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Taxonomic identification of dolphin love charms commercialized in the Amazonian region through the analysis of cytochrome *b* DNA

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*Previous studies identified the Amazon river dolphin (*Inia geoffrensis*) as the species frequently traded in public markets of Pará and Amazonas State (Brazil). In order to investigate the geographic origin and the taxonomic identification of these specimens we analysed 21 samples provided by residents of the city of Belém and vicinities (Pará State, Brazil), presumably purchased in the 'Ver-o-Peso' Market (Belém) or the Manaus Municipal Market. DNA was extracted from dehydrated genitalia or from genitalia conserved in alcohol and the cytochrome* b *gene was PCR-amplified. Phylogenetic analyses showed identical topologies in both MP and NJ trees, with* Sotalia *splitting into two groups, one clade comprising the estuarine dolphin (*S. guianensis*) and all eight haplotypes from market-samples and another one, comprising the tucuxi (*S. fluviatilis*) haplotypes from the GenBank. Our findings indicated that the marine species is under a stronger commercial pressure than the Amazonian river dolphin (*I. geoffrensis*) and the tucuxi (*S. fluviatilis*) in the region. Therefore population dynamic studies as well as population monitoring should be carried out in order to evaluate the effects of this commercial hunting on the species and its local populations. The marine dolphins have been incidentally captured in gill-nets in the region, suggesting that these activities must also be monitored at the same time that social programmes must be implemented in order to inform and clarify local community and people involved in fishery activity to avoid that more animals might be captured and killed.*

Keywords: Amazonian region; dolphins; market samples; mitochondrial DNA.

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INTRODUCTION

Dolphins have populated human imagination for centuries. In Brazil, they are strongly associated to cultural transmission, especially in the Amazon region. Three species of dolphins are found in the Amazonian river mouth: the Amazonian pink dolphin or 'boto' (*Inia geoffrensis*), the tucuxi (*Sotalia fluviatilis*) and the boto-cinza (*Sotalia guianensis*) (Siciliano *et al.*, 2005).

The trade of love charms of Amazonian dolphins represents a serious threat to the survival of this species. A previous study (Best & da Silva, 1989) identified the Amazonian river dolphin (*Inia geoffrensis*) as the species from which samples were frequently traded in two public markets, the 'Ver-o-Peso' of Belém (Pará State) and the Municipal Market of Manaus (Amazonas State, Brazil).

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Nowadays molecular markers are considered one of the best forensic tools to identify cetacean species and their illegal trade. With the progress of the molecular techniques, DNA can be recovered from almost any biological source, even products that have been dehydrated, cooked or canned (Baker & Palumbi, 1994).

In order to determine the taxonomic status as well as the geographic origin of these specimens, we improve a method of DNA extraction from samples of dolphin genitalia, which were either dehydrated or conserved in perfume tubes containing ethanol, and the cytochrome *b* from the mitochondrial DNA was analysed.

MATERIALS AND METHODS

Twenty-one samples provided by residents of the city of Belém and vicinities (Pará State, Brazil) (Figure 1), presumably purchased in the 'Ver-o-Peso' Market (Belém) or the Manaus Municipal Market were analysed. In addition, we compared our sequences to one sample collected from

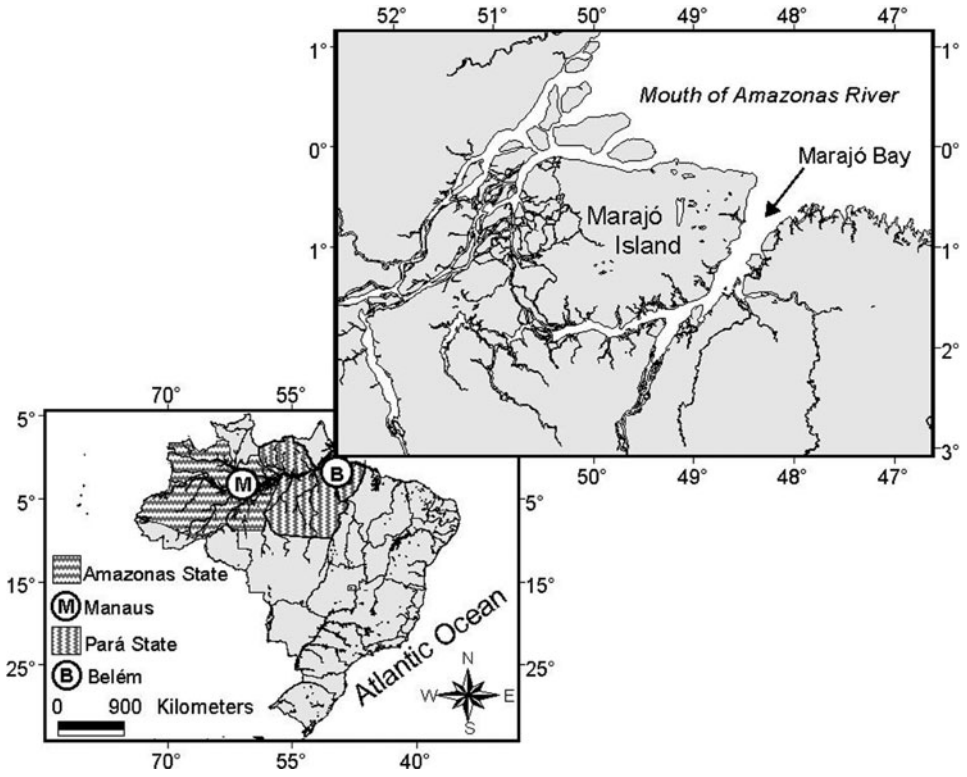

Fig. 1. Northern Brazilian region indicating the cities of Manaus and Belém. In detail the Marajó Bay area is indicated in the surroundings of Belém.

Sotalia fluviatilis captured in Rio Tarumã (GenBank Accession no. EF457551), an affluent of the Rio Negro (Amazonas State, Brazil) and one sample from an estuarine dolphin (*Sotalia guianensis*) incidentally captured off Amapá State (GenBank Accession no. EF457552). Tissue samples were removed from dehydrated genitalia or from genitalia conserved in alcohol and processed following a modified protocol of the standard phenol-chloroform extraction procedure (Sambrook *et al.*, 1989). Under continuous agitation, 30 mg of tissue were digested in 500 µl of lysis buffer (100 mM NaCl; 10 mM TRIS pH7,5), 25 µl of SDS 20%, 3 µl of Rnase (20 mg/ml) and 3 to 5 µl of Proteinase XIV (20 mg/ml) for 2 h, subsequently disrupted inside a 1.5 ml microcentrifuge tube with a plastic rod, and incubated at 37°C for at least 12 h. Following incubation, DNA extraction was carried out by the standard phenol-chloroform protocol (Sambrook *et al.*, 1989). DNA was diluted in 30 µl of sterile water.

The complete cytochrome *b* gene (1140 bp) was PCR-amplified with primers CB-out1 (5′AATGAYATGAA AARYCATCGTTG-3′) and CB-out2 (5′TCTTCCTTGAG TCTTAGGGAG-3′; Cassens *et al.*, 2000). Amplifications were carried out in 50 µl reactions containing 250 ng to 1.0 µg of DNA, dNTPs (0.5 mM/ml), primers (0.3 pmol/µl), Taq DNA polymerase (Invitrogen, 0.04 U/µl) and amplification buffer under the following conditions: 94°C (1 min), and 35 cycles at 94°C (1 min), 55 °C (1 min) and 72°C (90 s).

Four primers were used for sequencing: CB-out1 and CB-out2, which were used as external primers, and two internal primers: CB-in1 (5′-TTRTTRGATCCTGTTTCRT G-3′) and CB-in2 (5′-TGAGGACAAATATCATTYTGAG-3′; Cassens *et al.*, 2000). The PCR products were purified with 'GFX^{TM} PCR DNA and Gel Band Purification' kit (Amersham Pharmacia), and both strands were sequenced in ABI Prism 3730 automatic sequencer. Sequences were edited with Sequencing Navigator 3.3 (Applied Biosystems Inc., 1994).

The phylogenetic analyses were performed using the software Molecular Evolutionary Genetics Analysis (MEGA3; Kumar *et al.*, 2004) in order to construct a *p*-distance and a neighbour-joining (NJ) tree. Subsequently sequence data were analysed with PAUP* 4.0b (Swofford, 2003) and a phylogeny was constructed with a heuristic search with 100 random stepwise addition sequences and the Maximum Parsimony (MP) optimality criterion. Support for this phylogeny was estimated with 1000 bootstrap replicates.

The MP and NJ analyses also included sequence data from GenBank, one *S. fluviatilis* (AF304067), one *S. guianensis* (DQ086827), one *I. geoffrensis* (AF304068) and one *Tursiops truncatus* (AF084095) as outgroups.

RESULTS AND DISCUSSION

Analysis of the complete cytochrome *b* gene (1140 bp) of all 21 market-samples showed eight different haplotypes (Table 1). The MP and NJ analyses showed identical topologies, with *Sotalia* splitting into two strongly supported groups (Figure 2), one comprising the marine *S. guianensis* and the eight haplotypes from all market-samples and another, comprising the riverine *S. fluviatilis* haplotypes from GenBank.

Genetic *p* distance between haplotypes of the *S. guianensis* clade ranged from 0.0 to 0.004, showing a narrow range of variation as was the case of *S. fluviatilis* haplotypes (0.007), while distance estimates between *S. guianensis* and *S. fluviatilis* haplotypes ranged from 0.021 and 0.033 (Table 2), indicating that market samples belonged to

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Table 1. Cytochrome *b* haplotypes (Hap) of *Sotalia guianensis*.

Haplotypes	GenBank no.	Samples	Localities
Hap01	EF488216	Belem04, 05, 13, 14, 16, 19, 28, 29	'Ver-o-Peso' Market/ Belém
		CRB2848	Municipal Market/ Manaus
Hap02	EF488217	Belem06, 10, 12, 18, 20, 21	'Ver-o-Peso' Market/ Belém
Hap03	EF488218	Belem09	'Ver-o-Peso' Market/ Belém
Hap04	EF488219	Belem15	'Ver-o-Peso' Market/ Belém
Hap05	EF488220	Belem17	'Ver-o-Peso' Market/ Belém
Hap06	EF488221	Belem26	'Ver-o-Peso' Market/ Belém
Hap07	EF488222	Belem30	'Ver-o-Peso' Market/ Belém
Hap08	EF488223	Belem33	'Ver-o-Peso' Market/ Belém

marine specimens. These findings argued against the postulation of Best & da Silva (1989) who considered that samples of the 'Ver-o-Peso' Market (Belém) and the Municipal Market of Manaus corresponded to the Amazonian river dolphin (*Inia geoffrensis*), in agreement with information provided by local traders.

Our findings, that market samples originated from the estuarine dolphin (*S. guianensis*), indicated that this marine species is probably under higher pressure than the Amazonian river dolphin (*I. geoffrensis*) and the tucuxi (*S. fluviatilis*) and efforts should be done to monitor their populations. These marine dolphins have been incidentally captured in gillnets set off Marajó Bay and Amapá State coast, a region of intensive fishing activities. Thus, we suggest that such activities should also be monitored directly through regular onboard research or indirectly by the forensic analyses using molecular markers in the market products, in order to evaluate the real impact of these activities to the species conservation.

Nevertheless, long term social programmes and environmental education must also be implemented in the local community (fishery and non-fishery people) in order to intend to replace this commercial activity and change the local mentality in the near future. These complementary actions are the

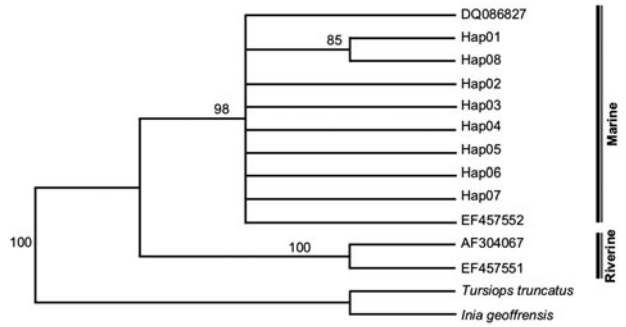

Fig. 2. Maximum Parsimony phylogenetic tree between eight market-sample haplotypes. Numbers are bootstrap values higher than 50%. See acronyms in Table 1.

Table 2. Genetic p distance × 100 between haplotypes of *Sotalia* (bold face), *Tursiops truncatus* and *Inia geoffrensis*.

	Taxon	GenBank	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*S. guianensis*	DQ086827													
2	*S. guianensis*	EF488216	**0.2**												
3	*S. guianensis*	EF488217	**0.1**	**0.3**											
4	*S. guianensis*	EF488218	**0.0**	**0.2**	**0.1**										
5	*S. guianensis*	EF488219	**0.1**	**0.3**	**0.2**	**0.1**									
6	*S. guianensis*	EF488220	**0.1**	**0.3**	**0.2**	**0.1**	**0.2**								
7	*S. guianensis*	EF488221	**0.2**	**0.4**	**0.1**	**0.2**	**0.3**	**0.1**							
8	*S. guianensis*	EF488222	**0.1**	**0.3**	**0.2**	**0.1**	**0.2**	**0.1**	**0.2**						
9	*S. guianensis*	EF488223	**0.3**	**0.1**	**0.4**	**0.3**	**0.4**	**0.4**	**0.4**	**0.4**					
10	*S. guianensis*	EF457552	**0.1**	**0.3**	**0.2**	**0.1**	**0.2**	**0.2**	**0.3**	**0.2**	**0.4**				
11	*S. fluviatilis*	AF304067	2.1	2.3	2.2	2.2	2.3	2.2	2.3	2.3	2.4	2.2			
12	*S. fluviatilis*	EF457551	0.3	3.2	3.1	3.0	3.1	3.1	3.2	3.1	3.3	3.1	0.7		
13	*T. truncatus*	AF084095	8.5	8.7	8.6	8.8	8.9	8.6	8.7	8.8	8.8	8.4	8.9	9.5	
14	*I. geoffrensis*	AF304068	16.9	17.1	17.0	17.4	17.4	17.0	17.1	17.7	17.3	16.8	17.0	17.7	18.6

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only way to avoid more dolphins being captured and killed. It is important to mention that cetaceans are protected against commercial whaling in Brazil since 1986 (*Portaria no N-11 de 21/fev./1986 e Lei no 7.643 de 18/dez./1987).*

The mtDNA analysis has been established since 1999 by the International Whaling Commission as the official method to control illegal trade of products from protected cetacean species. As previously pointed out by Baker et al. (2000) and Baker & Palumbi (1994), this report shows the utility of molecular techniques for identifying the origin of commercialized samples from cetacean species in South American markets.

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