GENETIC DIVERSITY AND GENE FLOW IN NINE-BANDED ARMADILLO (*DASYPUS NOVEMCINCTUS*) POPULATIONS OF PARAGUAY: IMPLICATIONS FOR CONSERVATION AND MANAGEMENT

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PREFACE

This study was conducted to provide knowledge on genetic variability within and among populations of nine-banded armadillo, *Dasypus novemcinctus*, in Paraguay. These results, and previous ecological studies on *D. novemcinctus* will aid the Paraguayan Government's conservation program by providing data for management of this species. Sequence analysis of mitochondrial DNA (mtDNA) cytochrome *b* was used to examine population structuring, haplotype and nucleotide diversity, and gene flow. Two approaches were undertaken to determine population structuring: ϕ-statistics, an analogue to Wright's *F* statistics and nested clade analysis. This thesis is written in the format of the Journal of Mammalogy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENETIC DIVERSITY AND GENE FLOW IN NINE-BANDED ARMADILLO POPULATIONS OF PARAGUAY: IMPLICATIONS FOR CONSERVATION AND MANAGEMENT POLICY

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ABSTRACT

In this paper we present an analysis of the mitochondrial DNA (mtDNA) variation in nine-banded armadillo (*Dasypus novemcinctus*) populations in Paraguay where the species is heavily hunted for food. Although *D. novemcinctus* is regularly hunted and constitutes an important protein resource in Paraguay the country lacks a management program for this species. To contribute toward conservation planning for this species we examined intra and interpopulation genetic variation in the mtDNA cytochrome *b* gene (1140 bp) across the range of the species in Paraguay. Twenty five haplotypes were identified among the 75 individuals examined. Gene diversity and nucleotide diversity ranged from 0.68 to 0.94 and 0.003 to 0.005

respectively. Genetic homogeneity was observed among all 5 populations indicating a lack of barriers to maternally mediated gene flow in the study area. Nested clade analysis reveals continuous range expansion, isolation by distance and long distance dispersal of female nine-banded armadillo.

Keywords: *D. novemcinctus*, genetic variability, Nested clade analysis, mitochondrial DNA

INTRODUCTION

In many countries the harvest of wildlife species constitutes an important human activity (Robinson and Redford 1991). For example, in remote areas of South America, subsistence hunting is an important occupation and a source of protein and income for indigenous and rural populations (Ojasti 1991; Prescott-Allen and Prescott-Allen 1982). Unfortunately, the classic ideology of subsistence hunting, in which hunter-gathers live in harmony with their environment, no longer prevails. Several studies have revealed that this activity has led to over-harvesting of many species (Alvard 1998; FitzGibbon 1998) and is aggravated by the increasing human population and concomitant anthropogenic factors (Spellerberg 1996).

Nine-banded armadillos serve as an important source of protein throughout most Latin American countries (Alvard

1995; Ayers et al. 1991; Frost 1977; Hill 1997; Mittermeier 1991; Vickers 1991). Considering that the harvest of wild species constitutes an important human activity in most Latin American countries (Oliver 1993; Robinson and Redford 1991) this activity is unlikely to change. Abundance (Cullen et al. 2000) and in encounter rates (Hill et al. 1997) of nine-banded armadillos keep decreased in some parts of Latin America where the species is hunted. Therefore, it is necessary to properly manage this species. Although the nine-banded armadillo is not currently listed as endangered by any international conservation agency (Emmons and Feer 1990) it is essential to amass base-line data. A combination of ecological and genetic factors can provide the necessary data for a proper management program that will ensure its long-term conservation. Additionally, more management options are available for non-endangered species compared to conservation efforts on endangered species thus, increasing the chances of management success. Finally, because historic information is encoded in DNA, a conservation management approach based on molecular data of natural populations of nine-banded armadillos would provide insight into their evolutionary history and therefore provide a better understanding of the processes that have been operating over evolutionary time.

Despite the fact that nine-banded armadillos are regularly hunted in Paraguay and represent an important source of protein, there is no management program for this species within the country. Therefore, the purpose of this study is to assess levels of genetic variation within and among populations of nine-banded armadillos in Paraguay. The goal of this study is to provide a better understanding of the biology of *D. novemcinctus*, which can be difficult to determine based on ecologic studies alone due to their burrowing and nocturnal or crepuscular behavior (Bider 1962, Kalmabach 1943; Newman 1913). Interpretation of genetic data in combination with ecologic data should lead to better management decisions and contribute to the long-term conservation of nine-banded armadillos in Paraguay.

MATERIALS AND METHODS

Study area and sample collections.--Samples for this study were collected in Paraguay, a land-locked country located in south-central South America, with the north-south flowing Paraguay River dividing the country in 2 distinct areas: the Eastern Region and the Chaco Region to the west (Hanratty and Meditz 1988; Fig. 1). Marked climatic conditions determine vegetation formation in both regions. Vegetation in the Chaco is mostly xerophytic consisting of a mosaic of grassland, savannas, open woodlands and xeric

thorn forest (Redford et al., 1990), whereas vegetation in the region east of the Paraguayan River is mostly subtropical humid forest, marshy plains, and grassland (Myers 1982). Annual precipitation increases in a northwest-southeast direction from 387 mm per year to 1760 mm per year. Annual temperature also varies along this gradient from 26°C in the northwest to 21°C in the southeast (Myers 1982).

Five sampling locations were selected (Fig.1). Three locations were established in the Chaco Region, west of the Paraguayan River, and 2 in the region east of the Paraguayan River. These locations were selected based on the following criteria: a) populations of nine-banded armadillos were present, b) locations were separated by approximately 100 km or more, and c) locations were in different ecosystems. The sampling locations in the Chaco Region were Casanillo (CA), Estancia Juan de Zalazar (EJZ), and Ruta Transchaco Km 106 (106). In the Eastern Region, collecting localities were Lago Ypoa (LY) and Estancia Golondrina-Caazapa (EGC; Fig. 1). In this paper a population is defined as a group of individuals from a single sampling location.

The numbers of *D. novemcinctus* sequenced from each collection site were: Casanillo = 18, Estancia Juan de Zalazar = 20, Ruta Transchaco Km 106 = 15, Lago Ypoa = 6, and Estancia Golondrina-Caazapa = 16 (Fig. 1).

Tissue samples (muscle, kidney, heart, liver, or carapace) from approximately 20 individuals at each collecting site were obtained with the help indigenous and local hunters during May - August, 1999. The samples were stored in 15 ml tubes containing 5 ml of lysis buffer (Longmire et al. 1997) and imported to the United States with legal documentation.

Laboratory Procedures.--Total genomic DNA was extracted from 75 individuals following the protocol of Longmire et al. (1997). The entire 1140 basepair (bp) cytochrome *b* gene was amplified via the polymerase chain reaction (PCR) using flanking tRNA primers L14724 and H15915 (Irwin et al. 1991). Each mixture contained approximately 550 ng of DNA in a final reaction volume of 50 µl containing 5 µl of 10X buffer, 0.52 mM of each primer, 1 mM of each dNTP, 2.5 mM $MgCl_2$, and 1.25 units of *Taq* DNA polymerase (Promega Corporations, Madison, Wisconsin). The thermal profile for amplification consisted of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 30 s, and 72°C for 90 s followed by a final extension of 72°C for 30 min. All amplifications were performed using a Perkin-Elmer Applied Biosystems GeneAmp PCR system 9600 (Foster City, California). Resulting amplicons were electrophoresed through 1% agarose gels stained with ethidium bromide and visualized with ultraviolet light.

Successful amplicons were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, Wisconsin). Both strands of the amplified products were sequenced using the flanking tRNA primers and cycle sequencing according to the manufacturer's instructions (BigDye™, Perkin-Elmer Applied Biosystems, Foster City, California). Cycling conditions were as follows: 25 cycles at 96°C for 10 s, 50°C for 5s, and 60°C for 4 min. Sequence products were electrophoresed on a Perkin-Elmer Applied Biosystems 377 Automated DNA sequencer. From these sequences, specific internal primers L7887 (5'-GCAACTCTAACACGCTTCTTCGCCT-3') and H7886 (5'-GACGAGATACCTGTGGGGTTGTTTG-3') were designed for complete double-stranded DNA sequencing.

AssemblyLIGN™ 1.0.9 (Oxford Molecular Group PLC, 1998) was used to assemble contiguous, overlapping fragments within individuals and a multiple sequence alignment of all individuals was performed using CLUSTAL X (Thompson et al. 1997). The multiple sequence alignment was subsequently imported into the computer program MacClade (Madison and Madison, 2000) for visual inspection and to group sequences into unique haplotypes.

Data Analysis.--Haplotype diversity (h), nucleotide diversity (π), and haplotype frequencies were calculated using Arlequin 2.0 (Stefan et al. 2000). The AMOVA procedure (Excoffier et al. 1992) implemented in Arlequin

was used to analyze nucleotide diversity [ϕ-statistic, an analogue to Wright's (1965) F_{ST}]. Arlequin was also used to compute pairwise ϕ_{ST}-values with significance levels calculated using 1,000 permutations. The effective number of dispersing females (Takahata and Palumbi 1985) was estimated from the approximation $N_fm = ((1/\phi_{ST}) - 1)/2$.

The program TCS (version 1.12; Clement et al. 2001) was used to generate an unrooted haplotype genealogy following the algorithm of Templeton et al. (1992) with ambiguities in the genealogy resolved following the recommendations of Crandall and Templeton (1993) and Crandall et al. (1994). This genealogy was converted into a nested design by grouping haplotypes into 1-step clades, 1-step clades into 2-step clades, and so on until all sub-clades are nested into a single clade using the procedure described by Templeton et al. (1987). The resulting nested clade design and geographic distance between all pairs of populations were analyzed using the program GeoDis (Posada et al. 2000). GeoDis calculates the clade distance (D_c), which measures the geographic range of a particular clade, and the nested clade distance (D_n), which measures how a particular clade is geographically distributed relative to its closest evolutionary sister-clade. D_c and D_n distances were then used to calculate the average interior distance minus the

average tip distances [$(I\text{-}T)_c$ and $(I\text{-}T)_n$]. These 4 statistics, with a key provided by the authors (Posada et al. 2000; http://biog.byu.edu/zoology/crandall_lab/geosis.htm), were used to infer a biological explanation of the results with the null hypothesis being no association between haplotype genealogy and geographic distribution. Rejection of this null hypothesis can provide insight into the role of historic and contemporary processes producing the observed patterns of haplotype distributions (Templeton et al. 1995).

RESULTS

For each individual, 18 bp from both the 5′ and 3′ ends of the sequence were removed because of their absence in 6 individuals. This reduction of sequence data does not represent a significant loss of information, as available sequences show no variable positions in the excluded region. Therefore, the final analysis consisted of 1,104 bp for the 75 individuals included in this study. All sequences were deposited in GenBank.

Sequence analysis identified 33 variable positions, representing 25 haplotypes (Table 1). Twenty-four percent, 3% and 73% of these substitutions were at 1^{st}, 2^{nd}, and 3^{rd} codon positions. Thirty of the substitutions were

transitions and 3 were transversions, resulting in 8 amino acid substitutions.

Haplotypes A, B, C, D, and E were the most frequent haplotypes (Table 2). Haplotypes A and B occurred in all 5 populations. Haplotypes C and D occurred in only 3 populations, but were present on both sides of the Paraguayan River. Haplotype E was restricted to a single population in the Chaco Region. Haplotypes G, H, and I occurred in 2 populations in the Chaco Region. Haplotype F and J were restricted to the Eastern and Chaco regions, respectively. The remaining 15 haplotypes occurred only in single individuals, 8 in the Chaco Region and 7 in the Eastern Region (Table 2).

Within population haplotype diversity (h), which represents number and frequency of haplotypes, was moderate to high, ranging from 0.68 to 0.94. In contrast, nucleotide diversity (π), which is based on frequency of haplotypes and sequence divergence among haplotypes within sites, was low in all 5 populations (Table 2).

Proportion of genetic diversity attributable to variation within populations and among populations within regions and between regions were 93.5%, 1.23%, and 5.24%, respectively. Only 6.5% of the differences in genetic diversity was attributable to differences among populations. Correspondingly ϕ_{ST}-values from pairwise population

comparsions revealed no significant geographic variation among populations of nine-banded armadillos after correcting the alpha level to obtain a type I error of 0.05 for multiple comparisons (Rice 1989; Table 3). Based on mean ϕ_{ST}, number of dispersing females ($N_f m$) was 7.2 individuals per generation.

Our parsimony haplotype network reveals haplotypes separated by up to 9 mutational steps (Fig. 2). Within this network, 2 loops indicate ambiguous connections. One loop contained haplotypes A, G, V, and Y, whereas the 2nd loop consisted of haplotypes A, P, and Y. Despite this ambiguity the logic of Crandall and Templeton (1993) indicate that haplotype Y is a tip haplotype derived from haplotype A by a single mutation.

Figure 2 shows the nested design and results of the statistical testing of the null hypothesis (no association between geographic distribution of haplotypes and mtDNA genealogy). Results of the nested clade analysis of geographical distance of the haplotypes are shown in figure 3. The null hypothesis was rejected for 3 nested clades (Table 4). The interpretation for clade 2-3 is that there was inadequate geographic sampling to discriminate between fragmentation and isolation by distance. The chain of inference for clade 2-4 indicates that the association between mtDNA genealogy and geographic distribution is due

to continuous range expansion, whereas results for clade 3-2 are interpreted as indicating restricted gene flow with some long distance dispersal.

DISCUSION

The genetic structure of nine-banded armadillos has been examined at various localities in South America (Huchon et al. 1999; Loughry et al. 1998), but this is the first such study of populations in Paraguay. In agreement with previous studies of nine-banded armadillos in South America (Huchon et al. 1999; Loughry et al. 1998), we found moderate to high levels of haplotype diversity. In contrast, nucleotide diversity within sites was low in our study. Thus Paraguayan populations have a high number of closely related haplotypes (Table 2). These results indicate that reduced haplotype diversity is not associated with polyembryony in this species supporting the conclusion reached by Huchon et al.(1999) for nine-banded armadillos from French Guiana.

The hierarchical analysis of genetic variation indicated that only 5.2% of the variation is attributable to differences among populations separated by the Paraguay River. Indicating that the river is a relatively weak barrier to dispersal of females possibly because the nine-banded armadillo is able to walk or swim across bodies of water (Kalmbach 1943; Taber 1939; Talmage and Buchanan

1954), and the water level of the Paraguay River can be dramatically low during extreme drought (http://www.rivdis.sr.unh.edu/cgi-bin/TileMap). Under the island model of gene flow (Wright 1965), the observed value of ϕ_{st} = 0.065 corresponds with 7.2 females exchanged among populations per generation. Number of migrants per generation necessary to prevent divergence among populations is 1 for the island model (Wright 1965) and 2-4 for the stepping stone model (Crow and Aoki 1982).

Few studies have assessed the Paraguay River as a barrier to gene flow. Frost et al. (1998) concluded that the Paraguay River was a barrier to gene flow for lizards of the genus *Tropidurus*.

The null hypothesis of no geographical association of haplotypes was rejected for 3 nested clades. First, the nested clade analysis indicated that the geographic distribution of Clade 2-3 indicated either fragmentation or restricted gene flow via isolation by distance. Anthropogenic factors, primarily due to livestock and agriculture have increased considerably in Paraguay during the past decades. However, such activities are unlikely to affect nine-banded armadillos. The species occurs throughout the heavily populated southern United States indicating that urbanization does not significantly affect this species. The main factors affecting nine-banded armadillos appear to

be drought and cold (Humphrey 1974). Therefore, because clade 2-3 contains haplotype C, which is the 3rd most abundant haplotype and found on both sides of the Paraguay River, a plausible explanation for rejecting the null hypothesis of random mating for this clade is isolation by distance and not fragmentation. Second, clade 2-4 contains 3 clades (1-7, 1-8, and 1-9) containing a mixture of haplotypes from both sides of the Paraguay River. Results of the nested clade analysis suggest that this clusters of clades and the associated frequencies and geographic distribution of haplotypes within these clades are due to continuous range expansion (Table 4).

The 2-step clades, 2-3 and 2-4, nested with in 3-2 indicate restricted gene flow with some long distance dispersal. This finding aids in elucidating the dispersal pattern of nine-banded armadillos. Nine-banded armadillos have been considered to be a sedentary species exhibiting site fidelity. However, their geographic range in the United States has expanded considerably over recent years. Loughry and McDonough (1997) studied the spatial pattern of nine-banded armadillo population over a 4 year period in Florida. They reported that nine-banded armadillo's mean movement within and between years to be less than 200 m. This value did not differ between years, by gender, or age. Genetic parentage studies in the same area are consistent

with this finding (Prodhohl et al. 1998). Prodhohl et al. (1998) revealed that mean spatial distance between plausible parents of offsprings were significantly lower than that of other pairs. However both studies have limitations. First, 2/3 of the armadillos were not resighted implying that mortality and/or emigration were high (Loughry and McDonough 1997). Second, not all litter mates of a clone were sampledand this can underestimate mean distance between parents if surviving juveniles emigrate from the study site. Results of our study support long distance dispersal, but it is unclear it is unclear if this is accomplished by adults or juveniles.

Conservation implications.--In Paraguay, several indigenous groups hunt nine-banded armadillos. Additionally the nine-banded armadillo is the most frequently sold armadillo species along the Ruta Transchaco, the main road to and from the Paraguayan Chaco in a SE-NW direction (Frutos, In litt.), and such activities probably have been going on for a very long time.

The nine-banded armadillo is hunted considerably over it's range especially in Central and South America. Anthropogenic factors in the Neotropics preceded European contact (Denevan, 1992). A number of studies reveal that subsistence hunting and other anthropogenic effects activities have deleterious effects on wildlife populations,

but there is no evidence that hunting has depleted populations of *D. novemcinctus*. Furthermore, the species seems resistant to anthropogenic factors since it is found throughout the southern United States and has been expanding its range northwards. Therefore, this species is ideal to harvest and manage. Althouhg advantages of conservation programs through sustainable harvest programs has been extensively outlined, harvest programs must monitor the sustainable levels of extraction.

The construction of the Hidrovia, a 2,000-mile-long waterway, that would straighten and deepen the Paraguay and Parana rivers to open an outlet to the sea for Paraguay and Bolivia may have devastating ecological effects (Brooke, 1995), especially on the wetlands and its biotic component. This study has revealed that the Paraguay River is not a barrier to gene flow. However, altering the watercourse and its seasonal variations may constitute a barrier to gene flow not only for nine-banded armadillos but for other species as well.

Although this study revealed interesting results on the dispersal behavior of female nine-banded armadillos, it is necessary also to understand male dispersal. Loughry and McDonough (1997) suggested from their study that males are more likely to exhibit philopatric behavior. However, they indicated there was little supporting evidence for this.

Therefore, it is recommended that genetic studies on biparentally inherited loci such as microsatellites and or paternally transmitted loci such as those on the Y-chromosome be conducted to obtain a more accurate and robust estimate of the metapopulation dynamics of nine-banded armadillos.

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Table 1.---Polymorphic sites within the mtDNA cytochrome *b* gene and haplotype designations (A-Y) of 75 nine-banded armadillos from 5 populations within Paraguay. Numbers at the top of the figure represent variable nucleotide positions. Identical nucleotide characters, with reference to the sequence of haplotype A, are indicated with a period (.).

	Variable nucleotide position																																
						1	1	1	1	1	2	2	2	3	4	5	5	5	6	6	6	7	7	7	7	7	8	8	8	9	9	9	1
	2	2	4	6	8	1	3	7	7	8	4	5	8	9	5	4	6	8	4	8	9	0	5	7	9	9	1	7	9	4	8	9	0
Haplotype	8	1	8	0	5	1	6	8	9	0	3	7	2	0	6	9	7	8	2	4	3	5	1	1	2	5	6	0	7	8	5	6	5
A	G	C	A	T	G	G	C	G	T	A	C	G	G	T	C	C	C	C	T	C	T	G	G	A	A	A	G	G	G	A	G	A	A
B	.	.	G	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	.	.	.	.
C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
D	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	G	A	.	.
E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	A	.	.	.	.	.	.
F	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	T	.	.	.	.	.	A	C	.	.	A	A	.	G	A	.	.
G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
H	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	A	A	.	G	A	.	.
I	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	C	.	.	A	A	.	.	.	.	.

Table 1.---Continued.

	Variable nucleotide position																																
						1	1	1	1	1	2	2	2	3	4	5	5	5	6	6	6	7	7	7	7	7	8	8	8	9	9	9	1 0
	2	2	4	6	8	1	3	7	7	8	4	5	8	9	5	4	6	8	4	8	9	0	5	7	9	9	1	7	9	4	8	9	0 0
Haplotype	8	1	8	0	5	1	6	8	9	0	3	7	2	0	6	9	7	8	2	4	3	5	1	1	2	5	6	0	7	8	5	6	5
J	.	.	G	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	C	T	.	.	.	C	C	.	A	A	T	.	.	.	C
K	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	G	A	.	.
L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
M	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	G	A	.	.
N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.
O	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	T	.	.	.	.	.	.	C	.	.	A	A	.	G	A	.	.
P	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Q	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	C	.	.	A	.	.	.	.	.	.
R	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.	.
T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
U	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
V	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
W	T	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	.	.	.	.
X	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	A	.	.	.	.	.	.
Y	T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 2.---Haplotype distribution and frequency, haplotype diversity ($h \pm SE$), and nucleotide diversity ($\pi \pm SE$) of nine-banded armadillos, *Dasypus novemcinctus*, within the 5 study sites; CA (Casanillo), EJZ (Estancia Juan de Zalazar), 106 (Ruta Transchaco Km 106), LY (Lago Ypoa), and EGC (Estancia Golondrina-Caazapa).

Sites	mtDNA haplotypes																											
	A	B	C	D	H	G	I	E	J	M	R	S	Q	U	W	Y	V	O	X	F	K	L	N	P	T	h	SE	π	SE
CA	4	1		2	1	1	1	3	2	1	1	1														0.93	0.04	0.005	0.003
EJZ	10	3	1										1	1	1	1	1									0.75	0.10	0.003	0.002
106	8	4		1	1	1	1																			0.68	0.10	0.004	0.002
LY	1	1	2															1	1							0.93	0.12	0.005	0.003
EGC	2	2	3	2																2	1	1	1	1	1	0.94	0.04	0.004	0.003
total	25	11	6	5	2	2	2	3	2	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1				

Table 4. - Mitochondrial genetic differentiation (ϕ_{ST}) ($P<0.05$) lower triangular matrix and estimate of gene flow (N_fm) upper triangular matrix for all pairwise comparison of 5 sites of nine-banded armadillo, *Dasypus novemcinctus*, from Paraguay. Statistically significant values are denoted with an asterisk (*).Populations are as follows CA (Casanillo), EJZ (Estancia Juan de Zalazar), 106 (Ruta Transchaco Km 106), LY (Lago Ypoa), and EGC (Estancia Golondrina-Caazapa).

Population	CA	EJZ	106	LY	EGC
CA	-----	11.13	7.83	20.33	28.91
EJZ	0.043	-----	∞	8.59	6.35
106	0.060	-0.027	-----	4.93	4.93
LY	0.024	0.055	0.092	-----	∞
EGC	0.017	0.073*	0.092*	-0.047	-----

Table 4.---The nesting clades containing 1 or more significant distance measures for nine-banded armadillo mtDNA haplotypes.

Nested clades	Steps followed using the Key Appendix 1	Final inference
2-3	1-2-3-9-10 no	Geographical sampling scheme inadequate to discriminate between fragmentation and isolation by distance
2-4	1-11-12 no	Continuous range expansion
3-2	1-2-3-5-6-7-yes	Restricted gene flow/dispersal but with some long distance dispersal

Fig. 1.--Locations of the 5 collection sites for nine-banded armadillo in Paraguay; CA (Casanillo), EJZ (Estancia Juan de Zalazar), 106 (Ruta Transchaco Km 106), LY (lago Ypoa), and EGC (Estancia Golondrina-Caazapa) and number of individuals per site (n). Instep shows Paraguay's location in South American continent.

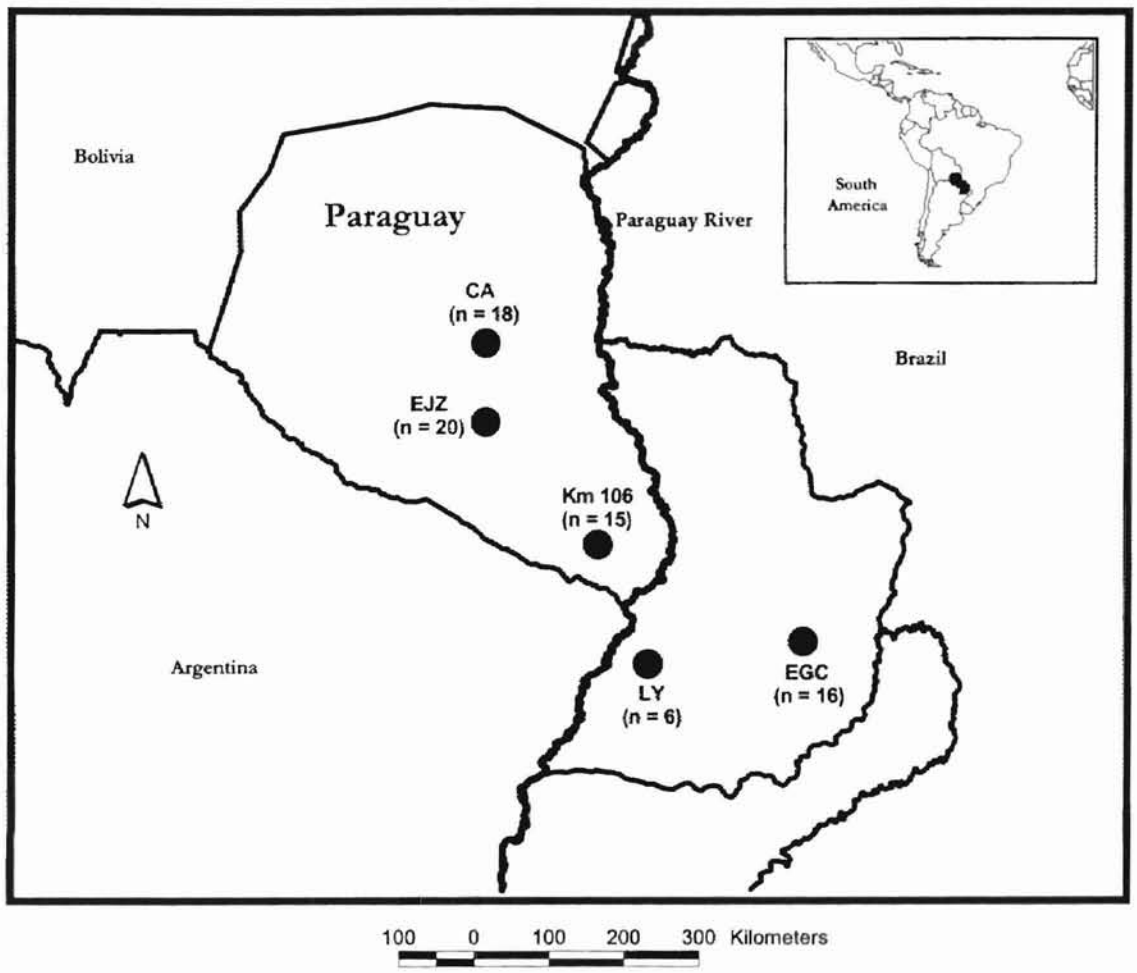
Bolivia
Paraguay
Paraguay River
South America
CA
(n = 18)
EJZ
(n = 20)
Brazil
N
Km 106
(n = 15)
Argentina
LY
(n = 6)
EGC
(n = 16)
100 0 100 200 300 Kilometers
Paraguay River
International boundary
Study Site Location

Fig. 2.--The unrooted mtDNA cytochrome b cladogram for nine-banded armadillos in Paraguay. Haplotypes are represented by the letters A-Y. Asterisk signify missing haplotypes that are intermediate between existing haplotypes but were not found in the sample. Solid lines connecting haplotypes indicate one mutational change. Interrupted lines indicate ambiguous relationship.

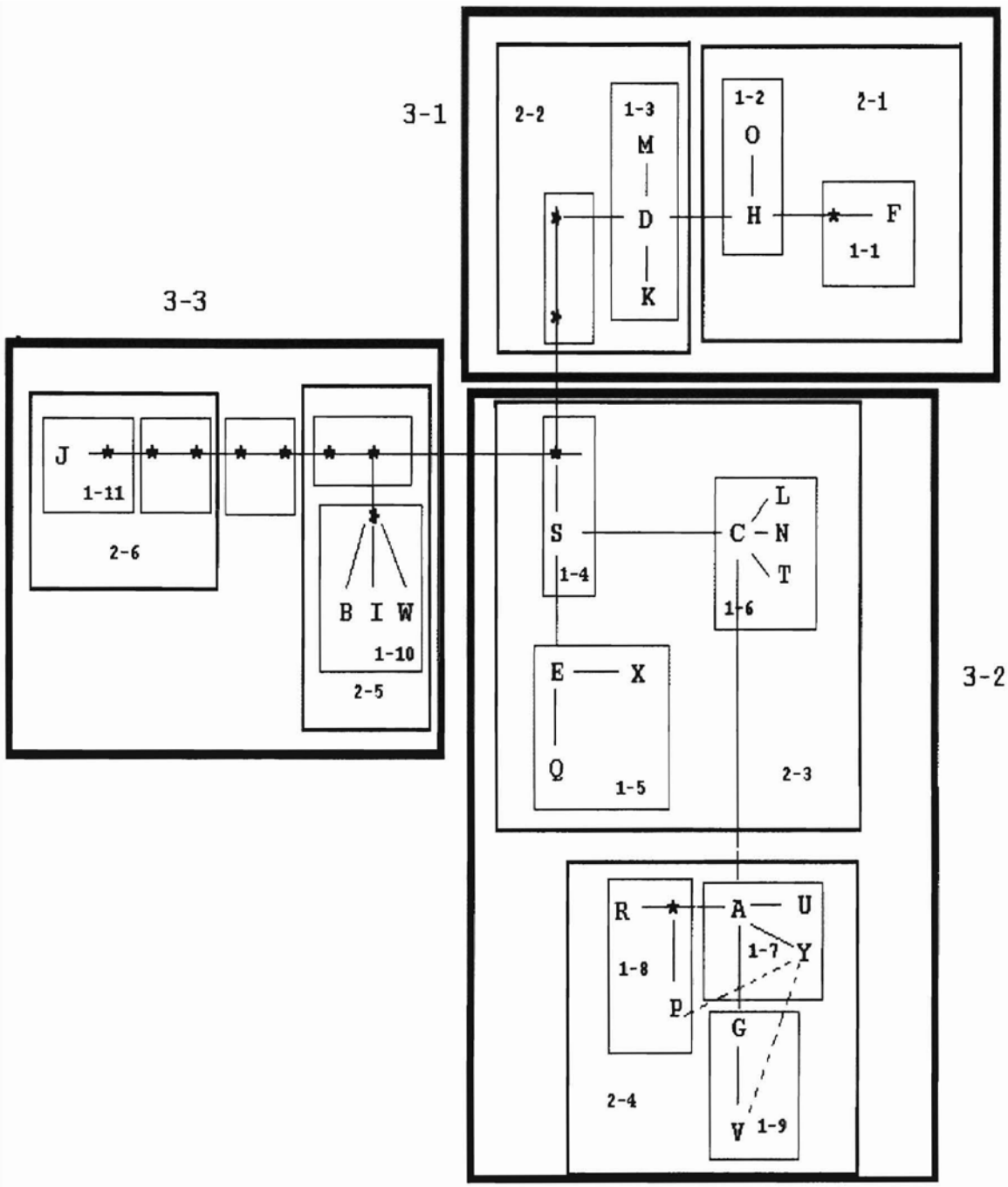
3-1
2-2
1-3
M
D
K
1-2
O
H
2-1
F
1-1
3-3
J
1-11
2-6
B I W
1-10
2-5
3-2
S
1-4
C
L
N
T
1-6
E
X
Q
1-5
2-3
R
1-8
P
A
U
1-7
Y
G
V
1-9
2-4

Fig.3.--Results of the nested clade analysis of geographical distance for the nine-banded armadillo mtDNA haplotypes. Haplotypes designations are at the far left and organized to depict the nested design shown on Fig.2. Boxes indicate nesting structure with higher level clade increasing toward the right.

1-Step clade (haplotype)				2-Step Clade				3-Step Clade				4-Step Clade			
Clade		**Dc**	**Dn**	**Clade**		**Dc**	**Dn**	**Clade**		**Dc**	**Dn**	**Clade**		**Dc**	**Dn**
F		---	---	1-1		0	176.08	2-1		161.96	183.56	3-1		214.25	225.64
H		147.09	181.99												
O		0	128.38	1-2		150.60	155.76								
	I-T	147.09.	53.61		**I-T**	150.60	-20.33								
M		0	290.94	1-3		---	---	2-2		244.67	244.44				
K		0	259.79						**I-T**	82.72	60.88				
D		232.03	232.91												
	I-T	232.03	-42.46												
S		---	---	1-4		0	367.48	2-3		204.46	242.51**L**	3-2		197.66	197.74
E		0	213.35	1-5		219.48	248.8								
Q		0	133.05												
X		0	252.90												
	I-T	0	20.37												
N		0	107.95	1-6		118.60	170.07**S**								
L		0	107.95		**I-T**	-154.63**L**	169.2851								
T		0	107.95												
C		115.01	122.08												
	I-T	115.01	14.13												
A		159.71	159.87	1-7		158.03	158.03	2-4		164.60**S**	169.77**S**				
U		0	131.21						**I-T**	39.86**L**	72.74**L**				
Y		0	131.21												
	I-T	159.71	28.66												
P		0	349.86	1-8		274.17**L**	286.93**L**								
R		0	116.31												
G		147.09	149.04	1-9		121.44	139.96								
V		0	59.52		**I-T**	-24.49	-40.36								
	I-T	147.0	89.52												
B		156.16	160.68	1-10		---	—	2-5		160.29	168.56	3-3		169.87	168.93
I		147.09	141.09										**I-T**	8.06	3.60
W		0	197.42												
J		---	---	1-11		---	---	2-6		0	244.65				
									I-T	160.29	-76.09				

VITA

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