Phylogeography and Pleistocene demographic history of the endangered marsh deer (*Blastocerus dichotomus*) from the Río de la Plata Basin

Alejandro Márquez[1,*], J. E. Maldonado[2], S. González[1], M. D. Beccaceci[3], J. E. Garcia[4] & J. M. B. Duarte[5]

[1]*División Citogenética-IIBCE, Unidad Asociada a Facultad de Ciencias, Av. Italia 3318, Montevideo, 11600, Uruguay;* [2]*Genetics Program, National Museum of Natural History, Smithsonian Institution, 3001 Connecticut Ave, NW, Washington, DC, 20008, USA;* [3]*Instituto de Medio Ambiente y Ecología, Universidad del Salvador., Rodríguez Peña 770 2°, Capital Federal, Buenos Aires, Argentina;* [4]*Departamento de Bioquímica, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, 6001, Londrina, SP, Brasil;* [5]*Departamento de Zootecnia, FCAV/UNESP, Via de Acceso Prof. Paulo Donato Castellane s/n, 14884-900, Jaboticabal, SP, Brazil (*Corresponding author: Fax: (598-2)-487-55-48; E-mail: ale@semic.fcien.edu.uy)*

Abstract

The marsh deer is the largest neotropical cervid with morphological and ecological adaptations to wetlands and riparian habitats. Historically, this now endangered species occupied habitats along the major river basins in South America, ranging from southern Amazonia into northern Argentina to the Paraná river delta. This particularly close association with wetlands makes marsh deer an excellent species for studying the effects of Pleistocene climatic changes on their demographic and phylogeographic patterns. We examined mitochondrial DNA variation in 127 marsh deer from 4 areas distributed throughout the Río de la Plata basin. We found 17 haplotypes in marsh deer from Brazil, Bolivia and Argentina that differed by 1–8 substitutions in a 601 bp fragment of mitochondrial control region sequence, and 486 bp of cytochrome *b* revealed only 3 variable sites that defined 4 haplotypes. Phylogeny and distribution of control region haplotypes suggest that populations close to the Pantanal area in central Brazil underwent a rapid population expansion and that this occurred approximately 28,000–25,000 years BP. Paleoclimatic data from this period suggests that there was a dramatic increase for precipitation in the medium latitudes in South America and these conditions may have fostered marsh deer's population growth.

Introduction

The endangered marsh deer (*Blastocerus dichotomus*, Illiger 1815) is the largest deer in South America (Pinder and Grosse 1991). The species has a range associated to most of the riparian marshlands from south of the Amazon River to northern Argentina (Pinder 1996; Tomas et al. 1997). However, an estimated reduction of 65% in its global range of distribution occurred in the last four decades (Weber and González 2003). It is currently distributed throughout southeastern Peru, Bolivia, Paraguay, Argentina and Brazil to the south of the Amazon rain forest (Pinder and Seal 1994; Figure 1). The distribution follows the three main South American river basins: (A) the Río de la Plata basin, which includes populations from Argentina, Brazil and Paraguay from the Paraná, Paraguay and Pilcomayo rivers. (B) The Amazon river basin, which includes populations mainly associated with the Tocantins, Araguaia, Xingú, Guaporé, and Juruena rivers. (C) The San
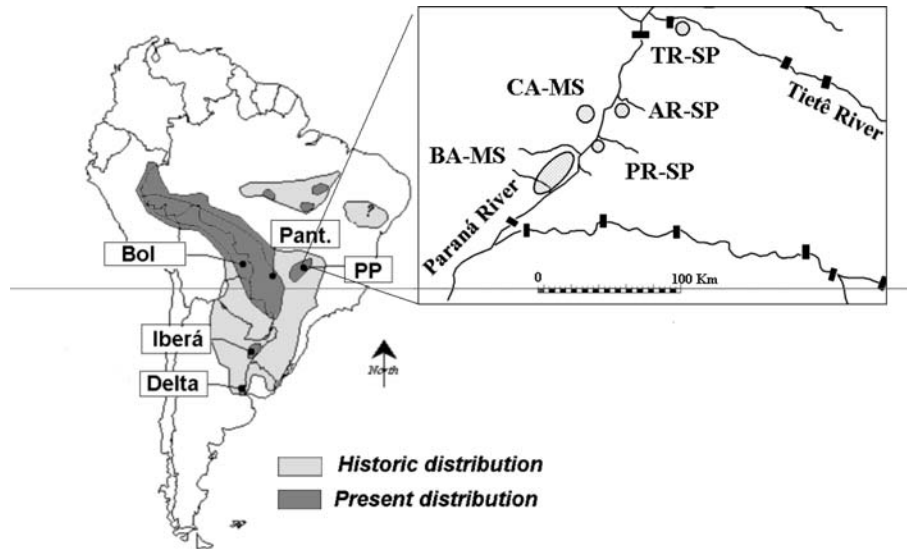
Figure 1. Historic and current distribution of the marsh deer. Sampling localities are shown with the following abbreviations: Pant: Pantanal, Bol: Bolivia, Iberá: Esteros del Iberá, Delta: Delta del Paraná, PP: Porto Primavera, SP and MS are Sao Paulo and Mato Grosso do Sul States, PR-SP: Peixe River, BA-MS: Bataguassu Area, CA-MS: Cisalpina Area, TR-SP: Tietê River, and AR-SP: Aguapei River.

Francisco river basin, although the status here is unknown.

The marsh deer is highly dependent on aquatic vegetation and prefers flooded areas in tropical and subtropical wetlands where the depth of the water does not exceed 60 cm and with low plant cover (Schaller and Vasconcelos 1978; Pinder 1999). Within seasonally flooded plains, the fluctuation on water level is probably a limiting factor and may determine the extension of seasonal movements of the species. Published reports on home range sizes vary greatly for this species ranging from 3 to 33 km^2 in females and from 8 to 64 km^2 in males (Pinder 1999; Piovezan 2004).

Current estimates of the size of the entire population do not exist, but it is certainly declining in all the areas where it is currently distributed (Weber and Gonzalez 2003). Several populations of marsh deer in the Paraná basin are on the brink of extinction (Pinder and Seal 1994; Duarte et al. 2003; Weber and González 2003). Habitat fragmentation because of habitat loss due to agricultural activities and dam constructions has resulted in a dramatic decline of the species throughout its range (Beccaceci 1994; Pinder and Seal 1994; Duarte et al. 2003). Diseases transmitted by domestic animals and poaching have also contributed to the decline and extinction of small and isolated populations of deer (Montassier et al. 2001; Szabó et al. 2003; Torres et al. 2003). In Uruguay, the species has been completely extirpated and it is probably extinct since the last known record was from 1958 (González 1994).

The taxonomy and systematics of the marsh deer subspecies have been poorly studied. Nuclear DNA studies using allozymes and microsatellite loci in a sample of individuals from Porto Primavera showed that this population had low levels of polymorphism (García and Duarte 2001; Oliveira et al. 2005) and cytogenetic studies showed that the marsh deer had no karyotypic variation (Bogenberger et al. 1987; Duarte and Giannoni 1995).

In order to analyze the effect of habitat fragmentation on gene flow and genetic variation, and thereby obtain criteria for defining priorities in the management of this endangered species, we undertook a molecular genetic study based on samples from throughout the Río de la Plata basin populations. We evaluate the phylogeographic pattern of differentiation of these populations revealed through the phylogenetic analysis of the control region and cytochrome *b* gene of mitochondrial DNA, and we consider the historic aspects that may have influenced the current distribution of the genetic lineages (Avise 1998). Using the criteria of

Moritz (1994) for conservation management, we evaluate the presence of different Conservation Units in the Río de la Plata basin.

Processes in the demographic history of populations, such as expansions or contractions, leave recognizable signatures in the pattern of molecular diversity (Harpending et al. 1998; Schneider and Excoffier 1999). Historic events that cause populations to decrease in size may result in low levels of genetic variation in current populations even though these populations do not currently show low population numbers. Likewise, a species may appear genetically homogenous because of the demographic history of its populations. A common origin and multiple expansions of small populations can mask the effects of recent population fragmentation (Aris-Brosou S and Excoffier 1996). On the other hand, climatic changes such as those observed during Pleistocene glaciations, have been responsible for the current patterns of genetic diversity observed in many species (Lessa et al. 2003). If a species is closely associated with a particular environmental condition it will be strongly dependent on environmental changes and the identification of these historical processes can lead to clues that will help us discover other species that have similar environmental requirements and that have been affected in similar ways. For these reasons, hypotheses on the demographic history of marsh deer populations are related with changes in precipitation that altered the moist environments of the Río de la Plata basin, associated with events occurring prior to the Last Glacial Maxima of the Pleistocene.

Materials and Methods

Sample collection

Blood and tissue samples were collected from 127 individuals from nine localities from the "Río de la Plata" basin (Figure 1). Blood was taken from animals that were captured from five Brazilian localities around Porto-Primavera Hydroelectric dam located between the states of Sao Paulo and Mato Grosso do Sul (22°26′S; 52°59′W) (n = 116) and from Brazilian Pantanal (n = 1). Marsh deer were captured as part of a research program supported by the São Paulo State Energy Company (CESP) to establish a captive population and to perform translocation experiments of animals that were affected by the flooding of the water reservoir. The "bulldogging" technique, with the aid of a helicopter, was used to capture animals as described by Duarte (2001).

Tissue and hair samples were also taken from dead animals found in the field from Esteros del Iberá (Corrientes, Argentina) (n = 6), and from Delta del Paraná (Buenos Aires, Argentina) (n = 2) and from two captive animals from an undetermined locality in the Pantanal region of Bolivia (n = 2).

Extraction, amplification and mtDNA sequencing

Procedures for DNA extraction from tissue samples were modified from those described in Medrano et al. (1990). Universal primers Thr-L15910 and DL-H16498 (Kocher et al. 1989) were used in PCR reactions to amplify a 601 bp fragment including the 5' domain and the conserved blocks F and E of the control region (Douzery and Randy 1997), following protocols described by González (1997). In addition, a 486 bp fragment of cytochrome *b* gene was amplified for 44 representative individuals from all localities using primers L14724 and H15149, as described in Maldonado et al. (1995). DNA extractions and PCR reactions were performed at the genetic facilities at IIBCE (Uruguay) and at Departamento de Zootecnia, FCAV/UNESP (Brazil). Purified PCR products were shipped to the Smithsonian's Genetics Program (USDA Import Permit #46747) and sequenced using the ABI Big Dye ready reaction kit and ran on an automated sequencer ABI 377 (Applied Biosystems). Sequences were aligned using Clustal X (Thompson it et al. 1997). Sequence data have been submitted to GenBank (accession numbers: AY326235–AY326251) and AY32623–AY326234 for control region and cytochrome *b* respectively). A complete list of all DNA sample numbers, locality, control region haplotype designation and the storage location is available as an Electronic Appendix I at the Deer specialist Group Website: http://www.iibce.edu.uy/citogenetica/deer

Relationship of mtDNA control region sequences

Levels of genetic diversity in the populations were evaluated using control region sequences by estimating the mean number of pairwise differences

along with nucleotide and gene diversity (Tajima 1983; Nei 1987). The number of mutations between DNA genotypes in pairwise comparisons was used to construct a minimum spanning network (MSN) using ARLEQUIN (Schneider et al. 2001).

The data set was tested for the most appropriate model of nucleotide substitution using the likelihood ratio test (Huelsenbeck and Crandall 1997) implemented in Modeltest 3.06 (Posada and Crandall 1998). We then used the estimated model to construct a neighbour joining tree of genetic distances and also a maximum likelihood tree using PAUP* 4.0b10 (Swofford 1999), including published sequences of South American Pampas deer, *Ozotoceros bezoarticus* (GenBank, accession numbers AF012558, AF012559, AF012599) and grey brocket deer *Mazama gouazoupira* (accession numbers AF012556, AF012557) as outgroups (González et al. 1998). Group supports in the neighbour joining and maximum likelihood trees were evaluated with 1000 and 100 bootstrap pseudoreplicates, respectively.

Patterns of geographic subdivision

The significance of geographic distribution of haplotypes was evaluated with an Analysis of Molecular Variance (AMOVA) implemented in ARLEQUIN. In order to evaluate affinities among sample localities and to see if molecular diversity distributes according to geography, we experimented with various groupings of populations. We evaluated the significance of separating or grouping together both Argentinean populations (Delta and Iberá), and the significance of leaving the Bolivian population alone as compared to grouping it with the Brazilian populations.

We assessed differentiation by distance by plotting pairwise log (average number of nucleotide differences of the control region) values against log (geographic distance) values. The significance of this correlation was assessed by generating a probability distribution with 1000 permutations using the program MANTEL 2.0 (Liedloff 1999).

Demographic fluctuations

We calculated a maximum likelihood estimate of current effective population size measured with the parameter θ, and the g parameter to evaluate if populations have experienced growth or decline

using the method proposed by Kuhner et al. (1995) and implemented in the program FLUC-TUATE. To do this, we used only individuals from Brazil, given that these populations had a larger sample size and to avoid confounding the effects of geographic structure. To estimate N_e of females we used a mutation rate (μ) of 2.5×10^{-8} per nucleotide site per year, proposed by González et al. (1998) for Neotropical deer and 3 years for generation time (Duarte and Capalbo 2004).

Fu's F_S test of neutrality (Fu 1997), which is particularly sensitive to population growth, was used to infer the population history. F_S tends to be negative when there is an excess of recent mutations (Excoffier and Schneider 1999).

Demographic history parameters of Brazilian populations were estimated using two different approximations, one based on the mismatch distribution (Rogers and Harpending 1992; Schneider and Excoffier 1999) and another based on the number of polymorphic sites (Wakely and Hey 1997). In the first approach, the estimated parameters of the expansion $\tau = 2ut$, $\theta_0 = 2N_0u$, and $\theta_1 = 2N_1$ are used to perform coalescent simulations of stepwise expansions, using θ_0 and θ_1 to describe the population sizes before and after the expansion, while τ is the time transpired since the expansion occurred (Excoffier and Schneider 1999). Schneider and Excoffier (1999) proposed a test to evaluate the validity of the demographic model obtaining the distribution of SSD statistic (Sum of the Squared Differences). The parameters were estimated with ARLEQUIN (Schneider et al. 2001). The second approach estimates the parameters of an hypothetic expansion from the expected numbers of different categories of polymorphic sites in a model of population history, in which a single population undergoes an instantaneous change in size (Wakeley and Hey 1997). The parameters were calculated using the program SITES.

Results

A total of 127 individuals were sequenced for 601 bp of the control region, yielding 17 haplotypes (Table 1). There were 18 polymorphic sites, 17 transitions and 1 transversion, with no indels or sites with more than two different bases. Haplotypes differed by 1–8 bp substitutions.

Table 1. Summary of the mitochondrial haplotype (control region and cytochrome *b*) distribution in all the sampled localities in Brazil, Bolivia and Argentina

Population	Control region	Cytochrome *b*
Peixe River, SP (Brazil)	A (12)	I (6)
	I (1)	II (3)
	K (2)	
	L (2)	
Bataguassu area, MS (Brazil)	A (30)	I (17)
	C (1)	
	H (2)	
	I (5)	
	J (15)	
	M (6)	
Tiete River, SP (Brazil)	A (8)	I (3)
Aguapei River, SP (Brazil)	A (12)	I (5)
Cisalpina area, MS (Brazil)	A (12)	I (5)
	P (6)	
	Q (2)	
Pantanal (Brazil)	A (1)	
Sta. Cruz Zoo (Bolivia)	N (1)	III (1)
	O (1)	
Iberá (Argentina)	D (1)	I (2)
	F (4)	
	G (1)	
Delta del Paraná (Argentina)	B (1)	IV (2)
	E (1)	
	127	44

Numbers in parenthesis refer to samples sizes.

The average number of paired differences ($\hat{\pi}$) in the Brazilian localities close to the Porto Primavera dam ranged from 0 (Tietê and Aguapei rivers) where only the A haplotype was found, to 0.851 (The Mato Grosso do Sul population in Porto Primavera). The Argentinean population of Iberá showed a somewhat higher value of $\hat{\pi}$ (1.667). Genetic diversity was similar between Iberá and Brazil (0.572 and 0.600 respectively). Nucleotidic diversity ($\hat{\pi}_n$) for all populations was 0.0017. The Brazilian localities showed lower values (0.0012) compared to Iberá (0.003) (Table 2). Only two individuals were sampled from the Delta population in Argentina and two from Bolivia, and therefore, genetic variability indices were not calculated.

By analyzing the Brazilian localities together as a group, we found that A was the most abundant haplotype, with a frequency of 64%, followed by the J haplotype with a frequency of 13% and the remaining haplotypes with frequencies lesser than or equal to 5%. The single individual from

Table 2. Control region diversity indexes of marsh deer. Abbreviations of locations as in Figure 1

Population	Nucleotide diversity ($\hat{\pi}_n$)	Gene diversity (H)	Mean number of pairwise differences ($\hat{\pi}$)
The species	0.0017	0.6367	
Brazil as a whole	0.0012	0.5720	0.0012
BA-MS			0.8510
PR-SP			0.4830
AR-SP			0.0000
CA-MS			0.6320
TR-SP			0.0000
Bolivia	–	–	–
Iberá	0,0030	0.6000	1.667
Delta del Paraná	–	–	–

Pantanal had the A haplotype and the two Bolivian individuals had haplotypes not found in other localities (N and O) but with only one base pair difference from the A haplotype. The Delta and Iberá samples also had haplotypes not found in other localities (Table 1).

The 486 bp fragment of the cytochrome *b* gene that was sequenced in 44 representative individuals from all localities yielded three variable sites that defined four haplotypes. Haplotype I occurred in high frequency (36 of 39 individuals) in the Brazilian populations and in the two individuals sequenced for this region from the Iberá population. The remaining Brazilian individuals (3) had haplotype II. A third haplotype (III) was found in the 2 individuals from Bolivia, while both individuals from the Delta population exhibited a unique fourth haplotype (IV). The extremely low diversity and the low levels of divergence (1 or 2 differences between haplotypes) found in cytochrome *b*, did not allow us to make any meaningful phylogenetic inferences using this marker.

Relationship between control region sequences

The relationship between the Brazilian haplotypes, the two Bolivian haplotypes, and at least one of those found in Iberá (Argentina) shows a star-shaped phylogeny in a minimum spanning network, typical in populations that undergo rapid demographic expansions (Rogers 1995; Schneider and Excoffier 1999) (Figure 2). In this part of the network, the most common A haplotype is one or
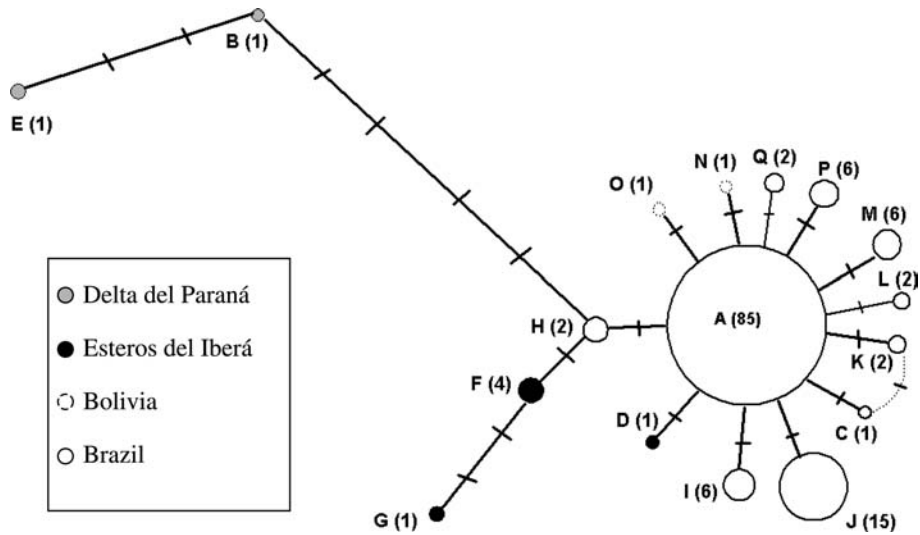
Figure 2. Minimum spanning network based on the number of substitutions among control region haplotypes. The circles are sized proportionally to the frequency of haplotypes and the absolute frequencies are indicated in brackets. Marks on the lines connecting each haplotype represent a single basepair substitution. Dotted lines represent alternative groupings.

two base pairs different from other 11 less common haplotypes.

The neighbour joining tree shows a strong support for the monophyly of *B. dichotomus* haplotypes with respect to the outgroups (Figure 3a) and no clear phylogeographic pattern of haplotype distribution was found. A relatively well-supported cluster included most haplotypes except those from the Delta del Paraná, and this

main cluster joined first with haplotype E from Delta del Paraná with a bootstrap support of 70% and then with the other haplotype (B) from Delta del Paraná. The Maximum likelihood tree showed the same topology as the neighbour joining tree but the consensus tree showed that the two Delta del Paraná haplotypes formed the only grouping with bootstrap support higher than 50% (Figure 3b). This separation is supported by the
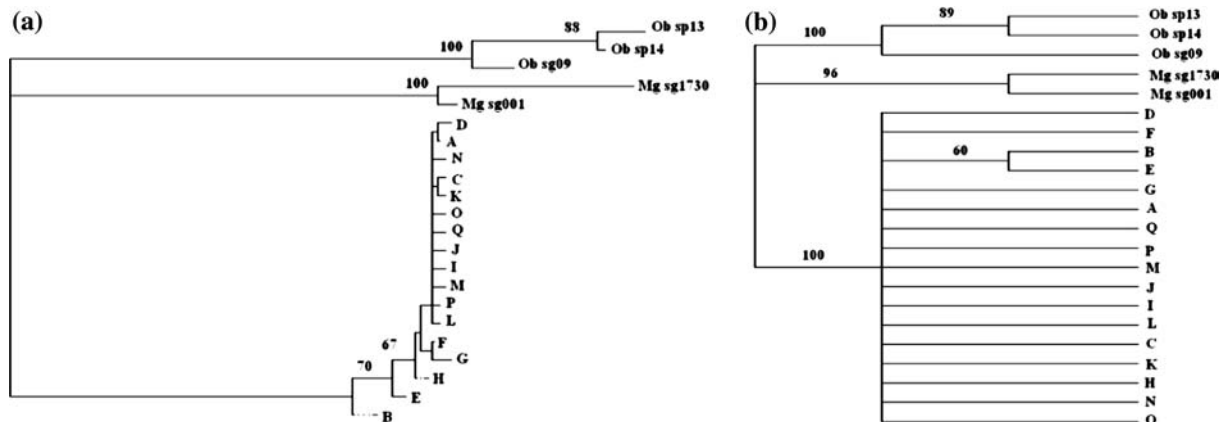

Figure 3. (a) Neighbour joining tree of control region sequences with a genetic distance based on a HKY85 model with a gamma distribution of variable sites (α = 0.20), utilizing *Ozotoceros bezoarticus* (Ob sp13, Ob sp14, Ob sg09) and *Mazama gouazoupira* (Mg sg001, Mg sg1730) as outgroups. Haplotypes B and E are from Delta del Paraná; D, F and G from Esteros del Iberá; N and O were present in Bolivia, while the remaining haplotypes are from Brazil. The numbers refer to nodes supported in 1,000 bootstrap pseudoreplicates. (b) Maximum likelihood consensus tree from bootstrap analyses based on 100 subreplicates (numbers refer to bootstrap support).

fact that haplotypes B and E share a change in the most conserved Pro-tRNA segment flanking the control region.

Patterns of geographic subdivision

Various populations groupings were assessed using an AMOVA analysis and the resulting Φ-statistics were used to assess the among group, among population within group and the within group population variance (Table 3). The grouping that includes the Bolivian haplotypes with those from Brazil (groupings 2, 5, 6) increased the among group variance and decreased the within group variance, based on whether it is considered alone or with the Argentinean populations (groupings 1, 3, 4, 7, 8), which shows the affinity of Bolivia with Brazil.

The two groupings separating the Argentinean populations (4 and 5) improved the explained variance values even more and were significant at the 10 % level. They presented the lowest intra-group variance values (2.73% and 2.86%, respectively), with greater between group variance when Bolivia is included with Brazil (grouping 5).

Grouping the Delta haplotypes with the others invariably decreased the efficiency in explaining molecular variance. The homogeneity of the

Table 3. AMOVA results for different subdivisions of analyzed localities. Groupings were made according to a geographic criterion and the results of phylogenetic trees. Fixation indices due to differences "among groups, AG" (Φ_{CT}), to "among populations within the groups, AP/WG" (Φ_{SC}) and to the differences "among populations according to its individuals with respect to the total individuals, AP" (Φ_{ST}). MS-PP includes both localities form the Mato Grosso do Sul of Porto Primavera: Bataguassu and Cisalpina areas; SP-PP includes the São Paulo samples of Porto Primavera: Peixe, Aguapei and Tietê rivers

Groupings	Variance component	% Total variance	Φ-statistics	P
(1)Brazil + Bolivia + Argentina	AG	56.25	Φ_{CT}: 0.56	0.02
	AP/WG	10.84	Φ_{SC}: 0.25	0.00
	WP	32.90	Φ_{ST}: 0.67	0.00
(2) (Brazil + Bolivia) + (Argentina)	AG	60.40	Φ_{CT}: 0.60	0.04
	AP/WG	10.11	Φ_{SC}: 0.25	0.00
	WP	29.48	Φ_{ST}: 0.70	0.00
(3)Brazil + (Bolivia + Argentina)	AG	46.91	Φ_{CT}: 0.47	0.02
	AP/WG	15.09	Φ_{SC}: 0.30	0.00
	WP	37.23	Φ_{ST}: 0.63	0.00
(4)Brazil + Bolivia + Iberá + Delta	AG	71.63	Φ_{CT}: 0.71	0.00
	AP/WG	2.73	Φ_{SC}: 0.10	0.00
	WP	25.84	Φ_{ST}: 0.74	0.00
(5) (Brazil + Bolivia) + Iberá + Delta	AG	74.59	Φ_{CT}: 0.75	0.07
	AP/WG	2.86	Φ_{SC}: 0.11	0.00
	WP	22.55	Φ_{ST}: 0.77	0.00
(6) (Brazil + Bolivia + Iberá) + Delta	AG	83.69	Φ_{CT}: 0.84	0.12
	AP/WG	4.45	Φ_{SC}: 0.27	0.00
	WP	11.86	Φ_{ST}: 0.88	0.00
(7) (Brazil + Iberá) + Bolivia + Delta	AG	73.08	Φ_{CT}: 0.73	0.03
	AP/WG	7.17	Φ_{SC}: 0.26	0.00
	WP	19.75	Φ_{ST}: 0.80	0.00
(8)Brazil + (Bolivia + Iberá) + Delta	AG	66.37	Φ_{CT}: 0.66	0.01
	AP/WG	5.49	Φ_{SC}: 0.16	0.00
	WP	28.14	Φ_{ST}: 0.71	0.00
(9) (Brazil + Bolivia + Delta) + Iberá	AG	53.37	Φ_{CT}: 0.52	0.26
	AP/WG	15.39	Φ_{SC}: 0.32	0.00
	WP	32.24	Φ_{ST}: 0.68	0.00
(10)MS-PP + SP-PP + Bolivia + Iberá + Delta	AG	42.39	Φ_{CT}: 0.42	0.02
	AP/WG	5.36	Φ_{SC}: 0.09	0.00
	WP	52.25	Φ_{ST}: 0.48	0.00

P is the probability value of the grouping respect to random.

Brazilian localities is shown in grouping 10, where the separation of these into two groups results in a decrease in between group variance.

Therefore, this analysis shows the homogeneity of the Brazilian localities, the similarity of the Bolivian haplotypes with the Brazilian haplotypes, and the differentiation of the haplotypes between the two Argentinean populations.

Furthermore, the degree of differentiation observed between localities appears to follow a predictable relationship with geographic distance. Mantel's test between Log (geographic) distance and Log (genetic) distance reveals a significant correlation ($r = 0.792$; $P < 0.05$)

Demographic history

The maximum likelihood estimate of θ was 0.0113 resulting in a present day effective number of reproductive females of 90,000 for the populations close to Pantanal. The exponential growth rate estimate of g was 2879 indicating that this population could have experienced a drastic increase of population numbers in a recent time of its history.

For Brazil, the F_S statistic value was negative and significant ($F_S = -8.70722$; $P = 0.0004$). The observed distribution of pairwise differences was unimodal, suggesting that the population would not be demographically stationary. The SSD statistic was significant ($P < 0.0001$) which would allow us to reject the null hypothesis and assume a process of demographic expansion.

Given that 601 bp were utilized in our analyses and based on the mutation rate per site described for neotropical cervids (González et al. 1998), the mutation rate per sequence utilized in the calculations of the absolute values was 1.50×10^{-5} year^{-1}. The absolute value of the expansion time, expressed in years, was around 28,000 years (Table 4). The 95% confidence limits were extensive, generating an interval between 5 and 37 thousand years. Assuming 3 years as generation time for calculating the absolute number of individuals before and after the expansion, the expansion dimension in the effective number of females is on the order of 10^7.

Using the estimated parameters τ, θ_A and θ_1 based on the Wakeley and Hey (1997) method, we found that the absolute expansion time was around 25,000 years and the magnitude of the expansion was estimated at 5.3×10^4 females (Table 4). Both methods estimate values of $\theta_1 = 0.0$, suggesting that the population must have been very small prior to the expansion process.

Discussion

Genetic variability

Genetic variability measured using different indices, was very low in the endangered South American marsh deer.

The cytochrome b sequences obtained confirm the low genetic variability observed in the control

Table 4. Estimated population expansion parameters. The values were obtained using two models that assume a recent expansion in the population. The first model is based on a mismatch distribution (Rogers and Harpending 1992; Schneider and Excoffier 1999) and the second model is based on the number and class of polymorphic sites (Wakeley and Hey 1997)

Estimated expansion parameters			Confidence interval (percentile method) $\alpha = 0.05$		
			Lower limit	Upper limit	Mean value
Model based on a mismatch distribution					
$\tau = 0.86$			$\tau = 0.34$	1.15	0.80
$\theta_0 = 0.00$			$\theta_0 = 0.00$	0.69	0.12
$\theta_1 = 1,457.80$			$\theta_1 = 2.19$	3907.31	1805.72
Absolute values					
$t = 28,752$ years B.P.			$t = 11.22$	38.14	26.56
$N_0 = 0$ females			$N_0 = 0$	7687.00	1287.00
$N_1 = 16,170,840$ females			$N_1 = 24,304$	43,342,263	20,030,117
Model based on the polymorphic sites					
τ	θ_A	θ_1			
0.737	0.000	4.783			
Absolute values					
$t = 24,526$ years B.P.	$N_A = 0$ females	$N_1 = 53,055$ females			

region, given that the greatest difference observed between two sequences was only two substitutions. These few differences did not allow us to assess phylogenetic relationships between the populations, however, given the slower rates of change of this region, this low divergence support the common origin of the populations we studied, prior to the processes of expansion and current differentiation revealed by the control region.

The low genetic diversity found in the marsh deer may be related to their small population sizes. Currently, the wetlands in South America, with the exception of the Pantanal, are not large enough to maintain large populations of marsh deer. Our estimate of present-day effective number of females of marsh deer (90,000) is twice the size of the entire population size of Pantanal, which is estimated at approximately 45,000 individuals (Mourão et al. 2000). Since N_e is an average of population sizes over generations, it reflects population sizes previous to the recent population declines caused by human factors.

Conversely, the genetic variation exhibited by the marsh deer is similar to that of species which have been hypothesized to have undergone a recent rapid demographic expansion from populations that underwent a bottleneck and survived in Pleistocene refugia (Kvist et al. 1999; Milá et al. 2000; Eizirik et al. 2001; Hundertmark et al. 2002). Therefore, the low levels of genetic diversity are probably due to the demographic history of the species, which would include periods of small population size during the Pleistocene.

The two haplotypes recovered from the Delta del Paraná are the most divergent in the minimum spanning network and in the phylogenetic trees. Considering that the mean divergence between sequences from the Paraná Delta population with the Brazilian populations utilized here is 1.0% (6.18 nucleotides) and based on the sequence divergence rate for the control region in South American Odocoileines of 5% per million years (González et al. 1998), the origin of genetic variation of Delta del Paraná can be estimated at around 200,000 years.

Analyses on population structure corroborate the relationship observed between haplotypes. AMOVA analyzes also show haplotypes from Brazil and Bolivia to be very close. On the other hand, the Paraná Delta population is separate from the rest and the Iberá population maintains

intermediate values, but with a greater affinity with Brazil than with the other Argentinean population. The similarity of Iberá to Brazil is supported by the shared cytochrome *b* haplotypes.

The genetic and geographic distance correlation analysis indicates that this relationship between populations may be due in part to limited gene flow caused by distance (Slatkin 1993).

Pleistocene History

The low levels of nucleotidic diversity observed in these populations of marsh deer indicate that at least the Brazilian populations close to the Pantanal withstood small population sizes during periods of their recent evolutionary history. Furthermore, the neutrality test, as well as the demographic history models, are in keeping with explosive population growth from small population sizes. The haplotypes from the Brazilian populations and those found in the Bolivian individuals show a star-shape phylogeny with a presumed ancestral A haplotype present in high frequency and the rest in lesser frequencies, differing by one or two bases from the ancestral haplotype.

Our expansion hypothesis is supported by paleoclimatic changes. In the mid-to-low latitudes, glacial advance principally depended upon the availability of moisture, reaching its peak advance between 30,000 and 27,000 B.P., indicating that there was an increase in precipitation in that period in South America (Clapperton 1993a). In addition, geochemical and palynologic studies (Salgado-Labouriau et al. 1997) furnished evidence that in central Brazil there was a great increase in moisture between 32,000 and 20,000 B.P. Ledru (1993) has also found evidence of increased precipitation in central and southern Brazil. Thus, this increase in general humidity in a region close to the source of the Paraná River coincides precisely with the times of population expansion for the central Brazilian populations analyzed here, estimated between 25,000 and 28,000 B.P.

It is interesting to note that even though habitat fragmentation may currently prevent movement of deer between sites along the Paraná river, there appears to have been no physical barriers to movement between the Delta del Paraná and other populations up to 300 years

ago before the process of habitat alteration by human activities. However, our results suggest that they are differentiated. A similar pattern was observed in two populations of Pampas deer in Uruguay currently separated by 400 km due to habitat fragmentation but that 300 years ago did not appear to have any barriers that would prevent gene flow. These populations showed significant genetic and morphological differentiation (González et al. 2002).

The phylogeographic break between the Delta del Paraná and other marshlands upstream from the Paraná River seems to be reflected in at least one other species associated with marshlands and of scarce distribution (i.e. the frog *Argenteohyla siemersi*) (Williams and Bosso 1994). The patterns of genetic differentiation observed in marsh deer from Delta del Paraná could be due to the presence of a refuge created by sea level changes in the Río de la Plata Area (Violante and Parker 1999). In addition, drier conditions to the North of Rio de la Plata during most of the Late Pleistocene (Clapperton 1993b; Panario and Gutiérrez 1999) could have isolated these populations from the rest of the populations in the Paraná River basin.

The genetic footprint of a demographic expansion detected in marsh deer suggest that wetland habitats in Eastern low and middle latitudes of South America may have been susceptible to the global climatic changes that occurred at the end of the Pleistocene and thus affected the patterns of genetic diversity of the species that inhabited them. Our results suggest that environmental changes that caused an increase in wetland habitat during the Pleistocene affected the population dynamics of marsh deer. Therefore, it is reasonable to assume that the same environmental processes could have affected other species with similar habitat requirements in the Pantanal region.

The observed differentiation of the Delta population, however, is based on only two samples and it must be corroborated by additional sequencing of more individuals and/or other nuclear genetics markers. This should also help to evaluate if the marsh deer that inhabit wetlands in Delta del Paraná and other southern regions in Uruguay and Southern Brazil share a different demographic history than that suggested for the more northern Pantanal wetlands.

Conservation Implications

The marsh deer is endangered in Argentina and Brazil. The largest extant populations exists in the Pantanal of Brazil and Eastern Bolivia. An estimate of the Brazilian Pantanal populations indicates a population size of 45,000 for the entire area (Mourão et al. 2000). The Argentinean Iberá wetland is the second largest wetland in South America (Beccaceci 1994) and aerial census at Iberá estimated that 1000 marsh deer inhabit a 12,000 Km^2 area. The current estimate of population sizes in the Paraná Delta in Argentina is approximately 400 individuals (Varela et al. 2001). Little is known about the population status in Bolivia and it is considered extinct in Uruguay (González 1994). Our results provide a strong mandate for the protection of the remaining populations in the Paraná basin, specifically for the Delta del Paraná given the potential differentiation of this population and its scarce number of individuals. The populations of Iberá, Delta del Paraná and Bolivia each showed unique haplotypes that were not shared with the Brazilian populations in the Porto Primavera dam area. However, this unique distribution of haplotypes in the populations is not reflected in the formation of as many different lineages as populations. The presence of different haplotypes among Delta de Paraná, Iberá, and Brazil, and the difference observed with AMOVA suggest that these populations represent three different reproductive stocks, and should be considered different *Management Units* (Moritz 1994). The two haplotypes found at Delta del Paraná were the most divergent ones and although our sample size is small it is important to stress that the remaining population sizes are also very small in this area (a few hundred individuals) and if the sequences obtained were rare in the population, it would be unlikely that these were the ones sampled. However, further sampling and additional information about population dynamic, reproduction, antler cycle and morphology are needed. This combined information will help us evaluate if the degree of genetic differentiation among these populations is also associated with behavioral, ecological and morphological variables and reevaluate our management strategies.

Several factors have contributed to the decline of this species: habitat loss by urbanization,

transmission of diseases by livestock, forestation with exotic tree species and poaching (Varela et al. 2001). Nevertheless, one factor that has had a big impact has been the construction of hydroelectric dams. For example, a marsh deer population in Porto Primavera estimated at about 1000 individuals before the dam operation in 1998, declined to 350 individuals in just two years (Piovezan 2004). For populations that are declining so rapidly as is the case of Porto Primavera several management actions were considered such as translocation experiments (Pinder 1999; Duarte and Torres 2003), reintroduction and captive breeding program (Duarte and Capalbo 2004; Figueira et al. 2005). A successful reintroduction in the state of Sao Paulo performed last 1998 (Figueira et al. 2005) showed that it is possible to undertake these management actions to increase the current range of the species in selected suitable areas where they were once abundant.

A captive breeding program was implemented in Brazil beginning with 30 founders and now has 60 animals from the Porto Primavera population (Duarte and Capalbo 2004). Another 50 animals derived from the Tietê river population are maintained in the Marsh Deer Conservation Center by CESP (Electric Power Company of São Paulo) and now these two populations will be joined in the same studbook. This captive stock will be used to sustain the reintroduction programs in areas where the marsh deer has gone extinct. However, it is extremely important to implement conservation measures at the *in situ* level to assure suitable habitat for the remaining marsh deer populations in the Paraná basin, and consider management actions to increase the quality of the habitat required by this species.

Acknowledgments

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