ID Artigo: 08-07
envio: 05/12/2007
Diagramador: Daniel
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Conservation genetics of the giant otter (*Pteronura brasiliensis* (Zimmerman, 1780)) (Carnivora, Mustelidae)

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Received April 2, 2007, Accepted July 24, 2007 – Distributed December 1, 2007

(With 3 figures)

Abstract

The giant otter (*Pteronura brasiliensis*) is an aquatic mammal of the Mustelidae family, endemic to South America. Its original distribution corresponds to the region from the Guyanas to Central-North Argentina, but it is extinct or on the verge of extinction in most of its historical range. Currently, the species is considered endangered by the World Conservation Union (IUCN). Based on its geographic distribution in the South American continent and on some morphological characters, two subspecies were suggested: *P. brasiliensis brasiliensis*, occurring in the Amazon and Orinoco River Basins, and *P. brasiliensis paranensis*, in the Paraná and Paraguai River Basins. However, there is no consensus on assuming this subspecies division and no detailed studies have been carried out to elucidate this question. This study aims to evaluate the genetic diversity and population structure of *Pteronura brasiliensis* along its range in Brazil to check the possibility of the existence of two distinct subspecies using also a reciprocal monophyly criterion. We analyzed the control region, and the Cytochrome b and Cytochrome c Oxidase subunit I genes of the mitochondrial DNA in several giant otter populations from the Amazon and Paraguai River Basins. Analyses have indicated some degree of geographic correlation and a high level of inter-population divergence, although the subspecies division is not highly supported. As we observed strong population structure, we cannot rule out the existence of further divisions shaping the species distribution. The results suggest that a more complex population structure occurs in *P. brasiliensis*, and the conservation practice should concentrate on preserving all remaining local populations.

Keywords: giant otter, mitochondrial DNA, conservation genetics, subspecies.

Genética da conservação da ariranha (*Pteronura brasiliensis*) (Carnivora, Mustelidae)

Resumo

A ariranha (*Pteronura brasiliensis*) é um mamífero aquático da família Mustelidae, endêmico da América do Sul. Sua distribuição original se estendia desde as Guianas até o centro-norte da Argentina, mas está extinta ou à beira da extinção na maior parte de sua distribuição histórica. Atualmente a espécie é considerada como ameaçada de extinção pela World Conservation Union (IUCN). Em função de sua distribuição no continente sul-americano e de algumas características morfológicas, duas subespécies foram sugeridas: *P. brasiliensis brasiliensis*, com ocorrência nas bacias do Amazonas e Orinoco, e *P. brasiliensis paranensis*, ocorrendo nas bacias dos Rios Paraná e Paraguai. Inexiste, contudo, um consenso sobre a validade da divisão em subespécies e nenhum estudo detalhado foi realizado para elucidar esta questão. Este trabalho tem o objetivo de avaliar a diversidade genética e a estrutura populacional de *P. brasiliensis* ao longo de sua distribuição no Brasil para verificar a existência de duas subespécies baseando-se também em um critério de monofilia recíproca. A região controle e os genes do Citocromo b e da Subunidade I da Citocromo c Oxidase do DNA mitocondrial foram analisados em diversas populações de ariranha que ocorrem nas bacias dos rios Amazonas e Paraguai. As análises indicaram um grau moderado de correlação geográfica e um alto nível de divergência inter-populacional, embora a divisão em subespécies não seja bem sustentada. Como uma forte estruturação populacional foi observada, não é possível descartar a existência de outras subdivisões nesta espécie. Os resultados indicam a presença de uma estrutura populacional mais complexa em *P. brasiliensis*, o que implica que medidas de conservação deveriam concentrar seus esforços preservando todas as populações locais remanescentes.

Palavras-chave: ariranha, DNA mitocondrial, genética da conservação, subespécies.

1. Introduction

The giant otter, *Pteronura brasiliensis* (Zimmermann, 1780), is an aquatic mammal of the Mustelidae family, order Carnivora, endemic to wetlands and river systems in forests of South America (Eisenberg, 1989). Its historical distribution corresponds to the area from the Guyanas to Central-North Argentina (Carter and Rosas, 1997). During the early 1970's, hunters decimated many populations to supply the international market with the species' valuable skin (Rosas, 2004). Today, the giant otter is believed to be extinct in most of its original range and is classified as endangered by the World Conservation Union (IUCN, 2000) While in the past, hunting strongly affected giant otter populations, currently the species is threatened by multiple anthropogenic factors derived from expanded colonization of tropical lowland rainforests and wetlands. Destruction of forests leading to soil erosion, decrease of prey abundance due to over-fishing, as well as illegal hunting of otters, are related to human colonization along rivers (Groenendijk et al., 2004).

The giant otter is the largest aquatic carnivore in South America, with a total body length ranging from 1.5 to 1.8 m. (Duplaix, 1980). It is a social species, with group sizes ranging from 2 to 12 individuals. Each individual can be identified since birth by the pale irregular pattern on its chin and throat (Foster-Turley, 1990).

Rengger (1830), later reviewed by Harris (1968), described the subspecies *Pteronura brasiliensis paranensis* based on morphological characters like dentition, throat color, and size, inhabiting the Paraná and Paraguai River Basins, as opposed to the *P. brasiliensis brasiliensis* occurring in the Amazon and Orinoco River Basins. However, no other taxonomic studies have been done to validate the subspecies division, and to this date no population genetic studies have been performed on this species.

According to Ridley (2006), subspecies can be defined as geographic populations, presenting clearly different phenotypes. However, it appears to be no clear phenotype differences documented between the suggested giant otter subspecies. Subspecies are formed by the evolution of at least two populations in relative isolation that accumulate genetic differentiation (Zink, 2004). Data on the level of population divergence in a particular species represent an important issue in conservation biology. Furthermore, population subdivisions such as subspecies are usually taken into account for different conservation management strategies, and are also frequently target of governmental protection, and focus of conservation efforts (Frankham et al., 2002). However, discrimination of subspecies at the phenotypic and genotypic level can be very difficult because of discordant patterns of intra- and inter-population diversity, as well as a complex population structure. Assuming that subspecies should present evolutionary distinctiveness, the use of a reciprocal monophyly criterion is also an important evidence of this taxonomic division, although it may not be the only one (Zink, 2004).

Different molecular markers can be used to access the diversity and genetic structure of natural populations (Avise, 1994). The mitochondrial DNA (mtDNA) has some peculiar characteristics such as maternal inheritance, no recombination, evolution rate about ten times faster than nuclear DNA, extensive intra-specific variation in Vertebrates, multiple copies per cell, and it is easy to isolate and study. Such factors make it an important tool for population and phylogenetic studies (Kocher et al., 1989). The non-coding control region (CR) is known to present the fastest evolution rate in the molecule, thus it is commonly used for intraspecific studies (Kvist et al., 2001; Rhymer et al., 2001). On the other hand, the Cytochrome b (Cyt-b) and Cytochrome c Oxidase subunit I (COI) are coding regions with slower evolution rate and therefore are frequently used to establish relationships among subspecies, species, genera, and families (Zink et al., 1998; Vilaça et al., 2006).

One major difficulty in genetic studies is the geographic sampling, especially when dealing with rare, threatened species that are difficult to capture or collect tissues. For some animals, methods requiring the capture and handling are sometimes not feasible, owing to logistical difficulties or ethical concerns. This problem is particularly acute for endangered carnivore species, which are usually scarce, live in low densities, and have elusive and secretive habits (Palomares et al., 2002). The use of DNA extraction protocols from materials such as feces, hair, skin, egg shells, among others, allow non-invasive sampling to overcome most of the restrictions (Kohn and Wayne, 1997). However, many problems have to get round, like the co-purification of contaminants (Litvaitis and Litvaitis, 1996), small amounts of DNA (Frantzen et al., 1998), degraded DNA leading to non amplification by PCR, identification of false alleles (allele dropout), and sporadic contamination (Gagneux et al., 1997; Kohn and Wayne, 1997; Taberlet et al., 1999). In the case of *P. brasiliensis*, the use of non-invasive techniques was facilitated by the presence of a viscous mucus containing mostly intestine epithelial cells and free of food residues, which can be deposited separately but near the feces or on its surface, making the mucus relatively easy to separate from fresh feces.

Our aim in this project was to carry out a pioneer study on the genetic diversity and population structure of the endangered species *Pteronura brasiliensis*, using mtDNA sequences obtained mainly by non invasive methods. This is the first approach to confront the initial suggestion, based on morphology, that there are two geographically separated subspecies, one in the North (Amazon and Orinoco River Basins), and another in the South (Paraná and Paraguai River Basins). The characterization of the population structure and the determination of taxonomic subdivisions in the giant otter are extremely important for the elaboration of management programs and conservation strategies.

2. Material and Methods

2.1. Sample collection

Thirty giant otter individuals were sampled in different areas - 22 from eight sampling sites located in the Brazilian States of Amazonas (AM) and Roraima (RR), both in the Amazon River Basin, and 8 individuals from the Brazilian Pantanal (Paraná and Paraguai River Basins) (Figure 1, Table 1). All samples (from captive or dead animals, and feces from latrines) were maintained at the Laboratory of Aquatic Mammals at INPA (Manaus, AM), at the Centre of Preservation and Research of Aquatic Mammals (CPPMA) of Manaus Energia S.A. (Vila de Balbina, AM), at the Institute of Sustainable Development Mamirauá (IDSM) (Tefé, AM), or at the Center of Research of Pantanal (CPP) (Cuiabá, MT). Blood samples from live captive animals (N = 4) were collected by anesthetizing individuals with Zoletil (Rosas, FW., unpublished data). Other samples (liver, kidney, heart) were obtained from animals (N = 7) that died in captivity between the years of 1992 and 2004, or from feces (mucus) collected from communal latrines of free-ranging giant otters (N = 13), or from pelts (N = 6) of known origin. The mucus quality and amount obtained from feces depended on the sample age. When feces were fresh it was possible to collect only the mucus using a syringe without needle. When feces were old or dry, the surface was scraped off with a spatula, targeting epithelial cells sloughed from the gut lining during gut passage of the fecal bolus. Both the mucus and dried feces were stored in 2.0 mL microcentrifuge tubes containing 70% ethanol. Other tissues were frozen or preserved in 92% ethanol. We have also used two samples of *Lontra longicaudis* (Olfers, 1818) as an outgroup.

2.2. Dna extraction and amplification

Total DNA from tissue and blood was extracted using a phenol:chloroform:isoamyl alcohol protocol (Sambrook and Russel, 2001) after digestion with Proteinase K. DNA concentration was estimated through visual evaluation of the band resulting from electrophoresis of 5 µL of DNA solution in 0.8% agarose gels stained with ethidium bromide. Various protocols were tested to extract the DNA from feces/mucus, such as the phenol:chloroform:isoamyl alcohol protocol, DNA Easy Qiagen Kit (Qiagen), QIAamp DNA Stool Mini Kit (Qiagen), and UltraClean DRY Soil DNA Kit for fecal samples (Gene Works). For extractions using feces/mucus, an average of 100-300 mg starting material was used after discarding the ethanol used for storage. For the majority of the extractions we used the protocol provided by the QIAamp DNA Stool Mini Kit (Qiagen). We weighted the fecal material in a 2 mL tube and placed it on ice, then added the ASL buffer, vortexed for 1 minute,

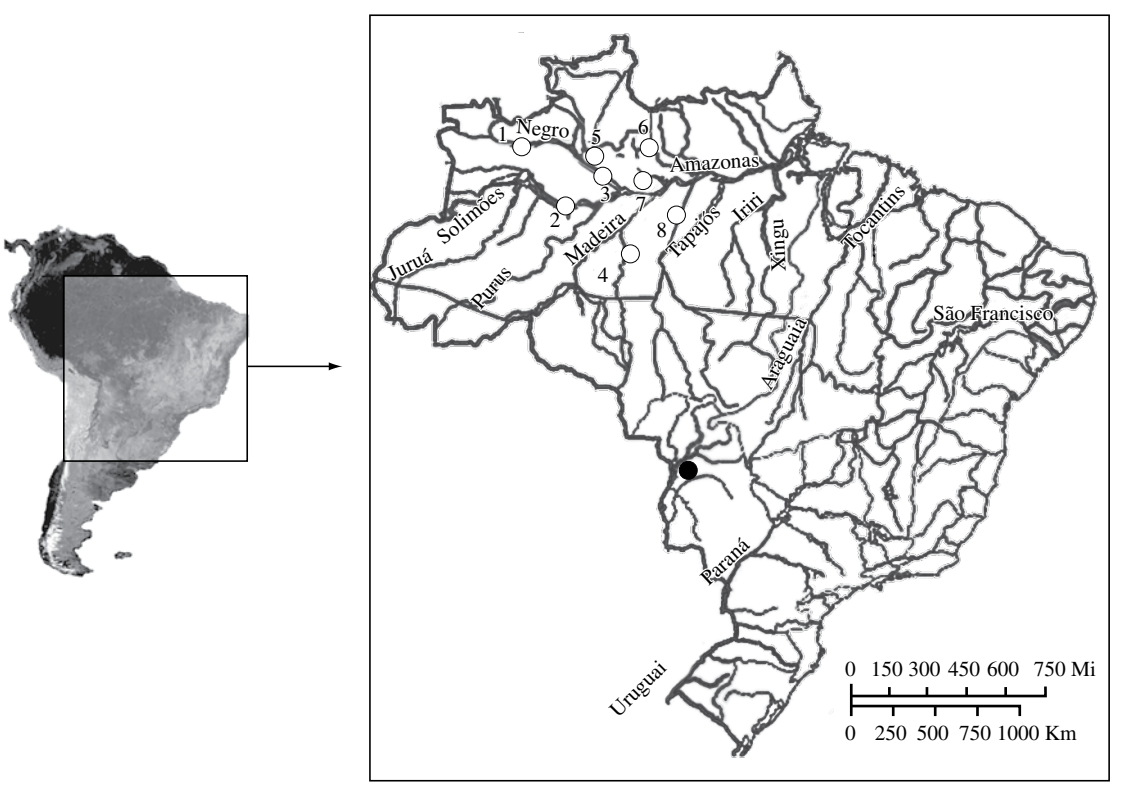

Figure 1. Sample localities for giant otter subpopulations in Brazil, on the Amazon River Basin (white dots) and Pantanal (black dot). In the Amazon, sampling sites are marked with numbers: 1) Negro River, 2) Tefé Lake, 3) Abonari River, 4) Uruá River, 5) Jauaperi River, 6) Nhamundá River, 7) Balbina Lake, and 8) Maués River.

Table 1. List of the analyzed samples, their respective sources, geographic origin, and haplotype (CR+Cyt-b).

ID	Sample	Geographic region	Haplotype
Pb 01	dried pelt	Negro River, Amazonas State	Hap_1
Pb 02	skin	Negro River, Amazonas State	Hap_2
Pb 03	muscle	Pantanal	Hap_1
Pb 05	tissue	Roraima State	Hap_3
Pb 06	tissue	Roraima State	Hap_4
Pb 07	tissue	Jauperi River, Roraima State	Hap_5
Pb 08	blood	Tefé Lake, Amazonas State	Hap_6
Pb 09	blood	Maués River, Amazonas State	Hap_7
Pb 10	blood	Nhamundá River, Roraima State	Hap_8
Pb 11	blood	Roraima State	Hap_4
Pb 13	skin	Negro River, Amazonas State	Hap_9
Pb 16	skin	Negro River, Amazonas State	Hap_12
Pb 17	skin	Maués River, Amazonas State	Hap_4
Pb 18	skin	Uruá River, Amazonas State	Hap_11
Pb 19	skin	Jauaperi River, Amazonas State	Hap_4
Pb 20	feces	Pantanal	Hap_10
Pb 23	feces	Pantanal	Hap_1
Pb 24	feces	Pantanal	Hap_1
Pb 30	feces	Pantanal	Hap_1
Pb 36	feces	Pantanal	Hap_1
Pb 38	kidney	Pantanal	Hap_1
Pb 40	feces	Pantanal	Hap_1
Pb 49	feces	Balbina Lake, Amazonas State	Hap_12
Pb 50	feces	Amazonas State	Hap_12
Pb 52	feces	Balbina Lake, Amazonas State	Hap_5
Pb 53	footpad	Abonari River, Amazonas State	Hap_5
Pb 54	footpad	Balbina Lake, Amazonas State	Hap_12
Pb 56	feces	Amazonas State	Hap_4
Pb 57	feces	Balbina Lake, Amazonas State	Hap_4
Pb 58	feces	Balbina Lake, Amazonas State	Hap_4

and spun in the centrifuge for 1 minute at full speed. The supernatant was transferred into a new 2 mL tube and the pellet was discarded. Next, an InhibitEX tablet (Qiagen) was added to each sample and vortexed immediately for 1 minute until the tablet was completely suspended. The suspension was incubated for 1 minute at room temperature. Samples were then centrifuged for 3 minutes at full speed, all the supernatant was transferred into a new 1.5 mL tube and the pellet discarded. The supernatant was incubated for 10 minutes at 70 °C with Proteinase K and AL buffer (Qiagen). Absolute ethanol was added to the lysate and vortexed. Spin filtering was performed until all extract has been filtered. Two washes with buffers AW1 and AW2 (Qiagen) were respectively performed, and DNA was finally dissolved in 200 µL buffer AE (Qiagen).

The CR and Cyt-b partial segments of about 900 base pairs (bp) were amplified using the specific primers XL14733 and H16498 designed by Kocher et al. (1989). PCR reaction mixes of 12.5 µL included 2 µL of genomic DNA (~ 40 ng), 1 U of Taq polymerase (Phoneutria®), 200 µM of dNTPs, 1X Tris-KCl buffer with 1.5 mM $MgCl_2$ (Phoneutria®), and 0.5 µM of each primer. The amplification program consisted of 2 minutes at 94 °C, followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 57 °C, 2 minutes at 72 °C, and a final extension step of 10 minutes at 72 °C.

We also amplified a 500 bp segment of the COI gene using the primers HCO2198 and LCO1490 described by Folmer et al. (1994). The PCR program cycle for these primers was the same as described above, except that the annealing temperature was changed to 40 °C. After amplification, PCR products were checked by running in 0.8% agarose gels and staining with ethidium bromide. Negative controls, where template DNA was omitted, were used in all amplification runs. Only products with a single and well-defined band were used in the sequencing reactions. Before sequencing, PCR products were

cleaned by precipitation using 20% polyethyleneglicol (PEG 8000). Sequencing reactions were performed using either of the primers described above, and were conducted in a final volume of 10 μL containing: 2-4 μL of purified PCR product, 1-3 μL of ultrapure water, 1 μL of one primer (5 μM), and 4 μL of sequencing kit (ET DYE Terminator Kit, GE Healthcare Life Sciences). The sequencing program consisted of 35 cycles of 95 °C for 25 seconds, 55 °C for 15 seconds, and 60 °C for 3 minutes Then, sequencing products were precipitated with ammonium acetate and ethanol, dried at room temperature, dissolved with formamide-EDTA, and run in the automatic sequencer MegaBACE 1000 (GE Healthcare Life Sciences).

To obtain high quality mtDNA sequences the following measures were undertaken: i) for each individual, at least two different PCR products were used in the sequencing reactions until, at least, two high quality and independent sequences could be obtained; ii) chromatograms were carefully checked for ambiguities; iii) consensus sequences were obtained with forward and reverse sequences; and iv) CR, Cyt-b, and COI sequences produced in the present study were aligned and also compared with others obtained from the GenBank, including sequences of other mustelid species, to check for the presence of any stop or nonsense codons, as well as alignment gaps.

2.3. Data analysis

Consensus sequences (GenBank accession numbers: EF488532-EF488561 and EF491161-EF491181) were obtained and checked through the programs Phred v. 0.20425 (Ewing et al., 1998), Phrap v. 0.990319 (http://www.phrap.org), and Consed 12.0 (Gordon et al., 1998). Alignments were done using Clustal X (Thompson et al., 1997), with manual edition whenever it was necessary. Genetic distances between different haplotypes were estimated using the Kimura 2-parameter (K2p) substitution model (Nei and Kumar, 2000), and used to build a Neighbor Joining (NJ) tree using the program MEGA 3.0 (Kumar et al., 2004). Branch support was checked with 10,000 bootstrap replicates. Genealogical relationships were also investigated by constructing a haplotype network using the Median Joining method through the program Network 4.0 (Bandelt et al., 1999). Haplotype (h) and nucleotide diversity (π) were estimated using the program DnaSP (Rozas and Rozas, 1997), and average nucleotide diversity between populations was calculated with the program MEGA 3.0. Other diversity indexes, analysis of molecular variance (AMOVA), population pairwise distances (Fst or Φ_{ST}), and an exact test of population differentiation were computed using ARLEQUIN 3.0 (Schneider et al., 2000). The significance of the population pairwise distances was evaluated by 1,023 permutations of the original data and the significance of the exact test of population differentiation was assessed by 10,000 Markov chain steps. Most of the analyses in Arlequin were performed using two groupings of populations: the suggested subspecies divided in populations of the Amazon and Pantanal River Basins, or comparing all local geographic populations defined by eight Amazon sites and a single site in Pantanal (Figure 1).

3. Results

3.1. Dna extraction from feces

The material derived from feces has a very low amount of cells as DNA source as well as some contaminants, which makes the extraction process very difficult. Different protocols were tested until satisfactory results and high quality sequences were obtained for many individuals. The best results were achieved using the kits QIamp DNA Stool Mini Kit (Qiagen) and UltraClean DRY Soil DNA Kit for fecal samples (Gene Works). Comparing the dry feces with mucus from fresh feces, the later one presented a greater DNA yield and also a higher amplification success (results not shown). The amplified products showed the expected length and good quality when compared to products derived from other tissue extractions.

3.2. CR and Cyt-b

High quality sequences of 816 bp long including partial segments of the control region and Cytochrome b were generated and analyzed for 30 individuals. We identified 21 variable sites and 12 haplotypes (Table 2). Nucleotide diversity (π) found for *P. brasiliensis* was 0.006 for the two combined mtDNA regions (CR + Cyt-b). The haplotype diversity index found was $h = 0.867 \pm 0.038$, and the average number of nucleotide differences was $k = 5.25$. We observed a high number of haplotypes in relation to the sample size (Amazon River Basin: n = 22 and 11 haplotypes; Pantanal River Basin: n = 8 and 2 haplotypes), which suggests the existence of a much larger number of non-sampled haplotypes. The phylogeographic analysis made through Network (Figure 2) revealed a distribution with some geographic correlation. Individuals from Pantanal (n = 8) presented two distinct haplotypes (Hap_1 and Hap_10) differentiated from each other by one mutation, and Hap_1 also included one individual from the Amazon. Eight out of twelve haplotypes encountered were represented by a single individual. The

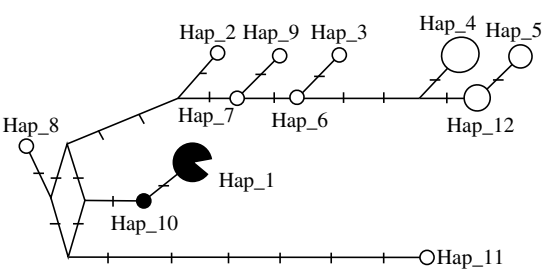

Figure 2. Phylogeographic network built by the median joining algorithm with the segment containing Cyt-b and CR nucleotide sequences. Black sectors correspond to Pantanal population and white to Amazon populations.

most frequent haplotypes were Hap_1 and Hap_4, occurring in 50% of the samples. The individuals from Amazon River Basin (n = 22) showed 11 haplotypes, including individuals from Roraima (RR), with difference among all haplotypes varying from one to sixteen mutations (Figure 2). The overall ϕst value calculated across all local populations divided in two subspecies groups was 0.737 (P < 0.00001), indicating a high and statistically significant population structure (Table 2). Although AMOVA analysis using this dataset indicated that most of the variation occurs among groups (Table 2), it is non-significant (P > 0.05), a result that is also observed using conventional Fst analysis and other datasets (Table 2). However, a large and significant variation is observed among and within populations (Table 2). Indeed, if we consider *P. brasiliensis* as one group of populations, we observe a high and significant ϕst (P < 0.0000) displaying 65% of variation occurring among populations (results not shown). Phylogenetic analyses were made with the two combined regions of the mtDNA (CR + Cyt-b), using sequences of the Neotropical river otter (*Lontra longicaudis*) as outgroup (Figure 3a). One individual from the Amazon (Rio Negro site) shared a haplotype (Hap_1) with seven individuals from Pantanal. Although presenting some geographic clustering, we found no reciprocal monophyly for the proposed subspecies in this tree. However, the Pantanal population appears to be a divergent subgroup of the Amazon diversity.

The pairwise Fst and ϕst´s (Table 3), as well as the exact test for population differentiation (results not shown), were shown to be significant (P < 0.05) for all comparisons between Pantanal and Amazonian populations that presented N > 3 individuals. However, we also observed a significant ϕst between two Amazonian populations, Negro River and Balbina Lake.

3.3. COI and CR+Cyt-b+COI

The sequencing of a 628 bp segment of the COI gene allowed the identification of four haplotypes and four variable sites (Table 2). The value found for haplotype diversity was h = 0.598 ± 0.003, and for nucleotide diversity π = 0.001. All the individuals from Pantanal presented the same haplotype (H_A) that is shared with some individuals (n = 4) from Amazon. Haplotypes H_A and H_B represented 80% of all samples (data not shown). Considering only the apportionment of diversity produced with COI data (Table 2), we observed a low and non-significant differentiation between Amazon and Pantanal groupings, which was also observed with the CR+Cyt-b+COI (1,444 bp) dataset (Table 2). The non-significant variation among groups appeared also when excluding from AMOVA all populations with N < 3 (results not shown). However, for all datasets (Table 2), the

Table 2. Population and sequence data, and AMOVA (ϕst with Tamura-Nei) results generated by Arlequin. The nine populations (see Figure 1) were grouped according to the suggested subspecies division. CR+Cyt-b+COI data set was analyzed for only seven Amazonian populations (excluding Maués).

	CR+Cyt-b	COI	CR+Cyt-b+COI
N (population size)	30	24	18
Number of loci (nucleotides)	816	628	1444
Number of polymorphic sites	21	4	24
Number of haplotypes	12	4	11
Overall Φ_{ST}	0.737	0.799	0.836
AMOVA - % among groups	48.11*	6.60*	29.18*
AMOVA - % among populations	25.63	73.38	54.44
AMOVA - % within populations	26.26	20.03	16.38

* Non-significant variance component (P > 0.05)

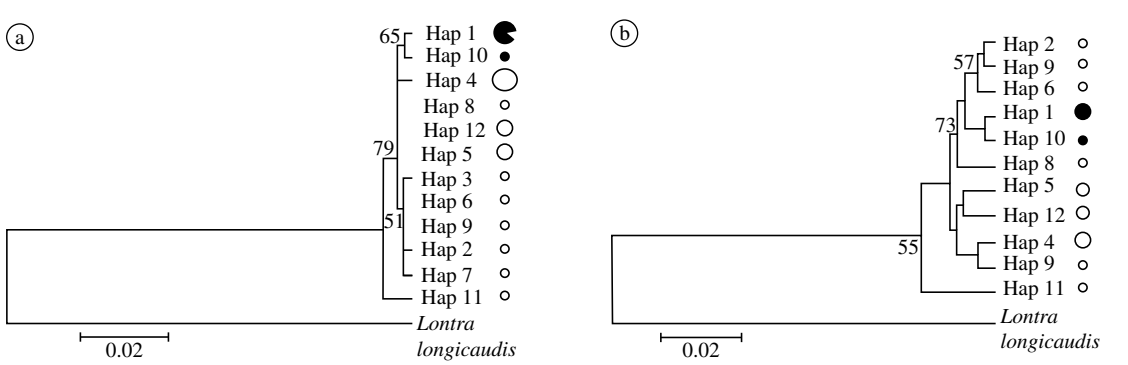

Figure 3. Neighbor joining trees with the different sets of mtDNA sequences. a) CR + Cyt–b; and b) CR + Cyt–b + COI *. Black sectors correspond to Pantanal population and white to populations. Bootstrap values higher than 50% are shown on the branches. *Haplotype CR+Cyt-b_Hap_3 represented by one individual that was not included on this tree because COI data could not be generated.

Table 3. Pairwise Fst (below diagonal) and ϕst (above diagonal) values calculated based on Cyt-b + CR data sets.

Pantanal	x	0.580*	0.972	0.949	0.972	0.901*	0.958	0.860*	0.964
Negro River - AM	0.306*	x	0.484	0.655	-0.077	0.236*	-0.650	0.085	-0.560
Uruá River - AM	0.750	0.000	x	1.000	1.000	0.872	0.100	0.777	0.487
Nhamundá River - AM	0.750	0.000	1.000	x	1.000	0.800	1.000	0.640	0.100
Abonari River - AM	0.750	0.000	1.000	1.000	x	0.167	1.000	0.729	0.100
Balbina Lake - AM	0.529*	-0.024	0.286	0.286	0.167	x	0.655	-0.019	0.544
Maués River - AM	0.750	0.000	0.100	0.100	0.100	0.285	x	0.315	0.100
Jauperi River - AM	0.034*	0.300	0.300	0.300	0.125	0.009	0.300	x	0.685
Tefé Lake - AM	0.750	0.000	1.000	1.000	1.000	0.286	1.000	0.300	x

* Significant pairwise distances ($P < 0.05$)

among populations apportionment of variance appeared high and significant.

3.4. Phylogeny of the complete set of mtDNA sequences

Phylogenetic trees were also constructed for data including CR+Cyt-b+COI sequences, using *Lontra longicaudis* as an outgroup (Figure 3b). Although in the CR+Cyt-b+COI NJ tree, the Pantanal haplotypes were apparently monophyletic, this can be explained by the absence in this tree of an individual from the Amazon that was not included due to the lack of COI data. This individual shared a CR+Cyt-b haplotype with Pantanal individuals (Figure 3a). In both analyses (Figure 3) Pantanal haplotypes appear as a subgroup of the Amazon population; however the suggested subspecies do not form reciprocal monophyletic groups that should be seen as separate phylogenetic clades, which is not the case.

4. Discussion

Reliable use of feces for genetic analyses is particularly important in studies where sampling is difficult due to animal size, behavior, conservation status, and ethical issues. The use of noninvasive samples such as feces and mucus for molecular genetic analyses has proven to be an informative DNA source to analyze *P. brasiliensis* populations.

In this study we have characterized the mtDNA diversity of giant otter populations in Brazil. The value found for nucleotide diversity ($\pi = 0.006$) in the species for the combined two DNA segments (CR + Cyt-b) was moderate. Using only control region (342 bp) data we found a diversity value ($\pi = 0.012$) that was similar to other endangered mustelids, like the European polecat ($\pi = 0.009$, Davidson et al., 2001), and the European mink ($\pi = 0.012$, Michaux et al., 2005). The average pairwise nucleotide diversity calculated by MEGA 3.0 between Amazon and Pantanal populations (0.9%) was much lower than values previously used (Avise, 1994) to indicate a possible limit for species (5-7%) and subspecies (2%) differentiation in mammals.

The significant pairwise distances (Table 3) and exact tests of differentiation found between Pantanal and some Amazonian populations are important data supporting the subspecies division. This is even more compelling when considering that only Amazonian populations with $N < 3$ presented non-significant differentiations with Pantanal, but a significant differentiation was also observed between at least two Amazonian sites. However, the AMOVA analysis and the sharing of haplotypes (between Negro River in the Amazon Basin and Pantanal) as well as the absence of reciprocal monophyly do not corroborate with the existence of two evolutionarily separate subspecies occurring in the Amazon and Pantanal. The observation of high population differentiation data and the great number of observed haplotypes, considering also the relatively small sample size, indicates that expansion of sampling areas is of extreme importance in further studies, not only to the characterization of new haplotypes but also to provide more robust data about highly heterogeneous giant otter populations in Brazil and other South American countries.

Thus, *P. brasiliensis* populations seem to be geographically structured, but the data do not completely agree with the suggested subspecies division, at least with our dataset. Although our results must be considered with care due to using only mtDNA, the sampling is representative of a large geographic distribution including both areas of occurrence for the proposed subspecies, and some important results were highly significant among populations, at least concerning the geographic structure of females. Nevertheless, the existence of a complex geographic structure due to differentiation of local populations should be further considered in conservation management.

In the near future, nuclear markers such as microsatellites or intron sequences may present limitations to be analyzed in DNA extracted from fecal material, but they may be also added to further characterize the genetic diversity of the giant otter populations. For now, our data indicates that conservation programs of the giant otter should focus on preserving all local populations, which seems not to cluster in major geographic groups but still retain a large heterogeneity.

Acknowledgments — D.M.G was supported by CAPES (Brazil), F.R.S., M.M. and F.W.R. were supported by CNPq (Brazil). This project received grants from CNPq (Proc.

47.6015/2004-9), FAPEMIG and Fundação o Boticário de Proteção à Natureza (FBPN), all from Brazil. We are grateful to R. Redondo (UFMG) for technical help and to Stella Maris Lazzarini (CPPMA, Manaus Energia S.A.) for providing some samples. We also thank for the comments of two anonymous referees that improved the quality of this manuscript.

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