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LONG-TERM VARIATION IN THE GENETIC DIVERSITY OF RED-HANDED HOWLERS *ALOUATTA BELZEBUL* (PRIMATES: PLATYRRHINI) FROM EASTERN BRAZILIAN AMAZONIA

Evonnildo C. Gonçalves[1]
Stephen F. Ferrari[2]
Diogo S. L. Medeiros[1]
Artur Silva[1]
Maria Paula C. Schneider[1]

Abstract. We evaluated evolutionary patterns of intron 7 of the *β-fibrinogen* nuclear gene, and its potential for the analysis of the genetic structure of the population of red-handed howlers (*Alouatta belzebul*) affected by the construction of the Tucuruí hydroelectric dam on the Tocantins river in eastern Brazilian Amazonia. Alignment of 45 sequences provided fragments of 792 base pairs, with 23 polymorphic nucleotide sites distributed among 22 haplotypes. Prior to the construction of the dam, nucleotidic diversity was slightly greater in the population on the left bank of the Tocantins. Seventeen years later, genetic diversity had increased slightly overall, possibly as a result of the admixture and recombination of different haplotypes present originally on opposite banks of the Tocantins. The results do not indicate any urgent need for the genetic management of remnant populations, except on the many small islands formed by the flooding, where effective population size has been reduced dramatically.

Key words: *Alouatta belzebul*, genetic diversity, *β-fibrinogen* intron 7, habitat fragmentation, population viability.

Resumo. Avaliamos os padrões evolutivos do intron 7 do gene nuclear *β-fibrinogênio*, e seu potencial para a análise da estrutura genética da população de guaribas-de-mão-vermelha (*Alouatta belzebul*) afetada pela construção da usina hidrelétrica de Tucuruí, no Rio Tocantins, na Amazônia oriental. O alinhamento de 45 seqüências forneceu 792 pares de base, com 23 sítios nucleotídicos polimórficos distribuídos entre 22 haplótipos. Antes da construção da represa, a diversidade nucleotídica foi ligeiramente maior na população da margem esquerda do Tocantins. Dezessete anos depois, a diversidade genética geral apresentou um pequeno aumento, possivelmente como resultado da mistura e recombinação de haplótipos diferentes presentes originalmente em margens opostas do Tocantins. O resultados não indicam qualquer necessidade de manejo genético das populações remanescentes, exceto nas muitas ilhas pequenas formadas pela inundação, onde o tamanho efetivo das populações tem sido reduzido drasticamente.

Palavras-chave: *Alouatta belzebul*, diversidade genética, intron 7 do *β-fibrinogênio*, fragmentação de habitat, viabilidade populacional.

[1]Laboratório de Polimorfismo de DNA, Universidade Federal do Pará, Caixa Postal 8607, 66075-970 Belém – PA, Brazil. Correspondence to Evonnildo Gonçalves; e-mail: ecostag@ufpa.br;
[2]Departamento de Biologia, Universidade Federal de Sergipe, São Cristóvão – SE, Brazil.

INTRODUCTION

Habitat fragmentation is a primary threats to wild primate populations, but while there is increasing interest in the ecological aspects of the phenomenon (e.g. Marsh, 2003), relatively few studies have focused on potentially important questions such as genetic variability. In Brazil, deforestation in the Amazon basin has yet to approach Atlantic Forest levels, but there are "hotspots" of devastation, such as the southern rim and eastern extremity, that threaten a number of endemic primates, in particular those with relatively restricted geographic ranges, such as the piebald tamarin, *Saguinus bicolor* (Gordo, 2005). On a more local scale, so-called "major projects" such as hydroelectric dams can have a considerable impact, but despite this, they may also provide a unique opportunity for a more systematic analysis of the effects of habitat fragmentation on primate populations.

The Tucuruí hydroelectric dam, which was constructed on the Tocantins river in eastern Amazonia between 1984 and 1985, is a case in point. The reservoir formed by the dam flooded an area of 2,850 km², most of which was primary *terra firme* forest. In addition to the loss of habitat, there was considerable fragmentation of the remaining forest, including the formation of over 1,600 islands. Eletronorte S.A. (the corporation responsible for the dam) implemented a series of compensatory measures, including surveys, a major rescue and release operation, nicknamed "Curupira", the collection of biological specimens, and the establishment of protected areas (Silva & Gribel, 2000).

The red-handed howler (*Alouatta belzebul*) was by far the most common mammal rescued during Operation Curupira, and remains abundant to the present day (Ferrari *et al*., 2002). This abundance was a primary determinant of the choice of the species for the present study, given the availability of blood samples collected during Operation Curupira, and the need to collect new specimens. Endemic to Brazil, *A. belzebul* ranges from the Tapajós River in southern Amazonia to the São Francisco in the northeastern Atlantic Forest (Hirsch *et al*., 2002). The species suffers intense hunting pressure and habitat fragmentation throughout most of its range, although its Amazonian populations – generally classified as distinct subspecies (Bonvicino *et al*., 1989; Rylands *et al*., 2000) – are still relatively abundant (but see Lopes & Ferrari, 2000). In the Northeast, however, the species has been reduced to a series of small, isolated remnant populations (Guedes *et al*., 2000).

In the present study, sequences of intron 7 of the *β-fibrinogen* nuclear gene from specimens collected in 2002 were analyzed comparatively with those from Operation Curupira, seventeen years earlier, in an attempt to evaluate the long-term effects of flooding and habitat fragmentation on the genetic structure of the local red-handed howler

populations. The results provide initial insights into the effects of habitat fragmentation on genetic variability, and their importance for the conservation and management of remnant populations, as well as the usefulness of the *β-fibrinogen* intron 7 for studies of this type.

METHODS

Sampling

The samples analyzed in the present study represent four spatially and temporally distinct populations of eastern Amazonian red-handed howler monkeys (*Alouatta belzebul*) that were affected by the construction of the Tucuruí hydroelectric dam in the Brazilian state of Pará. A total of 989 howlers were captured during the flooding of the reservoir, between 1984 and 1985. These animals were sedated and 3-6 ml of blood was collected via venal puncture. Aliquots of these samples were stored in the sample bank of the DNA Polymorphism Laboratory of the Pará Federal University (UFPA) in Belém. Most of these samples were used in the study of Schneider (1988), but unfortunately, for the present analysis, it was only possible to extract DNA from 21 of these specimens. It can nevertheless be assumed that these specimens represent a random sample of the original group. Of these specimens, 14 were collected on the left (west) bank of the Tocantins (Figure 1: Base 3), and seven were collected on the right bank (Base 4). Total DNA was obtained following the protocol of Sambrook *et al*. (1989). These populations were denominated ME and MD, respectively, in the analyses.

In 2002, howlers were captured live at two sites in the Tucuruí reservoir using a Pneu-dart rifle and tranquilizer darts containing 0.1 ml Ketamine hydrochloride per kg body weight (authorized by IBAMA through special license 080/02–DIFAS/DIREC/ IBAMA). Ten specimens were collected on the 129-ha Germoplasma island, close to the left bank of the Tocantins, and 14 howlers were captured at Base 4, an area of continuous forest on the right bank (see Martins, 2002).

Samples of approximately 3 ml of blood were collected from the femoral vein of anesthetized animals, and stored in Falcon tubes containing 300 µl of 0.02 M EDTA. The tubes were labeled and sent to the laboratory for DNA extraction. DNA was first isolated by enzymatic digestion using proteinase K, and then extracted with phenol-chloroform and precipitated with ethanol, following Sambrook *et al.*'s (1989) standard procedure.

Laboratory procedures

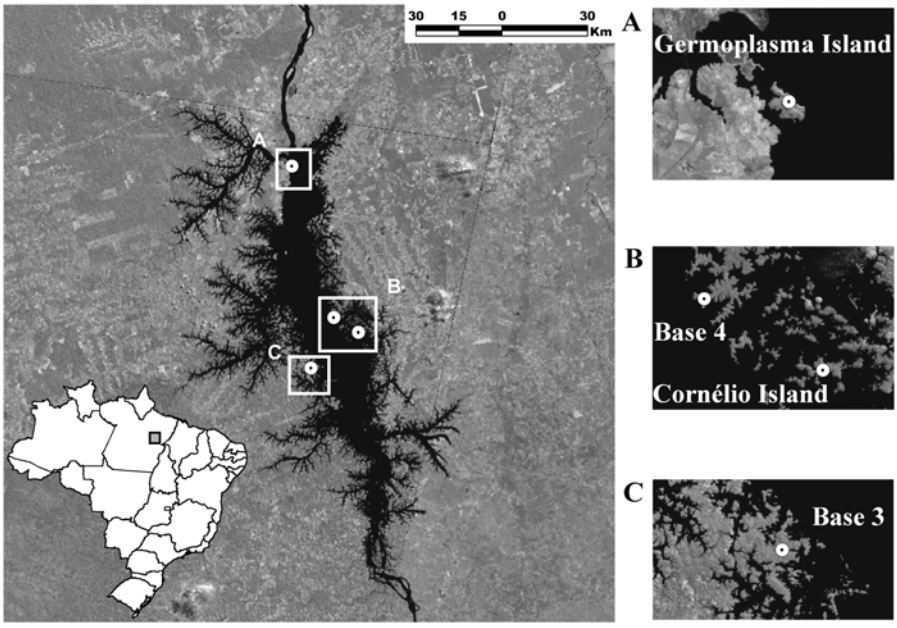

Figure 1. Collecting localities mentioned in the text. The Tocantins river and the Tucuruí reservoir are shown in black against a background of anthropogenic habitats (lighter gray) and remnant forest (darker gray).

Intron 7 of the *β-fibrinogen* nuclear gene was chosen for this study because of its relatively straightforward amplification using universal primers and, at least in birds, a phylogenetic signal similar to that observed in the cytochrome *b* mitochondrial gene (Prychitko & Moore, 1997), but without the problematic amplification of *numts* (Lopez *et al*., 1994), segments of nuclear DNA homologous to portions of the mitochondrial genome. Amplifications were carried out in a final reaction volume of 50 µl, containing 10 ng of DNA, 50 mM KCL, 1.5 mM MgCl2, 10 mM Tris-HCL, 50 µM of each DNTP, 0.5 µM of each oligonucleotide (FIB-17L/FIB-17U, Prychitko & Moore, 1997), and one unit of Taq DNA polimerase (Invitrogen). The following amplification procedure was used: 4 minutes at 94ºC for denaturation, followed by 30 one-minute cycles at 94ºC, 1 minute at 53ºC for association, 1 minute at 72ºC for extension, and a final cycle of 5 minutes at 72ºC to insure complete extension of the PCR products.

Amplification products were purified with the Qiaex II gel extraction kit (Qiagen) and cloned in *Escherichia coli* DH5α (Gibco) using the pGEM-T vector System I (Promega).The plasmid DNA of each clone was obtained using the QIAprep Spin plasmid Miniprep Kit (Qiagen) and sequenced automatically in an ALFexpress II (Amersham Biosciences), using the Cy5 thermo sequenase dye terminator kit (Amersham Biosciences), according to the maker's specifications. The nucleotide sequences were edited and aligned using BioEdit (Hall, 1999).

Data analysis

The DnaSP 3.53 program (Rozas & Rozas, 1989) was used to calculate levels of polymorphism and divergence for pairwise comparisons of sequences both within and between populations, and to test expected neutral evolution of the sequences. Variation of the sequences within each population was estimated through nucleotide (π) and haplotype (h) diversity, and neutral evolution was tested using Tajima's *D* (Tajima, 1989). This test is based on the difference between estimates of the product of the mutation rate and effective population size obtained from the number of segregating sites (θ), and the mean number of different nucleotides among sequences (π).

Genetic differentiation was evaluated using pairwise estimates of Nst, with Jukes & Cantor's correction (Lynch & Crease, 1990), which is analogous to Fst (Wright, 1978), but specific to nucleotide sequences. This parameter represents D_a (the number of nucleotide substitutions per site between populations) expressed as a proportion of overall divergence (mean nucleotide diversity for all comparisons within and among populations) and D_{xy} (mean number of nucleotide substitutions per site among populations). Phylogenetic relationships among haplotypes were evaluated using the maximum likelihood method (Felsenstein, 1981), run on PAUP, version 4.0b8 (Swofford, 1998), following the model selected by prior analysis of the data in the MODELTEST 3.04 program (Posada & Crandall, 1998).

RESULTS

Nucleotide composition and substitution of the β-fibrinogen *intron 7*

Alignment of the 45 nucleotide sequences of the *β-fibrinogen* intron 7 of the red-handed howler returned amplified fragments of 792 base pairs, 23 of which are polymorphic. Fifteen of these presented only two variants, while the remaining eight were informative for parsimony analysis. Of the 23 sites, 18 are transitions (9 A ↔ G and 9 T ↔ C), and five

transversions, 1 A ↔ T, 2 A ↔ C and 2 G ↔ T (Figure 2). Frequencies of the different bases – adenine (30.4%), cytosine (19.1%), guanine (19.4%) and thymine (31.1%) – did not vary significantly among populations ($\chi^2 = 0.9309$, P = 1.000, g.l. = 3). Values of Tajima's *D* indicated no deviations from neutral evolution for the sequences obtained (Table 1).

Genetic diversity and differentiation within and among populations

Mean pairwise distances (nucleotide diversity, π) among all individuals was 0.5%, and the probability that two individuals had different haplotypes (total haplotype diversity, h) was 87.0%. Within populations, values of π and h varied between 0.2% (Germoplasma) and 0.6% (Base 4), and 66.7% (Germoplasma) and 95.6% (Base 4), respectively (Table 1). The Germoplasma population thus presented the lowest nucleotidic and haplotypic diversity, followed by the two original populations in an intermediate position.

Values of Nst for between-population comparisons varied between 1.3% (Right Bank versus Left Bank) and 24.6% (Right Bank versus Germoplasma, Table 2). Two haplotypes – B401 and B405 – were found in all four populations, with overall frequencies of 33.3% and 15.6%, respectively. These appear to be the ancestral haplotypes of two genetic lineages, from which all others originated, via one or two mutations (Figure 2). Absent from Germoplasma, haplotype B408 was the third most frequent, at 6.70%, followed by haploytype B407 (4.4%). All other haplotypes were restricted to a single individual (2.2%) and, obviously, a single population.

Haplotypes phylogenetic analysis

Table 1. Variation in intron 7 of the *β-fibrinogen* nuclear gene and the results of Tajima's neutrality test for the howler populations from the Tucuruí Reservoir.

		Nucleotidic diversity	Haplotypic (genic) diversity		
	n	(π)	Number of haplotypes	*h* ± SD	Tajima's D
Total	45	0.005	22	0.869 ± 0.042	-0.692*
Original left bank (ME)	14	0.005	10	0.923 ± 0.060	0.432*
Original right bank (MD)	07	0.004	04	0.810 ± 0.130	1.725*
Germoplasma Island	10	0.002	05	0.667 ± 0.163	-0.754*
Base 4	14	0.006	11	0.956 ± 0.045	-0.177*

*$p > 0.10$.

Table 2. Estimates of Nst based on the sequences of intron 7 of the *β-fibrinogen* nuclear gene for pairwise comparisons of howler populations from the Tucuruí Reservoir.

	Original left bank (ME)	Original right bank (MD)	Germoplasma Island
MD	-0.01605		
Germoplasma	0.05069	0.24596	
Base 4	-0.02616	-0.07255	0.16363

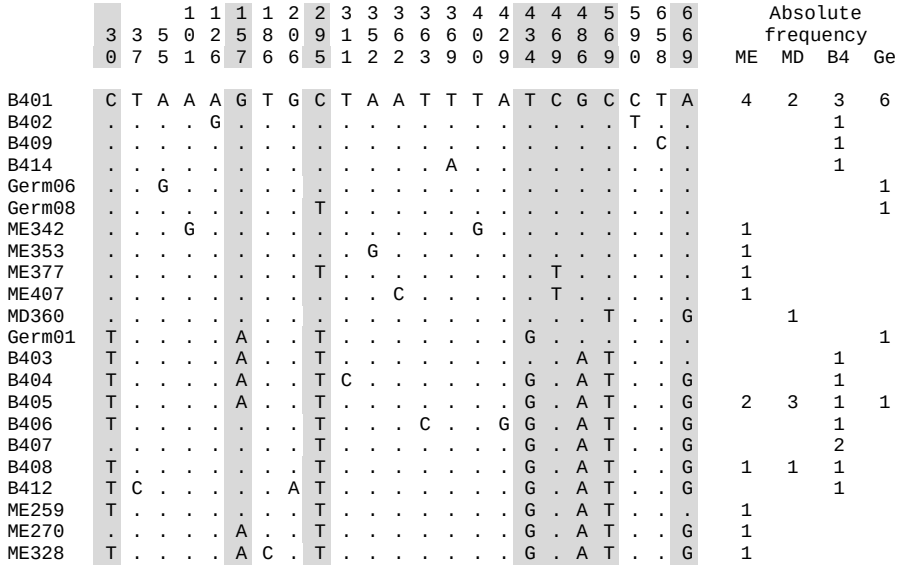

Figure 2. Alignment of the *β-fibrinogen* intron 7 haplotypes (GenBank accession numbers EF025062 - EF025083) obtained from 45 specimens of red-handed howlers from the area of the Tucuruí reservoir in eastern Brazilian Amazonia. Informative sites for parsimony are shaded in gray. ME = left bank, MD = right bank, B4 = Base 4, Ge = Germoplasma Island.

MODELTEST selected model HKY85 (Hasegawa *et al.*, 1985), with I (proportion of invariable sites) equal to 0.9329 for the maximum likelihood analysis using the 23 haplotypes. The mean transition/transversion rate was 6.6741. The HKY85 model permits unequal frequencies of bases, as well as unequal transition/transversion rates among the populations being analyzed. The heuristic analysis with 20 replicas and random addition of populations resulted in a tree with a score of 1221.668 (Figure 3). The topology of this tree suggests that the populations are not genetically structured, but that there are two distinct lineages, connected by haplotype Germ01.

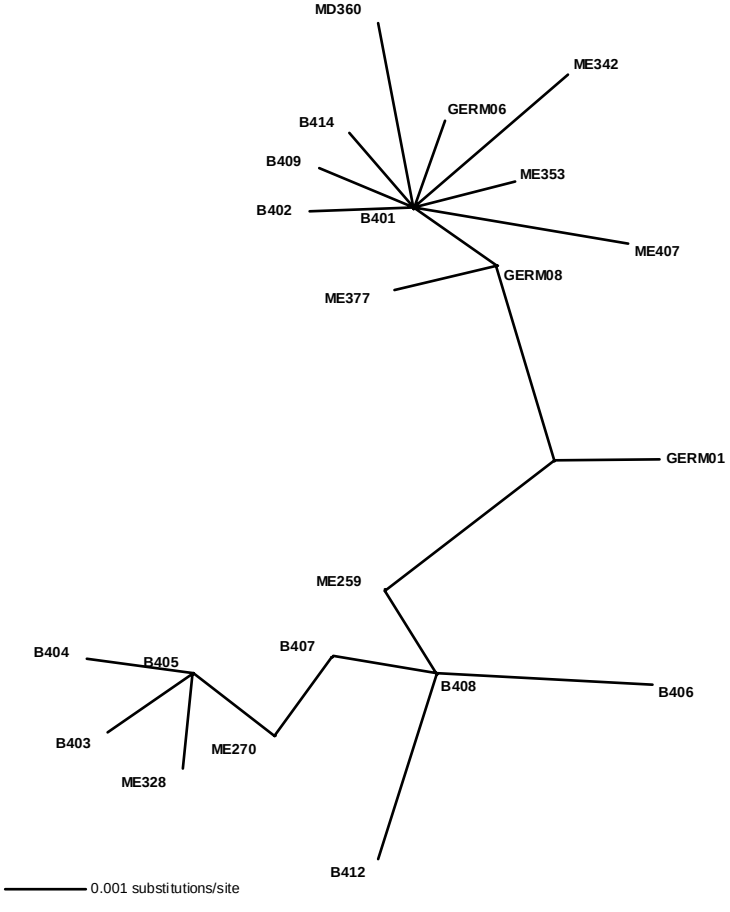

Figure 3. Unrooted phylogram obtained by the maximum verosimilitity method (-LnL = 1221.668) for the 22 haplotypes (45 sequences) of intron 7 of the β-fibrinogen nuclear gene of the howler populations from the Tucuruí Reservoir.

DISCUSSION

Variability of the β-fibrinogen intron 7

The levels of nucleotide diversity and evolutionary patterns observed here were similar to those recorded by Gonçalves (2002) and Ramos (2004) in their studies of the same *β-fibrinogen* intron 7 in populations of two bird species, *Calidris pusilla* (Semipalmated sandpiper) and *Eudocimus ruber* (Scarlet ibis), respectively. However, in *A. belzebul*, fragments were 200 bp shorter, and haplotypic diversity was approximately two-thirds lower. This may be related in part to generation length, which is two years, on average, in the bird species, but approximately four years in the howlers.

The results reconfirm the findings of Schneider *et al.* (1991), who also found greater diversity in 13 protein systems on the left bank of the Tocantins (mean Ho = 0.077) in comparison with the right bank (mean Ho = 0.043) prior to the flooding of the reservoir. Nucleotide diversity is greatly reduced in the present case, however, probably reflecting the more conservative nature of *β-fibrinogen* gene in this species.

Most studies of the genetic structure of populations are based on microsatellite markers or, in the absence of appropriate microsatellites, sequences of the mitochondrial cytochrome *b* gene. The present study is the first to assess the phylogenetic signal of *β-fibrinogen* intron 7 in primate populations, and has revealed a degree of potential as an alternative to mitochondrial genes, with the added advantage of avoiding *numts*, mitochondrial-type sequences which jumble the phylogenetic signal, and reduce the possible detection of gene flow mediated by both sexes.

Genetic structure of red-handed howler populations

The analyses of genetic diversity and differentiation presented here indicate that the present-day gene pool is distinct from that which existed in the study area prior to the flooding of the reservoir. In addition to the overall difference between the original and the present day populations of the right bank, there has been a considerable increase in genetic variability. A similar, but more pronounced tendency was observed by Bastos (2003) in his analysis of five microsatellite loci, the difference probably reflecting the higher evolutionary rate of these markers.

The increase in variability on the right bank contrasts with Germoplasma island, where diversity has decreased in comparison with the original left bank population. The most obvious difference between these two populations is that Germoplasma is relatively small (approximately 100 individuals) and isolated, whereas Base 4 is part of a much

larger, mainland forest, more or less contiguous with surrounding fragments, and probably represents a potential gene pool of thousands of individuals.

Obviously, both populations were subject to considerable change during the flooding of the reservoir, including the influx of animals retreating from the rising water, and others captured and released by Operation Curupira. In addition, while efforts were made to release animals at the same margin on which they were captured (especially important in the case of *Callicebus moloch*, which occurs naturally only on the left bank of the Tocantins), the numbers of howlers and their morphological heterogeneity made between-bank transfers inevitable.

This "passive" transfer between banks may partly account for the increased diversity at Base 4, although the reduced level of genetic differentiation between the two original populations (margins) suggests that they were not completely isolated from each other prior to the flooding. This might be expected, given the preference of Amazonian howlers for flooded forest ecosystems (Queiroz, 1995; Peres, 1997), and original topography of the area, which was characterized by a complex of fluvial islands. Theoretically, even a single migration per generation could uphold the genetic homogeneity of geographically distinct populations (Hartl & Clark, 1989), although gene flow between banks is probably all but nonexistent in the present day.

Whereas the population at Base 4 assimilated the influx of new haplotypes, the Germoplasma population may have been subject to one or more of a number of effects related to its relatively small size and isolation. One possibility is a founder effect, either from the isolation of the original population, or some bias in the origin of the animals released onto the island. Subsequently, the population may have been affected by inbreeding or genetic drift.

Overall, the results of this study indicate that present-day populations of red-handed howler in the area of the Tucuruí reservoir have maintained, or even increased their genetic diversity over the seventeen years subsequent to the flooding, an encouraging finding for their long-term conservation. However, the evidence also points to the potentially deleterious effects of small size and isolation in the Germoplasma population. Population density on this island is relatively high (approximately 100 individuals km^{-1}: Camargo & Ferrari, 2007), but its small size (1.3 km^2) restricts the total population to far less than 500 individuals, considered to be the threshold for long-term viability (Frankham *et al.*, 2002). The exact role of genetic factors in the extinction risk of natural population remains poorly understood, but there is increasing evidence of their importance (Frankham, 2003). The specific problem at Tucuruí is that there are many other islands of similar size to that of Germoplasma

which support howler populations so, whatever their current genetic diversity, the need for active management of these populations will almost certainly increase over time.

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