How far does it go along the coast? Distribution and first genetic analyses of the boto (*Inia geoffrensis*) along the coast of Pará, Amazon, Brazil

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Introduction

The boto or Amazon river dolphin (*Inia geoffrensis*) has been subject to intensive ecological studies during the last decade. Several authors consider the boto the best studied and most well known of the river dolphins. Despite such increase in knowledge in many parts of its range throughout the Amazon and Orinoco drainages, there is no information available on its distribution and ecology in the Amazon delta, including the south Amazon river channel, Marajó bay and Belém area, Pará state, Northern Brazil. In fact, the distribution of *I. geoffrensis* in the Marajó bay and near Belém has remained obscure for long time. Since November 2005 intensive surveys along the coast of Pará were conducted in search of stranded aquatic mammals. Two areas were selected for regular surveys: east coast of Marajó Island and Maiandeua Island. Additional surveys included several other locations, such as the northeast coast of Marajó Island and Curuçá, in the east coast of Pará state, which lacked any aquatic mammal survey and collection. Since then, several new records of *I. geoffrensis* have been confirmed. Concomitantly, there was an increase of biological material collected, resulting from the detection and recovery of carcasses during beach surveys. This material has been deposited in the mammal collection of Museu Paraense Emílio Goeldi. In order to analyze the existence of genetic variation for Amazon River dolphin, analysis of control region and cytochrome b were made to infer about subspecies of *Inia geoffrensis* for the east coast of Marajó Island and Curuçá Bay. Results of genetic analyses suggests that one specimen from the east coast of Pará state represent a different and isolated geographic form of boto, highly distant in terms of genes from the remaining subspecies proposed. The results of this study points to a common and regular presence of *I. geoffrensis* in the Marajó bay and the surrounding coastline of Marajó Island, to the east coast of Pará state.

Material and Methods

Study area and field work

Monthly beach surveys were regularly conducted since November 2005 in two separate areas along the coast of Pará (Figure 1). These areas are: (a) the east coast of Marajó Island and (b) around Maiandeua Island, Maracanã County, Pará state. The east coast of Marajó Island is formed by Marajó bay, which is ecologically a part of Amazon Basin (Goulding *et al.*, 2003), where important rivers flow as Tocantins, Mojú, Acará and Guamá. These rivers form the whole complex of Guajará Bay, and part of the discharge of the Amazon River (Isaac and Barthem, 1995; França and Souza Filho, 2003). Maiandeua Island is surrounded by Marapanim Bay to the west and Maracanã Bay to the east. Marapanim bay is formed by the estuary of Marapanim River, located in the northeastern of Pará state. The surveys were conducted along 68 km of beaches, including 58 km along the east coast of Marajó and 10 km around Maiandeua

Island. Additional monitoring surveys were conducted in the locality of Curuçá, since May 2012. The Curuçá estuary (00°10'S, 47°50'W) is located at the eastern tip of the mouth of the southern channel of the Amazon delta (Pará River). It comprises an extensive mangrove forest, a complex network of branching intertidal creeks with strong marine influence with little freshwater input, dominated by semidiurnal tides with a range of 3-4 m at neap tides and 4-5 m at spring tides (Giarrizzo *et al.*, 2011).

Local fishermen were interviewed in all visited villages for information on cetacean entanglement in gillnets and carcasses washed upon the beaches. Incidental sightings of *I. geoffrensis* were recorded during ship or ferryboat travel trips to and from Belém, as well as during navigation in Pará River delta and tributaries.

Sample collection and laboratory techniques

Three skin samples (MPEG 38764, 42055, 42122) were collected from stranded animals in Salvaterra, (00°52'59,1"S, 48°30'39,7"W), Soure, east coast of Marajó (00°40'01,9"S, 48°28'54,9"W) and Romana island (00°34'26"S, 47°51'29"W), Curuçá Bay, respectively (Figure 1, Table 1). DNA extraction was performed using an extraction kit Qiagen DNA Investigator [QIAGEN®]). Two different fragments were amplified by the polymerase chain reaction (PCR; Saiki *et al.* (1985): (i) the mitochondrial tRNAthr-control region was amplified using primers THR and TDKD (Kocher *et al.*, 1989) and (ii) the cytochrome c oxidase I gene (COI) a specific 658 bp portion of COI (Hebert *et al.*, 2003; Mueller, 2006; Rubinoff, 2006).

Primers THR and TDKD (Kocher *et al.*, 1989) were used to amplify an approximately 400 pb fragment of the mtDNA control region in 25 µL volumes containing: 1 µL of template DNA (100 ng/*µL*), with 1U/µl *Taq* DNA polymerase, 10X buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µl of each primer (20 µM each) and ultrapure water up to the final volume. The amplification profile was 93 °C for 1 min followed by 30 cycles of 93 °C for 30 sec, 60 °C for 1 min, 72 °C for 1 min, and a final extension in 72 °C for 5 min. PCR results were verified through electrophoresis of the amplicons on 1% agarose gels stained with ethidium bromide and visualized under UV transillumination. As well, the PCR products (10 µl) were purified by adding 0.5 µl (1U/ µl) Shrimp Alkaline Phosphatase (Promega AG, Wallisellen, Switzerland) and 0.5 µl (20U/ µl) Exonuclease I (New England Biolabs, Allschwil, Switzerland). This mix was incubated at 37 °C for 45 min, followed by a 10 min inactivation step at 80 °C.

We amplified of about 650 bp segment of the cytochrome c oxidase I (COI) gene using a cocktail of three forward and reverse primers described by (Ivanova *et al.*, 2006). The PCR reactions were run in a 25 µl volume using 1-2 µl of template DNA (100 ng/*µL)* with 0.2U/µl *Taq* DNA polymerase, 10X buffer, 1,5 mM $MgCl_2$, 0.4 mM of each dNTP, 1 µl of each primer (20 µM each) and ultrapure water up to the final volume. The PCR conditions for amplification were: 94 °C for 1 min, five cycles of 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 40 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplicons for both regions were submitted to direct sequencing at Macrogen (Macrogen Inc., Seoul, Korea), and each sample was sequenced in both directions.
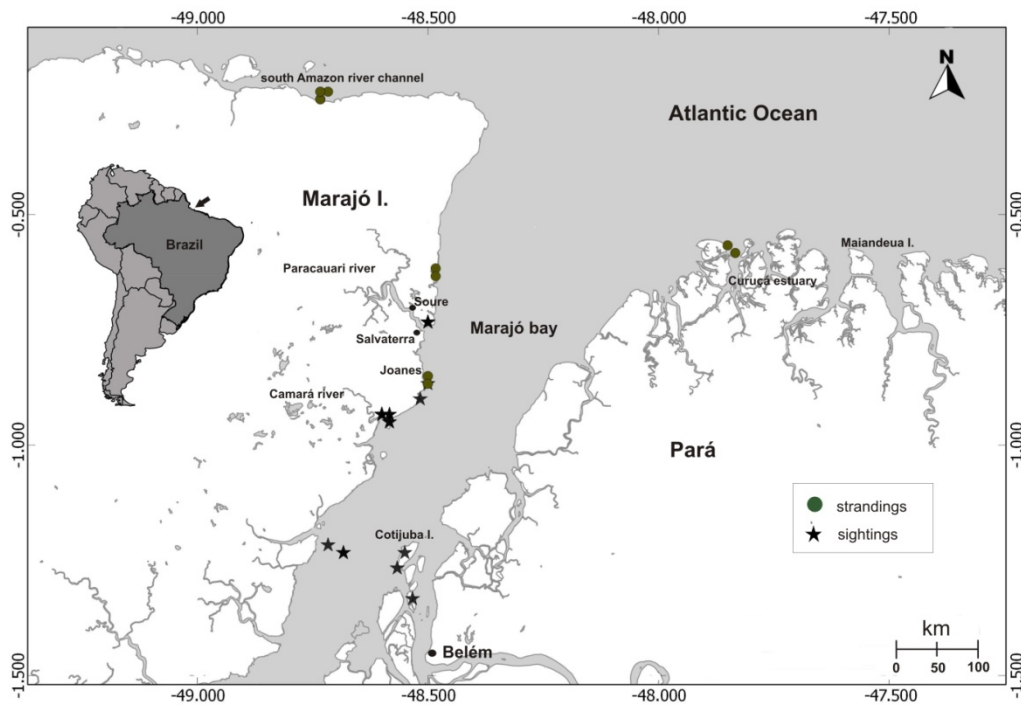
Figure 1. *Inia geoffrensis* strandings and sightings localities in Pará state coast, Northern Brazil.

Sequence analysis

Chromatogram quality was evaluated using Chromas Pro 1.5 (http://www.technelysium.com.au). Sequence similarity was obtained by comparison of similarity values to the sequences deposited at the GenBank using BLASTn (NCBI, available online). Sequence alignment was held with ClustalW implemented into Mega 5 (Tamura *et al.*, 2011) with later edition in *BioEdit* 5.0.9 (Hall, 1999). Consensus sequence was generated based on the alignment of two independent sequences. We also compare the obtained sequences with two available sequences of COI (AJ554059.1 and EU496359.1) and 20 of control region for *Inia geoffrensis* from GenBank.

Exploratory phylogeographic analyses were initially performed for each segment (control region and COI *b*) separately, to assess congruence between them. Haplotype networks were generated using the median-joining approach (Bandelt *et al.*, 1999) implemented in networkvv4.1.0.8 (www.fluxus-engineering.com).

The genetic variability was also analysed for each segment separately (control region and cytochrome *b*) and the molecular population diversity was estimated using indices such as haplotype (h) and nucleotide (π) diversity, calculated with Arlequin 3.1 (Schneider *et al.*, 2000). We also compare the genetic distances for control region sequences among our sample with the 20 samples from GenBank that represented Amazon system (Brazil, Peru and Ecuador), Orinoco (Venezuela and Colombia) and Bolivia by Kimura 2-parameters.

Results

Strandings

Strandings were confirmed in three distinct areas along the Pará state coast: (a) Soure and Salvaterra, east coast of Marajó Island [herein referred as 'ECM']; (b), south Amazon river channel [herein referred as SAC] and (c) Curuçá estuary [herein referred as CE] (Figures 1 and 2, Table 1). From 2007 to 2012, 14 specimens of *I. geoffrensis* were found on beaches and were recovered: 14% in CE, 43% in ECM and 43% in SAC (Figure 2). Skeletons and soft tissues are deposited in the mammal collection of Museu Paraense Emílio Goeldi (MPEG), in Belém, Pará. It is important to emphasize that the Amazon region has some unique features when dealing with the recovery of carcasses of marine mammals. Strong sunlight, quick action of scavenging birds and the difficulty of access to local beaches, making it difficult to collect biological data. As a result, most of the carcasses found during the surveys were in moderate to advanced decomposition. For these reasons, only one specimen (MPEG 42122) could be measured. It reached 2,52m in total length.

Table 1. Confirmed strandings of *I. geoffrensis* during monitoring surveys along the coast of Pará since November 2005. Categories are ECM (east coast of Marajó Island), CE (Curuçá estuary) and SAC (south Amazon River channel).

| Locality	Location	Date	Voucher Spec
Caju-una beach, Marajó Island	ECM	31 May 2007	MPEG 38748
Joanes beach, Marajó Island	ECM	28 Aug 2007	MPEG 38759
Port beach, Joanes, Marajó Island	ECM	28 Aug 2007	MPEG 38764
Céu beach, Marajó Island	ECM	17 Dec 2007	MPEG 39461
Água Boa beach, Marajó Island	ECM	01 Jan 2011	MPEG 42044
Pesqueiro beach, Marajó Island	ECM	21 Sep 2011	MPEG 42055
Taperebá, Romana Island	CE	25 May 2012	MPEG 42122
Areuá Island	CE	20 Jun 2012	MPEG 42139
Jaranduba farm beach, Marajó Island	SAC	09 Dec 2012	GEMAM 398
Jaranduba farm beach, Marajó Island	SAC	10 Dec 2012	GEMAM 402
Jaranduba farm beach, Marajó Island	SAC	10 Dec 2012	GEMAM 406
Jaranduba farm beach, Marajó Island	SAC	10 Dec 2012	GEMAM 407
Jaranduba farm beach, Marajó Island	SAC	10 Dec 2012	GEMAM 408
Jaranduba farm beach, Marajó Island	SAC	10 Dec 2012	GEMAM 409
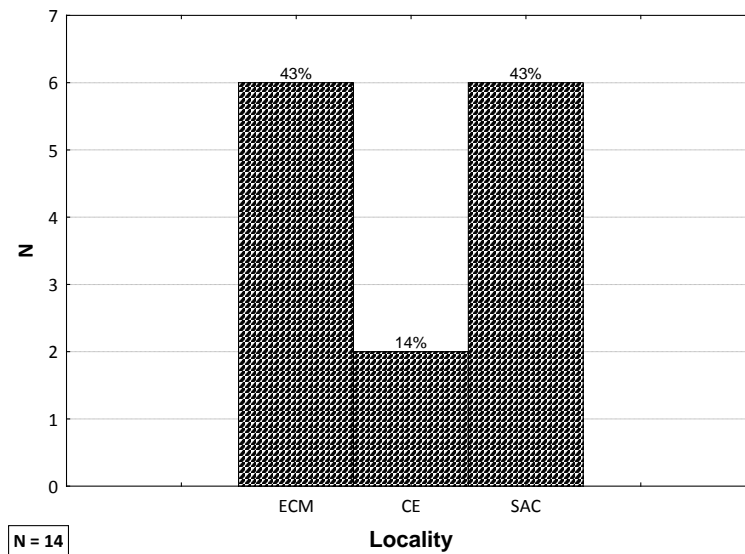
Figure 2. Amazon River dolphin stranding distribution in three distinct areas along the Pará state coast: ECM (Soure and Salvaterra, east coast of Marajó Island); CE (Curuçá estuary) and SAC (south Amazon River channel).

Sightings

Botos were sighted opportunistically in 25 occasions during boat travels from Belém to Camará port, in the east side of Marajó Island, from November 2005 to March 2013 (Table 2). A significant number of sightings (48%) was made in the port itself, in the Camará river mouth, while the boat or ferry was docking. Group size ranged from one to six individuals (mean ± SD: 1.5 ± 1.8) (Figure 3). General behaviour included moving (milling), fishing, socializing, and some aerial displays such as backslap and partial leaping. Observations were conducted both in the morning and in the afternoon, indicating possible unspecific pattern of activity.

Table 2. *I. geoffrensis* sightings between 2005 and 2013 in Marajó bay, Pará, North Brazil.

Locality	Category	Date	Number of individuals	Group size
Cotijuba island	CI	3 Nov 2005	1	1
Camará river	CR	3 Nov 2005	1	-
Joanes beach	MB	9 Sep 2006	3	2 adults, 1 young
Cotijuba island	CI	23 Sep 2006	1	1
Camará river	CR	23 Sep 2006	5	adult
Camará river	CR	24 Nov 2006	2	adult
Camará river	CR	28 Nov 2006	1	adult
Camará river	CR	9 Feb 2007	2	-
Camará river	CR	9 Feb 2007	1	-
Camará river	CR	9 Feb 2007	2	-
Camará river	CR	9 Feb 2007	3	-
Paracauari river	PR	11 Feb 2007	4	-

| Paracauari river	PR	11 Feb 2007	6	2 groups, 5 specimens
Cotijuba island	CI	30 Mar 2007	1	adult
Paracauari river	PR	01 Apr 2007	6	5 adults, 1 calf
Paracauari river	PR	02 Apr 2007	1	adult
Marajó bay	MB	08 Oct 2007	4	adults, young
Marajó bay	MB	09 Oct 2007	6	3 groups, 6 specimens
Marajó bay	MB	10 Oct 2007	1	adult
Joanes beach	MB	31 May 2011	2	adult
Camará river	CR	21 Aug 2011	1	adult
Camará river	CR	11 Jun 2012	1	-
Camará river	CR	04 Sep 2012	3	adults
Camará river	CR	01 Mar 2013	3	2 adults, 1 young
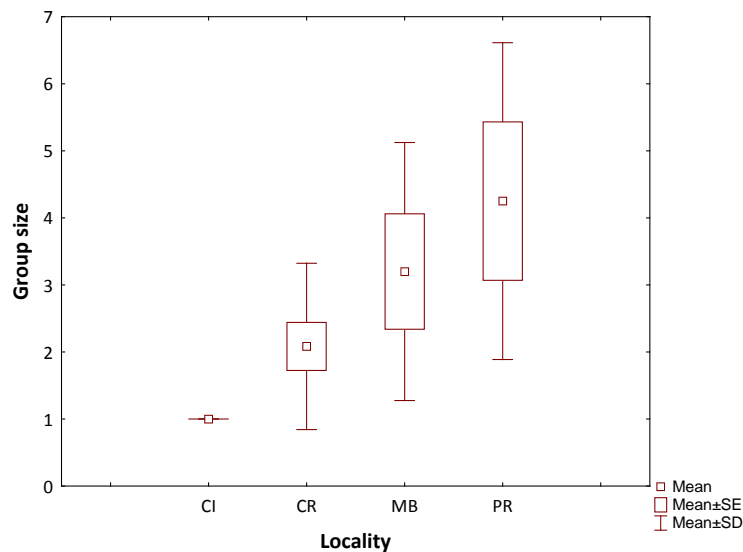
Figure 3. Distribution of group size of Amazon river dolphin, *Inia geoffrensis*, in Cotijuba Island (CI), Camará River (CR), Marajó Bay (MB) and Paracauari River (PR). All localities are in the east coast of Marajó Island, Pará state, Northern Brazil.

Genetics

A total of five sequences of mtDNA COI were analysed, which comprised 646 bp with just three different haplotypes (two for Amazonas river from GenBank and one from our samples from Pará region), but all of them highly distant from each other (Figure 4). High levels of genetic diversity were observed among individuals. The genetic diversity (h) and the nucleotide diversity (π) calculated for the species were $0,700 \pm 0,218$ and $0,02570 \pm 0,00778$, respectively.

Same scenario was observed for the mtDNA control region, a fragment of 400 bp with 20 different haplotypes. The genetic diversity (h) and the nucleotide diversity (π) calculated for the species were 0,995 $\pm 0,016$ and $0,04407 \pm 0,00369$, respectively. Once more, the only sample amplified from Pará for the

control region was highly distant from the remaining haplotypes (Figure 5), even more distant then the subspecies proposal for the species (Table 3).
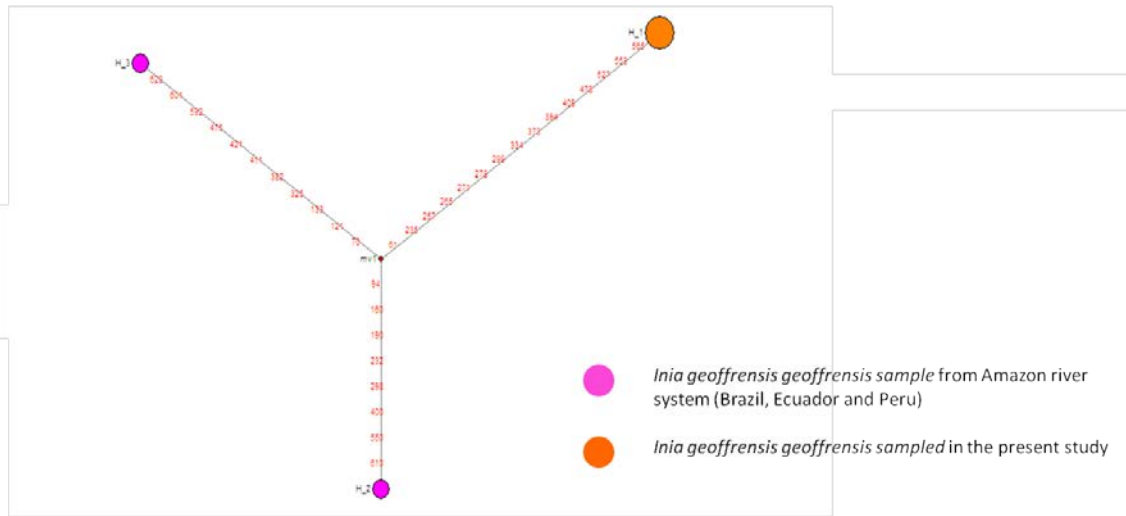

Figure 4. Median-joining networks of *Inia geoffrensis* mtDNA COI haplotypes generated with Network software.
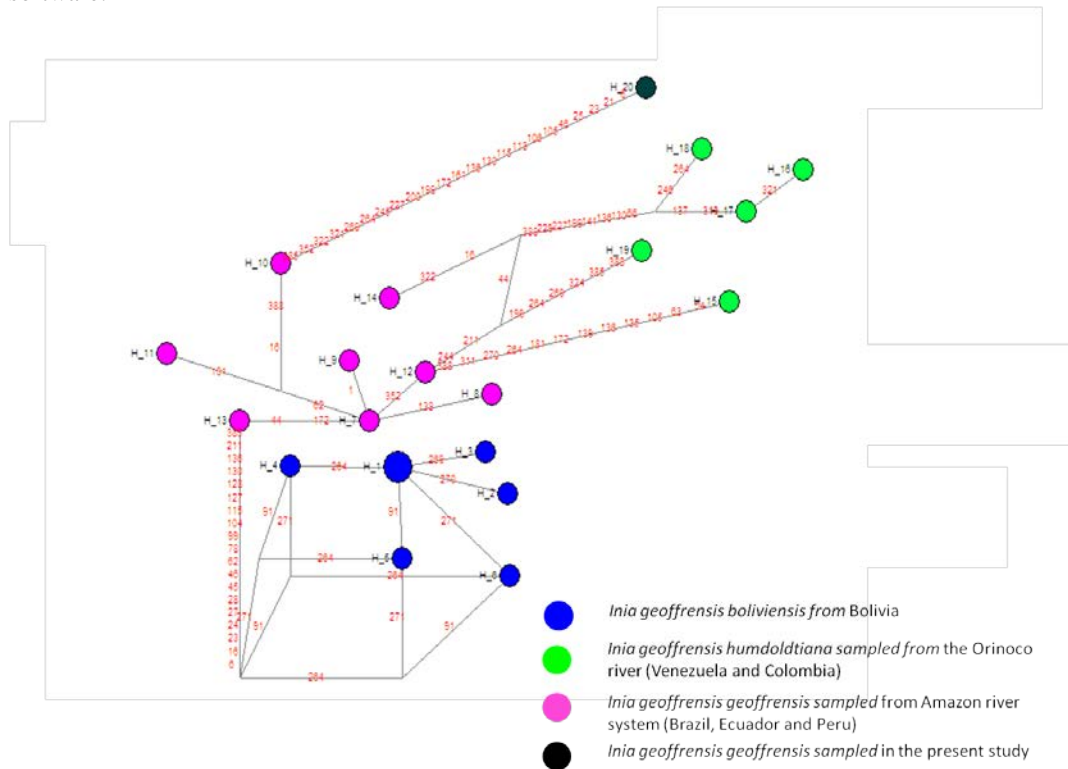

Figure 5. Median-joining networks of *Inia geoffrensis* mtDNA control region haplotypes generated with Network software.

Table 3. Genetic distances of the mtDNA control region by Kimura 2-Parameters, within and between subspecies of boto (*Inia geoffrensis*)

Subspecies	Within subspecies	Subspecies	Between subspecies
Inia geoffrensis geoffrensis	0,0094 ± 0,0028	*Inia geoffrensis geoffrensis* X *Inia geoffrensis humboldtiana*	0,0350 ± 0,0070
Inia geoffrensis humboldtiana	0,0328 ± 0,0062	*Inia geoffrensis geoffrensis* X *Inia geoffrensis boliviensis*	0,0632 ± 0,0121
Inia geoffrensis boliviensis	0,0036 ± 0,0015	*Inia geoffrensis geoffrensis* X **Present study**	**0,0686 ± 0,0131**
		Inia geoffrensis humboldtiana X *Inia geoffrensis boliviensis*	0,0794 ± 0,0131
		Inia geoffrensis humboldtiana X **Present study**	**0,0704 ± 0,0125**
		Inia geoffrensis boliviensis X **Present study**	**0,0750 ± 0,0138**

Discussion

Several authors, including some of them very familiar with the botos, have presented limited information on the distribution and ecological aspects of *I. geoffrensis* in the Amazon river delta, including the Marajó bay and the area near Belém, Pará state, North Brazil. Such impoverished information is clear in Best and Da Silva (1993) and Da Silva and Martin (2010) when mentioning: '*I. g. geoffrensis* is found in the Amazon river system, occurring from the delta, near Belém, into principal tributaries of the Solimões River, ...'. In addition, aquatic mammals' field guides and books such as Da Silva *et al.* (2008) and Jefferson *et al.* (2007) do not give any detailed information on the presence of botos in the Amazon delta itself, including Marajó Bay and near Belém. This makes clear the need for more accurate data on the distribution of the boto in the Amazon delta. Santos *et al.* (2012) provided data based on vantage point observation and transects effort for dolphins in Guamá River, in Belém, from September to October 2008 and from May 2009 to November 2010, respectively. There were 44 sightings, comprising 79 individuals. Among these, 56 (71%) were *I. geoffrensis* and 23 (29%) *Sotalia* sp. During the transect observations, they recorded 19 sightings comprising 50 dolphins. Among those, 36 (72%) were *Sotalia* and 14 (28%) were *Inia*.

Our observations suggest that the boto can preferably use river margins, confluences as well as open waters of Marajó bay. Da Silva and Martin (2010) and Gomez-Salazar *et al.* (2012) have reported the regular occurrence of *I. geoffrensis* in river margins and confluences. The use of areas of intense boat traffic was observed regularly during this study. In fact, three sightings were made during the monitoring of an oil spill incident in Marajó bay in October 2007 (Figure 1). It is noteworthy the sighting records in depths varying from 3 to 22m in Marajó Bay, indicating the ability of *I. geoffrensis* to use open waters with strong currents and windy conditions (Figure 6).

Most of our reported sightings and strandings of botos were made in typical areas of transition from freshwater to mangrove habitats. River discharge in Marajó Bay reaches its maximum during May-July when salinity drops abruptly because of high-water period of the Amazon River (Goulding *et al.*, 2003). It is supposed that botos take advantage of such seasonal conditions and displace to open waters of Marajó bay, reaching areas as far as the estuary of Curuçá River. The Curuçá Bay is surrounded by extensive mangroves forests, is dominated by a marine system formed by the confluence of the Curuçá River and the Muriá channel (Giarrizzo *et al.*, 2011). The occurrence of botos in a typical mangrove surrounded estuary contrast to what has been typically attributed to a river dolphin. In Curuçá estuary there are no measurable freshwater inputs from local rivers or from the Amazon, only by direct rainfall (Giarrizzo and Krumme, 2009). In Marajó bay, during the low-water period of the Amazon river, the water becomes brackish (Goulding *et al.*, 2003).

Our findings points to a common and regular presence of *I. geoffrensis* in the Marajó bay and the surrounding coastline of Marajó Island, to the east coast of Pará state. It is thus suggested that *I. geoffrensis* can use the tremendous discharge of rivers into Marajó bay to disperse to estuaries further outside the Amazon delta. This condition is similar to the movements of botos during dry and rainy seasons from rivers to lakes in the Amazon basin. As pointed out by Martin and Da Silva (2004) and Trujillo *et al.* (2010), the distribution of botos are affected by the water seasons as it changes water level, affect dissolved oxygen, habitat availability and productivity. Movements of fishes match the changes in the environment on rivers. Prey availability (Martin and Da Silva, 2004, Berta *et al.*, 2006, Da Silva *et al.*, 2008) and water seasons can direct determine the movement of botos. By consequence, the high and low water periods change the habitat of botos.

Complementing monitoring efforts along the coast of Pará, a cetacean awareness campaign was initiated in 2011 with the distribution of printed material requesting information on stranded aquatic mammals. Furthermore, fishermen were interviewed in all visited villages for information on cetacean entanglement in gillnets and carcasses washed upon the beaches. All these efforts have contributed to increase the number of records *I. geoffrensis*, and the information transmitted for communities are helping to achieve the goals for the species conservation.

The stranding records of *I. geoffrensis* in the Curuçá estuary suggest their presence as far as 160 km off Belém, well into a coastal environment with strong marine influence. The observations point that the presence of botos is usually associated to river discharges and ports, especially in the Camará river and may have some tolerance to waters with lower salinity levels. It is also noteworthy the intimate proximity of botos to ships, ferries and fishing boats, as well as fishing nets, suggesting a large tolerance to

disturbing environments. Far beyond this tolerance to disturbed areas and human activities, *I. geoffrensis* seems to occupy brackish conditions along the Pará state coast.

Moreover, the genetic data also suggests that the specimen from east coast of Pará state represent a different and isolated geographic form of boto, highly distant in terms of genes from the remaining subspecies proposed in the literature. Further analysis are necessary in terms of nuclear genes in order to propose that they represent a single Evolutionarily Significant Unit (Moritz, 1994), which could be crucial for the conservation of this special population.

Figures 6 and 7. Amazon river dolphin, *Inia geoffrensis*, observed in Paracauari River, east coast of Marajó Island, in 2007. Photos by A.F. Costa.

Fig. 8. Stranded *I. geoffrensis* on a beach in Romana Island, Curuçá, east coast of Pará State, on 25 May 2012 (Voucher specimen MPEG 42122). Photo by A.F. Costa.

Acknowledgments

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