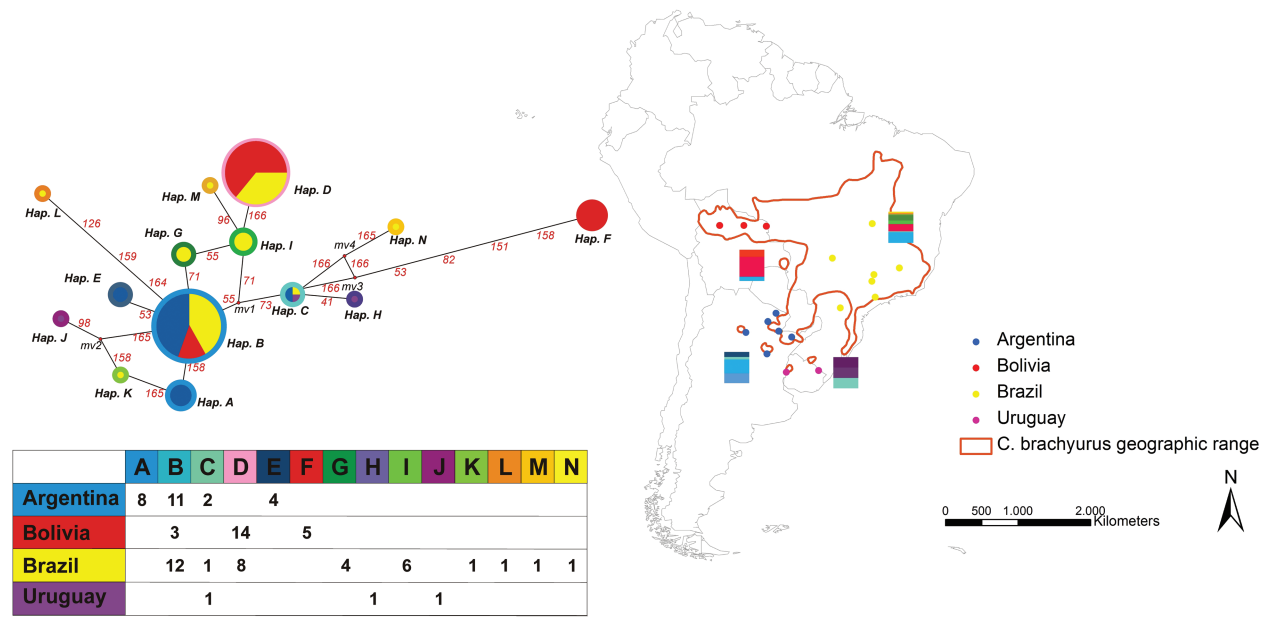


Figure 1. Genetic structure groups in the maned wolves based on mitochondrial DNA sequences. Approximate historical geographic range indicated in orange after Rodden et al. (2008). The colored dots on the map represent sampling localities: 1: Argentina (Blue), 2: Bolivia (Red), 3: Brazil (Yellow), and 4: Uruguay (Purple). The bars represent the haplotype diversity frequencies in each group. Minimum-spanning network of *D-loop* haplotypes using the molecular-variance parsimony algorithm, where circles represent haplotypes, letters within them correspond to haplotype designations, and circle sizes are proportional to the haplotype's frequency in the population. The numbers along the lines connecting the haplotypes show the substitutions sites. The Table inserted shows numbers and distribution of *D-loop* haplotypes within each of the 4 geographic groups (country).

hypothesis that maned wolves experienced historical demographic changes that mirrored changes in their habitat availability, such as increasing and decreasing population size when grasslands and *Cerrado* expanded and contracted under the influence of Pleistocene climatic fluctuations. To this end, we analyzed a fragment of the cytochrome *b* and a hypervariable fragment of the mitochondrial D-loop region that has been shown to be an ideal marker in other Neotropical mammal species with range distribution patterns similar to that of the maned wolf and with the power to unravel subtle signatures of historical genetic structure and demographic change (Márquez et al. 2006).

Material and Methods

Sampling

We collected 151 samples from 87 individual maned wolves from 9 geographic locations across the species' range in Argentina, Bolivia, Brazil, and Uruguay (Figure 1; Table 1; see details in Supplementary Table 1 online).

DNA Extraction, Amplification, and Sequencing

DNA was extracted following the protocol of Medrano et al. (1990) as modified in González et al. (1998). Fecal DNA extractions were performed using a DNeasy® kit (QIAGEN®) with sterile materials and filtered pipette tips in a room dedicated for DNA extraction from low quality samples and separate from polymerase chain reaction (PCR) product contamination. Extraction controls and no-template PCR controls were used in each reaction. Universal control region (D-loop) primers (*Tbr-L15910*: 5'-GAATTCCCCGCTCTTGTAAC-3' and *DL-H16498*: 5'CCTGAAGTAGGAACAGATG-3'; Vilà et al. 1999) were used to amplify a 460 bp fragment of the D-loop. All PCR amplifications of

the D-loop were performed in a final volume of 25 µl and contained 1× Invitrogen, 1.5 mM MgCl₂, 0.1 mM of each dNTP, 1 pmol/µl of each primer, 1.0 U Taq DNA Polymerase (Invitrogen), and approximately 50–100 ng of DNA. PCRs were performed in a programmable thermal cycler (Applied Biosystems; Model 2720), and profiles began with an initial hot start step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 60 s, annealing at 50 °C for 120 s, and extension at 72 °C for 90 s. The final step was one extension cycle at 72 °C for 7 min. In addition, we designed a new set of maned wolf specific D-loop primers (*DLMW234L* 5'-TTGACACCACCCACATTCAT-3' and *DLMW234H* 5'-GTTTCTCGAGGCATGGTGAT-3') using the program PRIMER3® (Rozen and Skaletsky 1996). The thermal profile included an initial denaturation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. A final extension step of 72 °C for 7 min concluded the profile. PCR amplifications for a 486 bp fragment at the 3' end of the *Cyt b* gene were performed with primers L14724:5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' and H15149:5'-AAACTGCAGCCCCCTCAGAATGATATTGTCCTCA-3' (Kocher et al. 1989). PCR conditions for this *Cyt b* fragment were as follows: 35 cycles consisting of a denaturalization at 94 °C for 45 s, annealing at 58 °C for 30 s, and extension at 72 °C for 50 s, plus an initial hot start step at 94 °C for 3 min and a final extension step of 72 °C for 7 min. PCR products were purified using the QIAquick® PCR Purification kit (QIAGEN®), sequenced in both directions using the ABI big dye ready reaction kit, and run on ABI 377 and 3100 Genetic Analyzers (Applied Biosystems).

Phylogeographic and Population Genetic Structure Analyses

Complementary strand sequences were analyzed using Sequencher® 4.1 (Gene Codes Corp); sequences were inspected and corrected by

Table 1. Summary of the distribution of mitochondrial haplotypes (*D-loop* and *Cytochrome b*) in the 4 sampled geographic groups (Argentina (1), Bolivia (2), Brazil (3), and Uruguay (4)) including the geographic coordinates for each locality within the group

Group	Locations	Mitochondrial marker	Unique D-loop haplotypes	Shared D-loop haplotypes	Shared Cyt b haplotypes
1	Argentina-Corrientes 27° 32' S; 59° 01' W	Cyt b (10); Dloop (20)	Hap A (7); Hap E (4)	Hap B (7); Hap C (2)	Hap I (10)
1	Argentina-Santa Fe 30° 14' S; 60° 47' W	Cyt b (1) Dloop (5)	Hap A (1)	Hap B (4)	Hap I (1)
2	Bolivia- National Park-Los Fierros 14° 33' S; 60° 55' W	Cyt b (7) Dloop (16)	Hap F (2)	Hap B (1), Hap D (13)	Hap II (7)
2	Bolivia- Mangabalito 13°47' S; 60° 32' W	Cyt b (1) Dloop (1)		Hap B (1)	Hap I (1)
2	Bolivia -El Refugio 14° 47' S; 61° 02' W	Cyt b (2) Dloop (5)	Hap F (3)	Hap B (2)	Hap I (1), Hap II (1)
3	Brazil- Emas National Park 18° 15' S; 52° 53' W	Dloop (13)	Hap I (1), Hap L(1), Hap M(1), Hap N(1)	- Hap B (4), Hap C (1), Hap D (4)	
3	Brazil- Araxá-MG (19° 58' S; 46° 97' W	Cyt b (2) Dloop (7)	Hap K(1)	Hap B (3), Hap D (3)	Hap I (2)
3	Brazil- Belo Horizonte-MG 19° 55' S; 43° 56' W	Cyt b (8) Dloop (8)	Hap G(1), Hap I(4)	Hap B (3)	Hap I (1), Hap II (7)
3	Brazil-Franca-SP 20° 32' S; 47° 21' W	Cyt b (7) Dloop (5)	Hap G(3), Hap I (1)	Hap D (1)	Hap I (1), Hap II (6)
3	Brazil-Chapada Do Soul-MS 18° 27' S; 52° 36' W	Cyt b (2) Dloop (2)		Hap B (2)	Hap I
4	Uruguay-Cerro Largo 32° 45' S; 56° 22' W	Cyt b (1) Dloop (2)	Hap J(1)	Hap C(1)	Hap I (1)
4	Uruguay-Rio Negro 32° 35' S; 58° 08' W	Dloop (1)	Hap H(1)	—	—

The number of individuals screened for each of the mitochondrial markers (*D-loop* and *Cytochrome b*) are shown inside parenthesis. Haplotypes that are unique to each of the 4 geographic groups and haplotypes that are shared between the 4 geographic groups are shown in separate columns.

eye. For phylogenetic analyses, sequences were aligned using *Clustal X* (Thompson *et al.* 1997); analyses were performed using *PAUP** (Swofford 2002) and *Mega 5* software (Tamura *et al.* 2011). We applied the maximum likelihood, neighbor-joining, and maximum parsimony methods and a 1000 pseudoreplicates of bootstrap to assess node support (Felsenstein 1985).

We estimated genetic distances among haplotypes using the *Kimura 2-parameter* model assuming a gamma distribution of nucleotide substitution (Kimura 1980). We also constructed a median-joining network using the software *Network 4.5* (Bandelt *et al.* 1999) to analyze the haplotype connections and to infer intraspecific phylogenies.

We assessed the significance of geographical subdivisions among local and regional population groupings with an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) using *Arlequin 3.5* (Excoffier and Lischer 2010). The significance of F-statistic analogs was evaluated by 1023 random permutations of sequences among populations. We experimented with various groupings of samples into populations suggested by the haplotype trees and those suggested by geographic isolation. The groupings that maximized values of Φ_{CT} , and were significantly different from random distributions of individuals, were assumed to be the most probable geographic subdivisions (Márquez *et al.* 2006). Finally, we calculated the genetic distances among groups using the neighbor-joining method, and Slatkin's linearized distance that computes the genetic distance derived from pairwise F_{ST} (Saitou and Nei 1987; Slatkin 1995).

We estimated gene flow within and among regions expressed as the number of female migrants per generation, Nm , where N is the

female effective population size and m is the female migration rate. The Nm parameter was approximated by the expression $F_{ST} = 1/(1 + 2Nm)$ (Weir and Cockerham 1984; Avise *et al.* 1988; Excoffier *et al.* 1992; Weir 1996).

Following Slatkin (1993), we assessed differentiation by distance by plotting pairwise log (Nm) values against log (geographic distance), applying Mantel's permutation test (Mantel 1967). The significance of this correlation was assessed by generating a probability distribution with 20 000 permutations using *Xlstat Microsoft® Excel 2010/XLSTAT®* (version 2014.1.08; Addinsoft, Brooklyn, NY). A significant association between geographic and genetic distance indicates population genetic structure and a limited dispersal of individuals (Slatkin and Maddison 1989; Slatkin 1993).

Genetic Diversity and Effective Population Size Change Analyses

We used *DnaSP* v. 5 (Librado and Rozas 2009) to calculate nucleotide diversity (π), defined as the average number of differences per site between any 2 sequences chosen randomly from the sample population. We also used this software to calculate haplotype diversity, which is a measure of the uniqueness of a particular haplotype in a given population $H = \frac{n-1}{n} \sum p_i^2$. We then used estimates

of θ based on the nucleotide diversity (Tajima 1996; Librado and Rozas 2009) and estimated female effective population size from the expression $\theta = 2Nef\mu$, where θ is a measure of haplotype diversity, Nef is the female effective population size, and μ is the mutation rate (González *et al.* 1998; Leonard *et al.* 2005). For estimating

the mutation rate, we used the maximum likelihood method based on the *Kimura 2-parameter* model to reconstruct the phylogenetic relationships among the maned wolf D-loop haplotypes in relation to the other Canidae species using *Mega 5* software (Tamura *et al.* 2011). The genbank database was queried for the following Canidae sequences: crab eating fox (*C. thous* gil125522681), pampas fox (*Lycalopex gymnocercus* gil125522709), hoary fox (*Lycalopex vetulus* gil125522705), domestic dog (*Canis lupus familiaris* gil189494080), gray wolf (*Canis lupus lupus* gil2467312), and coyote (*Canis latrans* gil301087587). For calibrating the maximum likelihood tree, we used the estimated divergence time between coyotes and gray wolves of 2 million years (Nowak 2003; Leonard *et al.* 2005). We also assumed the average maned wolf generation time to be 3 years (Paula *et al.* 2008; Emmons 2012).

We used *DnaSP* to test for pairwise differences among populations using a distance method (F_{ST}). We followed Wright's guidelines to interpret our F_{ST} values, where a value of 0–0.05 indicates little differentiation, 0.05–0.15 moderate differentiation, 0.15–0.25 great differentiation, and >0.25 very great differentiation (Wright 1978).

We used the mismatch distribution approach to test for genetic signatures of historical population expansion within the maned wolf populations (Rogers and Harpending 1992; Wakeley and Hey 1997). The distribution of the observed pairwise nucleotide site differences (mismatch distribution) and the expected values (for no recombination) in growing and declining populations were estimated using *DnaSP* (Rogers and Harpending 1992). The model is based on 3 parameters: theta initial (theta before the population growth or decline), theta final (theta after the population growth or decline), and tau (τ , the date of the growth or decline measured in units of mutational time) $\tau = 2\mu t$; (t is the time in generations, and μ is the mutation rate per sequence and per generation; Rogers and Harpending 1992). By setting theta final as infinite, it is possible to estimate theta initial and tau from the data (Rogers 1995). *DnaSP* gives estimates that can be used to obtain the expected values. We compared the observed frequency distribution of pairwise nucleotide differences among individuals with expected distributions from a population expansion using the program *Arlequin 3.5* (Excoffier and Lischer 2010). Populations at demographic equilibrium or in decline should provide a multimodal distribution of pairwise differences, whereas populations that have experienced a sudden demographic expansion should display a star-shaped phylogeny and a unimodal distribution (Slatkin and Hudson 1991; Rogers and Harpending 1992; Harpending and Rogers 2000). However, recent changes in population size may not be detectable in mismatch distribution analyses due to threshold effects, time lags, or earlier demographic events that may mask the effects of recent events (Rogers and Harpending 1992; Harpending and Rogers 2000).

In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses as follows: Sampling locations and supplementary materials with Dryad and DNA sequences: Genbank accessions KM406516–KM406517; KM406502–KM406515.

Results

Levels of Genetic Diversity

Analysis of a 421 bp fragment of the informative polymorphic segment of the cytochrome *b* gene for 41 individuals revealed low levels of diversity with only 2 haplotypes and only 1 transition (Genbank accession numbers: KM406516–KM406517). We detected both

haplotypes in the Brazilian and Bolivian samples (Table 1). Haplotype I was found in all the sampled locations, while haplotype II was restricted to the east central and northern populations. Because of the low levels of genetic diversity in the cytochrome *b* gene, we elected to exclude it from further analyses.

We then sequenced a 460 bp fragment of the hypervariable D-loop region and identified a 234 bp fragment that contained the majority of the parsimony informative substitutions. For this reason, we designed a new set of primers that allowed us to amplify this highly polymorphic region in samples with low DNA quality and concentration, such as those isolated from carcasses, museum specimens, and feces. We successfully amplified 137 of the 144 total samples that were collected and found 14 polymorphic sites that defined 14 haplotypes (Genbank accession numbers: KM406502–KM406515). In general, we found moderate levels of haplotype and nucleotide diversity, and each of the 14 D-loop haplotypes were found to be closely differing by 1–8 bp substitutions (Figure 1; Table 2; Supplementary Table 2 online).

Several of the most frequent haplotypes were unique and had distal positions in a haplotype phylogenetic network. We found that only 3 haplotypes (B, C, and D) were shared among the studied locations. The Brazilian maned wolves ($n = 37$) had the greatest level of variation, with 6 unique haplotypes, while those from Argentina ($n = 25$), Bolivia ($n = 22$), and Uruguay ($n = 3$) had 2, 1, and 2 unique haplotypes, respectively.

Phylogenetic Relationships and Phylogeographic Analyses

Our phylogenetic reconstruction and calibration estimates based on sequences from other South American canid species showed that the crab eating fox (*C. thous*) diverged around 2 800 200 years ago, and foxes in the genus *Lycalopex* around 2 000 000 years ago, with the maned wolves the last lineage to diverge around 500 000 years ago (Figure 2). The mtDNA D-loop sequence divergence values obtained for the splitting of each of these nodes were 0.80 for the crab eating fox, 0.55 for *Lycalopex* foxes, and 0.15 for the maned wolf.

When we analyzed the variation in a geographic context with AMOVA, we found that a structure of 4 groups had the best values of delta Φ_{CT} . The 4 groups were as follows: Group 1, individuals from Corrientes and Santa Fe Provinces, Argentina; Group 2, individuals from Bolivia; Group 3, individuals from Brazilian locations (Emas, Araxá, Belo Horizonte, Franca, and Chapada do Sul); and Group 4, individuals from Uruguay (Tables 1–3).

The fixation indices of the grouping structure indicated above were Φ_{SC} : -0.00982 , $P = 0.3675$; Φ_{ST} : 0.29852 , $P = 0.00000$; and Φ_{CT} : 0.30534 , $P = 0.00000$. The pairwise computations of Φ_{ST} using AMOVA indicated that populations had a high level of differentiation (Wright 1978) and were significantly differentiated ($P < 0.001$) relative to a random collection of genotypes (Table 3).

Our analysis of gene flow among the 4 groups, based on the pairwise values of migrants derived from estimates of Φ_{ST} , had values of lesser than one per generation (Table 3). The exception was between the Brazilian and Bolivian populations, which had 2 migrants per generation. Furthermore, when we correlated geographic with genetic distance using the Mantel test and/or number of migrants, we did not find significant association ($P = 0.428$; see Supplementary Table 3 and Figure 1 online) between genetic differentiation and isolation by distance.

The unrooted neighbor-joining tree, based on the average Slatkin genetic distance among populations, placed the Brazilian group in the central position. Two branches with the Bolivian and the Uruguayan

Table 2. The descriptive parameters of the 234bp of the mtDNA *D-loop* fragment analyzed in the 4 geographic groups

Statistics	Group 1-Ar	Group 2-Bol	Group 3-Br	Group 4-Uy	Mean	SD
No. of individuals	25	22	35	3		
No. of haplotypes	4	3	9	3		
Haplotype diversity	0.700	0.550	0.807	1		
Nucleotide diversity	0.00466	0.01400	0.03437	0.01196		
No. of transitions	4	8	7	4	5.750	2.062
No. of transversions	0	1	1	0	0.500	0.577
No. of substitutions	4	9	8	4	6.250	2.630
No. of indels	0	0	3	1	1.000	1.414
Theta_pi	1.04000	3.12121	1.98319	3.33333	2.36943	1.06628
SD theta_pi	0.79821	1.87739	1.27577	2.89742	1.71220	0.90515
Tau	1.13281	7.72852	2.44531	4.97852	4.07129	2.91415
Tau qt 2.5%	0.15625	0.00000	0.33594	0.00000	0.12305	0.15990
Tau qt 97.5%	2.00977	62.72852	4.14062	89.97852	39.71436	43.75410
Fu's FS (P)	1.07941; <i>P</i> > 0.10	1.43136; <i>P</i> > 0.10	-5.65557; <i>P</i> < 0.02	NA		
Tajima's D(P)	-0.04932; <i>P</i> > 0.10	0.88559; <i>P</i> > 0.10	-2.68232; <i>P</i> < 0.001	NA		

Estimated demographic expansion parameters for each group and for the entire maned wolf range. *Tau* is the coalescent time of expansion. The mismatch test values with significant *P* values are shown in bold. NA, not available estimation because 4 individuals are required.

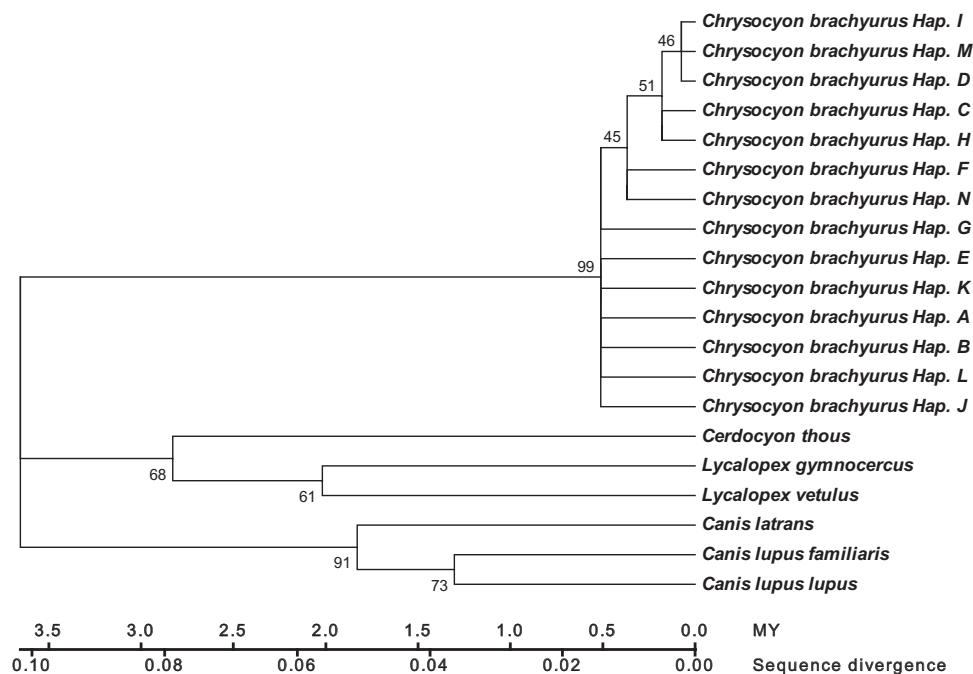


Figure 2. Phylogenetic reconstruction of mtDNA *D-loop* haplotypes by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the *Kimura 2-parameter* model, similar topology was obtained with Maximum Parsimony (Kimura 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). A discrete Gamma distribution was used to model evolutionary rate differences among sites (12 categories (+G, parameter = 0.9886)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 185 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

groups were more closely related, and a longer branch included the more distant Argentinian group (Supplementary Figure 2 online).

Demographic History

Our estimate of the effective number of reproductive females was 28246, assuming a rate of 2.7×10^{-8} substitutions per year for the *D-loop* region and based on the divergence time between coyotes and gray wolves of 2 million years (Nowak 2003; Leonard et al. 2005)

and the parameter $\theta = 2.36943$ (SD = 1.06628). This suggests that the total historical population size was about 90 000 individuals, 4 times larger than the current census estimates of about 23 600 individuals in the entire species range (Paula et al. 2008; Muir and Emmons 2012).

Mismatch Distribution Analyses

We compared the observed frequency distribution of pairwise nucleotide differences among individuals within our 4 maned wolf

structured groups (Rogers and Harpending 1992; Wakeley and Hey 1997). The estimated tau values among groups ranged from 7.72 for the Bolivian population to 1.13 for the Argentinian population (Table 2; Figure 3). However, the precision of this estimate was low because the confidence intervals for tau were large for a number of populations (Table 2). Mismatch distribution analyses indicated that the maned wolf populations recovered the genetic signal of a sudden expansion about 24 000 YBP. The Brazilian group had a detectable signal of sudden expansion in the mismatch distribution analyses (Table 2).

Table 3. Maned wolf sequence statistics for the 234bp fragment of mtDNA *D-loop*

	1-Ar	2-Bol	3-Br	5-Uy
1-Ar	<i>1.04000</i>	0.57622	1.35174	0.65921
2-Bol	0.86772	<i>3.120</i>	2.10433	1.32657
3-Br	0.36969	0.23761	<i>1.98319</i>	1.47850
5-Uy	0.75848	0.37691	0.33818	<i>3.33333</i>

The diagonal numbers in bold and italics correspond to the average number of pairwise differences (i.e. substitutions) within populations. Above the diagonal are the number of female migrants among populations. Below the diagonal are the Slatkin's linearized F_{ST} distance values among populations.

Discussion

Patterns of Molecular Evolution and Maned Wolf Population Differentiation

Our phylogenetic reconstruction and calibration resulted in a diversification time estimate for the maned wolf haplotypes recovered in our study of 504 659 YBP, which was similar to times estimated with a nuclear gene by Lindblad-Toh *et al.* (2005) and mitochondrial markers by Slater *et al.* (2009).

We found low to moderate levels of genetic variability in both mitochondrial markers; cytochrome *b* yielded 2 haplotypes and shallow divergences, and one of the haplotypes was restricted to the east-central and northern South American populations. This marker generally has an evolutionary rate 5 to 10 times slower than the *D-loop* region (Taberlet 1996). In addition, while the *D-loop* region had moderate levels of genetic diversity, the 14 haplotypes that we detected were closely related (Figure 1).

Pleistocene History

The demographic history of this species in South America was likely affected by Pleistocene climate changes. These involved dramatic habitat shifts that affected the expansion of the Amazonian forest and the reduction of the *Cerrado* (Mayle *et al.* 2000). A population expansion can occur if the geographic range of a population is initially restricted to

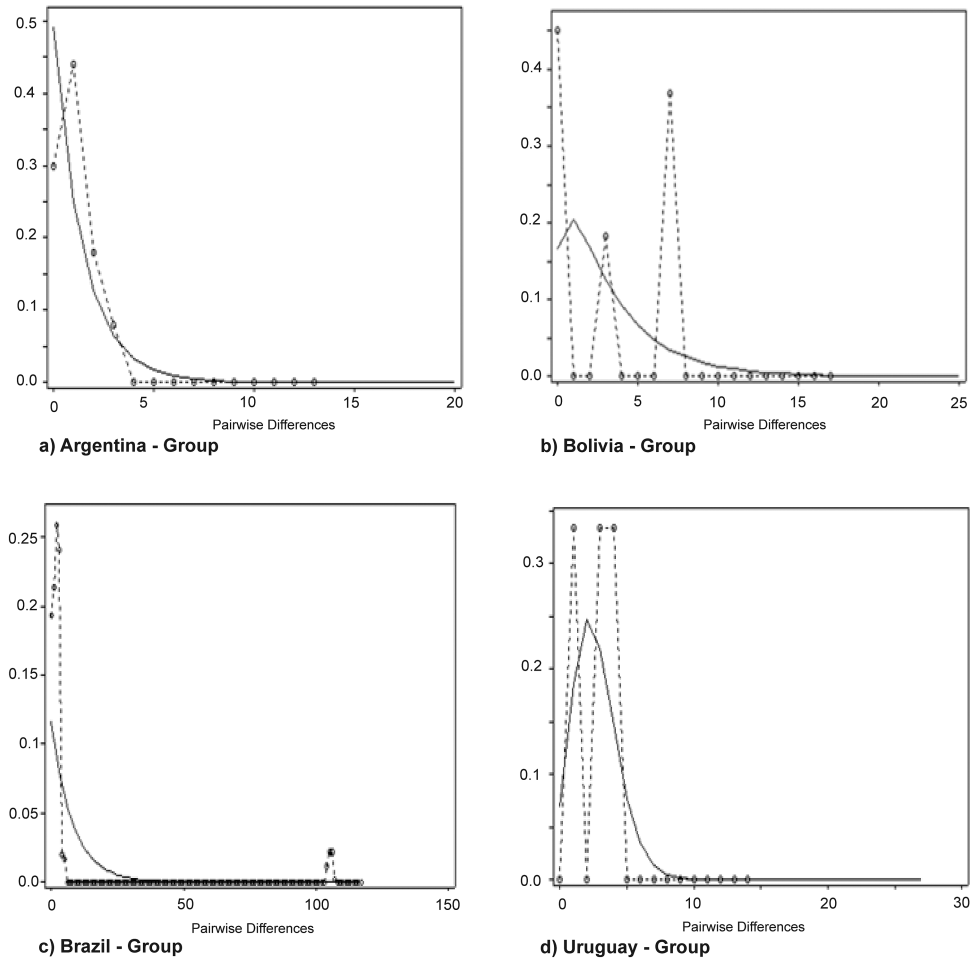


Figure 3. Mismatch distribution of pairwise differences of haplotypes for the maned wolf (*Chrysocyon brachyurus*) conservation genetic units a) Argentina-Group, b) Bolivia-Group, c) Brasil-Group, and d) Uruguay-Group. Shown are observed (dashed lines) and expected (solid lines) frequencies obtained under a model allowing for populations size change.

a small area, followed by a range increase over time and space. We recovered a significant genetic signature of demographic expansion in the mismatch analysis in maned wolves from populations in the Brazilian group (Table 2; Figure 3). This pattern was similar to that found in another endangered Neotropical mammal, the marsh deer (*Blastocerus dichotomus*). The marsh deer has a similar geographic range and has similar signatures of genetic diversity correlated with Pleistocene climatic events. Geochemical and palynologic studies (Salgado-Labouriau *et al.* 1997) suggest that central Brazil experienced a great increase in moisture between 32 000 and 20 000 YBP, which coincides with the timing of marsh deer population expansion in central Brazil between 25 000 and 28 000 YBP (Márquez *et al.* 2006). These results support our hypothesis that maned wolves experienced historical demographic changes that mirrored changes in their habitat availability.

In addition, the estimated coalescence date of maned wolf D-loop haplotypes places a lower boundary on the origin of extant lineages of about 24 000 YBP, suggesting that the inferred population expansion occurred around or before this time. It is important to recognize that these results are based on model inferences, and we should be cautious in extrapolating current patterns of genetic diversity for inferring past processes; however, the estimate of the timing of the coalescence of the haplotypic diversity coincides with the last glacial maximum period of the Pleistocene when maned wolf populations may have undergone severe bottlenecks. In periods of Pleistocene glaciation, the *Cerrado* covered most of what is now known as Amazonia, hugely expanding the range of maned wolves. Later, the *Cerrado* underwent contractions while the rainforest expanded in the southern margin of Amazonia during the late Quaternary (Mayle *et al.* 2000). Since then, the forest has continuously expanded and has progressively displaced savanna habitat, which is now at its smallest extent in 50 000 years (Mayle *et al.* 2000; Eizirik *et al.* 2001; Hundertmark *et al.* 2002; Márquez *et al.* 2006). This subsequent expansion of the Amazonian rainforest greatly reduced the suitable habitat available for maned wolves in this region of South America. Maned wolf populations would have experienced a population size reduction and fragmentation in their distribution resulting in moderately low levels of genetic subdivision as a result of the tendency of individuals to mate with geographically proximal rather than remote individuals.

Conservation and Management Implications

Large carnivores are difficult to manage because of conflicts with humans in livestock and agricultural areas (Treves 2009). However, under the *Convention on Biological Diversity* signatories are required to promote management guidelines to preserve the genetic diversity of wildlife within the “Aichi Targets” (<http://www.cbd.int/sp/>; Hoban *et al.* 2013). For endangered species such as the maned wolf, this will require an array of *in situ* and *ex situ* conservation initiatives such as population monitoring, habitat restoration, and management of captive stocks for future reintroduction programs (Rodden *et al.* 2008; Holland 2014).

At the *in situ* level, globally less than 4% of maned wolf population ranges overlap with an existing protected area (Vynne 2014). The main threats to the survival of maned wolf populations are directly linked to human activities and the conflicts that emerge from habitat conversion (Fonseca *et al.* 1994; Rodden *et al.* 2008; Emmons 2012; Vynne 2014). The Brazilian *Cerrado*, considered the heart of the species’ range, has lost native vegetation across approximately 50% of its extent, primarily due to habitat conversion for cattle ranching and agriculture. Recent biofuel production is accelerating the loss of the remaining *Cerrado* (Queirolo *et al.* 2011). The greatest range loss has been in the southern pampas of Argentina, Brazil, and Uruguay, which are among the most altered and degraded landscapes in South America (Bilenca and Miñaro 2004). Consequently, maned wolf populations in the southernmost portion of

their range are close to extinction (Queirolo *et al.* 2011). These populations are also the most genetically differentiated and face additional human impacts such as direct persecution and disease transmission from domestic animals (Deem *et al.* 2012; Muir and Emmons 2012).

Contrary to our initial prediction of no genetic differentiation, we detected 4 genetic units, which we feel do not reach the standard of evidence to be considered ESUs, but should for now be considered MUs, because they do not show reciprocal monophyly for mtDNA haplotypes, yet have diverged in haplotype frequency. Identifying MUs is significant for conservation because they represent populations connected by such low levels of gene flow that they are functionally independent and need to be carefully monitored for assessing possible population size fluctuations. These 4 MUs were: Group 1, Argentina-Corrientes and Argentina-Santa-Fe, including all the Argentinian populations and perhaps extending to Paraguay and western Brazil; Group 2, the Bolivian populations; Group 3, the Brazilian populations; and Group 4, the Uruguayan sampled population and likely including the southern Brazilian populations. Determining fine scale levels of genome-wide variation will further help clarify whether these should be considered ESUs as well as MUs but will require future genetic studies focused on increasing the number of individuals and populations sampled across their range, with more mitochondrial data as well as a large suite of microsatellite, SNPs, or other variable nuclear markers (Cronin *et al.* 2015). We recommend that until further studies are conducted, these MUs should be carefully monitored to detect how they evolve. Additionally, we recommend restoring connectivity among protected areas and private lands to ensure biological corridors. Conservation agencies should encourage compensation and conservation subsidies to guarantee the viability of these last remaining populations in private lands over a wide geographic area in South America.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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