

Amazon River dolphin love fetishes: From folklore to molecular forensics

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Traditional Amazonian folklore includes a host of supernatural beings believed to protect its forests and rivers. One of the most powerful and widely recognized of these beings is the Amazon River dolphin *Inia geoffrensis*, or boto. The boto is traditionally viewed as a mischievous and tempestuous being, both feared and respected. The most sensational folklore concerning botos is that they transform themselves at dusk into handsome Caucasian men, who are skilled at dancing and seducing young women. Botos are also believed to at times enter boats or households, paralyzing their occupants to engage in sexual intercourse. Before first light, the boto returns back into water, reverting into its dolphin form (Cravalho 1999). Indeed, unexpected teenage pregnancies in the Amazon region have been traditionally attributed to seduction by the boto (Cravalho 1999).

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It is the sexual nature of this folklore that leads to the perceived value of boto body parts as love charms. The most important of these are the eyes and genitalia (da Silva 1990) valued particularly by Amazonian city dwellers (Smith 1996). For example, holding the eye of the boto while conversing with a member of the opposite sex is thought to make one highly sexually attractive because no one can resist the boto's gaze (Salter 1994). Boto genitalia are considered even more powerful (Best and da Silva 1989). Grinding the dry penis of the boto, mixing it with talcum powder or perfume, and applying it to the genital area is believed to increase the pleasure that a woman can give a man. Similarly, the application of a ground dolphin's vagina to a man's penis is believed to increase his potency (Cravalho 1999). Other uses, such as those observed in Peru and Bolivia, are medicinal where dolphin oil is sold to cure chest ailments.

Demand for boto-derived fetishes is high and supposed boto body parts are sold in virtually all Amazonian city markets and are used mostly by city dwellers (Smith 1996). The indigenous and Caboclo (riverine peoples of mixed ancestry) societies appear not to use boto body parts, and largely appear to respect cultural taboos against killing these magical animals (Best and da Silva 1989). The ritualistic use of dolphin body parts has also been reported in the coastal regions of northern and northeastern Brazil (Siciliano 1994, Reeves *et al.* 2003), where genital organs and eyes of both the estuarine dolphin (*Sotalia guianensis*) and the freshwater tucuxi (*Sotalia fluviatilis*) are reportedly sold as amulets. The northern and northeastern coastal region of Brazil has a strong West African cultural heritage, including Afro-Brazilian religions such as Candomblé and Macumba. Unlike the case of folklore and traditional practices of the Amazon interior, these coastal populations and religions have no taboos against killing dolphins or using their body parts in rituals.

To determine the origin of the boto fetishes sold in major Amazonian markets, we sampled salted dried boto eyeballs at the Mercado Ver-o-Peso in Belém, Pará ($n = 22$), Mercado Central in Manaus, Amazonas ($n = 11$), and Mercado Municipal in Porto Velho, Rondônia ($n = 10$). Samples were transported to the laboratory where they were cleaned, an incision was made into them, and a sample of tissue previously unexposed to the environment was collected. We also analyzed samples obtained from reference individuals of *Inia g. geoffrensis*, *Sotalia fluviatilis*, and *Sotalia guianensis*; identification of reference individuals was based on the possession of diagnostic morphological characters. Forensic studies, such as the present study, have been, among other applications, very successful at tracking the origin of cetacean by-products and demonstrating the mislabeling of illegally harvested cetacean species as legal cetacean products (*e.g.*, Baker and Palumbi 1994, Baker *et al.* 1996, Palumbi and Cipriano 1998).

Total genomic DNA was extracted from tissue samples using a DNA extraction kit from GE Healthcare (São Paulo). Two blank extractions were carried out to check for contamination; no DNA contamination was observed. For determination of sex, we used the four primer polymerase chain reaction (PCR) protocol of Rosel (2003). Male and female positive control samples were used in molecular sexing. To determine the specific origin of the salted eyeballs, the mitochondrial control region was amplified *via* the PCR using the primers L15812 and H153 from Chivers *et al.* (2005) whereas

the cytochrome *b* region was amplified using primers L14725 (Pääbo 1990) and H15915 (Irwin *et al.* 1991).

The 25- μ L PCR reaction volumes were 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.2 mM each dNTP, 0.2 μ M forward primer, 0.2 μ M reverse primer, 0.05 units/ μ L LGC Biotecnologia Taq DNA Polymerase, and contained about 5–10 ng of genomic DNA. PCR conditions were as follows: denaturation at 93°C for 35 s, primer annealing at 50°C (cytochrome *b*) or 55°C (control region and SRY/SRX) for 35 s, and primer extension at 72°C for 90 s; these three steps were repeated 35 times.

Sex was inferred according to the method of Rosel (2003) with the modification that 10 μ L of the PCR product was electrophoresed on a 1.2% agarose gel run in 1 \times TBE buffer for approximately 60 min at 75 V, and 100 kb DNA ladder (Fermentas) was used as the size standard. Positive control individuals showed sex-specific banding. Of the 34 cetacean eyeball samples in our study, 10 eyeballs originated from males, and 20 originated from females; the sex of the remaining four cetacean eyeballs could not be determined unambiguously.

Control region and cytochrome *b* PCR products were purified using the GFX PCR DNA Kit (GE Healthcare) following the manufacturer's suggested protocol. The subsequent cycle sequencing reaction was performed in 10 μ L reaction volume that were 40 mM Tris-HCl pH 9.0, 1 mM MgCl₂, 0.4 μ M sequencing primer, and contained 4 μ L of amplified DNA product (~30 ng), and 1 μ L of DYEnamic ET Dye Terminator mix (GE Healthcare). Cycle sequencing PCR conditions were as follows: denaturation at 95°C for 15 s, primer annealing at 50°C for 35 s, and primer extension at 60°C for 120 s; these three steps were repeated 35 times. Resulting fluorescently labeled product was precipitated using a mixture of 70% ethanol and 175 mM ammonium acetate. Precipitated DNA product was resuspended in Hi-Di Formamide (Sigma), and resolved on a MegaBACE 1000 automatic DNA analysis system (GE Healthcare) using the manufacturer's recommended settings. Quality of sequences was checked using the Phred algorithm (Ewing and Green 1998, Ewing *et al.* 1998), and only those sequence portions with Phred Q values over 20 were used in further analyses. Of the 43 individual eyeballs analyzed, 37 could be amplified and sequenced with control region primers, and 29 could be amplified with cytochrome *b* primers. As expected, the control region and cytochrome *b* amplicons were approximately 500 bp and 750 bp, respectively. Four samples from Porto Velho failed to amplify most likely due to extensive degradation of DNA (neither our set of primers nor "universal" 16S primers resulted in PCR amplification of the targeted fragment size of 500–750 bp).

Determining species origin of the samples collected in the markets was accomplished by two methods. We used the basic local alignment search tool (BLAST) algorithm implemented in GenBank to compare our sequences to those of other species deposited in GenBank. BLAST analyses indicated that all eyeball samples from the Belém and Manaus markets most likely pertained to *Sotalia* spp. (100% similarity, E value = 0.0 for all 33 individuals; top 37 matches in Genbank were either *Sotalia guianensis* or *Sotalia fluviatilis* with 97%–100% sequence similarity to our query sequence), whereas only one sample from Porto Velho was identified as

Sotalia spp. (100% similarity, E value = 0.0), four were identified as pig (*Sus scrofa*) (99% similarity, E value = 0.0 for all four sequences), and one as a sheep (*Ovis aries*) (99% similarity, E value = 0.0). In no instance was one of our sequences more similar to the Amazon River dolphin (*Inia geoffrensis*) than to another cetacean or noncetacean species.

Those sequences that were determined to be cetacean-like, but could not be assigned to either of the species of the genus *Sotalia*, were subjected to phylogenetic and population aggregation analyses. For phylogenetic analyses we obtained control region sequence data deposited in GenBank for *Sotalia fluviatilis* (AY842465–AY842469 and EF027080–EF027092), *Sotalia guianensis* (AY842455–AY842464, AY842470, and EF027063–EF027079), *Lagenorhynchus obscurus* (AY821620), *Stenella coeruleoalba* (AY046543), *Steno bredanensis* (AY842471), *Tursiops aduncus* (AF287954), and *Delphinus delphis* (AY168602), and our positive control samples of *Sotalia guianensis* and *Sotalia fluviatilis* sequenced in our laboratory. We also included the control region sequences of *Inia geoffrensis* deposited in the GenBank (AF521113–AF521126), and positive control samples sequenced in our laboratory. Sequence data generated in this study as well as those obtained from GenBank were aligned using the algorithm Clustal W (Thompson *et al.* 1996) implemented in the program BioEdit (Hall 1999), and confirmed through visual inspection of the alignment. Clustal W alignment was done using the default gap opening and extension penalty parameters.

Phylogenetic relationships of the control region sequences were estimated using maximum parsimony implemented in PAUP* 4b10 (Swofford 2002) by heuristic tree search, with 25 random additions and TBR branch swapping. Robustness was assessed using 2,000 nonparametric bootstrap resamples. We also inferred topologies using the maximum likelihood algorithms implemented in PAUP* 4b10 (Swofford 2002) and Bayesian inference algorithm implemented in MRBAYES 3.01 (Huelsenbeck and Ronquist 2001) under the GTR model (Rodríguez *et al.* 1990) of molecular evolution with a portion of sites treated as invariable. The GTR + I model was suggested as the most appropriate by the software MODELTEST 3.7 (Posada and Crandall 1998). Maximum likelihood topology was estimated by a heuristic search, with 25 random additions and TBR branch swapping. Parameter values were estimated from the data. Robustness of the maximum likelihood phylogenetic hypothesis was assessed by 1,000 bootstrap replicates with one random addition and TBR branch swapping. For Bayesian inference of phylogenetic relationships, we ran 5,000,000 generations, sampling trees and branch length every 1,000 generations. Log likelihoods stabilized within the first 5% of the run, and we discarded these initial 250,000 trees in the computation of a 50% majority rule consensus tree. Sequences of *Inia geoffrensis*, which belongs to a different family than *Sotalia*, were too highly divergent, and resulted in an incorrect rooting of the *Sotalia* haplotypes; *Inia* was therefore removed from final phylogenetic analyses. All haplotypes obtained from the eyeballs form a statistically well-supported clade together with haplotypes from the marine *Sotalia guianensis* (Fig. 1). The monophyly of *Sotalia fluviatilis* is also well supported, as is the sister taxon relationship of *Sotalia guianensis* and *Sotalia fluviatilis* (Fig. 1).

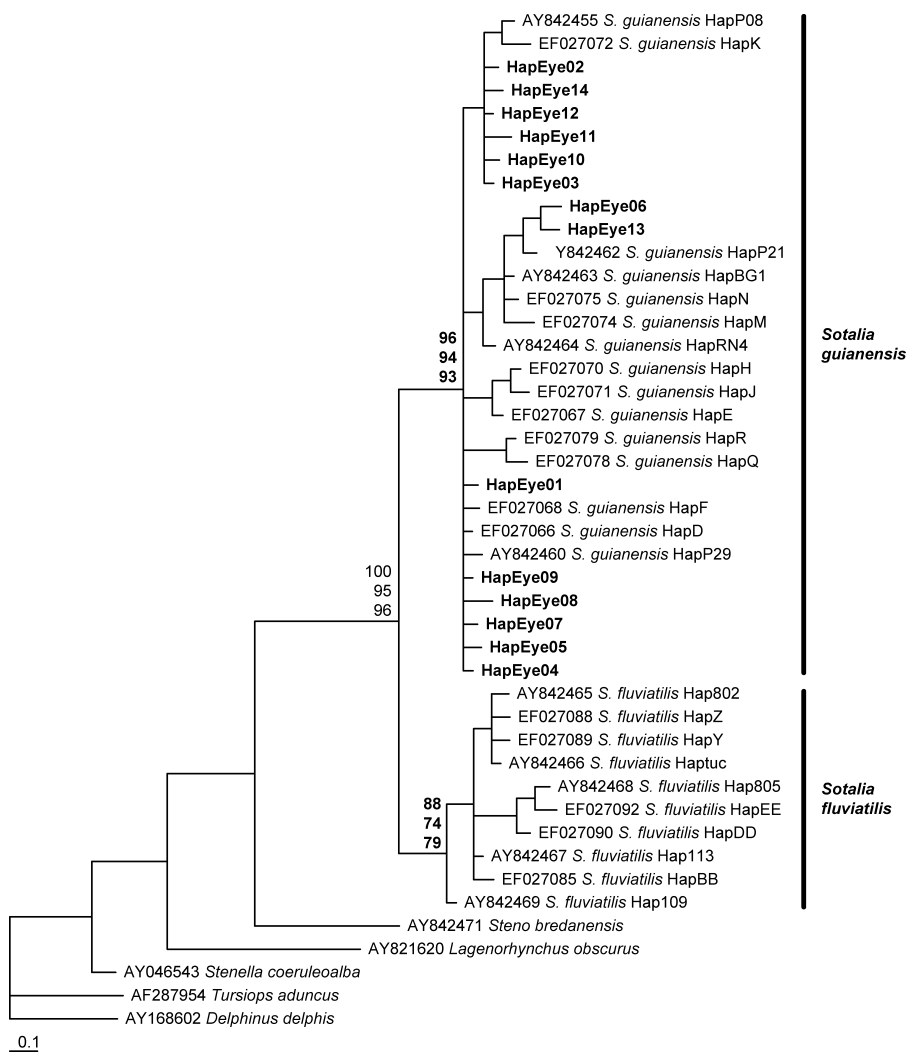


Figure 1. Maximum likelihood phylogenetic relationships of control region haplotypes derived from market bought eyeball samples and sequences obtained from known individuals. Phylogenetic relationships were identical in Bayesian inference, maximum likelihood and maximum parsimony estimates ($-\ln L = 1399.28612$; parsimony length = 141). Support values listed above nodes are Bayesian probabilities based on 5,000,000 resamples, maximum likelihood bootstrap support values based on 1,000 bootstrap replicates, and maximum parsimony bootstrap support values based on 2,000 bootstrap replicates. Support values are shown only for clades of *Sotalia guianensis* and *Sotalia fluviatilis* haplotypes, and for the monophyly of *Sotalia*. Branch lengths are proportional to maximum likelihood sequence divergence; scale indicates percentage of sequence divergence. Haplotypes 1–14, highlighted in bold, were found in our study and correspond to GenBank accession #EU022531–EU022544.

To further confirm the relationships of the unknown sequences, we performed a nonphylogenetic cladistic analysis. We constructed a matrix of autapomorphies for *Sotalia fluviatilis*, *Sotalia guianensis*, *Inia g. geoffrensis*, *Inia g. humboltiana*, and *Inia g. boliviensis* based on available sequence data in Genbank and our reference individuals, and carried out a population aggregation analysis (Davis and Nixon 1992). All eyeball-derived sequences determined by BLAST to be of cetacean origin shared species-specific autapomorphic character states indicative of molecular positional homologies with *Sotalia guianensis* and no other species, unambiguously assigning all eyeballs of cetacean origin to *Sotalia guianensis*. The control region and Cytochrome *b* data sets contain two and six diagnostic sites, respectively, that assign all cetacean eyeball samples to *Sotalia guianensis* and unambiguously distinguish it from *Sotalia fluviatilis* and *Inia* spp. (Table 1; online Appendix S1, S2).

In the 34 control region sequences determined in this study we observed a total of 14 haplotypes (GenBank #EU022531–EU022544). Seven of these haplotypes corresponded to those previously reported by Cunha *et al.* (2005) and/or Caballero *et al.* (2007) whereas the other seven were novel; no haplotypes corresponded to haplotypes of *Sotalia fluviatilis* (online Appendix S3). Haplotype 3 was also found in our positive control of *Sotalia guianensis*, and corresponded to *Sotalia guianensis* Hap11 (GenBank #AY842456) of Cunha *et al.* (2005), and HapB (GenBank #EF027064) and HapC (GenBank #EF027065) of Caballero *et al.* (2007). In the cytochrome *b* sequences we observed five *Sotalia* haplotypes (GenBank #EU022545–EU022549) that corresponded to *Sotalia guianensis* (online Appendix S4). The most common haplotype is identical to the complete cytochrome *b* haplotype of *Sotalia guianensis* reported by Cunha *et al.* (2005) whereas three other haplotypes corresponded to haplotypes reported by Caballero *et al.* (2007); one haplotype was novel.

It is clear that the “boto” amulets sold in markets of main Amazonian cities are not derived from the true boto (*Inia geoffrensis*). All amulets, if they are of dolphin origin at all, are unambiguously derived from the marine species *Sotalia guianensis*. This implies that the “boto” fetishes most likely originate in the coastal areas of North Brazil, and are then exported to the central Amazon cities for sale. In distant inland regions such as the city of Porto Velho, which is located some 4,000 km inland from Belém, a surprising 90% of the samples were either pig or sheep eyes. The fetishes in Porto Velho were also the most expensive (~US\$7.50/piece), approximately three times the price in Belém (~US\$2.50/piece) and more than twice the sale price in Manaus (~US\$4.00/piece). The high price of fetishes, and use of domestic animal eyeballs do not reflect regional scarcity of the boto, *Inia geoffrensis*, or the tucuxi (*Sotalia fluviatilis*), both of which are abundant near Porto Velho.

Since Amazonia was largely depopulated as a result of the introduction of Old World diseases and Portuguese slave raids (Hemming 2004), large numbers of the impoverished peoples from the north and northeastern regions of Brazil were resettled in the Amazon during the rubber boom (*e.g.*, Weinstein 1983, Anderson 1999, Dean 2002). It was apparently these migrants, and not the indigenous peoples of the Amazon, who brought with them and now maintain the cultural attitudes and practices that led to the use of boto fetishes. The indigenous populations do have a strong tradition of love magic, known widely as “pussanga” that includes botanical

Table 1. A matrix of control region and cytochrome *b* diagnostic molecular sites.

Variable position	Control region														Haplotype occurrence	BEL	MAO	PVH		
	39	136	139	160	175	231	256	269	270	362	381	387	404	463					504	559
<i>Inia</i> spp.	A	T	A	G	C	G	G/A	A	A	T	C	C/T	C	C/T	C	C/T	A	-	-	-
<i>Sotalia fluviatilis</i>	T	T	A	G	C/T	G	C/T	T	C	C/T	C	T	C	T	C	T	A	-	-	-
<i>Sotalia guianensis</i>	C	G/T	A/C	A	C/T	G/A	C/T	C/T	C/T	C/G	C/T	C/T	C/T	C/T	C/A	C/A	22	11	1	1
CR_Hap01	C	T	A	A	T	A	C	C	C	C	C	T	C	T	A	A	1	-	-	-
CR_Hap02	C	T	A	A	T	A	C	T	C	C	C	C	T	C	T	A	2	-	-	-
CR_Hap03	C	T	A	A	T	A	C	C	C	C	C	C	T	C	T	A	6	2	-	-
CR_Hap04	C	T	A	A	T	A	C	T	C	C	C	T	C	C	T	A	3	5	1	1
CR_Hap05	C	T	A	A	T	A	C	T	C	T	C	T	C	C	T	A	1	2	-	-
CR_Hap06	C	T	A	A	C	A	T	T	C	C	C	T	T	C	T	A	1	-	-	-
CR_Hap07	C	T	A	A	T	A	C	T	C	C	C	C	C	C	T	A	3	-	-	-
CR_Hap08	C	G	C	A	T	G	C	T	C	C	C	T	C	C	T	A	1	-	-	-
CR_Hap09	C	T	A	A	T	G	C	T	C	C	C	T	C	C	T	A	1	-	-	-
CR_Hap10	C	T	A	A	T	A	C	C	C	C	C	T	C	C	T	A	1	-	-	-
CR_Hap11	C	T	A	A	T	A	C	C	C	C	C	T	T	T	A	A	-	1	-	-
CR_Hap12	C	T	A	A	T	A	C	C	C	C	C	C	T	C	C	A	-	1	-	-
CR_Hap13	C	T	A	A	C	A	C	T	T	C	C	T	T	C	C	A	1	-	-	-
CR_Hap14	C	T	A	A	T	A	C	C	C	G	C	C	T	C	T	A	1	-	-	-
Cytochrome <i>b</i>																				
Variable position	49	78	124	297	367	396	462	501	528	531	561									
<i>Inia</i> spp.	G	T	A	C	G	C	T	T	A	C	T									
<i>Sotalia fluviatilis</i>	G	C	A	G	G	C	T	A	A	C	T									
<i>Sotalia guianensis</i>	G/A	C/T	G/A	A	G/A	C/T	C	G	C	T	C	16								
CB_Hap01	G	C	A	A	G	C	C	G	C	T	C	8								
CB_Hap02	A	C	A	A	G	C	C	G	C	T	C	1								
CB_Hap03	G	T	A	A	G	C	C	G	C	T	C	1								
CB_Hap04	G	C	G	A	G	T	C	G	C	T	C	3								
CB_Hap05	G	C	G	A	A	T	C	G	C	T	C	3								

Sites highlighted in bold unambiguously assign the unknown eyeball samples to *Sotalia guianensis*. Those not highlighted differentiate observed haplotypes. Control region haplotypes 1–14 correspond to GenBank accession numbers EU022531–EU022544, while cytochrome *b* haplotypes 1–5 correspond to GenBank accession numbers EU022545–EU022549. BEL = Belém, MAO = Manaus, PVH = Porto Velho.

and animal-based amulets and preparations, but it does not include the boto. As these immigrant populations, with their own largely African-derived traditions and beliefs surrounded with fetishes, merged with remnant indigenous populations, perhaps the use of love charms derived from the boto legend emerged. Despite these cultural changes, the people of the Amazon interior appear reluctant to supply boto body parts for the fetish trade, which has led to a long-distance trade of estuarine dolphin body parts or to outright falsification through substitution of domestic animal body parts.

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SUPPORTING INFORMATION

The following supporting information is available for this article online:

Appendix S1. A matrix of control region molecular autapomorphic characters for all species of *Sotalia* and *Inia*, and species-specific autapomorphies (highlighted in yellow) for *S. guianensis* and also observed in the analyzed eyeball samples.

Appendix S2. A matrix of cytochrome *b* molecular autapomorphic characters for all species of *Sotalia* and *Inia*, and species-specific autapomorphies (highlighted in yellow) for *S. guianensis* and also observed in the analyzed eyeball samples.

Appendix S3. Control region haplotypes found in each locality, and their correspondence to those reported in Cunha *et al.* (2005) and Caballero *et al.* (2007).

Appendix S4. Cytochrome *b* region haplotypes found in each locality, and their correspondence to those reported in Cunha *et al.* (2005).