Molecular Ecology (1998) **7**, 47–56

Conservation genetics of the endangered Pampas deer (*Ozotoceros bezoarticus*)

S. GONZÁLEZ,* J. E. MALDONADO,† J. A. LEONARD,† C. VILÀ,†
J. M. BARBANTI DUARTE,‡ M. MERINO,§ N. BRUM-ZORRILLA* and R. K. WAYNE†
División Citogenética, IIBCE, Av. Italia 3318, Montevideo, 11600 Uruguay; †Department of Biology, 621 Circle Drive South, University of California, Los Angeles, CA 90095–1606, USA; ‡Departamento Melhoramento Genético Animal, FCAVJ/UNESP, Rod. Carlos Tonanni km 5, 14870–000 Jaboticabal, SP, Brazil; §Estación de Cría de Animales Silvestres, CC 129, Villa Elisa, Buenos Aires, Argentina

Abstract

The Pampas deer (*Ozotoceros bezoarticus* L. 1758) is the most endangered neotropical cervid, and in the past occupied a wide range of open habitats including grassland, pampas, savanna, and cerrado (Brazil) from 5° to 41° S. To better understand the effect of habitat fragmentation on gene flow and genetic variation, and to uncover genetic units for conservation, we examined DNA sequences from the mitochondrial control region of 54 individuals from six localities distributed throughout the present geographical range of the Pampas deer. Our results suggest that the control region of the Pampas deer is one of the most polymorphic of any mammal. This remarkably high variability probably reflects large historic population sizes of millions of individuals in contrast to numbers of fewer than 80 000 today. Gene flow between populations is generally close to one migrant per generation and, with the exception of two populations from Argentina, all populations are significantly differentiated. The degree of gene flow was correlated with geographical distance between populations, a result consistent with limited dispersal being the primary determinant of genetic differentiation between populations. The molecular genetic results provide a mandate for habitat restoration and reintroduction of Pampas deer so that levels of genetic variation can be preserved and historic patterns of abundance can be reconstructed. However, the source of individuals for reintroduction generally should be from populations geographically closest to those now in danger of extinction.

Keywords: Cervidae, conservation, control region, mitochondrial DNA, *Ozotoceros bezoarticus*, phylogeography

Received 20 March 1997; revision received 1 August 1997; accepted 2 August 1997

Introduction

The Pampas deer (*Ozotoceros bezoarticus* L. 1758) is the most endangered neotropical cervid. Although it has a wide geographical distribution in south-eastern South America (from 5° to 41° S), the habitat required by this species has been greatly reduced by agriculture and urbanization. The Pampas deer formerly occupied a range of open habitats such as grassland, pampas, savanna, and cerrado (Brazil). However, the area encompassed by these habitats has been dramatically reduced to less than 1% of that present in 1900 (González 1993, 1996). Currently, Pampas deer populations are generally small and highly isolated (Jackson & Langguth 1987; González 1993; Merino 1994; Pinder 1994; Fig. 1). The largest extant populations are found in Brazil, in the north-east cerrado ecosystem where about 2000 individuals live, and in the Pantanal where 20 000–40 000 exist (Pinder 1994). In Uruguay there are two main populations: El Tapado (Salto Department) with about 800 individuals, and Los Ajos (Rocha Department) with about 200 deer (González 1996). At the turn of the century, the Argentinean population was probably very large as over 500 000 km^2 of grassland habitat was available. However, today only three small populations remain: Corrientes (Ituzaingo Department) with about 170 individuals (ML Merino & MD Beccaceci,

Correspondence: R. K. Wayne, Tel.: +1-310-206-0334; Fax: +1-310-206-3987; E-mail: rwayne@biology.ucla.edu

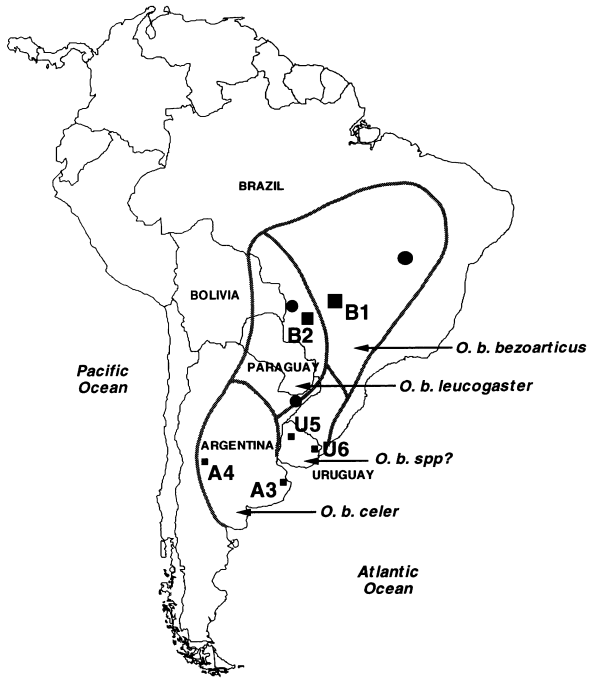

Fig. 1 Historic and current distribution of Pampas deer. Sampling localities are shown with squares. Brazil: Emas (B1) and Pantanal (B2), Argentina: Bahía de Samborombón (A3) and San Luís (A4), Uruguay: El Tapado (U5) and Los Ajos (U6). Other extant populations not sampled are shown with circles. The presumed historic distribution of subspecies is outlined by solid lines.

unpublished report), La Travesía (San Luís Province) with about 350 individuals (J Giullieti & M Maceira, unpublished report) and coastal Bahía de Samborombón (Buenos Aires Province) with about 200 individuals (Merino & Moschione 1995). A small population may still be extant in the south-eastern part of Bolivia.

The taxonomy and systematics of the Pampas deer have been based primarily on morphological data. Cabrera (1943) described the following subspecies: (i) *O. b. bezoarticus*: ranging from eastern and central Brazil south of Amazonia between the Mato Grosso plateau and the upper Rio San Francisco; (ii) *O. b. celer* inhabiting the entire Argentinean pampas from the Atlantic coast to the Andean foothills and southward to the Rio Negro; (iii) *O. b. leucogaster* living in south-western Brazil, south-eastern Bolivia, Paraguay and northern Argentina (Fig. 1). The Uruguayan pampas may contain a distinct subspecies (González *et al*. 1989, 1992). Nevertheless, cytogenetic studies on Pampas deer found no geographical variation in chromosome number or morphology (Bogenberger *et al*. 1987; Neitzel 1987; Spotorno *et al*. 1987; González *et al*. 1992; Duarte & Giannoni 1995; Duarte 1996).

In order to deduce genetic units for conservation (Moritz 1995) and to better understand the effect of habitat fragmentation on gene flow and genetic variation, we initiated a molecular genetic study of the Pampas deer based on samples from throughout their geographical range. To determine levels of genetic differentiation among isolated populations, we examined DNA sequence from the mitochondrial control region of 54 individuals from six localities (Fig. 1). The control region is a hypervariable, noncoding segment of the mitochondrial genome that is often used in studies of genetic population structure (Avise 1992). These data represent the first molecular systematic study of Pampas deer and, although sample sizes are limited, our results suggest that mitochondrial control-region sequence variability in this species is remarkably high, reflecting large historic population sizes. Pampas deer populations are significantly differentiated with respect to control-region sequences.

Materials and methods

Sample collection

Samples were collected from 54 individuals from six localities (Fig. 1, Table 1). Brazilian samples were obtained from Emas National Park (B1; 18°15′ S, 52°53′ W; n = 14) and from Pantanal da Nhecolandia (B2; 19°59′ S, 56°39′ W; n = 13), the largest Pampas deer population. Heparinized blood samples were obtained when the animals were captured to be fitted with radio collars.

The Uruguayan samples were obtained from El Tapado (U5; 31°65′ S, 56°43′ W; n = 10) and Los Ajos (U6; 33°45′ S, 54°02′ W; n = 7). Blood and hair samples were obtained from captive animals from El Tapado stock at San Carlos, Piriápolis, Durazno and Salto Zoos. Samples from wild individuals consisted of skin, hair, muscle and bone from dead animals.

The Argentinean samples were from Bahía de Samborombón (A3; 35°30′ S, 56°45′ W; n = 6) and from San Luís (A4; 34°22′ S, 65°44′ W; n = 4). The majority of the samples from the Argentinean populations consisted of tissues collected from dead animals. Blood samples were also collected when the animals were captured to be fitted with radio collars, and from one individual (SG24) from a captive breeding facility (Estación de Cría de Animales Silvestres).

Leukocytes were isolated by centrifugation and then resuspended in buffer (DMSO and PVP as cryopreservatives) and stored in liquid nitrogen. To put divergence values among Pampas deer sequences in perspective, we also sequenced samples from two brown brocket deer *Mazama gouazoubira* from Uruguayan zoos (Minas and San Carlos).

DNA extraction and PCR amplification

Tissue samples (50 mg or 100 μl) were transferred to

Table 1 Distribution of control region haplotypes among populations. Individuals with identical haplotypes within each population are listed in the same column

Population	Unit	Genotypes/Individuals											No. of genotypes/no. of individuals
Emas Brazil	B1	SP13 SP12	SP14 SP18	SP15	SP17 SP20	SP52	SP54	SP55	SP56	SP51	SP53	SP19	11/14
Pantanal Brazil	B2	SP36	SP38	SP41	SP42	SP43	SP44 SP45 SP47 SP48 SP49	SP40	SP46	SP50			9/13
B. Samborombón Argentina	A3	SG39	SG40	SG42	SG43	SG24	SG52						6/6
San Luís Argentina	A4	SG66	SG67	SG68	SG18								4/4
El Tapado Uruguay	U5	SG01	SG04	SG16	SG10	SG11	SG17	SG09	SG49	SG20	SG1623		10/10
Los Ajos Uruguay	U6	SG02	SG19 SG15	SG07 SG13	SG34	SG1738							5/7

1.7 mL eppendorf tubes containing 550 µl of lysis buffer composed of 50 mM Tris-HCl (pH 8), 50 mM EDTA, 1% sodium dodecyl sulphate, 100 mM NaCl, 1% beta-mercaptoethanol, and 20 µl of proteinase K (20 mg/mL). Samples were placed in a shaker-bath overnight at 40 °C. Genomic DNA was precipitated in 5 M NaCl with ethanol and resuspended in ultra-pure water (Medrano *et al*. 1990). Tissue extraction was performed with sterile materials and filtered pipette tips in a separate room utilized for that purpose only. Extraction controls and no-template PCR controls were used in each amplification. Universal primers Thr-L15910 (5'-GAATTCCCCGGTCTTGTAAACC-3') and DL-H16498 (5'-CCTGAACTAGGAACCAGATG-3') (based on Kocher *et al*. 1989) were used to amplify a 603 bp fragment of the control region. Each PCR mixture contained about 100 ng of sample DNA, and 1 mM dNTP in a reaction buffer of 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), and 2.5 U of Taq DNA polymerase in a total volume of 50 µl. Reactions contained 25 pmoles of each primer. Thirty-five cycles of amplification were performed in a programmable thermal cycler (Perkin Elmer-Cetus, Model 480). Each cycle consisted of denaturation at 94 °C for 60 s, annealing at 50 °C for 120 s, and extension at 72 °C for 90 s. Double-stranded products were separated in a 2% NuSieve (FMC Corp.) agarose gel in TAE buffer. After staining with ethidium bromide, the appropriate band was excised, the DNA extracted using a GeneClean Kit (BIO 101), dried by speed-vacuum, and eluted in 11 µl of ddH_2O.

DNA sequencing

Direct sequencing of double-stranded DNA was carried out using modifications of DMSO-based protocols (Green *et al*. 1989; Winship 1989) and a Sequenase Version 2.0 kit (US Biochemicals). The sequencing reaction products were separated by electrophoresis in a 6% polyacrylamide gel for 3 h at 55 W in a Stratagene Base Ace Sequencing apparatus. The gels were then dried and exposed to autoradiographic film (Kodak Biomax) for 1–3 days. Sequence autorads were scored on an IBI gel reader, and entered into the MacVector computer program (IBI-Kodak). A total of 35 individuals were sequenced for 453 bp within the left domain of the d-loop (Saccone *et al*. 1987. In addition, a sample of 21 individuals was sequenced using an ABI automated sequencer. In total, we sequenced 54 Pampas deer individuals (27 from Brazil, 10 from Argentina and 17 from Uruguay) and two brocket deer (*Mazama gouazoubira*) from Uruguay using the mitochondrial primers Thr-L and DL-H. Sequences were aligned first by eye, then by using Clustal V (Higgins & Sharp 1989), and rechecked by eye. Sequence data have been submitted to GenBank (accession numbers: AF012556–AF012602).

Relationship of control-region sequences

The number of mutations between DNA genotypes in pairwise comparisons was used to construct a minimum spanning network in which sequences are the nodes of a

network rather than the terminal tips of a tree. Networks may more effectively portray the relationships among sequences for populations in which many sequences may be derived from the same ancestral genotype (See examples in Excoffier *et al*. 1992). Using the number of substitutions in pairwise comparisons as input to the program supplied by Excoffier (Department of Anthropology, University of Geneva), we calculated a minimum spanning tree and its alternative links and scaled the length of the lines in proportion to the number of substitutions. We also calculated genetic distances between genotypes assuming a gamma distribution of substitution rates across nucleotide sites (Tamura & Nei 1993; Wakeley 1993). A value of $a = 0.36$ was estimated from our sequence data for the gamma distribution parameter in the Tamura & Nei (1993) model. We also calculated the average sequence divergence between populations and used these pairwise values to construct a neighbour-joining tree (Saitou & Nei 1987).

Patterns of geographical subdivision and gene flow

We used AMOVA (analysis of molecular variance) to deduce the significance of geographical divisions among local and regional population groupings (Excoffier *et al*. 1992). AMOVA is a hierarchical approach analogous to analysis of variance (ANOVA) in which the correlations among genotype distances at various hierarchical levels are used as *F*-statistic analogues, designated as ϕ statistics. Thus, ϕ_{ST} is the correlation of random genotypes within a population relative to those from the whole species and is analogous to F_{ST} of Wright (1951). The statistic ϕ_{CT} is the correlation of random genotypes within a group of populations relative to those drawn from the entire species and measures the proportion of genetic variation among groupings of populations. Lastly, ϕ_{SC} is the correlation of random genotypes within populations relative to those within a regional grouping of populations and measures the proportion of variation among populations within a region. The significance of these *F*-statistic analogues was evaluated by 500 random permutations of sequences among populations. We experimented with various groupings of populations suggested by the analysis of DNA sequence and population trees, and those suggested by taxonomy and geographical isolation. The groupings which maximized values of ϕ_{CT} and were significantly different from random distributions of individuals were assumed to be the most probable geographical subdivisions.

Gene flow within and among regions was approximated as *Nm*, the number of female migrants occurring between population units per generation, and was estimated using the expression $F_{ST} = 1/(1 + 2Nm)$ where N is the female effective population size and m is the female migration rate (Slatkin 1987, 1993; Baker *et al*. 1994). We used pairwise estimates of ϕ_{ST} as surrogates for F_{ST} among regional groupings of populations (e.g. Stanley *et al*. 1996). Following Slatkin (1993), we assessed differentiation by distance by plotting pairwise log (*Nm*) values against log (geographical distance). The significance of the association was determined by applying a Mantel's permutation test (Mantel 1967). A significant association between *Nm* and distance indicates genetic structuring in populations and that dispersal of individuals is limited (Slatkin 1993). In addition, an independent cladistic method was utilized for estimating *Nm* (Slatkin & Maddison 1989).

Results

The control region of the Pampas deer is one of the most polymorphic of any mammal (see Arctander *et al*. 1996a). We sequenced all individuals for a 453 bp region with primers Thr-L/DL-H. We found 45 different haplotypes in the 54 Pampas deer from the six localities defined by base-pair substitutions (Table 1). Additionally, we found a polymorphic dinucleotide TA repeat sequence within the 453 bp fragment that had four to eight tandem repeats beginning at nucleotide position 186, with position 1 as the first nucleotide of our control-region sequence. The Emas population, in Brazil, showed the highest degree of tandem repeat polymorphism with four different alleles, and the Argentinean populations the least, with only one allele (Table 2). The same allele sizes were found in divergent sequences from geographically distant populations (e.g. Emas and Los Ajos, Table 2). Consequently, because of the high degree of homoplasy, we excluded the tandem repeat region from the analysis leaving 432 bp of DNA sequence to be analysed.

Geographic distribution of control-region sequences

The sequenced haplotypes were perfectly segregated because no locality shared haplotypes (Table 1). The Brazilian haplotypes differed by two (0.4%) to 24 (5.5%)

Table 2 Distribution of the number of tandem repeats in Pampas deer populations. Sample size in parentheses

Repeats	B1 Emas (14)	B2 Pantanal (13)	A3 Sambor. (6)	A4 San Luis (4)	U5 Tapado (10)	U6 Los Ajos (7)	Total
4	–	1	–	–	–	–	1
5	6	12	6	4	8	5	41
6	4	–	–	–	2	1	7
7	3	–	–	–	–	1	4
8	1	–				–	1

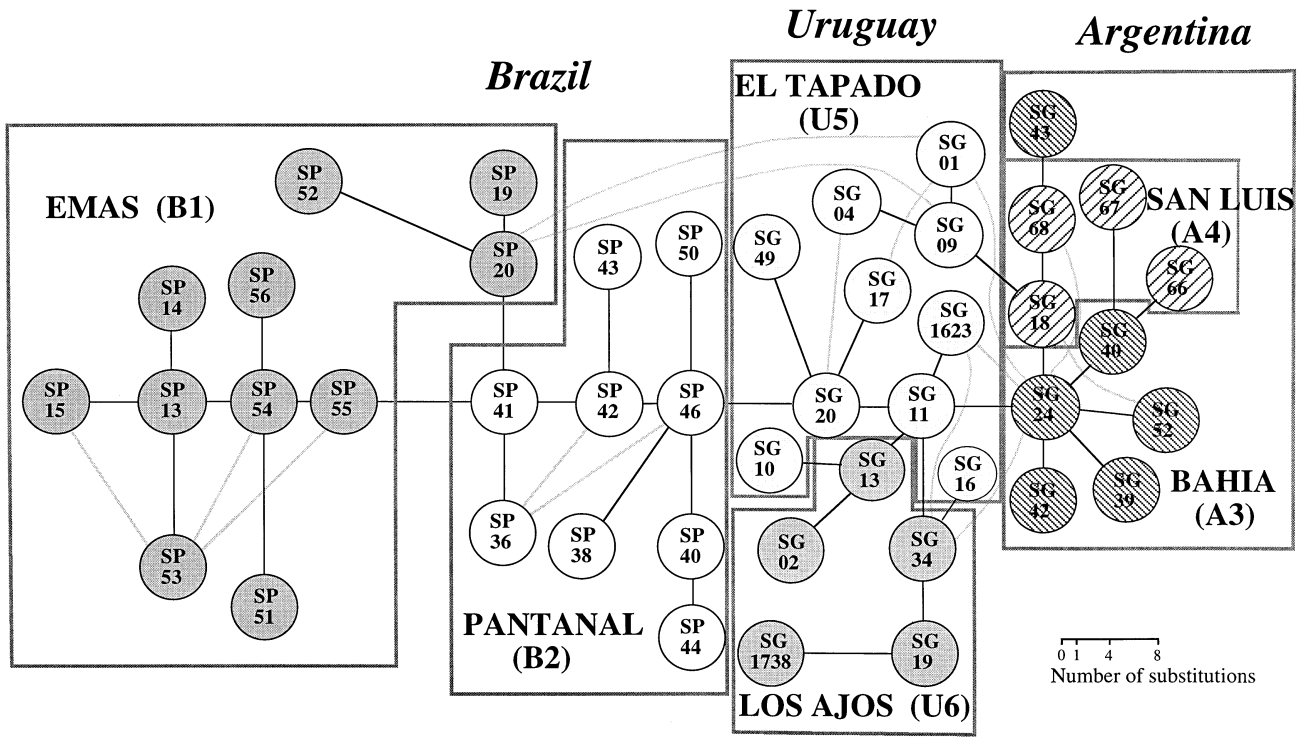

Fig. 2 Minimum spanning network based on the number of substitutions among control-region genotypes. The consistency and homoplasy index of this network is 0.667 and 0.333, respectively. Shadow lines indicate groupings in alternative minimum spanning trees all of which have a lower consistency index and a higher homoplasy index.

substitutions, the Argentinean differed by two (0.4%) to 12 (2.7%) substitutions, and the Uruguayan differed by one (0.2%) to 24 (5.5%) substitutions (distance matrix available from the authors by request). The nucleotide diversity (Nei 1987) within populations ranged from 0.011 to 0.025 and was lowest in the Argentinean populations (Table 3). The average sequence divergence between different populations ranged from 0.8% (A3 vs. A4) to 3.1% (B1 vs. A4). However, the maximum sequence divergence between genotypes was 8.2% which is a large value compared to that found in other mammal species (but see Arctander *et al*. 1996a,b).

Sequences from the same locality tend to be clustered together in minimum spanning networks (Fig. 2). Moreover, a north-to-south cline is apparent in the relationships of populations with the majority of links between sequences from the same or neighbouring populations. Sequences from the two Brazilian localities, Emas (B1) and Pantanal (B2), have two links between each other and a sequence from the latter population (SP46) is linked to a sequence (SG20) from El Tapado, Uruguay. Sequences from both Uruguayan populations have four links to each other and two El Tapado sequences, SG09 and SG11, are linked to the Argentinean sequences SG18 from San Luís and SG24 from Bahía de Samborombón, respectively. The Argentinean populations have four links to each other. Alternative networks constructed from these sequences show a similar array of connections between populations (Fig. 2). These general geographical patterns of relationships among sequences from different populations are further supported by an unrooted neighbour-joining tree based on genetic distance between populations (Fig. 3). Additionally, in this tree, the Argentinean populations

Table 3 Sequence statistics for Pampas deer for a 432 bp fragment of d-loop sequence, with tandem repeat omitted. Diagonal numbers in italics: Nei's (1987) nucleotide diversity within populations. Lower left-hand corner: average sequence divergence between populations; upper right-hand corner: number of migrants per generation (*Nm*)

	B1	B2	A3	A4	U 5	U6
B1	*0.019*	0.962	0.644	0.631	1.043	0.988
B2	0.024	*0.021*	0.456	0.480	1.123	1.029
A3	0.027	0.028	*0.011*	7.313	1.366	0.769
A4	0.031	0.029	0.008	*0.015*	1.161	0.753
U5	0.023	0.019	0.015	0.018	*0.019*	2.901
U6	0.027	0.023	0.024	0.028	0.016	*0.025*

Abbreviations as in the legend to Fig. 1.

clearly are more closely related to each other than are those from Brazil or Uruguay.

Gene flow and genetic units for conservation

With two exceptions, pairwise computations of ϕ_{ST} using AMOVA indicate that populations are significantly differentiated relative to a random collection of genotypes (Table 4). The clearest exception is for the low ϕ_{ST} between Argentinean populations (ϕ_{ST} = 0.064, P = 0.166). The lack of significant differentiation between these populations might have been predicted from the low levels of divergence between sequences from the two populations (Fig. 3, Table 3). Of marginal nonsignificance is the comparison of the two Uruguayan populations (ϕ_{ST} = 0.147, P = 0.054). However, the average sequence divergence between these two populations is approximately two times larger than that between the Argentinean populations (1.6% vs. 0.8%, Table 3) and the sample size is smaller, suggesting that a larger population sampling might reveal significant differences. Because the power of randomization tests is low if sample sizes are small (Excoffier *et al*. 1992), these results suggest that, conservatively, the Uruguayan populations should be considered distinct whereas the two populations from Argentina should be considered part of the same interbreeding population.

The degree of gene flow can be estimated from the number of phylogenetic links between populations (Slatkin & Maddison 1989). Using the minimum spanning network in Fig. 2, and after grouping both Argentinean populations as one, we determined that nine links existed between sequences from different populations. The value of *Nm* implied by this number of links is 0.5 migrants per generation. An independent estimate of gene flow can be derived from the ϕ_{ST} values based on haplotype frequency and genetic distance (Table 4) and assuming an island model of migration (Wright 1965). The value of ϕ_{ST} across populations is 0.37 yielding a value of Nm = 0.87, similar to that derived from consideration of the minimum number of migration events.

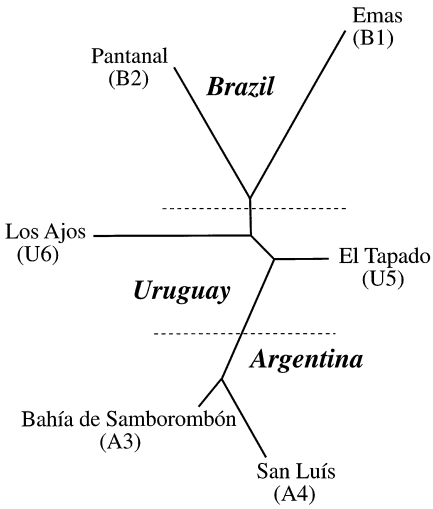

Fig. 3 Unrooted neighbour-joining tree based on average sequence divergence between populations.

Table 4 Matrix of ϕ_{ST} (lower left) and associated P- values (upper right) between populations

	B1	B2	A3	A4	U 5	U6
B1	–	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
B2	0.342	–	< 0.002	< 0.002	< 0.002	< 0.002
A3	0.437	0.523	–	0.166	< 0.002	< 0.002
A4	0.442	0.510	0.064	–	< 0.002	< 0.002
U5	0.324	0.308	0.268	0.301	–	0.054
U6	0.336	0.327	0.394	0.399	0.147	–

Abbreviations as in the legend to Fig. 1.

In general, pairwise values of *Nm* based on ϕ_{ST} are close to or less than one, with the exception of the two Argentinean (Nm = 7.31) and Uruguayan populations (Nm = 2.9, Table 3). The degree of differentiation observed between localities appears to follow a predictable relationship with geographical distance (Fig. 4, r = 0.60, P = 0.014, Mantel's test). The outlier in this regression is the comparison of the two Argentinean populations which the AMOVA analysis suggests may form part of the same interbreeding population. Although they are 720 km apart, the estimated number of migrants per generation, *Nm*, is 7.3. If these localities are treated as a single population the correlation coefficient between distance and *Nm* increases to 0.66 (P = 0.024, Mantel's test). Therefore, the divergence between Pampas deer populations is consistent with a model of differentiation with distance (Slatkin 1993), which suggests that limited dispersal and distance largely explain the level of genetic differentiation existing between deer populations.

Discussion

Levels of genetic variation

The origin of the Pampas deer may be associated with a substantial cooling event dated about 2.5 million years ago at the boundary between the Gauss and Matuyama chrons (Bonadonna & Alberdi 1987). This event was associated with the first appearance of steppe mammals and the extinctions of and changes in the distribution of some marine species. The direct ancestor of the Pampas deer first appeared in the Pampean Formation during the Pleistocene about 2 Ma (Marshall *et al*. 1984). As bovids

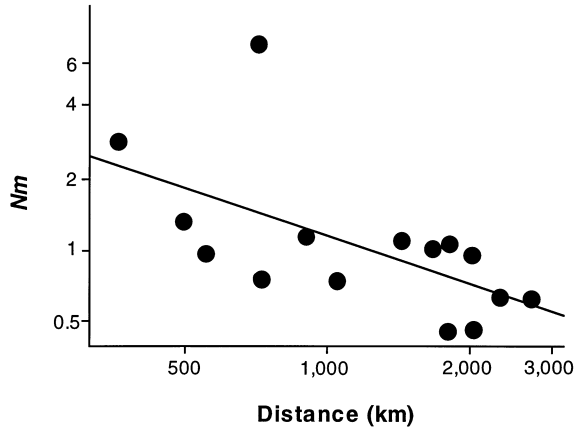

Fig. 4 Scatterplot of *Nm* and geographical distance (logarithmic scale) between the six populations. Regression statistics: log (*Nm*) = 2.07–0.67 (log distance).

were not present in South America, cervids radiated to fill grazing and browsing niches that otherwise might have been occupied by bovids (Eisenberg 1987). The large sequence divergence between some Pampas deer haplotypes supports an origin close to that suggested by the fossil record. The mean sequence divergence between the Pampas deer and the closely related brocket deer is 10.1% and, given a minimum divergence time between the two taxa of 2 Myr (see above), the rate of sequence divergence is 5% per million years or less. The maximum divergence between any two Pampas deer sequences is 8.2%; consequently, the coalescence of the observed haplotypes occurred earlier than 1.6 Ma.

The large number of haplotypes and high level of nucleotide diversity in the Pampas deer suggest that it was a more abundant and widespread species in the recent past. Four species of African bovids, Grant's gazelle (*Gazella granti*), waterbuck (*Kobus ellipsiprymnus),* wildebeest (*Connochaetes taurinus*) and impala (*Aepyceros melampus*), have been studied for variation in the same control-region fragment as sequenced in this study (Arctander *et al*. 1996a,b). In these four species, polymorphism was also high. For example, the nucleotide diversity ranged from 0.014 to 0.037. The comparable range for Pampas deer is 0.011 to 0.025. In the four African species, the mean sequence divergence between populations ranged between 0.2% and 1.4%, with the exception of Grant's gazelle which had a maximum divergence between populations of 14.2%. In the Pampas deer, sequence divergence between populations ranged between 0.4% and 3.1% (Table 3). All four of the African bovids studied by Arctander *et al*. (1996a) have census population sizes greater than one million individuals (Murray 1982). Levels of genetic diversity are similar among these African bovids despite marked difference in social system, density, distribution and habitat requirements. This suggests that if the dynamics of control-region sequences in Pampas deer is similar to that in bovids, then the degree of control region polymorphism in the Pampas deer implies that more than a million deer existed in the recent past, greater than 10 times their number today (Pinder 1994; Merino & Moschione 1995; González 1996; J Giullieti & M Maceira, unpublished report; ML Merino & MD Beccaceci, unpublished report).

The effective population size can be estimated based on the relation $\theta = 2N\mu$ where N is the effective number of females and μ is the mutation rate per site. Using coalescent likelihood methods incorporated in the COALESCE program by Kuhner *et al*. (1995), the parameter θ can be calculated from a population sample of DNA sequences. Our estimate of $2N\mu$ is 0.173 and assuming a mutation rate of 2.5×10^{-8} per nucleotide site per year for the control region (based on sequence divergence between the Pampas and brocket deer, as above), the effective number of breeding females would be about 3 460 000. The total census size of females is probably at least double this value (e.g. Nunney & Elam 1994). Therefore, both comparative and theoretical estimates indicate that a substantial reduction in population size has occurred as the total present day number of deer is between 64 000 and 80 000 individuals (González *et al*. 1994).

The existence of high levels of variation despite present-day low numbers of deer indicates that the decrease in population size was recent, as variability would be lost rapidly in populations that had maintained a small size for long periods of time (Ballou 1994). Historically, Pampas deer were numerous, as suggested by the high frequency of *Ozotoceros bezoarticus* in archaeological sites from the Buenos Aires province (Tonni *et al*. 1992). Pampas deer were probably a significant part of the diet of the native human population. In addition, in the 18th and 19th centuries vast numbers of Pampas deer pelts were exported. For example, from 1860 to 1870 an estimated 2 130 000 skins were exported from Rio de la Plata (Thornback & Jenkins 1982). The large number of pelts supports the genetic estimates of a population size in the millions that was recently decimated through hunting and loss of habitat. The decline in population size has been so recent that it has not yet been reflected in levels of polymorphism (Lavery *et al*. 1996).

Genetic differences between populations

The AMOVA analysis showed that all populations, except the two Argentinean populations, are differentiated from each other. The surprisingly high number of migrants per generation estimated between the two Argentinean populations, which are 720 km apart, could be explained by

translocation events; however, none have been reported. Alternatively, the two populations could have had a high rate of migration through a habitat corridor no longer apparent today.

With the exception of the two Argentinean populations, the degree of differentiation between populations is strongly associated with geographical distance (Fig. 4). This suggests that genetic differentiation is largely explained by the limited dispersal abilities of deer rather than the presence of long-standing ecological or geographical barriers. For example, gene flow has occurred between the eastern Argentinean population of Pampas deer (A3) and those in Uruguay (U5, U6; Nm = 1.4 and 0.77, respectively) despite the presence of the Uruguay river that separates populations in the two countries. Genetic units for conservation have been based on criteria such as reciprocal monophyly (evolutionary significant units) or differences in genotype frequency (management units; Moritz 1995). The numerous reticulations in the minimum spanning network (Fig. 2) show that none of the neighbouring Pampas deer populations are reciprocally monophyletic and indicates the occurrence of past episodes of migration. Thus, they should not be considered independent evolutionary units and have not been isolated for a period of time sufficient for reciprocal monophyly to occur.

However, with the exception of the Argentinean populations, all the other populations are significantly or marginally differentiated, thus they might be classified as management units experiencing low to modest rates of gene flow. A pronounced sequence divergence exists between Brazilian populations from Emas and Pantanal (Fig. 3), corresponding to the different subspecific designation recognized by Cabrera (1943). These populations may have been historically isolated in different habitats. In fact, the population in Emas (B1) is located in the cerrado of central Brazil, with a 650–1000 m elevation, which has a distinct dry season, whereas the population in El Pantanal (B2) is found in wetlands below 100 m. Differentiation between these populations is supported by discrepancies in their physiology. In the Emas population, antlers are shed in April, whereas in the Pantanal this occurs in June and July (Rodrigues & Monteiro-Filho, in press). Similar differences are found in other populations (Jackson 1986). Although based on limited evidence, these physiological differences may indicate differences in the timing of the reproductive cycle and hence, if of genetic origin, may be an important reason why the populations should not be interbred or used as a source for cross-translocation. For example, the ibex (*Capra ibex*) became locally extinct in the Tatra Mountains of the Slovak Republic through overhunting. Ibex were successfully reintroduced from nearby Austria which had a similar climate. However, later translocations used animals from Turkey and the Sinai, and hybrids between them and the Tatra ibex rutted in the early fall instead of the winter, resulting in progeny born in February, the coldest month of the year. Consequently, the entire population became extinct (Greig 1979).

Implications for conservation

Several populations of Pampas deer in Uruguay and Argentina are on the brink of extinction (Thornback & Jenkins 1982). Plans have been developed in Uruguay to translocate deer to impoverished habitats from areas where they are more abundant (González *et al*. 1994). In general, translocation from a wild population is preferable over the use of captive raised animals because of disease threats and lack of survival skills (Snyder *et al*. 1996). However, the surveyed Pampas deer populations are small, isolated and genetically differentiated (with the exception of the two Argentinean populations). The genetic data imply that neighbouring Pampas deer populations separated by a few hundred kilometres had levels of gene flow close to one migrant per generation (Table 3, Fig. 4). Consequently, the recent fragmentation of Pampas deer habitats has given an artificial appearance of subdivision that may not have existed historically. The relationship of gene flow and geographical distance (Fig. 4) provides a guideline for translocation as human-mediated migration can attempt to mimic historic levels of gene flow (e.g. Hedrick 1995).

For populations that are declining rapidly, translocation of a few individuals per generation will not be sufficient to increase numbers, and captive breeding of individuals from declining populations might then be considered. Recently an introduction experiment was carried out successfully in Uruguay with seven animals from captive stocks from Durazno and Piriápolis Zoos moved to a protected area in Rocha about 100 km east of the population in Los Ajos. The parental generation of the introduced animals was obtained from El Tapado. The genetic results suggest that Los Ajos may have been a more appropriate source of stock for reintroduction as both populations are marginally differentiated.

The Pampas deer is endangered in Uruguay and Argentina, with fewer than 1800 individuals. The levels of genetic diversity in populations from these countries suggest that historic population sizes were several orders of magnitude larger, and that populations have recently decreased dramatically, thus providing a strong mandate for restoration and augmentation. This population decline was due to habitat loss and unregulated hunting beginning in the last century and, most recently, to control efforts by ranchers who believe that deer compete with livestock. Pampas deer numbers might increase if protected from hunting in areas where natural habitats remain and if some grazing land, as a buffer, could be designated

for dual use by deer and livestock. The genetic data suggest that Pampas deer have the potential to exist over a much greater area and historical data demonstrate a much wider distribution for the species. Therefore, if the goal of conservation is to maintain long-term population stability and preserve genetic variation, conservation efforts should focus on the restoration of deer habitats and the reintroduction of deer over a wide geographical area.

Acknowledgements

Part of this research was supported by Programa de Desarrollo de Ciencias Básicas (PEDECIBA) from Uruguay. Special thanks to Mr Carlos Arrate, Mr José Pedro Castro and Mr Guillermo Constantín for authorising access to field sites. J. Carnahan, K. Koepfli, M. H. Kohn, G. Roemer and S. E. L. West provided helpful comments which improved the quality of this manuscript. Thanks also to Dr Ulysses S. Seal from the Conservation Breeding Specialist Group/IUCN and Dr Richard Fariña for encouragement in this project. Sample collection in Brazil was undertaken by the Veado Campeiro project, Fundo Nacional do Meio Ambiente do Brasil, and IBAMA granted the permits.

References

Arctander P, Kat PW, Simonsen BT, Siegismund HR (1996a) Population genetics of Kenyan impalas-consequences for conservation. In: *Molecular Genetic Approaches in Conservation* (eds Smith TB, Wayne RK), pp. 399–412. University Press, Oxford.

Arctander P, Pieter WK, Rashid AA, Siegismund HR (1996b) Extreme genetic differences among populations of *Gazella granti*, Grant's gazelle, in Kenya. *Heredity*, **76**, 465–475.

Avise JC (1992) Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. *Oikos*, **63**, 62–76.

Baker CS, Slade WR, Bannister JL et al. (1994) Hierarchical structure of mitochondrial DNA gene flow among humpback whales, *Megaptera novaeangliae*, world-wide. *Molecular Ecology*, **3**, 313–327.

Ballou J (1994) Small population overview. In: *Vortex Users Manual. A Stochastic Simulation of the Extinction Process* (eds Lacy RC, Hughes KA, Kreeger TJ), pp. 2–11. IUCN SSC/CBSG.

Bogenberger JM, Neitzel H, Fittler F (1987) A highly repetitive DNA component common to all Cervidae: its organization and chromosomal distribution during evolution. *Chromosoma*, **95**, 154–161.

Bonadonna FP, Alberdi MT (1987) The N/Q Boundary at 1. 64 Ma? *Mediterranea Series Geología*, **6**, 115–130.

Cabrera A (1943) Sobre la sistemática del venado y su variación individual y geográfica. Revista del Museo de la Plata (NS). Tomo III. *Zoology*, **18**, 5–41.

Duarte JMB (1996) Analise citogenetica de diferentes populaçôes de veado campeiro (*Ozotoceros bezoarticus*). In: *Relatorio final de pesquisa (Projeto Veado Campeiro Ozotoceros bezoarticus)* (ed. FUNEP), pp. 80–84. FUNEP, Jaboticabal.

Duarte JMB, Giannoni ML (1995) Cytogenetic analysis of the Pampas deer *Ozotoceros bezoarticus* (Mammalia, Cervidae). *Brazilian Journal of Genetics*, **18**, 485–488.

Eisenberg JF (1987) The evolutionary history of the Cervidae with special reference to the South American radiation. In: *Biology and Management of the Cervidae* (ed. Wemmer C), pp. 60–64. Smithsonian Institute Press, Washington, D.C.

Excoffier L, Smouse PE, Quatro JM (1992) Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

González S (1993) Situación poblacional del venado de campo en el Uruguay. In: *Pampas Deer Population and Habitat Viability Assessment*, *Section 6* (ed. CBSG/IUCN), pp. 1–9. Workshop Briefing Book, Apple Valley, Minnesota.

González S (1996) El Tapado Pampas deer population. *IUCN Deer Specialist Group Newsletter*, **13**, 6.

González S, Gravier A, Brum-Zorrilla N (1992) A systematic subspecifical approach on *Ozotoceros bezoarticus* L. 1758 (Pampas deer) from South America. In: *Ongules/Ungulates 91 Proceedings of the Intrernational Symposium* (ed. Spitz F, Janeau G, González G, Aulagnier S), pp. 129–132. SFEPM-IRGM, Toulouse, France.

González S, Gravier A, Kaladjian R (1989) Estudio craneométrico de *Ozotoceros bezoarticus* ('venado de campo'). *Boletín de la Sociedad Zoológica del Uruguay (2a ep.)*, **5**, 29–30.

González S, Merino M, Gimenez-Dixon M, Ellis S, Seal US (1994) Population and habitat viability assessment for the pampas deer (*Ozotoceros bezoarticus*) (ed. IUCN), pp. 171. Workshop Report CBSG/IUCN, Apple Valley, Minnesota.

Green PM, Bentley DR, Mibashan RS, Nilson IM, Gianelli F (1989) Molecular pathology of haemophilia B. *European Molecular Biology Organization Journal*, **8**, 1067–1072.

Greig JC (1979) Principles of genetic conservation in relation to wildlife management in Southern Africa. *South African Journal of Wildlife Research*, **9**, 57–78.

Hedrick PW (1995) Gene flow and genetic restoration: the Florida panther as a case study. *Conservation Biology*, **9**, 996–1007.

Higgins DG, Sharp PM (1989) Fast and sensitive multiple sequence alignment on a microcomputer. *Cabios*, **5**, 151–153.

Jackson J (1986) Antler cycle in pampas deer (*Ozotoceros bezoarticus*) from San Luís, Argentina. *Journal of Mammalogy*, **67**, 175–176.

Jackson JE, Langguth A (1987) Ecology and status of pampas deer (*Ozotoceros bezoarticus*) in the Argentinian pampas and Uruguay. In: *Biology and Management of the Cervidae* (ed. Wemmer C), pp. 402–409. Smithsonian Institute Press, Washington, D.C.

Kocher TD, Thomas WK, Edwards A et al. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequecing with conserved primers. *Proceedings of the National Academy of Sciences USA*, **86**, 6196–6200.

Kuhner MK, Yamato J, Felsenstein J (1995) Estimating effective population size and mutation rate from sequence data using Metropolis-Hastings sampling. *Genetics*, **140**, 1421–1430.

Lavery S, Moritz C, Fielder DR (1996) Genetic patterns suggest exponential population growth in a declining species. *Molecular Biology and Evolution*, **13**, 1106–1113.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, **27**, 209–220.

Marshall LG, Berta A, Hoffstettes R *et al.* (1984) *Mammals and Stratigraphy: Geochronology of the Continental Mammal-bearing Quaternary of South America.* Laboratoire de palontologie des vertebres de l'Ecole pratique des hautes etudes, Montpellier.

Medrano JF, Aasen E, Sharrow L (1990) DNA extraction from nucleated red blood cells. *Biotechniques*, **8**, 43.

Merino ML (1994) Situación del venado de las Pampas (*Ozotoceros bezoarticus*, Linneus 1758) en la República Argentina. In:

Population and Habitat Viability Assessment for the Pampas Deer (Ozotoceros bezoarticus) (ed. González S, Merino M, Gimenez-Dixon M, Ellis S, Seal US), pp. 145–156, Workshop Report CBSG/IUCN, Apple Valley, Minnesota.

Merino ML, Moschione FN (1995) Estimación del tamaño poblacional del venado de las pampas (*Ozotoceros bezoarticus celer*, Cabrera 1943) en la Bahía Samborombón, Buenos Aires. *Resumenes X Jornadas de Mastozoología*, La Plata Argentina.

Moritz C (1995) Uses of molecular phylogenies for conservation. *Philosophical Transactions of Royal Society of London B Biological Sciences*, **349**, 113–118.

Murray MG (1982) Home range dispersal and clan system of the impala. *African Journal of Ecology*, **20**, 253–269.

Nei M (1987) *Molecular evolutionary genetics*. Columbia University Press, New York.

Neitzel H (1987) Chromosome evolution of Cervidae: karyotypic and molecular aspects. In: *Cytogenetics: Basic and applied aspects* (eds Obe G, Basler A), pp. 90–112. Springer-Verlag, New York.

Nunney L, Elam DR (1994) Estimating the effective population size of conserved populations. *Conservation Biology*, **8**, 175–184.

Pinder L (1994) Status of Pampas deer in Brazil. In: *Population and Habitat Viability Assessment for the Pampas Deer (Ozotoceros bezoarticus)* (ed. González S, Merino M, Gimenez-Dixon M, Ellis S, Seal), pp. 157–162. Workshop Report CBSG/IUCN, Apple Valley, Minnesota.

Rodrigues FHG, Monteiro-Filho ELA (in press) Commensalistic relation between the pampas deer (*Ozotoceros bezoarticus*, Mammalia, Cervidae) and rheas (*Rhea americana*, Aves, Rheidae). *Biotropica*, in press.

Saccone C, Attimonelli M, Sbisà E (1987) Structural elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. *Journal of Molecular Evolution*, **26**, 205–211.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.

Slatkin M (1987) Gene flow and the geographic structure of natural populations. *Science*, **236**, 787–792.

Slatkin M (1993) Isolation by distance in equilibium and non-equilibrium populations. *Evolution*, **47**, 264–279.

Slatkin M, Maddison WP (1989) A cladistic measure of gene flow infered from the phylogenies of alleles. *Genetics*, **123**, 603–613.

Snyder NFR, Derrickson SR, Beissinger SR et al. (1996) Limitations of captive breeding in endangered species recovery. *Conservation Biology*, **10**, 338–348.

Spotorno A, Brum-Zorrilla N, Di Tomaso MV (1987) Comparative cytogenetics of South American deer. *Fieldiana Zoology*, **39**, 473–483.

Stanley HF, Casey S, Carnahan JM et al. (1996) Worldwide patterns of mitochondrial DNA differentiation in the harbor seal (*Phoca vitulina*). *Molecular Biology and Evolution*, **13**, 368–382.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Thornback J, Jenkins M (1982) *The IUCN Mammal Red Data Book Part II*. IUCN, Gland, Switzerland.

Tonni EP, Alberdi MT, Prado JL, Bargo MS, Cione AL (1992) Changes of mammal assemblages in the Pampean region (Argentina) and their relation with the Plio–Pleistocene boundary. *Palaeogeography, Palaeoclimatology, Palaeocology*, **95**, 179–194.

Wakeley J (1993) Substitution rate variation among sites in hypervariable region I of human mitochondrial DNA. *Journal of Molecular Evolution*, **37**, 613–623.

Winship PR (1989) An improved method for directly sequencing PCR amplified material using DMSO. *Nucleic Acid Research*, **17**, 1266.

Wright S (1951) The genetical structure of populations. *Annual Eugenics*, **15**, 323–354.

Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution*, **19**, 395–420.

This study is part of the PhD. research of Susana González, who has been assisted and directed by Jesús Maldonado in all laboratory and data analyses. Jennifer Leonard helped in the laboratory procedures. Carles Vilà contributed to the data analysis and in the revision of the manuscript. José Mauricio Barbanti Duarte and Mariano Merino provided samples from Brazil and Argentina, respectively. The initial phase of the study was conducted from Nadir Brum-Zorrilla's lab in Montevideo, and most of the laboratory analyses and all the data analyses were performed in the Conservation Genetics laboratory of Dr Robert K. Wayne in Los Angeles.
