

## MITOCHONDRIAL DNA VARIATION AND SYSTEMATICS OF THE GUANACO (*LAMA GUANICOE*, *ARTIODACTYLA*: CAMELIDAE)

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Guanacos (*Lama guanicoe*) are the most important native herbivorous species in the South American steppes and the dominant ungulate in a fauna rich in rodents but poor in large mammal species. Between 2 and 4 subspecies are usually recognized within *Lama guanicoe*, based on subtle morphological differences and geographic distribution. To evaluate whether molecular variation is consistent with the latter hypotheses, we analyzed the complete cytochrome-*b* and partial control region mitochondrial DNA sequences of *L. guanicoe* from 22 localities in Peru, Bolivia, Argentina, and Chile. Sequence analyses of both genes support the monophyly of the species but failed to distinguish the occurrence of subspecies along the geographic range. Despite that, the northernmost populations (Peru and northern Chile) showed some degree of genetic differentiation with respect to southern representatives from Argentina, Bolivia, and rest of Chile. Analysis of genetic diversity also showed a strong signal of past low population size and a recent population expansion.

Key words: camelids, cytochrome *b*, d-loop, demographic history, *Lama guanicoe*, phylogeography, subspecies

The guanaco (*Lama guanicoe*) is the largest wild artiodactyl in South America. Fossil remains of *L. guanicoe* have been found from Argentine Pleistocene deposits (Cabrera 1932; Menegaz et al. 1989) dated about 2 million years ago (Webb 1974). Fossil remains have also been found in Tarija, Bolivia (Hoffstetter 1986), in strata dated 97,000–73,000 years ago (MacFadden et al. 1983), but the species may not have spread into the high Andean puna ecosystem before the establishment of modern climatic conditions 12,000–9,000 years ago (Hoffstetter 1986). Before European

settlement in South America, guanacos were found along an altitudinal gradient from the Pacific coast to the high Andes, and from northern Peru to Tierra del Fuego and Isla Navarino. To the east, they extended to the Paraguayan Chaco and across the pampas as far as the Atlantic coast of Argentina (Tonni and Politis 1980; Torres 1985). Neither fossil nor recent guanaco remains have been found in the Andes of Ecuador and Colombia.

Four subspecies of guanacos have been traditionally recognized (Wheeler 1995) based on their distribution, size, and coloration (Fig. 1): *L. guanicoe guanicoe* Müller, 1776; *L. g. huanacus* Molina, 1782; *L. g. cacsilensis* Lönnberg, 1913; and *L. g. voglii* Krumbiegel, 1944. A recent review by González et al. (2006) provides extensive information on the morphology and biogeography of the 4 subspecies. Scattered, relict populations of the smallest guanaco, *L. g. cacsilensis*, are

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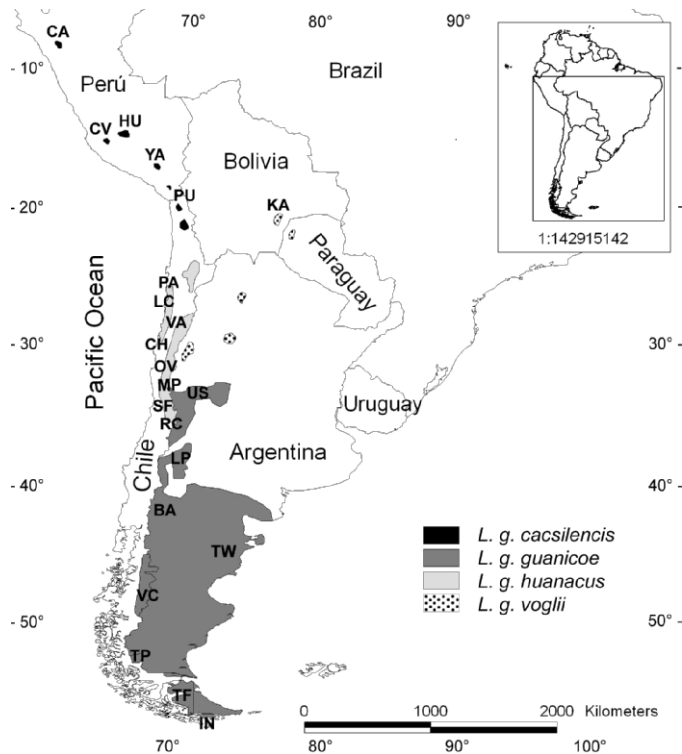


FIG. 1.—Geographic distribution of subspecies of *Lama guanicoe* in South America (based on González et al. 2006). Numbers correspond to sampling localities listed in Table 1.

found both at high elevation and along the western slope of the Andes to the coast in southern Peru (Hoces 1992) as well as in the Andean foothills of northern Chile (Torres 1985). To the south, *L. g. huanacus* is restricted to Chile on the western slope of the Andes between 22°S and 38°S (Cunazza 1992). Small populations of *L. g. voglii* are found in southeastern Bolivia (Villalba 1992), northwestern Paraguay (Torres 1985), and on the eastern slopes of the Argentinean Andes between 21°S and 32°S (Puig 1992). Finally, the largest guanaco, *L. g. guanicoe* is found mainly on the eastern slope of the Andes, south of 35°S, and its distribution extends throughout Patagonia to Tierra del Fuego and Isla Navarino (Cunazza 1992; González et al. 2006; Puig 1992; Wheeler 1995).

Ponce del Prado and Otte (1984) postulated the occurrence of an undescribed coastal subspecies in Peru, and Franklin (1982) speculated on the possible occurrence of 2 different taxa separated by the salt plans of southern Bolivia and the crests of the Andean chain. The 1st subspecies, composed of the smaller, lighter-colored *L. g. cacsilensis*, is restricted to the northwestern slopes of the Andes between 8°S and 41°S, whereas the 2nd, larger and darker *L. g. guanicoe* is located on the south eastern side of the Andes between 18°S and 55°S (Franklin 1982, 1983; Raedeke 1979). Nonetheless, neither study presents sufficient evidence on geographic variation in morphology, behavior patterns, and genetic diversity to validate either hypothesis.

Based on the carrying capacity of the territory they occupied, Raedeke (1979) has estimated that pre-Hispanic guanaco

populations totaled 30–50 million individuals. Starting with the European conquest, indiscriminate hunting led to a rapid decline in numbers and by 1954, Dennler de la Tour warned of the imminent extinction of the Patagonian guanaco if hunting of yearling “chulengos” was not controlled and reserves established. In 1969, Grimwood reported that the Peruvian guanaco was on the edge of extinction, and in 1971 the Peruvian government responded by declaring it an endangered species. In 2006, it was determined that fewer than 3,000 guanacos survived in Peru, with an estimated time to extinction of <30 years (Bruford et al. 2006). In the early 1970s, the Chilean government undertook a conservation program for Patagonian populations (Bonacic et al. 1993). In 1974, the International Union for the Conservation of Nature and Natural Resources (IUCN) declared *L. guanicoe* a vulnerable species (Thornback and Jenkins 1982). Currently, populations of guanacos are estimated to number a little less than 1 million individuals (González et al. 2006) and they are currently protected in 22 reserves: 14 in Argentina, 4 in Chile, 3 in Peru, and 1 in Bolivia, leaving only the Paraguayan populations unprotected. The IUCN South American Camelid Specialist Group has urgently recommended increasing protection for the guanaco in general, but especially for *L. g. cacsilensis* (Torres 1985), a highly endangered subspecies, which is virtually unknown to science.

Previous molecular research on South American camelids has focused primarily on the origin of the domestic alpaca (*Lama pacos*) and llama (*Lama glama*). Stanley et al. (1994) and Palma et al. (2001) used cytochrome-*b* sequences, and Kadwell et al. (2001) used both mitochondrial and nuclear microsatellite markers to show that llamas constitute the sister species to guanacos, and alpacas to vicuñas (*Vicugna vicugna*), leading to the reclassification of the alpaca as *Vicugna pacos* (Kadwell et al. 2001). However, these studies did not examine whether molecular data are consistent with the proposed subspecies of *L. guanicoe* (Wheeler 1995), or with only 2 subspecies as currently accepted by several authors: the smaller, lighter-colored subspecies (*L. g. cacsilensis*), and the larger, darker animals (*L. g. guanicoe*) found at the southern limits of their distribution (Cabrera 1961; Grubb 2005). In the present study, we have used partial sequences of the mitochondrial DNA (mtDNA) control region and the complete cytochrome-*b* gene to examine the phylogeographic structure of *L. guanicoe* throughout its geographic range in order to evaluate the taxonomic validity of the 4 historically described subspecies.

## MATERIALS AND METHODS

**Sample collection.**—Material suitable for DNA analysis was collected from guanacos of all 4 nominal subspecies throughout their distributional range. DNA was extracted from blood samples taken from 97 wild-caught adults following chemical immobilization (Sarno et al. 1996) at 14 localities. DNA was extracted from muscle samples from 6 dead animals found at 4 localities, from bone marrow samples from the epiphysis of 2 carcasses of young animals at Reserva Nacional Rio Cipreses,

**TABLE 1.**—South American subspecies of *Lama guanicoe* sampled for analyses of mitochondrial DNA. Capital letters indicate the type of sample (B, blood; F, fecal; M, muscle; BM, bone marrow; and L, liver).

Taxon	Sample type	Locality, country (locality abbreviation)	Geographic location	No. cytochrome <i>b</i> analyzed (total = 43)	No. control region analyzed (total = 176)
<i>L. g. cacsilensis</i>	F	Calipuy National Reserve, Peru (CA)	08°27'S, 78°16'W	0	2
	F	Chavin, Peru (CV)	14°05'S, 75°22'W	0	3
	B	Huallhua, Peru (HU)	14°39'S, 74°24'W	2	10
	F	Yarabamba, Peru (YA)	16°04'S, 71°24'W	0	6
	B, F	Putre, Chile (PU)	18°20'S, 69°35'W	3	18
<i>L. g. voglii</i>	F	Kaa-Iya National Park, Bolivia (KA)	20°15'S, 62°26'W	3	20
<i>L. g. huanacus</i>	F	Paposo and Pan de Azucar National Park, Chile (PA)	26°06'S, 70°38'W	4	6
	B, M	Llanos de Challe National Park, Chile (LC)	28°10'S, 71°05'W	2	3
	B	Huasco, Chile (VA)	28°31'S, 70°56'W	2	0
	B	Vallenar, Alto del Carmen, Chile (VA)	28°31'S, 70°56'W	2	4
	F	Llanos Pueblo de Choros, Chile (CH)	29°14'S, 71°20'W	2	4
	B	Ovalle, Chile (OV)	30°35'S, 70°11'W	2	4
	B	Illapel, Minera Pelambres, Chile (MP)	31°71'S, 70°51'W	2	5
	B	Putendo-San Felipe, Chile (SF)	32°30'S, 70°25'W	2	5
	F, BM	Río Cipreses National Reserve, Chile (RC)	34°30'S, 70°20'W	1	6
	B	Uspallata, Argentina (US)	32°43'S, 69°13'W	2	5
	B	La Payunia Reserve, Argentina (LP)	36°10'S, 68°49'W	2	9
	B	Bariloche, Argentina (BA)	41°09'S, 71°19'W	2	5
	B, M	Trelew-Sector Bajada del Diablo, Argentina (TW)	42°49'S, 67°42'W	2	4
	L	Valle Chacabuco, Chile (VC)	47°36'S, 72°27'W	2	18
	B	Torres del Paine National Park, Chile (TP)	51°03'S, 72°55'W	2	19
B	Porvenir, Tierra del Fuego, Chile (TF)	53°18'S, 70°11'W	2	16	
F, M	Isla Navarino, Chile (IN)	67°15'S, 55°05'W	2	4	

Chile, and from 53 fresh fecal samples from different dung piles obtained from 8 localities. DNA also was obtained opportunistically from liver samples of 18 adult males slaughtered in Valle Chacabuco (Valchac Ltd.), Chile, under a sustainable use program authorized by the Chilean government. That population belongs to *L. g. guanicoe*, which is not classified as endangered by the IUCN (González et al. 2006; IUCN 2006). Locations of sites sampled and the geographic position of individuals collected at each site are given in Fig. 1 and Table 1. All samples were stored at  $-70^{\circ}\text{C}$  in the Laboratorio de Genómica Evolutiva, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile, and at CONOPA in Lima, Peru. We followed guidelines approved by the American Society of Mammalogists during the collection and handling of animals used in this work (Gannon et al. 2007).

**DNA extraction, polymerase chain reaction amplification, and sequencing.**—Total genomic DNA was extracted from blood and bone marrow using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) following the manufacturer's instructions. DNA from liver and muscle samples was purified using proteinase K digestion, phenol, phenol–chloroform, and ethanol precipitation (Sambrook et al. 1989). DNA was extracted by using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, California) in another laboratory.

The mitochondrial cytochrome-*b* gene ( $\approx 1,200$  base pairs [bp]) and  $\approx 600$  bp of the control region) were amplified via the polymerase chain reaction (Saiki et al. 1988) using primers LGlu ARTIO: 5' TCT AAC CAC GAC TAA TGA CAT G 3'–

HThr ARTIO: 5' TCC TTT TTC GGC TTA CAA GAC C 3', and LThr ARTIO: 5' GGT CTT GTA AGC CGA AAA AGG A 3'–HLOOP550G: 5' ATG GAC TGA ATA GCA CCT TAT G 3', respectively (Marín et al. 2007b). These primers were designed by aligning consensus sequences obtained from several artiodactyl taxa available in GenBank.

Polymerase chain reactions containing 10 ng of DNA were amplified in a total volume of 50  $\mu\text{l}$ , using a 5-min denaturing step at  $95^{\circ}\text{C}$ , followed by 30–35 cycles of  $95^{\circ}\text{C}$  for 45 s,  $55^{\circ}\text{C}$ – $58^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 45 s, and a final extension at  $72^{\circ}\text{C}$  for 5 min. Polymerase chain reaction products were purified with the QIAquick PCR Purification Kit (Quiagen). A total of 1,140 bp of cytochrome *b* and 410 bp of control region (5' region) were sequenced using an ABI-377 and an ABI-3100 semi-automated DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, California). Polymerase chain reaction products were sequenced in both directions at least twice to ensure sequence fidelity. Sequencing primers for the cytochrome-*b* genes were: LGlu ARTIO, L400: 5' GGG CTA TGT ACT CCC ATG AGG 3', LBE-02: 5' CTC CGT AGA TAA AGC CAC CC 3', and the HThr ARTIO primers. Sequencing and polymerase chain reaction primers for the d-loop were LThr ARTIO: 5' TCC TTT TTC GGC TTA CAA GAC C 3', Hloop550G: 5' ATG GAC TGA ATA GCA CCT TAT G 3', Lloop0007G: 5' GTA CTA AAA GAA AAT ATC ATG TC 3', H362: 5' GGT TTC ACG CGG CAT GGT GAT T 3', and H15998: 5' CCA GCT TCA ATT GAT TTG ACT GCG 3' (Marín et al. 2007b). Sequences were deposited in GenBank with accession numbers AY535173–AY535256 and AY856157–AY856269.

*Genetic variation.*—Sequences were aligned using the programs Clustal X 1.8 (Thompson et al. 1997) and SEQUENCHER (GeneCodes Corporation, Ann Arbor, Michigan), and by eye. The number of nucleotide substitutions was obtained using the program MEGA3 (Kumar et al. 2004), and the transition–transversion ratios were estimated in PAUP\* 4.0b8a (Swofford 2002). Levels of genetic variation within subspecies of *L. guanicoe* were measured in terms of number of polymorphic sites, nucleotide diversity ( $\pi$  per nucleotide site, i.e., the probability that 2 randomly chosen homologous nucleotides are different—Nei 1987), haplotype diversity ( $h$ ), and number of private haplotypes using ARLEQUIN 2.0 (Schneider et al. 2000).

*Geographic structure of genetic diversity.*—Intraspecific d-loop gene genealogies were inferred using the method of statistical parsimony (Templeton 2001) implemented in the program TCS (Clement 2000). This program allows the user to estimate phylogenetic relationships when there are low levels of divergence and provides a 95% plausible set for all haplotype connections. The null distribution to test significance of the variance components and the pairwise  $F$ -statistic equivalents ( $F_{ST}$ ) were constructed from 10,000 permutations of the data.

Genetic differentiation between subspecies was expressed as the mean number of pairwise differences per site ( $d_{xy}$ ) and as pairwise fixation indices ( $\Phi_{ST}$ s), taking into account the variation in haplotype frequencies among subspecies and the genetic distance based on nucleotide variation. Distribution of genetic variance of population structure was obtained using analysis of molecular variance (AMOVA—Excoffier et al. 1992; Weir and Cockerham 1984) and spatial analysis of molecular variance (SAMOVA—Dupanloup et al. 2002). AMOVAs were conducted in the program ARLEQUIN (Schneider et al. 2000) for testing our hypothesis of currently recognized subspecies. Different population clustering designs based on taxonomic and geographic criteria were used for maximizing the “among-group” component and were applied to 4 proposed subspecies (*L. g. cacsilensis*, *L. g. voglii*, *L. g. huanacus*, and *L. g. guanicoe*), 3 lineages, and combinations of 2 subspecies. Additionally, groups of populations were defined under criteria of geographical homogeneity and maximal differentiation from each other using the program SAMOVA version 1.0 (Dupanloup et al. 2002) in order to look for new genetic patterns. We tested between 2 and 10 groups using the entire data set. Statistical confidence in variance estimates was determined by comparing the observed  $\Phi$  statistics against a distribution of estimates generated from 10,000 permutations of data (Dupanloup et al. 2002; Excoffier et al. 1992). To statistically test the existence of a pattern of isolation by distance, the correlation between geographic distances and mean genetic distances for each pair of populations was computed using Mantel test included in the program ARLEQUIN and performing 10,000 permutations.

*Phylogenetic and phylogeographic analyses.*—We searched for the model of DNA substitution that best fit the data using a hierarchical likelihood ratio test as implemented in the program MODELTEST 3.7 (Posada and Crandall 1998). For

cytochrome-*b* sequences the model that best fit the data was the HKY+G model ( $-\ln L = 4,000.9604$ ,  $G = 0.578$ ), whereas for control region sequences it was the K8luf+I+G model ( $-\ln L = 890.8987$ ,  $I = 0.595$ ,  $G = 0.715$ ). The individual and combined phylogenetic analyses were performed through PAUP\* 4.0b10 (Swofford 2002) using maximum parsimony with the heuristic search option, neighbor joining (Saitou and Nei 1987), and maximum likelihood. The best-fit model for maximum likelihood was obtained through the Akaike information criterion (AIC—Akaike 1974) using the program MODELTEST (Posada and Crandall 1998). In addition, we performed a Bayesian analysis using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) according to the model proposed by the program MrModeltest (Nylander 2004), with the evaluation of at least 1 million generations and a “burn-in” region of 2,000 trees. The confidence values for each node for the first 3 analyses were measured by a nonparametric bootstrap (Felsenstein 1985) with 1,000 replications (except for the maximum-likelihood analysis, where only 500 replications were considered). For the Bayesian analysis the posterior probability of each clade on the 50% majority-rule consensus tree was calculated. To test for congruence between the 3 data partitions, cytochrome *b*, hypervariable domain I, and the conserved domain of the control region, 1,000 replicates of the partition homogeneity test (PHT,  $P$ -value = 0.074—Farris et al. 1994), as contained in PAUP\* 4.0b10, were performed. The model that best fit the data for the total-evidence data set was K8luf+I+G ( $-\ln L = 3,696.1836$ ,  $AIC = 7,406.3672$ ;  $I = 0.634$ ,  $G = 0.727$ ).

*Genetic inference of demographic history.*—Tajima’s  $D$ -test (Tajima 1989) and Fu’s  $F$ -test (Fu 1997) tests were performed to detect departures from neutrality or from a Wright–Fisher population model. The existence of historical demographic expansions was investigated through examination of the frequency distribution of pairwise differences between control region sequences (mismatch distribution) within species and subspecies (Rogers and Harpending 1992; Slatkin and Hudson 1991). We performed this analysis by means of a least-squares approach (Schneider and Excoffier 1999) implemented in ARLEQUIN. For distributions that did not differ significantly ( $P > 0.05$ ) from the expectations of the sudden-expansion model, we estimated the parameter  $\tau$ , the time since expansion expressed in mutational time units (Slatkin and Hudson 1991).

## RESULTS

*Genetic variation.*—We obtained sequence data for the complete mitochondrial cytochrome-*b* gene (1,140 bp) from 43 specimens from 20 localities along the range of the species. Average base composition was A = 29.1%, C = 27.7%, G = 14.2%, and T = 28.9%. Alignment of the cytochrome-*b* sequences exhibited 40 (3.51%) sites parsimoniously informative, 67 (5.87%) polymorphic sites, and 21 haplotypes ( $h = 0.956$ ). The observed transition–transversion ratio was 7.31.

Thirty-eight variable positions (7.41%) from 513 nucleotides and 38 haplotypes ( $h = 0.89$ ) were identified in 176 partial sequences of the 5′ end of the control region in *L. g. cacsilensis*,

TABLE 2.—Alignment of 38 control region haplotypes showing variable sites only. Subspecies, locality, and sample size for each haplotype also are included in the table.

Table with columns for Haplotype, sequence positions (1-30), Taxa, Localities, and sample size (n). Haplotypes H1-H38 are listed with their corresponding nucleotide sequences and associated data.

a Putative subspecies: c = Lama guanicoe cacsilensis; v = L. g. voglii; h = L. g. huanacus; g = L. g. guanicoe. b Localities are given in Table 1.

L. g. voglii, L. g. huanacus, and L. g. guanicoe. The distribution of haplotypes within 22 localities is given in Table 2. Average base compositions were A = 28.0%, C = 26.5%, G = 17.9%, and T = 27.6%. Among variable sites, only 8 (1.56%) were phylogenetically informative. Inter- and intraspecific variation.—The minimal spanning network obtained from control region sequences showed the relationship among 38 haplotypes connected through a maximum of 12 mutational steps (Fig. 2). The network did not exhibit a clear genetic partition among all subspecies,

showing 5 haplotypes shared by 2 of the 4 taxa at least. Both predominant haplotypes (H37 = 29 and H38 = 41) were shared among samples covering a wide distribution (Table 2), identified as L. g. voglii, L. g. huanacus, and L. g. guanicoe. Haplotype 35 was found in specimens from coastal populations of north-central and southern Chile (Pan de Azúcar National Park, Llanos de Challe near Puerto Choros, and Tierra del Fuego; Table 1). Among 12 haplotypes found in L. g. cacsilensis, only 1 (H12) was shared with L. g. huanacus. This haplotype was exclusively found in Putre and Pan de

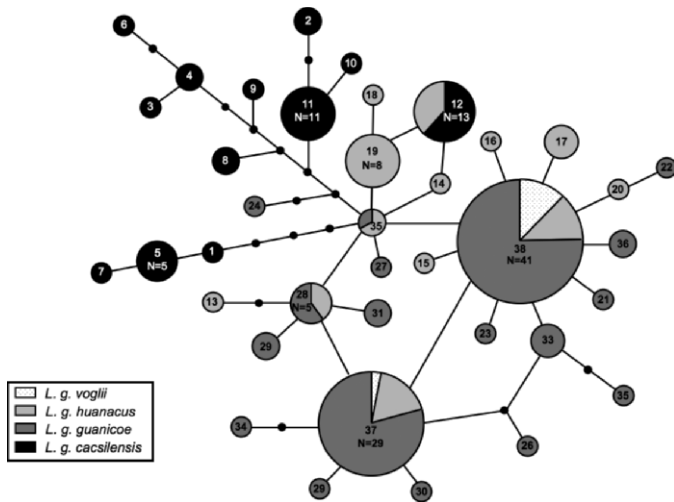


FIG. 2.—Minimum spanning network for *Lama guanicoe* representing the relationships between 38 control region haplotypes. Circle sizes correspond to haplotype frequencies.

Azúcar, corresponding to the southern and northern distributional boundaries for *L. g. cacsilensis* and *L. g. huanacus*, respectively. All other haplotypes of *L. g. cacsilensis* were restricted to this taxon and grouped in 2 closed clusters.

Fixation indices ( $\Phi_{ST}$ s) revealed a high degree of genetic structuring. Pairwise  $\Phi_{ST}$  comparisons showed statistical significance ( $0.45156, P < 0.001$ ) between *L. g. cacsilensis* and each of the remaining subspecies, revealing a strong differentiation between the northernmost taxon and the others. In contrast, the differentiation between *L. g. huanacus* and *L. g. guanicoe* ( $0.27816, P < 0.001$ ) showed a lower level of genetic structure (Table 3).

Nevertheless, AMOVA and SAMOVA of the control region of *L. guanicoe* did not show a clear structuring pattern. Populations grouped by any combinations of subspecies showed significant low levels of structuring in AMOVA; nevertheless maximum difference between groups was reached only when *L. g. cacsilensis* was compared with the remaining 3 subspecies (Table 4). In fact, the highest differentiation indices between groups ( $\Phi_{CT}$ ) was observed when populations from Calipuy, Chavin, Huallhua, Yarabamba, and Putre (described as *L. g. cacsilensis*) were separated from the other samples of guanacos designated as *L. g. huanacus*, *L. g. voglii*, and *L. g. guanicoe* (model B,  $\Phi_{CT} = 38.91\%$ ). Lower indices also were observed in the other partition models, particularly when sample localities were grouped according to the 4 subspecific taxa (model F,  $\Phi_{CT} = 28.37\%$ ), or their geographic location on

either northern against southern taxa (model C,  $\Phi_{CT} = 29.61\%$ ) or the eastern against western side of the Andes (model D,  $\Phi_{CT} = 21.42\%$ ). On the other hand, nonsupervised clustering by SAMOVA grouped populations significantly but did not follow the subspecific geographical pattern. Instead, when the number of groups was increased, partitioning was executed by extracting populations in a north-to-south direction (Chavin followed by Arequipa, Putre, Pan de Azucar, Calipuy, Huallhua, Yarabamba, and so on), and leaving the main Patagonian and Bolivian populations intact (data not shown).

Clustering by SAMOVA was consistent with AMOVA when structuring of the northernmost population of guanacos is taken into account. A Mantel test revealed a slight, but significant, correlation between genetic and geographic distances when all populations were considered ( $r = 0.2692, P = 0.0194$ ), indicating a pattern of isolation by distance. However, values of the correlation coefficient were nonsignificant ( $r = 0.0502, P = 0.3351$ ) when the populations of *L. g. cacsilensis* were excluded from the analysis, indicating a lack of structuring in the Patagonian groups.

*Phylogenetic and phylogeographic analysis.*—Maximum-likelihood analysis of the combined data set for both mtDNA genes revealed that all populations of guanacos from Peru, Bolivia, Argentina, and Chile constitute a monophyletic group (Fig. 3). However, phylogenetic analysis using parsimony, distance, and Bayesian analyses (trees not shown) did not reveal clusters corresponding to the 4 nominal subspecies of guanaco. In contrast, the maximum-likelihood phylogenetic tree indicated the existence of shared haplotypes among the subspecies *L. g. voglii*, *L. g. huanacus*, and *L. g. guanicoe*. Basal branches corresponded mostly to individuals from the northernmost populations, whereas central and Patagonian populations occupied the most derived position in the phylogenetic tree. Finally, at the base of the tree we recovered a clade including individuals from different localities in Chile, although the most basal haplotypes were those from the northern Chilean localities of Putre and Pan de Azucar National Park.

*Historical demography.*—Tajima's *D*- and Fu's *F*-values were negative and statistically significant for *L. guanicoe* and for the Patagonian subspecies *L. g. guanicoe* (Table 4). Furthermore, Fu's test, which is considered a powerful test to detect past population expansion (Fu 1997; Ramos-Onsins and Rozas 2002), was negative and significant for the subspecies *L. g. huanacus*, indicating an excess of lower frequency haplotypes than predicted under the Wright-Fisher model ( $F_s = -5.424; P < 0.01$ ).

The mismatch distribution was unimodal when *L. g. voglii*, *L. g. huanacus*, and *L. g. guanicoe* were grouped, reflecting the reduced number of mutational steps in these groups that may be attributed to a recent expansion event (Fig. 4A). Assuming the

TABLE 3.— $F_{ST}$ -values obtained from pairwise comparisons of control region haplotypes between subspecies of *Lama guanicoe* ( $P < 0.05$ ).

Taxon	<i>L. g. cacsilensis</i>	<i>L. g. voglii</i>	<i>L. g. huanacus</i>	<i>L. g. guanicoe</i>
<i>L. g. cacsilensis</i>	—	0.00079 + -0.0003	0.00000 + -0.0000	0.00000 + -0.0000
<i>L. g. voglii</i>	0.30050	—	0.04346 + -0.0021	0.51440 + -0.0051
<i>L. g. huanacus</i>	0.27816	0.13339	—	0.00000 + -0.0000
<i>L. g. guanicoe</i>	0.45156	-0.01389	0.17542	—

**TABLE 4.**—Analysis of 6 models (A–F) for molecular variance at 22 localities for *Lama guanicoe*. Variance components: AG = among groups; AP = among populations within groups; WP within populations. All  $\Phi$  values are significant at  $P < 0.001$  (10,000 random permutations of sequences among populations).

Model	Taxa <sup>a</sup>	Localities <sup>b</sup>	Variance component <sup>c</sup>	% variance
(A) 1 group; 22 localities	(1) c, v, h, g	(1) CA, CV, HU, YA, PU, KA, PA, LC, VA, CH, OV, MP, SF, RC, US, LP, TW, BA, VC, TP, TF, IN	$\Phi_{ST} = 0.46074$	AP = 46.07 WP = 53.93
(B) 2 groups	(1) c (2) h, v, g	(1) CA, CV, HU, YA, PU (2) KA, PA, LC, VA, CH, OV, MP, SF, RC, US, LP, TW, BA, VC, TP, TF, IN	$\Phi_{CT} = 0.38914$ $\Phi_{SC} = 0.32595$ $\Phi_{ST} = 0.58825$	AG = 38.91 AP = 19.91 WP = 41.17
(C) 2 groups	(1) c, v (2) h, g	(1) CA, CV, HU, YA, PU, KA (2) PA, LC, VA, CH, OV, MP, SF, RC, US, LP, TW, BA, VC, TP, TF, IN	$\Phi_{CT} = 0.29608$ $\Phi_{SC} = 0.36307$ $\Phi_{ST} = 0.55165$	AG = 29.61 AP = 25.56 WP = 44.84
(D) 2 groups	(1) c, h (2) v, g	(1) CA, CV, HU, YA, PU, PA, LC, VA, CH, OV, MP, SF, RC (2) KA, US, LP, TW, BA, VC, TP, TF, IN	$\Phi_{CT} = 0.21421$ $\Phi_{SC} = 0.38215$ $\Phi_{ST} = 0.51450$	AG = 21.42 AP = 30.03 WP = 48.55
(E) 3 groups	(1) c (2) hg (3) v	(1) CA, CV, HU, YA, PU (2) PA, LC, VA, CH, OV, MP, SF, RC (3) KA, US, LP, TW, BA, VC, TP, TF, IN	$\Phi_{CT} = 0.31220$ $\Phi_{SC} = 0.30100$ $\Phi_{ST} = 0.51922$	AG = 31.22 AP = 20.70 WP = 48.08
(F) 4 groups	(1) c (2) h (3) v (4) g	(1) CA, CV, HU, YA, PU (2) PA, LC, VA, CH, OV, MP, SF, RC (3) KA (4) US, LP, TW, BA, VC, TP, TF, IN	$\Phi_{CT} = 0.28368$ $\Phi_{SC} = 0.31299$ $\Phi_{ST} = 0.50788$	AG = 28.37 AP = 22.42 WP = 49.21

<sup>a</sup> Putative subspecies: c = *Lama guanicoe cacsilensis*; v = *L. g. voglii*; h = *L. g. huanacus*; g = *L. g. guanicoe*.

<sup>b</sup> Localities are given in Table 1.

<sup>c</sup>  $\Phi_{ST}$  = fixation index;  $\Phi_{CT}$  = between-group fixation index;  $\Phi_{SC}$  = between-localities/within groups fixation index.

instantaneous stepwise demographic expansion model described by Rogers and Harpending (1992), time since expansion was estimated to  $\tau = 1.814$ . In the case of *L. g. cacsilensis*, mismatch distribution presented a multimodal pattern that did not indicate a past population expansion. When observed separately, mismatch distributions for *L. g. huanacus* and *L. g. guanicoe* both suggested past demographic expansion (Figs. 4C and 4D), even if the comparison of  $\tau$ -values indicates that expansion of *L. g. guanicoe* may have occurred more recently than for *L. g. huanacus* ( $\tau = 1.102$  and  $\tau = 2.141$ , respectively).

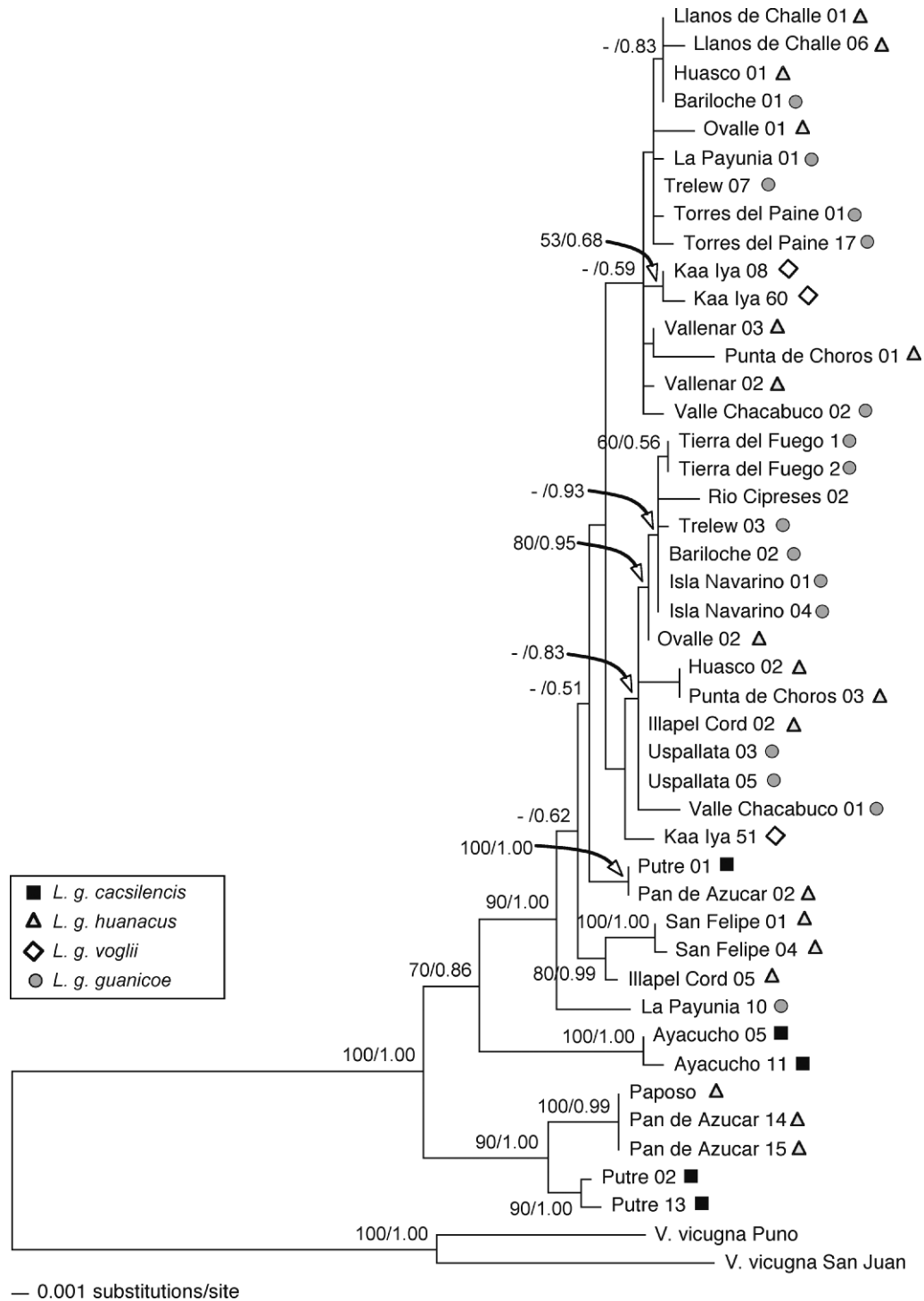
## DISCUSSION

**Genetic variation.**—Patterns of variation in mtDNA in guanacos have been shaped by a combination of historic and contemporary ecological factors. Based on our control region sequences (513 bp), the first 300 bp contiguous to the tRNA-Pro contained 25 of the 28 polymorphic sites. Therefore, this segment would possibly correspond to the hypervariable domain I of the control region, although significantly shorter than that proposed by Maté et al. (2004). This 300-bp fragment would be also the most useful in further population studies in this species.

Despite their extensive distribution along the southern part of South America, guanacos exhibit a low genetic diversity for the cytochrome-*b* gene ( $\pi = 0.01013$ ) and the control region ( $\pi = 0.00627$ ), when compared with other related species with

extensive distributions (see below). Analyses of partial sequences for the control region (513 bp) revealed only 38 haplotypes among 176 specimens from 23 localities covering most of the present species distribution. Other artiodactyls such as moose (*Alces alces*), reindeer (*Rangifer tarandus*), and antelope (*Kobus kob*) exhibit nucleotide diversities of 1.8%, 3.4%, and 4.6%, respectively, for the mitochondrial control region (Birungi and Arctander 2000; Gravlund et al. 1998; Hundertmark et al. 2002). These values are greater than the value of 0.48 observed in this study for guanacos. However, the amount of genetic diversity was not equally distributed in the species. Among the sampled localities, the northernmost subspecies *L. g. cacsilensis* reveals the highest diversity indices (Table 5). In contrast, *L. g. guanicoe* was characterized by much lower values, particularly the southern forms from Tierra de Fuego and Isla Navarino that shared a unique haplotype (38).

**Inter- and intraspecific variation.**—Despite the higher genetic diversity, populations of *L. g. cacsilensis* were not separated in the minimum-spanning tree obtained for the control region haplotypes. In contrast, the other 3 subspecies shared central and dominant haplotypes. Among them, the most abundant haplotypes 38 and 37 were found in *L. g. voglii*, *L. g. huanacus*, and *L. g. guanicoe*, distributed along more than 4,000 km, from the Bolivian Chaco to Isla Navarino in southern Chile (Table 2). The broad distribution of such common haplotypes could be interpreted as the result of colonization processes by northern ancestral haplotypes after the last glacial maximum. However, pairwise  $F_{ST}$ -values



**Fig. 3.**—Maximum-likelihood tree for *Lama guanicoe* constructed from the “total evidence” data set (K81uf+I+G model). Bootstrap values (500 replications) and Bayesian probabilities are shown above each node. Values are not given for nodes with <50% bootstrap or <0.50 posterior probability.

indicated a significant differentiation among *L. g. cacsilensis*, *L. g. huanacus*, and *L. g. guanicoe* (Table 3) because of the existence of numerous taxon-specific haplotypes. *L. g. voglii* appears to be less differentiated with respect to other subspecies, but this might be because we analyzed a single population. Even though all pairwise comparisons were highly

significant (except for *L. g. voglii*, see above), the highest  $F_{ST}$  values always involved the *L. g. cacsilensis* group, particularly when compared to the Patagonian *L. g. guanicoe*. Similarly, the AMOVA component was maximized when samples of *L. g. cacsilensis* were contrasted with the group formed by all remaining guanacos (Table 4). Nevertheless, this pattern was



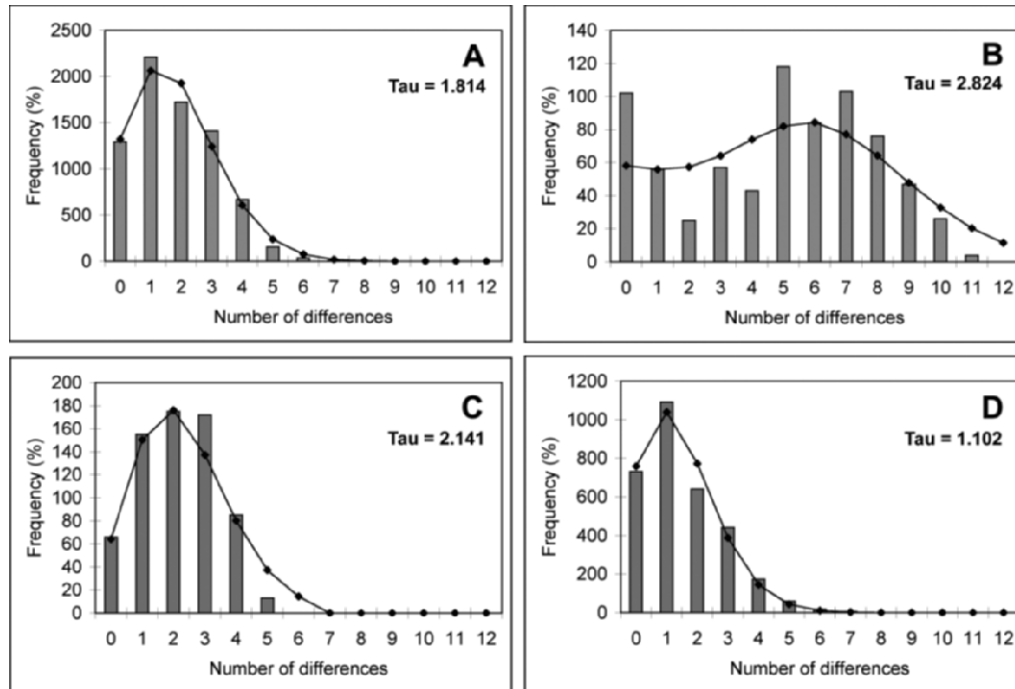


FIG. 4.—Mismatch distribution of pairwise nucleotide differences among control region sequences of A) *Lama guanicoe*, B) *L. g. cacsilensis*, C) *L. g. huanacus*, and D) *L. g. guanicoe*. Solid lines indicate expected distribution under Rogers' (1995) sudden population expansion model.

not supported by SAMOVA. These results partially agree with the suggestions of both Cabrera (1961) and Franklin (1982) that 2 different populations exist, separated by the salt plains of southern Bolivia, but are not different at the subspecific level such as found in *V. vicugna* inhabiting the high plateau at the same area (Marín et al. 2007a).

With a single mutational step from the ancestral haplotype, 8 haplotypes found in guanacos from central Chile and southern Argentina show a signature of population expansion. The subspecies *L. g. voglii*, *L. g. huanacus*, and *L. g. guanicoe* follow the same pattern of exchange present in other taxa from these xeric habitats during the recent past (Mares 1985). Finally, between 1 and 13 mutational steps separate haplotypes of *L. g. cacsilensis*, demonstrating a greater differentiation than the other subspecies.

**Phylogenetic relationships.**—Phylogenies recovered using both mtDNA markers showed *L. guanicoe* as a monophyletic group. Although the basal portion of the tree, representing northwestern populations, was more structured phylogenetically, we did not recover clustering of subspecies. Indeed, the basal part of the tree showed a group of haplotypes

representing *L. g. cacsilensis* and *L. g. huanacus* with representatives of both forms present in other branches of the phylogenetic tree (Fig. 3). We believe that this structuring might be due to fragmentation of populations (as shown in the distributional range of the species in Fig. 1) and isolation by distance. In fact, Mantel tests showed a low positive correlation when all populations were considered, but relationships between geographic distances and mean genetic distances disappeared across the distribution of guanacos when populations of *L. g. cacsilensis* were excluded from the analysis.

Historically, dispersal of populations of *L. guanicoe* may have occurred southward along the western and eastern slopes of the Andes. Postglacial events in the Quaternary may have further exacerbated this dispersal scenario after glacial retreat, producing subsequent differentiation of peripheral populations along both sides of the mountains. In fact, the southern dispersal of guanacos through the Chilean and Argentinean Andes may have occurred at this time (Rabassa and Clapperton 1990).

In contrast to the gene tree that did not support the occurrence of subspecific lineages in *L. guanicoe*, results of

TABLE 5.—Genetic diversity indices from control region sequences by subspecies of *Lama guanicoe*. Values in parentheses are standard deviation for nucleotide diversity ( $\pi$ ) and haplotype diversity ( $h$ ), and probability ( $P < \text{observed}$ ) for Tajima's  $D$ -test and Fu's  $F$ -test.

Taxon	$n$	No. polymorphic sites	No. haplotypes	No. private haplotypes	$\pi$	$h$	Tajima's $D$	Fu's $F$
<i>L. guanicoe</i>	176	38	38	—	0.0062 (0.0036)	0.8900 (0.0157)	-1.540 (0.029)	-10.417 (0.004)
<i>L. g. cacsilensis</i>	39	19	12	11	0.0094 (0.0052)	0.8623 (0.0332)	0.266 (0.658)	-0.265 (0.497)
<i>L. g. voglii</i>	20	1	2	0	0.0007 (0.0008)	0.3333 (0.2152)	0.722 (0.852)	0.976 (0.545)
<i>L. g. huanacus</i>	37	10	13	8	0.0041 (0.0026)	0.9009 (0.0241)	-0.321 (0.423)	-5.424 (0.005)
<i>L. g. guanicoe</i>	80	19	18	14	0.0029 (0.0020)	0.7687 (0.0353)	-1.779 (0.015)	-12.084 (0.000)

analyses of genetic structuring were significant when grouping was done by nominal subspecies. This structuring was maximized in a 2-lineage scenario when comparing northwestern (Peru and Chile) and southern-Patagonian populations (e.g., Bolivian Chaco, north-central Chile, Patagonian Argentina to Tierra del Fuego, and Navarino Island; AMOVA). As we stated above, the structuring of northern populations may be due to isolation of populations in an initial phase of genetic differentiation. The reverse is true regarding structuring, because the pattern of genetic structuring in south-central and Patagonian populations of *L. guanicoe* showed a homogeneous pattern of variation, probably due to marked gene flow. Furthermore, the connectivity among populations would be a balance between geographic distance and the impact of social structure in guanacos, within which polygamous dominant males normally control between 4 and 12 females (González et al. 2006), restricting the effective population size. The frequent occurrence of shared haplotypes among different taxa is another indication of connected populations, with no active geographic barriers.

Consequently, examination of our molecular data does not support the occurrence of 4 subspecies along the distributional range of *L. guanicoe* other than the structuring of northern populations. Maybe the use of a more variable molecular marker (e.g., microsatellites) could show a clearer pattern of genetic variation among populations to detect subspecies, and maybe it would be necessary to reassign the taxonomic status of some populations, at least in the northern distribution of guanacos.

*Historical demography.*—Currently, the demographic trajectories of populations of guanacos are very different along their geographic range. *L. g. cacsilensis* and *L. g. voglii*, located in the north, live in small, fragmented groups. Local populations from Huyallhua (Ayacucho, Perú) and Putre (Tarapaca, Chile) have been estimated to be <2,000 individuals (Cunazza 1992; D. Hoces, Darwin Initiative for the Survival of Species, pers. comm.). Furthermore, the population of the Bolivian Chaco comprises <200 individuals (Cuellar and Fuentes 2000). In contrast, large populations characterize the southern *L. g. huanacus* and particularly *L. g. guanicoe*.

In the Chilean and Argentinean Patagonia, population sizes have been estimated to be more than 500,000 animals (Amaya et al. 2001; Bas and González 2000; Cunazza and Benoit 2000; González et al. 2006). The large southern populations are associated with low genetic diversity, whereas the small, fragmented populations from northern Chile and Peru held the highest haplotype and nucleotide diversity (Frankham et al. 2002; Schmitt and Hewitt 2004). The low genetic diversity found in *L. g. guanicoe*, with populations located on both sides of the Strait of Magellan, may be explained by the “bridge” established during glacial cycles of the Pleistocene that connected Patagonia and Tierra del Fuego (Holling and Schilling 1981; McCulloch et al. 2000; Moreno et al. 2001). Guanacos possibly moved southward because of the low sea levels associated with glacial advances (Sarno et al. 2001). This suggests that, as in many other species, historical processes have strongly molded the spatial and temporal patterns of genetic diversity of guanacos, particularly the cyclic paleo-

climatic changes during the Pleistocene and Quaternary (Avice 2000; Templeton 2004). The series of paleoenvironmental changes in southern Patagonia and Tierra del Fuego also may have affected food resources, triggering local faunal extinctions, a process perhaps accelerated by Paleo-Indian hunters, who may have dealt the final blow to some species (e.g., megatherium, glyptodon, and saber tooth cat among others) while the generalist guanaco survived (Markgraf 1985).

The inference of a severe bottleneck or extinction of the southernmost populations of guanacos, followed by recolonization from refugial areas in northern Patagonia (Premoli et al. 2000; Smith et al. 2001), is strongly supported by genetic data. The low nucleotide diversity, low genetic structure, and starlike haplotype network of *L. g. guanicoe*, and to a lesser extent of *L. g. huanacus*, support this hypothesis. Signals indicating demographic expansion also were detected in the unimodal and leptokurtic mismatch distribution pattern in both taxa. Moreover, the fact that the dominant haplotypes were found in all subspecies except *L. g. cacsilensis* suggests a southern expansion following the last glacial maximum.

In contrast, populations of *L. g. cacsilensis* revealed high genetic diversity, and signals of demographic expansion were not detected. A plausible explanation for these results is that the high Andean region, recognized as the center of the origin and diversification of guanacos, had stable populations over long time periods, thus allowing an accumulation of genetic diversity. The fragmentation and reduction of these populations, as we know them now, is likely to be a very recent process linked directly to human activities. Thus, the loss of genetic diversity by drift might be still in action if the present situation is maintained.

## RESUMEN

El guanaco es el herbívoro nativo más importante de las estepas de Sudamérica y el ungulado dominante en una fauna rica en roedores, pero pobre en mamíferos de gran tamaño. Usualmente, entre 2 y 4 subspecies de guanaco han sido reconocidas dentro de *Lama guanicoe*, basadas en sutiles diferencias morfológicas y en su distribución geográfica. Para evaluar si la variación molecular es consistente con la hipótesis de la existencia de subspecies, analizamos el gen completo de citocromo b y la secuencia de la región control del DNA mitocondrial en *L. guanicoe* a partir de muestras provenientes de 22 localidades de Perú, Bolivia, Argentina y Chile. El análisis de la secuencia de ambos genes apoya la monofilia de la especie pero no distinguen la existencia de subspecies a lo largo del rango geográfico. A pesar de esto, las poblaciones más septentrionales (Perú y norte de Chile) muestran algún grado de diferenciación con respecto a los representantes de Argentina, Bolivia y resto de Chile. El análisis de la diversidad genética también demuestra una reducción poblacional en el pasado, seguida de una expansión reciente de la población.

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