Extreme population divergence and conservation implications for the rare endangered Atlantic Forest sloth, *Bradypus torquatus* (Pilosa: Bradypodidae)

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ABSTRACT

The maned sloth (*Bradypus torquatus*) is an endangered species endemic to the Atlantic Forest of eastern Brazil. This biome has been reduced to 7% of its original extent and the remaining forests are highly fragmented. We analyzed 70 samples from the largest remnant populations in the states of Bahia, Espírito Santo and Rio de Janeiro to characterize their geographic structure and to produce estimates of genetic diversity. The analysis indicated that the remnant populations are reproductively isolated and extremely divergent. The populations present a very discontinuous distribution, with divergent genetic clusters specific to different geographical regions, probably caused by allopatric fragmentation. This pattern is likely related to Pleistocenic climatic and vegetation changes, and indicates the presence of at least two independent evolutionary units. The analyses also indicate that populations separated by more than 100 km should be considered different management units. Thus, devastation of the Atlantic Forest leads to an unrecoverable loss of genetic diversity in this species. These conclusions should direct conservation actions aimed at preserving the distinctiveness of each evolutionary unit, as well as to preserve the demographic isolation of different management units.

1. Introduction

It is broadly accepted that genetic variability plays an important role in the persistence and adaptation of populations to changing environments (Lande and Shannon, 1996; Frankham and Ralls, 1998; Frankham et al., 2002). This variability is usually related to the effective population size (N_e), a fact that raises concerns regarding the survival of many species since studies with natural populations have indicated effective sizes quite below expectations based on census size (Frankham, 1995). Moreover, it has been shown that spatially structured populations display even lower N_e since mating is not random as in a panmictic population (Hanski and Gilpin, 1991; Gilpin, 1991).

In order to maximize species persistence in nature, conservation genetics can diagnose historical and present day processes affecting population structure. The study of the current patterns in the distribution of genetic diversity in a species can help to establish management units (MUs, *sensu* Palsboll et al., 2006) in order to design adequate management actions that help to maximize the probabilities of population survival and increase the extent of genetic diversity to be

preserved. On the other hand, the detection of historical processes that resulted in a long history of population isolation also indicates the need to manage populations (or groups of populations) as distinct evolutionary significant units (ESUs) that represent independent diversity components of the species (Moritz, 1994, 2002; Palsboll et al., 2006). Observed patterns of high inter-population divergence may indicate that isolation should be maintained in order to avoid unwanted exogamic depression effects due to likely adaptive (local or epistatic) differences between populations (Frankham et al., 2002). As a consequence, the delineation of ESUs and MUs that encompass the patterns and processes that characterize the diversity of a species emerges as a priority for biodiversity conservation (Crandall et al., 2000; Moritz, 2002; Palsboll et al., 2006).

Bradypus torquatus is endemic of the Brazilian Atlantic Forest and is considered "Endangered" by the IUCN (IUCN, 2007). It is the most threatened sloth species of the South American continent because of its small geographical range and the disturbed and fragmented nature of its habitat (Aguiar and Fonseca, 2008). The Atlantic Forest biome has been severely fragmented and degraded (Myers et al., 2000; Fundação SOS Mata Atlântica and INPE, 2002) particularly in the region where the species dwells. Today, the species is restricted to some remaining forest fragments in the states of Sergipe (SE), Bahia (BA), Espírito Santo (ES), Rio de Janeiro (RJ) and probably in the northeastern portion of Minas Gerais (MG) (Oliver and Santos, 1991; Aguiar and Fonseca, 2008). However, the largest remaining populations are so far found in southeastern BA and central-south ES. The species seems to be absent in a region between Doce (north-central ES) and Mucuri (southern BA) rivers (Oliver and Santos, 1991), turning the remaining populations in BA and ES historically discontinuous (Fig. 1). Similarly, since pastures and agriculture dominate most of northern RJ and southern ES, the few surviving populations in RJ are likely to be isolated from those of ES (Fig. 1). As a result, both the historical process (i.e. allopatric fragmentation) and the recent fragmentation due to habitat loss have likely influenced the current pattern of maned sloth distribution.

Few studies produced recent data about the ecology, behaviour and biology of the species (see Chiarello, 2008 for a review). The diet of each individual is restricted to few available plant species (Chiarello, 1998a). Besides, food preference is probably inherited in a matrilineal way in each population, since the infants learn what plant species they can eat during their first months of association with the mother (A.G. Chiarello pers. obs.). Maned sloths were shown to be the largest of all *Bradypus* species and individuals from higher altitudes (600–1000 m.a.s.l.) are significantly heavier than those from warmer lowland (<350 m.a.s.l) (Lara-Ruiz and Chiarello, 2005). No information is available so far on the dispersal patterns of the species, but given its strictly arboreal habits, the short distances it travels daily, and its characteristic lethargy (Chiarello, 1998b), it is likely that the species has limited dispersion ability.

Unfortunately, there is little information regarding the genetics of Xenarthran species and populations, which could be used to improve the knowledge of their conservation status (Garcia, 2003; Prodöhl et al., 2008). Thus far, the maned
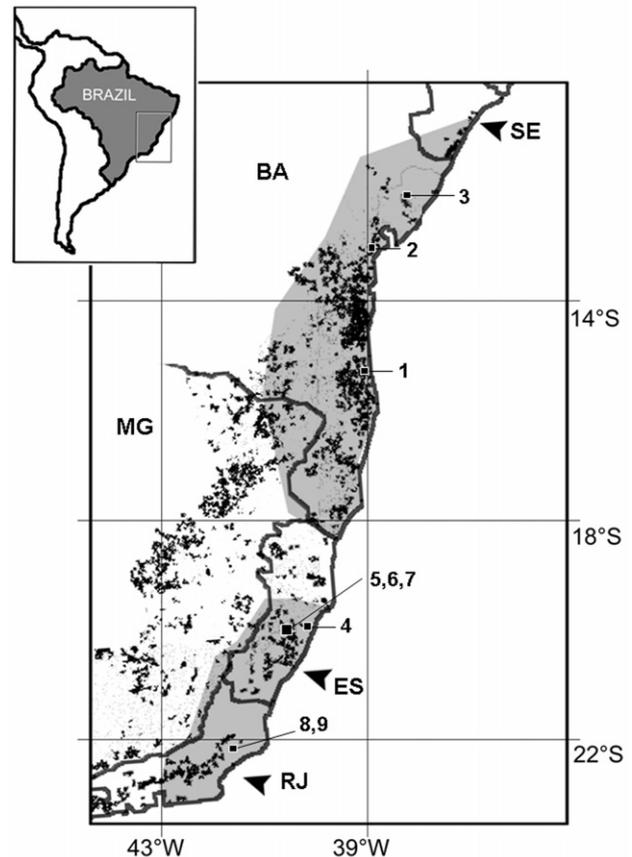
Fig. 1 – Current distribution limits of the maned sloth *Bradypus torquatus* in the Atlantic Forest of eastern Brazil (Shaded) and forest fragments remaining in the area (Black). Numbers indicate the municipalities in the Brazilian States of Bahia (BA), Espírito Santo (ES) and Rio de Janeiro (RJ) where samples were collected. Southeastern BA: Una (1); northeastern BA: Pratigi (2) and Mata de São João (3); lowland ES: Aracruz (4); highland ES: Santa Teresa (5), Santa Maria (6) and Itarana (7); RJ: Cassimiro de Abreu (8) and Silva Jardim (9). Adapted from Fundação SOS Mata Atlântica and Instituto de Pesquisas Espaciais (2002).

sloth has been included in a single study (Moraes-Barros et al., 2006). However, this study was focused mainly on *B. variegatus* populations and the *B. torquatus* samples were limited to two individuals captured in ES and 17 samples from individuals of unknown origin from a rehabilitation facility in BA.

Here, we present the results of the first extensive conservation genetics study carried out with the endangered Atlantic Forest sloth *B. torquatus*. Our sample contains representatives of wild animals from the main remnant populations of the species, which were analyzed by sequencing mitochondrial DNA (mtDNA) fragments. We aim to assess the conservation status of the species by evaluating the genetic diversity within and among remnant populations. Using a phylogeographic approach, we will be able to investigate the likely influence of recent (anthropogenic) and historical (vicariant) events leading to the present geographic distribution. The results will provide baseline information that

can be used as guidelines for the establishment of future management actions such as translocations or reintroductions.

2.   Materials and methods

2.1.   Sampling localities and sample collection

A total of 70 specimens were sampled between 2002 and 2006 in 16 localities of montane (highland) and lowland Atlantic Rain Forest (*sensu* Rizzini, 1963) from nine municipalities of the Brazilian states of BA, ES and RJ, where the largest remnant populations occur (Fig. 1, Table 1). The samples were taken from animals captured at two forest reserves in Santa Teresa (ES, Santa Lúcia Biological Station and São Lourenço Municipal Park), from two privately owned fragments in Santa Maria de Jetibá (ES), from five privately owned fragments in Aracruz (ES), from two forest reserves (REBIO Una and Ecoparque) in Una (BA) and from one locality in each of the remaining sampled municipalities. Sampled areas were chosen on the basis of previous knowledge about the species occurrence, size and protection status of conservation units or forest fragments, local accessibility, availability of field volunteers and transport to the areas. Detailed information about 14 of these sampled localities is found in Lara-Ruiz and Chiarello (2005). The two new localities, sampled later to increase the geographic coverage of our sample, are: the Camarujipe/Passagem Grande Reserve (1329 ha), north of Salvador (Municipality of Mata de São João, BA) and the Restinga de Pratigi (32,000 ha, south of Salvador, Municipality of Pratigi, BA). The former is a privately owned forest reserve (RPPN) that represents one of the largest remaining blocks of Atlantic Forest north of Salvador (capital of the state of Bahia) and the latter is a protected area (APA) that encompasses several forest types including rainforest, mangroves and sandbanks.

The degree of forest connectivity is high in the region of lower montane ES (municipalities of Santa Teresa, Santa Maria de Jetibá and Itarana >600 m.a.s.l.) but is much lower between this montane region and Aracruz municipality (lowland ES). Additionally, individuals from these two regions differ in body size and weight (Lara-Ruiz and Chiarello, 2005). Thus, for the analyses, we classified samples from ES into two distinct populations, one from lower montane populations

and the other from the lowlands (hereafter highland ES and lowland ES, respectively). A similar reasoning was applied to samples from Bahia. Due to large distance (hundreds of kilometres) and lack of forest connectivity between southern BA (Una) and northern BA (Pratigi and Mata de São João), samples from these two regions were considered to belong to distinct populations (hereafter southeast BA and northeast BA, respectively). The two forest reserves surveyed in Rio de Janeiro state are currently isolated although separated by only 50 km. Because of this and the fact that an extensive search resulted in only three captures, the two municipalities sampled in RJ were joined together for the analysis.

All samples were collected during field trips by a team of trained observers that carried out nearly 400 h of active search of sloths along trails, secondary roads and forest edges. Once found, sloths were captured manually via climbing trees and brought to the forest floor inside cloth bags. No anesthesia was necessary; instead, physical containment was achieved by wrapping Velcro® strips around each claw. Some of the sampled animals were being monitored with radio collars as part of independent projects in ES and BA. Blood samples (~1 ml) were taken from the cephalic vein, stored in tubes with absolute ethanol (1:1) and placed on ice. After blood collection, samples were taken to the laboratory where all genetic analyses were carried out.

2.2.   Sample processing and sequencing

DNA extraction was performed following the standard phenol–chloroform procedure (Sambrook and Russell, 2001) with modifications (detailed protocols available at http://www.icb.ufmg.br/lbem/protocolos). PCR amplification of a 370 bp fragment of the mitochondrial DNA (mtDNA) control region (HVI) was performed using the Primers L_0 (Douzery and Randi, 1997) and E_3 (Huchon et al., 1999), using a first denaturing step of 3 min at 94 °C followed by 35 steps of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and a final extension step of 10 min at 72 °C. The amplification of a 632 bp fragment of the cytochrome oxidase subunit I (COI) gene was done using the primers and thermocycling conditions described in Folmer et al. (1994) or using the same conditions and Xenarthran-specific primers   COXI-L2   (5'-TGTCTTTAGATTTACAGTCTAATGC-3')

Table 1 – Sampled municipalities, geographical location, sample size (number of sampled individuals; n) and distribution of the observed HVI and COI haplotypes found in the 70 wild B. torquatus individuals

State (region)	Municipality[a]	n	HVI haplotypes						COI haplotypes					Latitude/longitude
			1	2	3	4	5	6	1	2	3	4	5	(decimal degrees)
Bahia (northeast)	(3) Mata de São João	6						6				6		−12.50/−38.05
	(2) Pratigi	1						1				1		−13.65/−39.04
Bahia (southeast)	(1) Una	13			9	4			10	3				−15.14/−39.08
Espírito Santo (highland)	(5) Santa Teresa	13	13						13					−19.93/−40.59
	(6) Santa Maria	18	15	3					18					−20.03/−40.70
	(7) Itarana	1	1						1					−19.87/−40.87
Espírito Santo (lowland)	(4) Aracruz	15	15						15					−19.79/−40.20
Rio de Janeiro	(8) Silva Jardim	1				1						1		−22.62/−42.43
	(9) Cassimiro de Abreu	2				2						2		−22.55/−42.28
Total		70	44	3	9	4	3	7	47	10	3	3	7	

a Numbers refer to labels in Fig. 1.

and COXI-H (5'-ACTTCAGGGTGTCCGAAGAATCA-3') designed in our laboratory.

All reactions were carried out including positive and negative controls (template-free reactions) in order to test for contamination and to assure the fidelity of the PCR amplifications (Innis et al., 1990). The PCR products were cleaned using Polyethyleneglycol 8000 (20% PEG, 2.5 M NaCl) and sequenced using the same primers used for PCR and the ET Dye terminator Cycle Sequencing Kit (Amersham Biosciences) following the manufacturer recommendations. Reactions were run in an automated MegaBACE 1000 DNA sequencer. At least two independent PCR products from each sample were sequenced using both forward and reverse primers. The chromatograms were analyzed using the software Phred 0.020425 (Ewing et al., 1998) and the sequences were aligned and edited to produce high-quality consensus sequences for each individual using Phrap 0.990319 (www.genome.washington.edu/UWGC/analysistools/phrap.htm) and Consed 14.0 (Gordon et al., 1998). Consed and Sequence Analyzer (Amersham Biosciences) were also used to visualize the chromatograms and verify the quality of the sequences and the base assignment in the observed polymorphic sites.

2.3. Data analysis

Alignments of consensus sequences were made using the default parameters in the algorithm Clustal X (Thompson et al., 1997) implemented in the software MEGA 3.1 (Kumar et al., 2005) in order to define polymorphic sites and haplotypes. Haplotypes were deposited in the GenBank under accession numbers EU301711 to EU301721. Genetic distances between haplotypes and haplogroups were calculated using MEGA. The software Modeltest (Posada and Crandall, 1998) was used to find the nucleotide substitution model that best fits the data. Phylogenetic trees were generated by Neighbor Joining, Minimum Evolution and Parsimony methods available in MEGA, using a *B. tridactylus* sequence (GenBank number AY960979) as the out-group. Estimated time of divergence was calculated with MEGA using the lowest substitution rate values estimated for mammals (Pesole et al., 1999) for the mtDNA HVI region (only the rate for the ETAS domain) and for the synonymous substitutions in protein coding genes. MEGA was also used to perform a phylogenetic analysis including the *B. torquatus* HVI sequences from individuals of unknown origin produced by Moraes-Barros et al. (2006).

Genetic and molecular diversity indexes for the species and for each population sampled were generated using Arlequin 3.11 (Excoffier et al., 2005). Analyses of molecular variance (AMOVA) and exact tests of population differentiation (100,000 steps in the Markov Chain and 10,000 dememorization steps) were also carried out in Arlequin. The AMOVA analysis considers haplotype frequencies as well as molecular distances between them to generate Φ_{ST} values (analogous to Wright's F_{ST}) and their associated variances to estimate how the variability is distributed among and between populations. The population pairwise genetic distances (Φ_{ST}) were calculated using 10,000 permutations to assess the significance of the associated P values. The exact test of population differentiation (analogous to Fisher's exact test) tests the null hypothesis of panmixia by estimating the probability of obtaining a random distribution of the observed alleles. Arlequin was also used to perform Fu's and Tajima's tests for evidences of population expansion.

The relationships among haplotypes were inferred using median joining networks (Bandelt et al., 1999) implemented in the program Network 4.2 (Fluxus Technology, 2004) and also using the TCS algorithm (Clement et al., 2000) implemented in the ANeCA software (Panchal, 2007). The Nested Clade Analysis (NCA) (Templeton et al., 1987) was performed in order to look for processes that could be influencing population genetic structure. By analyzing the patterns in the distribution of genetic diversity (nested haplotype networks and associated geographical information), the analysis is able to distinguish between historical events (past allopatric fragmentation) and current ones (recent dispersal and gene flow) using a coalescent approach. The haplotype networks were nested following the method outlined in Templeton (1998) and the NCA was performed in ANeCA using the software GeoDis (Posada et al., 2000) to test for significant geographical associations of haplotypes.

In addition, the web-based software IBDWS v 3.09 (Bohonak, 2002) was used to test for evidence of isolation by distance (IBD) and limited ongoing gene flow among remaining populations caused by habitat fragmentation. This software has been used to identify the effect of such processes on other speciés distributions (Ovenden et al., 2004; Trizio et al., 2005; Proudfoot et al., 2006; Vandergast et al., 2007). The program uses a Mantel test to asses the significance of the correlation obtained when regressing (RMA regression) genetic pairwise distances (Φ_{ST}) against geographic distances among sampled locations. A positive correlation indicates isolation by distance and it is expected under limited ongoing gene flow while no correlation will be expected when there are extreme dispersal limitations (i.e. allopatric fragmentation followed by a lack of gene flow). As the IBDWS software presents some limitations for the analysis of sequence data when there is more than one sample with $n = 1$, we performed the analysis defining populations as samples belonging to ES, RJ, northeast BA and southeast BA. In this analysis, the two populations from ES (highland and lowland) were joined together, despite their current isolation and their ecological differences (see Lara-Ruiz and Chiarello, 2005), since the AMOVA analysis did not indicate any population differentiation between them. In order to study the correlation in more detail (i.e. using geographic data on all the nine sampled municipalities), we performed the same Mantel test using genetic (p-distances between municipalities) and geographic distance matrices. In this analysis, we also included a third matrix of indicator values (option available in the IBDWS software) to take into account the "gap" in the species distribution found between northern ES and southern BA while assuming that, before anthropogenic forest fragmentation, there was suitable habitat between all populations north and south of this gap. The indicator matrix consists of a pairwise matrix in which indicator values are entered: value one (to describe all pairs of populations north or south of the gap) and value zero (for population pairs, including one population located north of the gap and another located south of it). The program runs partial correlation tests between the matrices to asses the correlation between geographical and genetic distances when

controlling for the existing gap and the correlation between the indicator matrix (gap) and the genetic distances when controlling for geographic distances.

3. Results

Among the 70 samples, only five COI haplotypes (632 bp) defined by 48 variable sites (45 Ts: 3 Tv) and six HVI haplotypes (370 bp) defined by 21 variable sites (19 Ts: 2 Tv) were found (Table 2). Genetic and molecular diversity indexes showed extremely low levels of diversity in all populations sampled but moderate values were found when considering all samples as belonging to a single population (HVI: $h = 0.5797$, $\pi = 0.017634$; COI: $h = 0.5226$, $\pi = 0.030209$), with a large nucleotide diversity value due to the observed differences in the sequences between populations (Table 2).

The inspection of HVI and COI sequences revealed a strong pattern of isolation among populations from the three states analyzed. The genetic distances among haplotypes belonging to the same state varied from 0 (ES and RJ) to 0.005 (BA) for HVI, and from 0 (ES and RJ) to 0.003 (BA) for COI. On the other hand, distances between groups of haplotypes from different states are considerably greater, varying from 0.011 (ES–RJ) to 0.053 (RJ–BA) for HVI and from 0.008 (ES–RJ) to 0.077 (BA–ES) for COI.

All three states harbored private haplotypes and no shared haplotypes were found among them (Table 1). The same was observed within the state of Bahia, where there is a considerable distance between northeast (Mata de São João and Pratigi municipalities) and southeast populations (Una municipality). Despite the extensive sampling effort made in ES

($n = 47$; 4 municipalities), only two HVI haplotypes and one COI haplotype were found. The common haplotype (44 of 47) found in ES was observed in all municipalities sampled and is the only one found in the population from Aracruz (lowland ES, $n = 15$) that is spatially isolated from the populations in the highlands (Itarana, Santa Maria de Jetibá and Santa Teresa municipalities). Due to this isolation and the observed morphological differences registered by Lara-Ruiz and Chiarello (2005), we kept the distinction between ES highland and ES lowland for some analyses. The analysis of the COI amino-acid sequences revealed that there is a distinct protein sequence characterizing samples from each state. These sequences are product of four non-synonymous codon changes (first position substitutions) that produce amino acid substitutions in the COI protein product.

For both sets of data, Modeltest found the HKY85 (Hasegawa et al., 1985) to be the best nucleotide substitution model to describe the data. As this model is not available for analysis in MEGA or Arlequin, all the analysis was run using the Tamura and Nei (1993) substitution model that is the most similar to the HKY. The phylogenetic relationships between haplotypes are found in Fig. 2 and show two different monophyletic groups representing the haplotypes from the northern portion of the distribution (Bahia state, north of the distribution gap) as opposed to the group formed by southern (RJ and ES) haplotypes. The tree constructed using HVI sequences also suggests that the RJ sequence is derived from the common ES haplotype. The inferred divergence times using the two different data sets suggested a late Pliocene (COI: 2.48 million years ago, MYA) or early Pleistocene (HVI: 1.44 MYA) divergence of the north (BA) and south (RJ–ES) clades, while the

Table 2 – Molecular diversity indexes generated by Arlequin for HVI (370 bp) and COI (632 bp) sequences from several B.torquatus populations. Southeastern Bahia (SE BA: Una municipality); northeastern Bahia (NE BA: Pratigi and Mata de São João municipalities); Rio de Janeiro (RJ: Cassimiro de Abreu and Silva Jardim municipalities); Espírito Santo highland (ES H: Itarana, Santa Maria and Santa Teresa municipalities); Espírito Santo lowland (ES L: Aracruz municipality)

	N	NH[a]	S[b]	Ts[c]	Tv[d]	h[e]	π[f]	k[g]
HVI (370 bp)								
NE BA	7	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
SE BA	13	2	1	1	0	0.4615 ± 0.1096	0.001247 ± 0.001312	0.461538 ±0.431834
ES H	32	2	1	1	0	0.1754 ± 0.0841	0.000474 ± 0.000713	0.175403 ±0.237076
ES L	15	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
RJ	3	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
Overall	70	6	21	19	2	0.5797 ± 0.0628	0.017634 ± 0.009354	6.524638 ± 3.122159
COI (632 bp)								
NE BA	7	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
SE BA	13	2	1	1	0	0.3846 ± 0.1321	0.000609 ± 0.000687	0.384615 ± 0.386303
ES H	32	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
ES L	15	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
RJ	3	1	0	0	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
Overall	70	5	48	45	3	0.5226 ± 0.0643	0.030209 ± 0.015007	19.092340 ± 8.555816

a Number of haplotypes (NH).
b Number of substitutions (S).
c Transitions (Ts).
d Transversions (Tv).
e Haplotype diversity (h).
f Nucleotide diversity (π).
g Average number of pairwise differences (k).
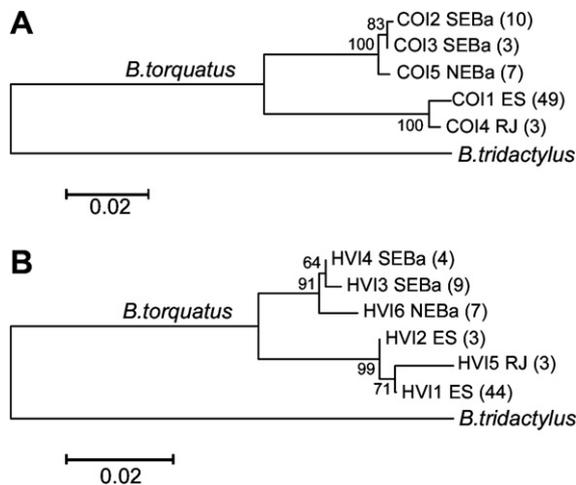
Fig. 2 – Neighbor Joining (Bootstrap: 10000 replications) phylogenetic hypothesis for mtDNA COI haplotypes (A) and control region haplotypes (B) found in several *B. torquatus* populations. Substitution model:Tamura-Nei. Numbers in parenthesis indicate sample size. Southeastern Bahia (SE Ba); Northeastern Bahia (NE Ba); Rio de Janeiro (RJ); Espírito Santo (ES). Parsimony and ML approaches have also resulted in the same topologies.

Fig. 3 – Statistical parsimony network and nested clade analysis of *B.torquatus* COI haplotypes. The circles represent the different haplotypes found and circle sizes are proportional to frequencies of haplotypes in Table 1. Black circles represent missing haplotypes. The total cladogram is represented by two independent parsimony networks (A: Bahia; B:Espírito Santo + Rio de Janeiro) separated by 42 mutation steps.

divergence between populations in ES and RJ and between the two BA clades (northeast and southeast) appears to be more recent (late Pleistocene: 0.32–0.13 MYA).

The shorter HVI sequences (330 bp) retrieved from Moraes-Barros et al. (2006) clearly grouped together with the haplotypes from Bahia (BA) populations in the phylogenetic analyses (not shown) suggesting that, at least in the period where samples were taken from the rehabilitation facility in Itabuna (BA), there were no captive animals belonging to southern (ES and RJ) populations. When both data sets were analyzed together, there were six haplotypes found in BA that clustered into two distinct groups. A group formed by HVI3, HVI4 (haplotypes found in southeast BA) and a new haplotype (S) and a group formed by HVI6 (haplotype found in northeast BA) and two new ones (N1 and N2). Of the 17 specimens analyzed by Moraes-Barros et al. (2006), one presented haplotype HVI3, five presented haplotype HVI4, and three presented the S haplotype indicating southeastern Bahia as their most probable origin. Other four specimens displayed haplotype HVI6, while three presented the N2 and one the N1, indicating a most likely northeastern origin.

The relationships among haplotypes, revealed by the Network and TCS analyses evidenced a high degree of genetic differentiation among populations from the different states indicating that they are distinct units (Fig. 3; only COI network shown). The two networks retrieved were identical except for the presence of two HVI haplotypes in the ES population. Both analyses were concordant, showing that the southern (ES+RJ) group is highly divergent from the northern (BA) group to the extent that the TCS analysis retrieved (for both data sets) two independent statistical parsimony networks separated by 12 (HVI) or 42 (COI) mutation steps. This, together with the phylogenetic analysis, indicates that populations from ES and RJ might belong to the same phylogroup even though they are

genetically distinct (pairwise $\Phi_{ST} > 0.96$, see below). The NCA revealed significant geographical associations for all clades and indicated a past allopatric fragmentation process as the cause of the observed pattern in the total cladograms. We also tested this analysis including in GeoDis input data several intermediate locations (this causes GeoDis to assume that suitable intermediate habitats are present in order to evaluate the effects of inadequate sampling). The results of the NCA in this case suggested "inadequate sampling" for the network containing ES and RJ clades but the final inference outcome, for the total cladogram, continued to be "allopatric fragmentation".

The geographic segregation of populations was tested by the AMOVA analysis, which indicated that the populations belonging to each of the three sampled states are deeply differentiated from each other, as evidenced by the extremely high Φ_{ST} values obtained (0.987 for HVI and 0.998 for COI). The pairwise comparison between populations (northeast BA, southeast BA, ES highland, ES lowland and RJ) produced highly significant Φ_{ST} values for all comparisons, except the one between the two ES populations (Table 3). Accordingly, the exact test of population differentiation rejected the null hypothesis of panmixia ($p < 0.01$) but did not find significant differences between the two ES populations. Both Tajima's and Fu's tests performed by Arlequin did not indicate any signal of population expansion.

As suggested by the phylogenetic (Fig. 2) and nested clade analyses (Fig. 3), the grouping of southern populations (ES and

Table 3 – Population pairwise Φ_{ST} and significance (p < 0.01) of associated p-values (*) calculated by Arlequin (10–100 permutations) for HVI (below diagonal) and COI (above diagonal) data sets

	ES highland	ES lowland	BA northeast	BA southeast	RJ
ES highland		0.00000	1.00000*	0.99779*	1.00000*
ES lowland	0.02323		1.00000*	0.99634*	1.00000*
BA northeast	0.99114*	1.00000*		0.93320*	1.00000*
BA southeast	0.98175*	0.98492*	0.91857*		0.99297*
RJ	0.96054*	1.00000*	1.00000*	0.97870*	
Distance method: Tamura & Nei.					

RJ), separated from the northern ones (BA), resulted in a higher between groups divergence (HVI Φ_{SC}: 0.90; COI Φ_{SC}: 0.96) than the grouping of populations by states (HVI Φ_{SC}: 0.86; COI Φ_{SC}: 0.94). Other groupings (ES+BA/RJ or RJ+BA/ES) presented non significant Φ_{SC} and Φ_{CT} (data not shown).

Using the raw sequence data of the four distinct populations identified by the exact test of differentiation (ES, RJ, northeast BA, and southeast BA), the RMA regression analysis performed in the IBDWS software retrieved an R^2 value of 0.47 for the correlation between Φ_{ST} values and geographic distances (Fig. 4A). However, the Mantel test failed to find a significant correlation between them. Using distance data rather than raw DNA sequences in order to include all municipalities sampled, the correlation was high and significant ($R^2 = 0.92$, $p < 0.001$) when no indicator values where included (Fig. 4B). Using an indicator matrix to take into account the gap in the species distribution the IBD analysis found the same significant correlation between genetic and geographic distances. It also found a high and significant partial correlation between genetic distances and the indicator matrix when controlling for the effects of geography ($R^2 = 0.88$, $p < 0.001$) while the partial correlation between genetic and geographic distances when controlling for the gap was high but not significant. These results suggest that geographic distance alone does not fully explain the observed pattern of genetic differentiation.

4. Discussion

Genetic diversity data obtained by the analysis of mtDNA sequences have shown low levels of genetic variability in several mammalian species (Eizirik et al., 1998, 2001; Garcia-Rodriguez et al., 1998; Huchon et al., 1999; Fernando et al., 2000; Freeman et al., 2001; Frutos and VanDenBussche, 2002). However, since mutation rates can vary widely among different lineages, the comparison of these values is not straightforward. Nevertheless, the average diversity values obtained in the present study for *B. torquatus* populations are among the lowest registered for mammals in the literature. The values are moderately high only when all samples ($N = 70$) are considered to belong to a single demographic population, an assumption that goes against the expectations for a species with such low dispersal capabilities. With the exception of the southern BA population, all other populations studied are monomorphic for control region haplotypes, suggesting that matrilineages tend to remain in their area of origin.
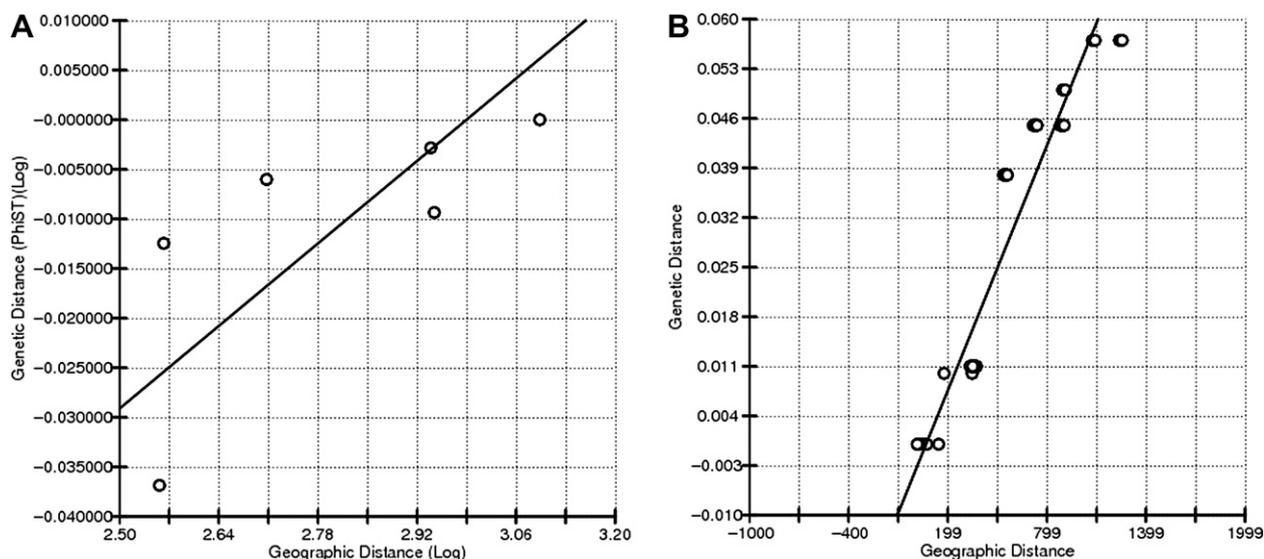
Fig. 4 – Reduced Mayor Axis (RMA) regression showing the slope and intercept for the correlation between geographic and genetic distances calculated considering populations as samples belonging to the same state (A) and considering all municipalities sampled as different populations (B). The mantel test found a significant correlation only in case B.

Even though an extensive sampling has been carried out in ES (four municipalities, N = 47) only two HVI haplotypes and one COI haplotype were found in this sample, suggesting that the observed low genetic variability of local populations is not an artifact of small sample sizes. The low diversity in the ES population is remarkable, especially considering that lowland and highland subpopulations show significant phenotypical (body size) and probably adaptive differences (Lara-Ruiz and Chiarello, 2005). On the other hand, the results suggest that the Bahia populations have higher diversity, especially when the information on the 17 samples used by Moraes-Barros et al. (2006) collected in a rehabilitation facility in southern Bahia is included. However, it is likely that these samples taken from the rehabilitation facility represent several monomorphic populations scattered among remaining Bahia forest fragments. This would be concordant with the high degree of genetic differentiation between populations observed in this study and in the study by Moraes-Barros et al. (2006) for populations of both B. variegatus and B. torquatus.

The phylogeographic analysis revealed an extremely discontinuous distribution, with three distinct clusters, each representing populations belonging to one of the sampled states. The concordance of our results with the political (state) boundaries is partially explained by the observed pattern of isolation by distance and the fact that the boundary between ES and BA is located in the region where the gap in the distribution of the species is found. As outlined above, haplotypes from each cluster are highly divergent indicating that populations to the north and south of the gap and/or separated by distances greater than 60 km (maximum distance between localities found to be genetically homogeneous) have been historically isolated. The divergence between BA and southern populations is the most ancient separation, since RJ shares a more recent ancestor with the most common ES haplotype. The higher levels of genetic diversity in BA, along with the phylogeographic pattern observed and the phylogenetic clustering of RJ and ES populations, suggest that these populations originated after a range expansion from southern BA and subsequent isolation, leading to interpopulation divergence. Alternatively, it may be that all intermediate haplotypes have gone extinct or were not sampled because some areas were not covered by our survey. The latter explanation should not be ruled out as finding a sloth in the field is difficult and populations might indeed exist between sampled localities. However, recent efforts to locate maned sloth populations in northern ES and eastern and extreme northeastern MG (i.e., half way through southern BA and lower montane ES) have proved unsuccessful (Chiarello, 1999; unpublished data).

The NCA (Fig. 3) suggested that the observed divergence is due to a historical process of isolation and restricted gene flow (i.e. allopatric fragmentation). Furthermore, population structure analyses also indicate that a great portion of the variation found in the species as a whole, resulted from divergence accumulated in isolated populations in remnant Atlantic Forest fragments. Divergence between northern and southern components of Atlantic Forest fauna has been observed in several taxonomic groups, including plants (Mori et al., 1981), birds (Cabanne et al., 2007) and mammals (de Vivo, 1997a, b; Costa, 2003), and has also been related with observed centers of endemism (Kinzey, 1982; Costa et al., 2000).

Moreover, Moraes-Barros et al. (2006) also found differences between northern and southern clades of the common sloth B. variegatus, explained by isolation and restricted gene flow among populations. However, B. variegatus is a common and widespread species found over most of the Atlantic Forest, while B. torquatus has a much narrower distribution. Thus, sampling gaps can explain much of the inter-population divergence observed in B. variegatus, but not in B. torquatus. The topologies of the phylogenetic trees (Fig. 2) are concordant with the hypothesis that southern (ES–RJ) clade originated from a northern population. A past range expansion towards the southern Atlantic Forest coupled with subsequent founder effects could explain the lower levels of diversity observed in southern populations. Thus, B. torquatus' natural history suggests that there are different conservation concerns for southern and northern populations. Southern populations (RJ+ES) are product of historical bottlenecks, while northern populations (BA) are more heterogeneous within them, deserving independent management.

Our time estimate for the divergence between northern and southern clades (late Pliocene to early Pleistocene) seems plausible since sloths have an apparently old evolutionary history. The first split between Bradypus lineages likely occurred 21 MYA (Delsuc et al., 2004), and the separation between B. variegatus and B. torquatus was estimated to happen 7.7 MYA (Barros et al., 2003). The estimated split between the two Bradypus torquatus lineages occurred likely during the Gelasian (2.5–1.8 MYA), a period characterized by the last stages of a global cooling trend that led to the quaternary ice ages (International Commission on Stratigraphy, 2007). As quaternary climatic oscillations have been related to a series of contractions and expansions of habitats and species ranges all over the world (see review in Hewitt, 2000), it could be expected that the global cooling at the end of the Pliocene also led to forest contractions that ultimately resulted in the current observed distribution of B. torquatus populations. Indeed, some authors (Haffer, 1969; de Vivo and Carmignotto, 2004) have proposed that these climatic oscillations produced the forest retraction that isolated the northern and southern portions of the Atlantic Forest since the Pliocene. Thus, if the current population structure is the product of ancient vicariant processes as suggested, conservation practices should focus on managing separately the remaining populations from each Brazilian state.

The IBD analysis indicated that the correlation found between geographic and genetic distances is highly influenced by the existence of a gap in the distribution of the species, thus spatial distance alone can not fully explain the pattern observed. This is likely due to the effects of genetic drift in historically isolated populations that can overcome the differentiation processes resulting from restricted current gene flow (Frankham et al., 2002). In the case of highly sedentary species such as sloths, the high levels of divergence found might be the result of an interaction between historical processes of isolation, small population sizes and isolation by distance due to limited ongoing gene flow between populations. These results are likely related to the low dispersal capabilities of these forest dwellers and their sedentary habits. Moreover, if some degree of female philopatry exists in these species, we would expect a higher level of mtDNA differentiation as observed here. Female philopatry is difficult

to demonstrate in cryptic species such as maned sloths, however, some degree of site fidelity can be inferred by the information available on the feeding habits of this species, which follow a matrilineal path such as mtDNA. Despite being considered generalists (*B. torquatus* feeds on a variety of plant species), less than 10 plant species can account for more than 90% of the diet of each animal, and there are individual differences in the ten top species consumed (Chiarello, 1998a, 2008). As each individual learns its feeding preferences during the time that it is associated with its mother, it is probable that these feeding habits also limit the ability of the individuals to disperse since changes in the floristic composition of the area could diminish the probability of find suitable (known) sources of food. Likely changes in vegetation during Pliocene/Pleistocene and Pleistocene/Holocene transitions could also have affected the dispersal of matrilineages, leaving their signatures both in the feeding preferences and mtDNA haplotype distributions.

Thus, our results support the existence of two evolutionary significant units (ESUs) with likely adaptive differences caused by a long period of isolation (Palsboll et al., 2006), and also the existence of several management units (MUs), which have to be managed separately in order to maintain their demographic distinctiveness. Although we observed highly significant results, our conclusions should be taken with care because they are based on a single locus, and can therefore be reasonably modified after the addition of further nuclear markers to these analyses.

The extreme inter-population divergence observed implies that the loss of any of these populations will greatly reduce the overall diversity of the species. The spatial distribution and phylogenetic history of mtDNA lineages indicated that the populations belonging to northern and southern clades should be considered distinct ESUs (*sensu* Moritz, 1994, 2002) that must be preserved to maintain the evolutionary potential of the species. Furthermore, this work also revealed several genetically distinct populations (northern BA, southern BA, ES and RJ) within the species distribution, whose management as independent units is mandatory in order to preserve their demographic distinctiveness and minimize the genetic risks to *B. torquatus* conservation (inbreeding and outbreeding depressions). Presently, displaced individuals due to habitat loss are recovered by the Brazilian government and other organizations, and relocated to other areas, as part of the conservation program. As translocations should reflect the historical relationships between populations in order to avoid the disruption of patterns of population subdivision and local adaptation, it is also very important to manage these populations in a way that maintains the historic pattern of subdivision between the northern and southern clades.

5. Conclusions

Considering (i) that the split between the two clades (and populations) occurred long ago, (ii) that *B. torquatus* presents highly specialized, maternally inherited feeding preferences of each individual, (iii) that the species has limited dispersal ability, and (iv) that there are differences in floristic composition of the Atlantic Forest from the different states where the

populations occur, it is probable that there are adaptive differences among these populations. Indeed, although mtDNA is (likely) a neutral marker, it is transmitted in a matrilineal fashion in the same way as feeding preferences and thus can mirror different adaptive responses between populations that are highly important for their survival.

We can interpret these data following the conservation categories proposed by Crandall et al. (2000). This proposal considers both historical and recent processes affecting the genetics and ecology aspects in terms of connectivity among populations (exchangeability *sensu* Crandall et al. 2000) in order to define eight different scenarios (Cases 1–8) upon which management units can be established. As a consequence, for these *B. torquatus* populations, not only the historical and recent genetic exchangeability criteria will be rejected, but at least the recent ecological exchangeability will be rejected too (Case 1). If managers are to follow the recommendations of Crandall et al.'s, the studied populations should be treated (and managed) as distinct species. Moreover, even the subpopulations within Espírito Santo state (which belong to the same mtDNA cluster) would be classified in Crandall et al. (2000) scheme as a "Case 2" (absence of historical and present day connectivity). This is because there is a significant and probably adaptive differentiation in size (Lara-Ruiz and Chiarello, 2005), plus an absence of present-day genetic and ecological interchange due to forest reduction and fragmentation in that state. Recommended management actions for this Case 2 are also to treat the populations as distinct species.

The conservation suggestions for management include preserving all remaining populations and preventing the eventual admixture of northern and southern populations to maintain the history of long-term isolation, which once lost, will not be recovered. However, it is important to highlight the need to perform further studies using morphological characters and autosomal DNA markers for assessing not only the taxonomic status of these populations but also to obtain population diversity and kinship data that could provide detailed information on inbreeding coefficients and how these relate to adaptive characteristics and survival. In the case when translocation of individuals for genetic restoration may be required, it is advisable to avoid admixture of individuals from genetically distinct populations (MUs). The exchange of individuals should also take into account the altitudinal differences (there are lowland and highland populations in ES and RJ), evaluating dietary preferences to guarantee that translocated animals will recognize at least some plant species as suitable food.

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