

INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA - INPA  
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BIOLOGIA EVOLUTIVA

O BOTO VERMELHO (*Inia* spp.) NOS RIOS MADEIRA, MAMORÉ E  
GUAPORÉ: DISTRIBUIÇÃO, EVOLUÇÃO E ESTRUTURA POPULACIONAL.

WALESKA GRAVENA

Manaus, Amazonas

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**Sinopse:**

Atualmente são reconhecidas duas espécies de boto, *Inia geoffrensis* e *I. boliviensis*, que até recentemente imaginava-se que permaneciam separadas pelas barreiras (corredeiras) existentes no alto rio Madeira. Foram utilizados marcadores mitocondriais e nucleares (microssatélites) para analisar aspectos populacionais dos botos dos rios Guaporé, Mamoré e Madeira, além de inferir quais os mecanismos históricos e filogeográficos foram responsáveis pela diversificação dos botos nessas regiões.

**Palavras-chave:** Amazônia, corredeiras, Cetacea, populações, DNA mitocondrial, marcadores microssatélites.

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*“Quando o homem aprender a respeitar até o menor ser da criação, seja animal ou vegetal, ninguém precisará ensiná-lo a amar seu semelhante.”*

*Albert Schweitzer*



## RESUMO

Este estudo teve como objetivos avaliar a estrutura das populações e distribuição histórica dos botos nos rios Madeira, Mamoré e Guaporé. No primeiro capítulo, foi possível avaliar se as barreiras geográficas presentes no alto rio Madeira eram potenciais barreiras à distribuição das duas espécies de boto (*Inia boliviensis* e *I. geoffrensis*) presentes nessa região. Foi observado que a espécie *I. boliviensis* ocupa não somente os rios da Bolívia acima das corredeiras, mas também grande parte do rio Madeira sendo encontrada até a localidade de Borba, 870 Km abaixo das corredeiras. O fluxo gênico entre essas populações é unidirecional sempre em direção a jusante, e o tempo de divergência das populações entre essas localidades foi estimado em cerca de 122 mil anos atrás. O segundo capítulo apresentou a caracterização genética da população localizada entre as corredeiras, com o objetivo de verificar se existe conexão entre os botos que foram encontrados entre as corredeiras e os botos coletados a montante e a jusante das mesmas. A maioria dos animais foram identificados a partir de sequências de DNA mitocondrial como pertencentes à espécie *I. boliviensis*, no entanto, abaixo da principal e mais alta cachoeira (Teotônio) a maioria dos indivíduos apresentaram grande parte dos alelos nucleares pertencentes à espécie de boto da Amazônia (*I. geoffrensis*), caracterizando a ocorrência de híbridos. Por possuírem alelos exclusivos e baixa taxa de migração entre as outras localidades, os animais encontrados entre as corredeiras evoluíram independentemente gerando uma unidade evolutiva significativa (ESU) da espécie de boto da Bolívia. Já no terceiro capítulo foi investigada a ocorrência de hibridização entre as espécies de boto e identificado quais os prováveis eventos biogeográficos que levaram a esse resultado. Foi observada a existência de uma zona híbrida de aproximadamente 870 Km, onde são observados animais que possuem genoma mitocondrial da espécie da Bolívia e a maior parte do genoma nuclear pertencente a espécie da Amazônia. Ainda não se pode estimar há quanto tempo as espécies entraram em contato, mas podemos afirmar que elas permaneceram separadas por algum tempo, e a separação das espécies não se deu devido à formação das corredeiras como já foi sugerido por outros autores, e sim devido à descontinuidade do rio durante a sua formação. É provável que o boto da Bolívia tenha colonizado grande parte do rio Madeira, provavelmente até a confluência dos rios Manicoré e Madeira, durante o Plioceno. A formação do restante do rio até a sua confluência com o Amazonas é mais recente, do fim do Pleistoceno, provavelmente a época que houve o encontro das duas espécies, revelando que a barreira geográfica que impediu o encontro entre as espécies não foram as corredeiras e sim a própria formação do rio Madeira.

## ABSTRACT

### **The boto (*Inia* spp.) from the Madeira, Mamoré and Guaporé Rivers: Distribution, Evolution and Populational Structure.**

This study aimed to evaluate the population structure and historical distribution of the boto of the Madeira, Mamoré and Guaporé Rivers. In the first chapter, I investigated if the geographic features of the upper Madeira River act as potential barriers to the distribution of botos (*Inia boliviensis* and *I. geoffrensis*). I observed that *I. boliviensis* occupies not only the Bolivian rivers above the rapids, but also a large part of the Madeira River until the Borba locality, 870 km below the rapids. The gene flow between populations is unidirectional downriver. The divergence time between upstream and downstream populations was estimated as approximately 122,000 years. The second chapter presented the genetic characterization of the population located between the rapids, evaluating the possibility of connectivity with the dolphins found upstream and downstream. Most individuals between the rapids were identified as *I. boliviensis* based on mitochondrial DNA analysis; however, the nuclear alleles of individuals below the highest waterfall (Teotônio) were characteristic of the Amazon river dolphin species (*I. geoffrensis*), therefore proving the occurrence of hybrids in this area. Because of the unique alleles and low migration rate I believe that the group found between the rapids evolved independently and became an evolutionary significant unit (ESU) of the Bolivian pink river dolphin. In the third chapter I investigated the occurrence of hybridization between the two species and identified which biogeographic events have led to the observed result. I found a hybrid zone of about 870 km, where individuals have mitochondrial genome of the Bolivian species and most of the nuclear genome belonging to the Amazon species. Although we can not estimate for how long the species are in contact, I fell confident to affirm that the species remained separated not due to the formation of the rapids, as has been suggested by other authors, but because of the river discontinuity during its formation. It is likely that the Bolivian pink river dolphin has occupied the Madeira River up to its confluence with Manicoré River during the Pliocene. While the formation of the rest of the river until its confluence with the Amazon River is recent, dated from the end of the Pleistocene. Thus, the contact between the two species is likely related to a more recent time, revealing that the geographical barrier that hid this secondary contact were not the rapids, but the formation of the Madeira River itself.

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**Figura 7.** Gráfico obtido pela PCA, utilizando a PC1, eixo x, e PC5 no eixo y. As amostras foram agrupadas em três grupos, Bolívia (animais coletados acima das corredeiras), Rapids1 (animais coletados entre Abunã e a cachoeira do Teotônio), e um grupo composto pelos subgrupos Rapids2 (animais coletados entre a 131

cachoeira de Teotônio e a corredeira de Santo Antônio) e Madeira (animais coletados no restante do rio Madeira).

**Figura 8.** Gráfico empregando o componente com maior variação obtido pela PCA, juntamente com as distâncias geográficas entre as localidades amostradas. Cada círculo corresponde a um indivíduo coletado, totalizando 146 botos desde a localidade de Baía Grande no rio Guaporé (0Km) até a localidade correspondente a confluência entre os rios Madeira e Amazonas (cerca de 1900Km após a primeira localidade de coleta). As linhas mostram o resultado do cálculo de regressão linear para cada um dos grupos obtidos. 132

### Síntese

**Figura 1.** Barras representando os rios analisados de forma linear. Cada barra representa o resultado obtido, apenas com marcador mitocondrial (DNAmt), apenas com marcador nuclear (DNAu) e utilizando os dois tipos de marcadores em conjunto (Espécies/ESU). As siglas abaixo das barras pretas indicam as localidades de Baía Grande (BGR), Abunã (ABN), cachoeira de Jirau (JIR), corredeira do Caldeirão (CLD), cachoeira do Teotônio (TEO), corredeira de Santo Antônio (STA), Borba/Guajará (GJR), confluência entre os rios Amazonas e Madeira (BCA), e a localidade do rio Solimões utilizada para comparação, Mamirauá (MMI). As linhas pontilhadas em vermelho indicam onde foram construídas as duas hidrelétricas, UHE Jirau e UHE Santo Antônio. As cores das barras ilustradas representam as espécies ou unidades evolutivas significantes (ESUs). Os três tons de azul representam a espécie *I. boliviensis*. O tom azul observado abaixo da cachoeira de Teotônio na barra que representa os resultados de DNAmt, indica a presença de haplótipos diferenciados das localidades a montante. O tom azul diferenciado nas localidades entre corredeiras observado nos resultados de 137

DNAu, indicam a presença de alelos exclusivos dessas localidades, representando uma ESU. Pode-se notar a existência de um gradiente da cor azul para a cor rosa na segunda barra ilustrada, indicando a mudança na constituição alélica dos animais encontrados a montante da cachoeira de Teotônio (TEO). Unindo os resultados obtidos nota-se a existência da cor verde que representa a ESU encontrada entre as corredeiras e a cor laranja que representa a zona híbrida entre as espécies. Em todas as barras nota-se que a cor rosa representa a espécie *I. geoffrensis*.

## INTRODUÇÃO GERAL

O Rio Madeira é o mais longo dos tributários da Bacia Amazônica, totalizando 1,4 milhões de Km<sup>2</sup>, o que corresponde a 20% da Bacia Amazônica. Esse rio é formado pela confluência de três rios principais, Beni, Mamoré e Madre de Dios, e é o único afluente da margem direita do rio Amazonas que nasce na Cordilheira dos Andes. Cerca de 50% da sub-bacia do Rio Madeira está na Bolívia, 40% no Brasil e 10% no Peru. O Brasil e a Bolívia compartilham cerca de 1.000 Km de suas águas ao longo dos Rios Mamoré e Guaporé/Iténez, que constituem as divisas entre esses países (Goulding *et al.* 2003).

Nos rios Mamoré e Madeira, entre os municípios de Guajará-Mirim, na divisa com a Bolívia, e Porto Velho, em uma extensão de 290 Km, são observadas 18 corredeiras ou cachoeiras (Cella-Ribeiro *et al.* 2013). Até recentemente essas corredeiras eram consideradas barreiras a navegação nessa região e, de acordo com Goulding *et al.* (2003) muitas espécies de peixes não conseguiam ultrapassá-las. Já Best e da Silva (1989) chegaram a considerar que as espécies de boto poderiam transpor as corredeiras durante as cheias do rio Madeira, quando algumas corredeiras ficavam submersas, como acontece com os grandes bagres migradores (Goulding 1981) e outros vertebrados que habitam a região (Pearse *et al.* 2006; Farias *et al.* 2010).

Em 1834, D'Orbigny analisou um espécime de boto vermelho do Rio Iténez e relatou que o mesmo possuía diferentes características morfológicas quando comparado com botos do restante da bacia Amazônica. Assim, aos botos que ocorriam nessa região foi designada uma nova espécie, *Inia boliviensis*. Desde então, vários trabalhos tem sido realizados para discutir as relações filogenéticas entre as duas espécies de boto (Pilleri e Gihl 1977; da Silva 1994; Hamilton *et al.* 2001; Banguera-Hinestroza *et al.* 2002; May-Collado e Agnarsson 2006). Apesar de alguns autores ainda adotarem a existência de uma única

espécie, *Inia geoffrensis*, com três subespécies (*I. g. geoffrensis*, *I. g. humboldtiana* e *I. g. boliviensis*) com distribuição distinta (Best e da Silva 1989; Rice 1998), no presente trabalho foi adotada a classificação proposta por Pilleri e Gehr em 1977, onde foram descritas duas espécies *Inia geoffrensis* e *I. boliviensis*, provavelmente separadas pela sequência de corredeiras do alto rio Madeira. Os autores utilizaram análises morfológicas para verificar as diferenças descritas por D'Orbigny (1834). Essa classificação já foi confirmada por outros trabalhos que utilizaram análises morfométricas (da Silva 1994) e moleculares (Hamilton *et al.* 2001; Banguera-Hinestroza *et al.* 2002; Vianna *et al.* 2010).

Embora já existam diversos trabalhos que separam as espécies de boto, nunca se soube qual seria a barreira geográfica limítrofe que separaria a espécie que ocorre nos rios da Bolívia da espécie que ocorre no restante da bacia Amazônica. Sabe-se que as espécies de boto têm como principais limites à sua distribuição, cachoeiras e corredeiras intransponíveis (Best e da Silva 1993), por isso, imaginava-se que os 290 Km onde ocorrem as corredeiras seriam barreiras à distribuição das duas espécies. Outros autores concordaram com essa possibilidade, descrevendo que as corredeiras e cachoeiras encontradas nos rios Mamoré e alto Madeira poderiam manter a espécie *I. boliviensis* restrita aos rios da Amazônia Boliviana, enquanto que a espécie *I. geoffrensis* só seria encontrada abaixo dessas corredeiras e no restante da bacia Amazônica (Pilleri e Gehr 1977; Casinos e Ocaña 1979; Pilleri e Gehr 1981; Grabbert 1983; da Silva 1994; Hamilton *et al.* 2001; Banguera-Hinestroza *et al.* 2002).

Apesar dos diversos trabalhos realizados com o boto vermelho dos rios da Bolívia e dos rios da bacia Amazônica (Pilleri e Gehr 1977; da Silva 1994; Banguera-Hinestroza *et al.* 2002; Martínez-Aguero *et al.* 2006; Ruiz-García 2010; Hollatz *et al.* 2011), a maioria dos trabalhos não analisou exemplares da região das corredeiras e ao longo do rio Madeira, suposta área de contato entre as espécies de boto.

Banguera-Hinestroza *et al.* (2002) utilizando marcadores mitocondriais para medir o fluxo gênico entre as populações de botos dos rios da Bolívia e da Amazônia central, descreveram que as corredeiras do rio Madeira constituíam uma barreira significativa ao fluxo gênico das populações e que provavelmente existiu um isolamento por distância que levou a diferenciação das mesmas. No entanto, as amostras utilizadas para esse trabalho são muito distantes geograficamente entre si, assim como nos outros estudos, já citados anteriormente. As coletas de material foram realizadas principalmente em regiões da Colômbia, no rio Tefé (Amazônia Central) e no rio Mamoré (Bolívia). Na região crítica para as duas espécies, onde ocorrem as cachoeiras, e no restante do rio Madeira, não foram realizadas coletas de animais e, portanto, os resultados não mostravam a situação real das espécies de boto ao longo dessa região.

Nessa região crítica, com o objetivo de fornecer energia para o Brasil e a Bolívia, foram construídas duas usinas hidroelétricas (UHEs), nas corredeiras de Santo Antônio e Caldeirão, UHE Santo Antônio e UHE Jirau, respectivamente. A UHE Santo Antônio fechou suas comportas em agosto de 2011, e a UHE Jirau em dezembro de 2012. As duas usinas agora mantêm represadas as águas do rio Madeira, formando reservatórios de aproximadamente 350 Km<sup>2</sup> e 303 Km<sup>2</sup> (Energia Sustentável do Brasil 2010; Santo Antônio Energia 2011). Esses reservatórios ainda não estão em seus níveis máximos e mesmo assim já submergiram nove das 18 corredeiras existentes nessa região, transformando completamente o ambiente.

Por isso, nos três capítulos apresentados a seguir, foram utilizados marcadores moleculares que possuem diferentes modos de herdabilidade e taxas de mutação, para inferir quais mecanismos evolutivos influenciaram na estrutura populacional observada atualmente nos botos dos rios Madeira, Mamoré e Guaporé. As amostras foram coletadas em 27 localidades ao longo desses rios e foram analisadas em três capítulos com diferentes objetivos. No primeiro capítulo, quando ainda não havia amostras de botos da região das

corredeiras, foi realizada a identificação molecular, baseado em marcadores mitocondriais de todas as amostras obtidas até então, com o intuito de definir, principalmente, se as corredeiras poderiam ser consideradas barreiras geográficas à dispersão dos botos. As amostras dos indivíduos coletados entre corredeiras foram utilizadas no segundo capítulo, onde foi realizada descrição molecular dos indivíduos que habitavam essa região antes do fechamento das comportas das hidrelétricas construídas. Além disso, todas as amostras coletadas foram utilizadas no terceiro capítulo, onde foi possível inferir quais eventos podem ter dado origem aos padrões observados atualmente nas populações de botos, utilizando marcadores mitocondriais e nucleares.

Os resultados aqui apresentados fazem parte dos primeiros estudos envolvendo espécimes de boto em toda a sub-bacia do rio Madeira, incluindo a região de corredeiras.



## OBJETIVOS

Os objetivos gerais de cada capítulo foram os seguintes:

Capítulo I – Caracterizar molecularmente os indivíduos das espécies *Inia boliviensis* e *Inia geoffrensis* para atribuir a qual espécie pertencem os botos coletados no rio Madeira e testar se as 18 corredeiras presentes no alto rio Madeira representam uma barreira geográfica entre as duas espécies.

Capítulo II – Caracterizar a população de botos que ocupava a região entre corredeiras do rio Madeira utilizando marcadores mitocondriais e nucleares, e prever os possíveis impactos das hidrelétricas construídas nessa região nessas populações.

Capítulo III – Elucidar mecanismos evolutivos e fatores geográficos que levaram ao padrão observado nas duas espécies de botos que ocorrem no rio Madeira, caracterizando os processos evolutivos que ocorreram ao longo da história das populações dessa região.

## Capítulo 1

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Gravena, W.; Pires, I. P.; da Silva, M. N. F.; da Silva, V. M. F.; Hrbek, T. Looking to the past and the future: were the Madeira River rapids a geographical barrier to the boto (Cetacea: Iniidae)? *Conservation Genetics*. 2014

1 Looking to the past and the future: were the Madeira River rapids a  
2 geographical barrier to the boto (Cetacea: Iniidae)?

3

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17 Running Head: Rapids as geographic barriers for the boto

18 **ABSTRACT**

19 In the present study we tested if a series of 18 rapids on the upper Madeira River  
20 form an effective barrier to gene flow, and in particular if they delimit the  
21 distribution of the boto *Inia boliviensis* - which it is believed to occurs only in the  
22 Bolivian sub-basin, above the rapids - and *I. geoffrensis*, which occurs  
23 throughout the Amazon basin and below the upper Madeira River rapids. We  
24 analyzed 125 individuals from the Madeira River basin sampled from upstream  
25 and downstream of the rapids. As the two species are morphologically similar,  
26 we used diagnostic molecular characters from known reference specimens to  
27 assign individuals to species. We observed that all individuals of *Inia* from the  
28 Bolivian sub-basin up to almost the mouth of the Madeira River basin belong to  
29 the species *I. boliviensis*. Therefore we concluded that the rapids do not delimit  
30 the distribution of *I. boliviensis* upstream and *I. geoffrensis* downstream of the  
31 rapids as previously hypothesized. Since we registered *I. boliviensis* along  
32 almost the entire length of the Madeira River, we estimated gene flow, time of  
33 divergence and effective population sizes of the upstream (Bolivian) and  
34 downstream (Madeira River) groups of *I. boliviensis* using IMA2. We concluded  
35 that gene flow is uni-directional from the upstream to the downstream group.  
36 Divergence time between the two groups was estimated to have occurred  
37 approximately 122 thousand years ago. The coalescent effective population size  
38 for the upstream group was estimated at approximately 131 thousand individuals,  
39 while for the downstream group it was estimated at approximately 102 thousand

40 individuals. Recently two dams have been constructed in the region of the rapids;  
41 neither has a mechanism that will maintain connectivity between the upstream  
42 and downstream regions, and together with anthropogenic alterations to the  
43 hydrodynamic regime and ecology of the river will likely pose serious long-term  
44 and short-term consequences for *I. boliviensis* and other aquatic taxa.

45

46 **Keywords**

47 *Inia*; Amazon River dolphin; gene flow; species limits; species distribution

## 48 INTRODUCTION

49 Popularly known as the pink river dolphin, Amazon River dolphin, or boto,  
50 the species of the genus *Inia* are widely distributed in the rivers of the Amazon  
51 and Orinoco basins. In both basins, botos are found from the headwaters to the  
52 delta (da Silva 1994). Principal limits to the distribution of the *Inia* dolphins are  
53 large rapids and waterfalls (Best and da Silva 1993).

54 The two currently described species of *Inia* - *I. geoffrensis* and *I.*  
55 *boliviensis* - have allopatric distributions, and no external morphological  
56 characters distinguish the two species. *Inia geoffrensis* occurs in the rivers of the  
57 Orinoco and Amazon basins (Pilleri and Gehr 1977; da Silva 1994; Banguera-  
58 Hiestroza et al. 2002). In the Madeira River it is thought to occur below the  
59 Teotônio rapids located just upstream of Porto Velho (Pilleri and Gehr 1977; Best  
60 and da Silva 1993; da Silva 2009). *Inia boliviensis* on the other hand has a much  
61 more restricted distribution and is only found in the rivers of northern and north-  
62 eastern Bolivia, including the Abunã, Guaporé/Iténez and Mamoré Rivers that  
63 form a political boundary between Bolivia and Brazil (Pilleri and Gehr 1977; Best  
64 and da Silva 1993; da Silva 2009). Between the municipalities of Guajará-Mirim  
65 (located on the Mamoré River), and Porto Velho (located on the Madeira River),  
66 there is a series of 18 rapids along a 290 km stretch of the river (Cella-Ribeiro et  
67 al. 2013). These rapids are thought to act as a barrier to the movement of the two  
68 species, thus restricting the distribution of *I. boliviensis* upstream of the rapids,  
69 and *I. geoffrensis* downstream of the rapids. Individuals of the genus *Inia*,

70 however, are also found in between the rapids, but it is unclear which species  
71 occurs in this 290 km inter-rapid section. Lack of information from the region of  
72 the rapids is due to low densities of the animals, and the difficulty of capture; the  
73 river is deep and fast flowing, with steep banks, large stones and few beaches or  
74 sand bars. Despite a lack of convincing data, most authors follow Pilleri and Gühr  
75 (1977) in supposing that the Teotônio rapid, the largest and highest of the 18  
76 rapids (Cella-Ribeiro et al. 2013), delimits the geographic distribution of both  
77 species. On the other hand, authors such as Best and da Silva (1993) suggest  
78 that during the rainy season dolphins can, and on occasion do cross the upper  
79 Madeira River rapids (including the Teotônio rapid). Indeed, one of us (WG)  
80 observed dolphins crossing the Santo Antônio rapids from the downstream to the  
81 upstream direction. Therefore the rapids may not represent a complete physical  
82 barrier to animal movement, and consequently to gene flow.

83           In terms of conservation, incidental or deliberate capture appears to be the  
84 principal source of threat to botos in the various Amazonian countries where they  
85 are found (Leatherwood 1996; Portocarrero-Aya et al. 2010; Trujillo et al. 2010).  
86 In the region of the Madeira River rapids this threat may soon be surpassed by  
87 large-scale aquatic habitat modification resulting from the construction of two  
88 hydroelectric dams. Severe ecosystem change associated with the construction  
89 of hydroelectric dams and barrages (Finer and Jenkins 2012) have been the  
90 principal source of risk for Asian freshwater dolphins (Reeves and Leatherwood  
91 2004) due to fragmentation of natural populations (Smith & Reeves, 2012;  
92 Reeves & Leatherwood, 2004).

93 We used three mitochondrial markers to: (1) characterize representative  
94 individuals of the species *I. boliviensis* and *I. geoffrensis*; (2) make a species  
95 level assignment of individuals sampled from the Madeira River basin; (3) test if  
96 the series of 18 rapids on the upper Madeira River represent the geographic limit  
97 to the distribution of *I. boliviensis* and *I. geoffrensis*; and (4) test if the rapids form  
98 an effective barrier to gene flow between the upstream and downstream  
99 populations. Using these data, we also estimated temporal divergence of the  
100 upstream and downstream populations, and their effective population sizes.  
101 Further, we discuss the implications of our findings for the conservation of these  
102 animals.

103

## 104 **METHODS**

### 105 **Study area, samples and DNA extraction**

106 Between the years 2007 and 2011 we sampled 125 dolphins from 6  
107 localities along the Guaporé (N = 34) and Mamoré (N = 14) rivers (Fig. 1;  
108 localities 14-19), and 13 localities along the Madeira River (n = 77) (Fig. 1;  
109 localities 1-13). The distance between the furthest sampling sites was  
110 approximately 1500 km.

111 Samples were removed from margins of caudal lobes of animals captured  
112 using the procedures of da Silva and Martin (2000). Capture occurred between  
113 the months of September and December, when rivers are at their lowest levels



114 and animals are concentrated in river channels. Samples were preserved in 95%  
115 ethanol and deposited in the official tissue collection (Coleção de Tecidos de  
116 Genética Animal – CTGA) of the Federal University of Amazonas (Universidade  
117 Federal do Amazonas – UFAM). We also used 14 additional samples of animals  
118 collected in the Mamirauá Sustainable Development Reserve located at the  
119 confluence of the Japurá and Amazonas Rivers, as reference specimens of *I.*  
120 *geoffrensis*. Since the type locality of *I. geoffrensis* is “South America, probably  
121 upper Amazon of Brazil”, and *I. geoffrensis* is diagnosable by only few subtle  
122 osteological and dentition differences which cannot be easily observed on live  
123 animals, we chose specimens from the Mamirauá Sustainable Development  
124 Reserve which is located in the upper Amazon of Brazil where *Inia* of this reserve  
125 have been confirmed to be *I. geoffrensis* (Banguera-Hinestroza et al. 2002;  
126 Martin and da Silva 2006). The 14 individuals collected from Porto França – 5.5  
127 km upstream of Príncipe da Beira, the type locality of *I. boliviensis* – (Fig. 1)  
128 served as representative specimens of this species. Total DNA was extracted  
129 from all samples using a standard phenol/chloroform protocol (Sambrook et al.  
130 1989).

131

## 132 **Molecular markers**

133 We amplified three portions of mitochondrial DNA (mtDNA): the control  
134 region (CR), the complete cytochrome *b* gene (*cytb*), and the DNA barcode  
135 region of the cytochrome *c* oxidase I gene (COI) (Hebert et al. 2003). To amplify

136 the CR and the cytb gene we used the primers HDH6 and LTRO (Banguera-  
137 Hinestroza et al. 2002), and the primers IniaGluF1 (5'-  
138 GACCAATGACATGAAAAATCATCG-3') and IniaProR1 (5'-  
139 CAGCTTTGGGTGTTGGTGGTGA-3') developed for this study, respectively. The  
140 COI region was amplified using the COIF\_Inia (5'-  
141 CTCAACAAACCACAAAGACATYGG-3') and COIR\_Inia (5'-  
142 TATACTTCGGGGTGGCCAAAGAATCA-3') developed for this study.

143 All the regions were amplified in a final volume of 15  $\mu$ l and contained 7.6  
144  $\mu$ l of ddH<sub>2</sub>O, 1.2 of 25 mM MgCl<sub>2</sub>, 1.2  $\mu$ l of 10 mM dNTPs, 1.5  $\mu$ l of 10x buffer  
145 (750 mM Tris-HCl – pH 8.8 at 25°C, 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% (v/v) Tween 20),  
146 1.5  $\mu$ l of each primer (2  $\mu$ M), 0.3  $\mu$ l of Taq DNA Polymerase (1 U/ $\mu$ l) and 1  $\mu$ l of  
147 DNA (concentration varied between 10 ng and 30 ng). PCR reagents were  
148 obtained from Fermentas (Vilnius, Lithuania). PCR consisted of an initial  
149 denaturation step at 94°C for 90 seconds followed by 35 cycles of denaturation at  
150 93°C for 60 seconds, primer annealing at 50°C for 40 seconds, and primer  
151 extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5  
152 minutes. PCR products were purified using EXO-SAP (Exonuclease – Shrimp  
153 Alkaline Phosphatase) following the manufacturer's suggested protocol (Werle et  
154 al. 1994).

155 To sequence the CR and COI gene regions we used the amplification  
156 primers, and for sequencing the cytb gene we used the internal primers  
157 IniaCytbF1 (5'-CTACCCTGAGGACAAATATCATT-3'), IniaCytbF2 (5'-

158 CCCGACCTACTAGGAGACCC-3') and IniaCytbR1 (5'-  
159 GAAGAAGCGTGTTAGTGTTC-3') developed for this study. Sequencing was  
160 performed on ABI 3130xl automatic DNA analysis system (Applied Biosystems)  
161 using the manufacturer's recommended settings.

162

### 163 **Analysis**

164 Sequence products were edited, concatenated and aligned using the  
165 program Geneious 5.6.3 (Drummond et al. 2012). Each CR, cytb and COI gene  
166 sequence was then compared to our reference sequences of bona fide *I.*  
167 *geoffrensis* and *I. boliviensis*, and based on the sharing of diagnostic sites, was  
168 assigned to a species. Given that *I. geoffrensis* occurs only near the mouth of the  
169 Madeira River (see results), all subsequent analyses were performed on *I.*  
170 *boliviensis* occurring upstream (n = 48) and downstream (n = 56) of the Madeira  
171 River rapids in the Bolivian sub-basin and the Madeira River, respectively.

172 Phylogenetic relationships among haplotypes were visualized via a  
173 haplotype network in the program HaploViewer (Salzburger et al. 2011), with  
174 relationships among haplotypes being inferred under the maximum likelihood  
175 criterion using the program dnaml of the PHYLIP package (Felsenstein 1993).  
176 The most appropriate model of molecular evolution for the mtDNA dataset was  
177 inferred using the corrected Akaike Information Criterion (Akaike 1974) in  
178 jModelTest (Posada, 2008).

179 We measured  $F_{ST}$  and tested its significance to assess whether  
180 haplotypes were distributed randomly with respect to their geographic occurrence  
181 upstream or downstream of the Madeira River rapids. All tests were performed in  
182 the software Arlequin 3.5.2.1 (Excoffier and Lischer 2010).

183 To measure gene flow, infer the historical contribution to haplotype  
184 sharing, and estimate the time of isolation of the two groups upstream and  
185 downstream of the Madeira River rapids, we used the isolation-with-migration  
186 framework implemented in the program IMA2 (Hey and Nielsen 2007). We ran 50  
187 chains with dynamic heating (-ha 0.99 -hb 0.45) and uniform priors (-q 100 -m  
188 100 -t 100), collecting 100,000 from 10,000,000 generated topologies (i.e. one  
189 topology in every 100th was sampled). Topologies were collected after an initial  
190 burn-in period of 2 million topologies, when parameter estimates had stabilized.  
191 Convergence of parameter estimates was inferred from the effective sample size  
192 (ESS) of the parameters, from analyzing trendline plots, and from comparing  
193 parameter estimates based on the first half and second half of the MCMC run.  
194 More restricted demographic models, e.g. symmetric gene flow, were tested  
195 against the full isolation with migration model using a hierarchical likelihood ratio  
196 test as implemented in IMA2.

197 The parameters obtained by IMA2 ( $\theta$ ,  $m$  and  $t$ ) were converted to the  
198 demographic parameters: coalescent effective population size, gene flow and  
199 divergence time in years between populations above and below the rapids, using  
200 the substitution rate of  $1.26 \times 10^{-8}$  (range  $0.94 \times 10^{-8} - 1.58 \times 10^{-8}$ ) for the CR,

201 and  $1.03 \times 10^{-8}$  (range  $0.90 \times 10^{-8} - 1.16 \times 10^{-8}$ ) for *cytb* and COI genes (Pesole  
202 et al., 1999), and generation time of 10 years (Taylor et al. 2007). The  
203 substitution rates are estimated values for similar sized mammals (Pesole et al.  
204 1999), but are faster than those reported for the humpback whale (Alter et al.  
205 2007). Humpback and other baleen whales are inferred to have relatively slow  
206 rates of molecular evolution, approximately 8 to 10 times slower than medium  
207 sized mammals (Jackson et al. 2009). The substitution rate of mammalian  
208 protein-coding mtDNA (Pesole et al. 1999) also corresponds closely to the  
209 substitution rate of  $1.01 \times 10^{-8}$  per site per year, as estimated from the calibrated  
210 cytochrome *b* phylogeny of Xiong et al. (2009). It should be noted that the  
211 estimated substitution rates are likely to be conservative, as actual intraspecific  
212 substitution rates tend to be higher than interspecific substitution rates (Ho et al.  
213 2005; Ho et al. 2007). This, in turn, implies that actual divergence times and  
214 effective population size estimates are likely to be smaller than reported.

215

## 216 **RESULTS**

217 For the 125 samples collected between 2007 and 2011 in the Guaporé,  
218 Mamoré and Madeira rivers and the samples of *I. geoffrensis* collected from the  
219 Mamirauá Sustainable Development Reserve (Amazon/Japurá rivers), we  
220 generated a concatenated sequence of 2382 bp, including the CR (621 bp), the  
221 *cytb* (1241 bp) and the COI (520 bp). All sequence data were deposited in  
222 GenBank under the accession numbers KF657433 – KF657536 and KF802277 –

223 KF802312 (CR), KF657537 – KF657640 and KF802313 – KF802348 (cytb), and  
224 KF657329 – KF657432 and KF802241 – KF802276 (COI).

225 Sequences were assigned to species by examining diagnostic sites  
226 shared with reference samples of *I. geoffrensis* from the Mamirauá Sustainable  
227 Development Reserve, and additional reference samples available in GenBank.  
228 The two species differed at 18 positions in the CR (Table 1a), 38 positions in the  
229 complete cytb gene (Table 1b), and 28 positions in the DNA barcode region of  
230 the COI gene (Table 1c).

231 Using the 14 reference samples of *I. geoffrensis*, and the 14 reference  
232 samples of *I. boliviensis*, we observed haplotypes of both *I. geoffrensis* (21  
233 samples) and *I. boliviensis* (104 samples) in the 125 samples collected in the  
234 Madeira River basin. All sequences from the Guaporé and Mamoré rivers, and  
235 unexpectedly, most from the Madeira River, belong to *I. boliviensis*. Thus,  
236 molecularly identified *I. boliviensis* was found to occur throughout nearly the  
237 complete length of the Madeira River all the way to and including the Guajará  
238 locality (Fig. 1, locality 3); this locality is situated approximately 10 km upstream  
239 of the city of Borba and 870 km downstream of the Santo Antônio rapid and 890  
240 km downstream of the Teotônio rapid. From Guajará (Borba) to the mouth of the  
241 Madeira River, only *I. geoffrensis* was observed (Fig. 1). *Inia boliviensis* occurred  
242 exclusively in all localities upstream the rapids and in 10 localities downstream  
243 the rapids, while *I. geoffrensis* occurred exclusively in two localities closest to the  
244 mouth of the Madeira River (Fig. 1; locality 1-2). In Guajará (Borba) (Fig. 1;

245 locality 3) both species occurred. Of the nine individuals from Guajar (Borba),  
246 three were identified as *I. geoffrensis* and six as *I. boliviensis*.

247 In the 104 *I. boliviensis* individuals from upstream and downstream the  
248 rapids, we observed 41 haplotypes of which 23 were singletons (Fig. 2). The  
249 majority of the haplotypes, besides the two most frequent ones, were restricted to  
250 either upstream or downstream of the rapids. The two most common haplotypes,  
251 occurring in 15 and 11 individuals, respectively, had different frequencies  
252 upstream and downstream of the rapids (Fig. 2). Given that we sampled *I.*  
253 *boliviensis* in both upstream and downstream localities, we also tested the effect  
254 of the 18 rapids in the upper Madeira River as a population structuring factor.  
255 The  $F_{ST}$  values observed were relatively low, but indicate that the haplotypes  
256 have non-random distribution ( $F_{ST} = 0.169$ , 95% CI 0.073 – 0.275,  $P < 0.001$ )  
257 between the upstream and downstream groups of *I. boliviensis* separated by  
258 rapids. However, the series of rapids does not act as complete barrier to gene  
259 flow. Although we infer zero migration rate in the downstream to upstream  
260 direction ( $\mu = 0$ , 95% HPD 0.000 –  $1.150 \times 10^{-3}$ ), the most likely migration rate in  
261 the upstream to downstream direction is positive ( $\mu = 1.350 \times 10^{-3}$ , 95% HPD  
262  $0.350 - 2.950 \times 10^{-3}$ ). This resulted in an inference of no gene flow ( $2Nm =$   
263  $0.000$ , 95% HPD 0.000 – 2.801) in the downstream to upstream direction and  
264 gene flow of 7.24 effective female migrants (95% HPD 2.351 – 14.890) in the  
265 upstream to downstream direction (Fig. 3). The hypothesis of zero bi-directional  
266 migration rate and thus no gene flow in either direction, is strongly rejected using  
267 the hierarchical likelihood ratio test ( $2\Delta\ln(\text{Pr}) = 487.80$ ,  $df = 2$ ,  $P < 0.001$ ).

268 Isolation between the upstream and downstream groups is not recent; the  
269 two groups only share the two most common haplotypes (Fig. 2). The IMA2  
270 analysis resulted in an approximate age of isolation of 122 thousand years (95%  
271 HPD  $32 - 283 \times 10^3$ ) using a harmonic mean of the average mammalian  
272 substitution rates (Pesole et al. 1999) for the three analyzed gene regions. When  
273 using the upper and lower bound substitution rate values from Pesole et al.  
274 (1999), see Materials and Methods, values ranged from a mean of 97 (95%  
275 HPD  $25 - 226 \times 10^3$ ) to 163 (95% HPD  $43 - 379 \times 10^3$ ) thousand years.

276 Values of theta estimated in IMA2 for the downstream and upstream  
277 regions were 10.250 (95% HPD 5.950 – 16.950) and 7.950 (95% HPD 4.450 –  
278 13.150), respectively. Demographic estimates of coalescent effective population  
279 sizes were approximately 131 thousand individuals (95% HPD  $76 - 217 \times 10^3$ )  
280 downstream and 102 thousand individuals upstream (95% HPD  $57 - 168 \times 10^3$ )  
281 using the mean mammalian substitution rate (Pesole et al. 1999).

282

## 283 **DISCUSSION**

284 Since the description of *I. boliviensis* from Príncipe da Beira, Guaporé  
285 River (D'Orbigny 1834), the real limits of the geographic distribution of this  
286 species remained unknown. Previous studies, whether employing morphological  
287 analyses or molecular analyses (Pilleri and Gühr 1977; da Silva 1994; Hamilton et  
288 al. 2001; Banguera-Hinestroza et al. 2002; Hollatz et al. 2011) used samples of *I.*



289 *boliviensis* from the Bolivian sub-basin, Guaporé and Mamoré rivers, and of *I.*  
290 *geoffrensis* from the central Amazon basin, Solimões River near the city of Tefé  
291 and other rivers, but not the Madeira River. In spite of the Bolivian basin and the  
292 region of Tefé being separated by more than 1900 km and various potential  
293 barriers to gene flow besides the upper Madeira River rapids, all these studies  
294 assumed that the rapids on the upper Madeira River delimited the distribution of  
295 *I. boliviensis* and *I. geoffrensis* (Pilleri and Gihl 1977; Casinos and Ocaña 1979;  
296 Pilleri and Gihl 1981; da Silva 1994; Hamilton et al. 2001; Banguera-Hinestroza  
297 et al. 2002).

298         Although this study uses exclusively maternally inherited markers, we  
299 demonstrate that *I. boliviensis* is neither restricted to the Bolivian sub-basin, nor  
300 upstream of the Teotônio or other rapids on the upper Madeira River. In fact, *I.*  
301 *boliviensis* is found at least as far downstream as the city of Borba on the lower  
302 Madeira River. The two *I. boliviensis* groups found upstream and downstream of  
303 the upper Madeira River rapids are differentiated from each other despite being  
304 connected by unidirectional gene flow. Fifteen haplotypes were restricted to the  
305 upstream group while 24 haplotypes were restricted to the downstream group  
306 with only two haplotypes shared between the two groups. Excluding rare  
307 haplotypes, singletons which could have recently arisen via a mutational process  
308 and did not yet have time to expand geographically from their area of origin, one  
309 still observes five medium frequency haplotypes restricted to the upstream  
310 locality and 11 to the downstream locality.

311 Borba is located 870 km downstream of the rapids. It is worth noting that  
312 upstream from Borba, of the 50 *Inia* individuals collected in the 10 sampling  
313 localities, not a single individual had an *I. geoffrensis* haplotype. Thus, it is  
314 reasonable to assume at this time that only *I. boliviensis* occurs in the Madeira  
315 River upstream of Borba. On the other hand, in the localities downriver from  
316 Borba to the mouth of the Madeira River, only haplotypes of *I. geoffrensis* were  
317 observed. The region of Borba seems to represent an area of contact of both  
318 species. Of the nine individuals collected at this locality, three had haplotypes of  
319 *I. geoffrensis* while six of *I. boliviensis*. Since the lower Madeira has no actual  
320 and no apparent historical physical barriers, it is unclear why the two species  
321 replace each other at or near the city of Borba, nor if the two hybridize.

322 An extensive ichthyological survey of the Madeira River (Queiroz et al.  
323 2013) demonstrated that besides the upper Madeira River rapids, there is a  
324 sharp ichthyofaunal break at the confluence of the Aripuanã and Madeira Rivers.  
325 The confluence of these two rivers is 135 km upstream of the city of Borba, and  
326 therefore the substitution of *I. boliviensis* by *I. geoffrensis* and the substitution of  
327 fish species and communities may be related to a common historical event. It is,  
328 however, unclear what this event may have been.

329 As noted above, although *I. boliviensis* is divided into two groups  
330 physically separated by the rapids and waterfalls of the upper Madeira River, and  
331 these groups are significantly differentiated ( $F_{ST} = 0.169$ , 95% CI 0.073 – 0.275),  
332 these rapids do not present a complete barrier to dispersal and gene flow for

333 females. Dispersal and gene flow is apparently unidirectional in the upstream to  
334 downstream direction. The region of rapids is characterized by relatively fast  
335 water, deep channels and steep banks, with little suitable habitat for *Inia*. There  
336 are also sections of the river (e.g. between the Morrinho and Teotônio rapids)  
337 where dolphins were never observed in river surveys, or have been reported  
338 during interviews with local inhabitants held during the quarter-annual surveys  
339 carried prior to construction in 2004 and prior to filling in 2010 and 2011 of the  
340 hydroelectric reservoirs (FURNAS et al. 2005; Energia Sustentável do Brasil  
341 2012). Therefore, given that one observes relatively high levels of upstream to  
342 downstream gene flow, the dispersal events that mediate this gene flow must be  
343 rare and sporadic, but involve a large numbers or groups of individuals.

344 *Inia boliviensis* was observed by one of the authors (WG) to move across  
345 the rapids during high water season, a time during which a number of fish  
346 species have their annual spawning migrations (Goulding 1979). Most of the  
347 rapids are emerged during the dry season while some are emerged even during  
348 the rainy season. However, during periods of extreme high water such as have  
349 occurred in the years 1996, 2006, 2008, 2009 and 2012 (ANA/CPRM, 2012), all  
350 rapids become submerged, allowing a potentially unhindered movement of  
351 animals. While extreme high water potentially facilitates the bi-directional  
352 movement across the rapids, it also increases the velocity of the river. The deep  
353 channels with steep banks and high velocity current characteristic of the rapids  
354 region become even more of a barrier for upstream movement of animals, while  
355 the same phenomenon probably facilitates downstream movement and gene

356 flow. Period of extreme high waters, therefore, most likely facilitate uni-directional  
357 connectivity between the upstream and downstream groups of *I. boliviensis*.

358         The effective population size is some 131 thousand individuals  
359 downstream and 102 thousand individuals upstream. Given that these population  
360 sizes are similar, it reinforces the inference that differences in gene flow are due  
361 to differences in migration rates rather than differences in effective population  
362 sizes – gene flow is a product of migration rate and effective population size. The  
363 coalescent effective population size estimates for the *I. boliviensis* from the  
364 Bolivian Basin are larger than the census size reported by Tavera et al. (2010)  
365 who estimated a census size of approximately 80 thousand individuals in the  
366 Bolivian basin (upstream of the rapids). The two estimates measure two very  
367 different things thus it is not surprising that the estimates diverge. The effective  
368 population size represents a number of individuals in an ideal population that  
369 would results in the observed time to the most rescent common ancestor  
370 (TMRCA), while the second represent a number of individuals observed in a  
371 given area extrapolated to the total potential area inhabited by the species. It is  
372 also not surprising that the effective population size is larger than the census  
373 size. While generally census sizes are thought to be an order of magnitude larger  
374 than effective population sizes (e.g. Frankham et al. 2004) this is not always the  
375 case (e.g. Templeton 2006).

376         The upstream Bolivian sub-basin group was estimated to have diverged  
377 from the downstream Madeira River group approximately 122 thousand years

378 ago. This divergence has not resulted from the formation of the upper Madeira  
379 River rapids, but rather from another yet unknown event. The formation of the  
380 rapids most likely resulted from the rise of the Fitzcarald Arch in the mid to late  
381 Pliocene, no earlier than 4 million years ago (Espurt et al. 2007; Espurt et al.  
382 2010). Interestingly, 122 thousand years ago is near the boundary of the  
383 penultimate interglacial cycle of the Pleistocene, the transition from the Middle to  
384 the Late Pleistocene (Richmond and Fullerton 1986; Roy et al. 2004), thus the  
385 divergence of the Bolivian sub-basin and the Madeira River group may be related  
386 to past climate change and or changes in sea levels, both of which would have  
387 affected the hydrological dynamics of the Madeira River.

388         The effects of the Madeira River rapids have been studied previously in  
389 other organisms. There are a number of species of fish for which the rapids  
390 delimit distributions (e.g. *Semaprochilodus insignis* and *Arapaima gigas*)  
391 (Goulding 1979), and/or in which apparent sister species occur on either side of  
392 the rapids (e.g. *Chalceus guaporensis* and *C. epakros* (Queiroz et al. 2013)). In  
393 this second case, the apparent sister species replace each other  
394 upstream/downstream of the Teotônio rapid, the largest of the rapids on the  
395 Madeira River, as was thought to have occurred with the two species of *Inia*. In  
396 morphologically cryptic species, the Teotônio rapid also delimits distinct  
397 mitochondrial lineages as observed, for example, in the genus *Cichla* (Willis et al.  
398 2012) and crocodylians (Hrbek et al. 2008; Muniz 2012). In other cases the rapids  
399 restrict, but do not impede gene flow, such as for *Colossoma macropomum*  
400 (Farias et al. 2010), which shows a similar pattern of gene flow to *I. boliviensis*.

401 However, for the aquatic turtle *Podocnemis expansa*, the rapids do not present a  
402 barrier to gene flow (Pearse et al. 2006).

403 It is clear that the Madeira River rapids had an influence on the  
404 evolutionary history of the aquatic fauna of the Madeira River basin; however, it  
405 is also apparent that each aquatic species perceives this geological formation  
406 differently, and therefore demographic histories may differ among taxa. It should  
407 also be taken into account that this study is based on mitochondrial genes only,  
408 and thus may not represent the evolutionary history of *Inia boliviensis* in its  
409 entirety.

410

#### 411 **Conservation**

412 Until 2008, when construction work began on two hydroelectric dams in  
413 the rapids area, the Madeira River was free flowing. The dams created two  
414 reservoirs of approximately 300 and 350 km<sup>2</sup> (Energia Sustentável do Brasil  
415 2010; Santo Antônio Energia 2011). These flooded areas submerged 11 of the  
416 18 rapids that occurred in this area.

417 Trying to facilitate movement of animals in this area, Jirau and Santo  
418 Antônio hydroelectric dams have designed different mechanisms of fish  
419 transposition, both of which in theory are suppose to function as ecological filters  
420 (Tuomisto et al. 2003; Jabot et al. 2008), but have completely different physical  
421 characteristics. Species of *Inia* are capable of sustained swimming in fast waters

422 for short periods of time after which they need to recuperate (Best and da Silva  
423 1989a; Best and da Silva 1989b). For this reason they are capable, and have  
424 been observed to cross certain rapids. The animals use short bursts of energy to  
425 cross the rapid followed by a period of rest in an area with little or no current. The  
426 fish transposition mechanisms have been designed to be shallow and narrow,  
427 channeling a high velocity stream, and thus with near certainty will not be used  
428 by *I. boliviensis*. Thus the two dams have completely isolated the upstream and  
429 downstream populations of *I. boliviensis*, as well as have created an isolated  
430 population between the Santo Antônio and Jirau dams. More importantly,  
431 however, is that both dams will modify the hydrological dynamic of the river,  
432 which affects fish community structure and abundance of individual species  
433 (Torrente-Vilara et al. 2011; Finer and Jenkins 2012), in turn affecting food  
434 availability for *Inia*.

435 Dams and other works that impede selective connectivity in riverine  
436 systems have had large impact on river dolphins from other continents, such as  
437 in the case of the Indus River bhulan (*Platanista gangetica minor*) (Khan and  
438 Naizi 1989), and the Ganges River shushuk (*Platanista gangetica gangetica*)  
439 (Shrestha 1989). Both subspecies are classified as endangered (EN) by the  
440 IUCN (Smith and Braulik 2012), principally as a result of more than a 50%  
441 decline in census population size in the last three generations due to decrease in  
442 the area of occupancy as a result of dam/barrage construction. Even more  
443 worrying is the extinction of the Chinese baiji (*Lipotes vexillifer*) (Turvey et al.  
444 2007) caused by extensive modifications of the Yangtze River including dam

445 building, unregulated boat traffic, noise pollution and fishing practices (Peixun  
446 and Yuanyu 1989).

447 Increased water traffic, industrial pollution, noise pollution and decrease in  
448 available prey have directly contributed to the extinction of the baiji (Turvey et al.  
449 2007; Turvey 2008). The construction of the Santo Antônio and Jirau dams also  
450 aims to create a waterway that will allow the transportation of agricultural  
451 products from central Brazil to global markets via the Amazon. Therefore, the  
452 planned construction of locks is likely to increase river traffic and associated  
453 noise and environment pollution, as well as accelerate the human occupation  
454 and settlement of the basin (Finer and Jenkins 2012).

455 While the prognosis for *I. boliviensis* is bleak if no action is taken, it is not  
456 too late to seek solutions. Both the upstream and downstream populations of *I.*  
457 *boliviensis* have relatively large effective population sizes and large number of  
458 alleles compatible with genetically healthy populations (Frankham et al. 2004).  
459 The Santo Antônio and Jirau dams were also closed only in August of 2011 and  
460 December of 2012, respectively, with various construction projects are still  
461 ongoing. There is therefore time to include an engineering solution that would  
462 maintain the gene flow observed in *I. boliviensis*, as well as other aquatic  
463 species. Additionally, aquatic units of conservation should be implemented.  
464 Protected as well as relatively undisturbed areas have been shown to have  
465 higher densities of river dolphins than unprotected areas (Gomez-Salazar et al.  
466 2012). Such units should preserve complexity, diversity and richness of existing



467 aquatic habitats upstream and downstream of the rapids, and limit or completely  
468 bar boat traffic. These conservation units would also maintain and serve as  
469 repositories of genetic diversity. Additionally, and perhaps most importantly the  
470 hydrological dynamic and the annual cycles of flooding and low water needs to  
471 be maintained since these cycles trigger migrations, and reproduction in fishes,  
472 and maintain ecological complexity and diversity (Junk et al. 1989).

473

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## Tables

**Table 1a.** Species level diagnostic characters observed in the Control Region. *Inia boliviensis* is diagnosable from *Inia geoffrensis* by 18 molecular synapomorphies. **1b.** In the mitochondrial cytochrome *b* gene, *I. boliviensis* is diagnosable from *I. geoffrensis* by 38 molecular synapomorphies. **1c.** In the cytochrome *c* oxidase I **DNA barcode region**, *I. boliviensis* is diagnosable from *I. geoffrensis* by 28 molecular synapomorphies. First line indicates position of the character within the cytochrome *b* gene, the control region and the cytochrome *c* oxidase I DNA barcode region.

Table 1a – Control region

	36	53	54	57	58	75	76	92	108	129	134	145	157	158	160
<i>I. geoffrensis</i>	C	A	G	G	T	C	C	G	T	A	T	T	C	C	C
<i>I. boliviensis</i>	T	G	A	A	C	T	A	A	C	G	C	C	T	T	T
	166	415	477												
<i>I. geoffrensis</i>	T	T	C												
<i>I. boliviensis</i>	C	G	T												

Table 1b – Cytochrome b

	45	51	120	138	198	201	244	258	291	306	365	375	574	576	585
<i>I. geoffrensis</i>	T	A	T	A	T	T	T	A	C	G	T	C	T	A	G
<i>I. boliviensis</i>	C	T	C	G	C	C	C	G	T	A	C	T	C	G	C
	589	654	657	690	702	724	771	792	807	813	819	825	873	891	909
<i>I. geoffrensis</i>	C	C	A	A	A	T	C	C	G	G	C	G	G/C	T	T
<i>I. boliviensis</i>	T	T	T	G	G	C	T	T	A	A	T	A	A	C	C

	924	985	1008	1047	1048	1080	1089	1113
<i>I. geoffrensis</i>	C	T	C	C	G	C	G	C
<i>I. boliviensis</i>	T	A/G	A	T	A	T	A	T

Table 1c – Cytochrome Oxidase I barcode region

	24	30	51	60	99	125	159	177	180	231	270	276	279	288	291
<i>I. geoffrensis</i>	A	C	A	C	C	A	T	C	A	G	G	T	A	T	G
<i>I. boliviensis</i>	G	T	G	T	T	G	C	T	G	A	A	C	G	C	A

	309	327	353	381	417	426	435	498	507	519	531	552	582
<i>I. geoffrensis</i>	C	T	T	G	A	G	A	T	A	A	T	A	T
<i>I. boliviensis</i>	T	C	C	A	G	A	G	C	G	G	C	G	A

## Figures

**Fig 1.** Distribution map of *Inia boliviensis* and *I. geoffrensis* in the Madeira River basin. Dashed line across locality 3 (Guajará/Borba) indicates the region of transition between the two species. Bars represents the two major waterfalls, Teotônio and Jirau, while black spots indicate other minor rapids. Sampling localities are: 1) Madeira River, mouth – AM; 2) Madeira River, mouth of Canumã River – AM; 3) Madeira River, cove at the mouth of Guajará creek, Borba – AM; 4) Madeira River, island in front of Novo Aripuanã – AM; 5) Madeira River, Uruá canal – AM; 6) Madeira River, cove upstream of Liberdade creek – AM; 7) Madeira River, cove at the mouth of Acará Lake – AM; 8) Madeira River, Paracanatuba cove – AM; 9) Madeira River, Três Casas cove – AM; 10) Madeira River, São Carlos community, mouth of Jamari River – AM; 11) Madeira River, Mirarí community – AM; 12) Madeira River, Belmonte Creek – RO; 13) Madeira River, downstream of Santo Antônio rapids – RO; 14) Mamoré River, mouth of Mercedes Lake – RO; 15) Guaporé River, mouth of Cautário River – RO; 16) Guaporé River, Porto Acre – RO; 17) Guaporé River, Porto França – RO; 18) Guaporé River, Contra Fiado – RO; 19) Guaporé River, Baía Grande – RO; 20) Solimões River, Mamirauá Sustainable Development Reserve – AM.

**Fig. 2.** Maximum-likelihood haplotype network of the 41 haplotypes observed in the 104 samples of *Inia boliviensis*. Size of each haplotype is correlated with the number of individuals carrying this haplotype. Colors/shades denote geographic distribution of these haplotypes.

**Fig. 3.** Bayesian estimate of gene flow between localities upstream and downstream of the rapids situated on the upper Madeira River. Gene flow ( $2Nm$ ) in the upstream direction is zero, while in the downstream direction is 7.24.

Fig 1.

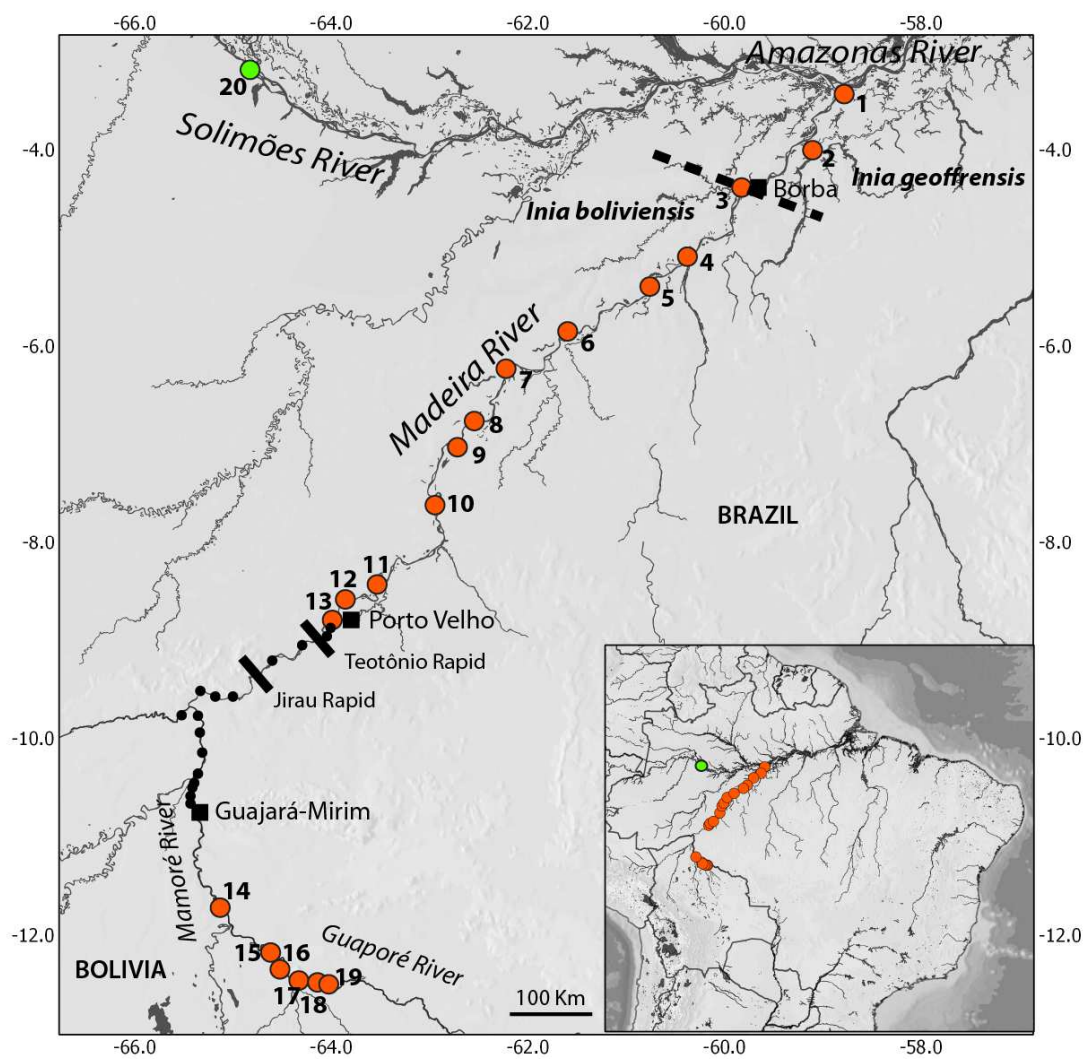


Fig 2.

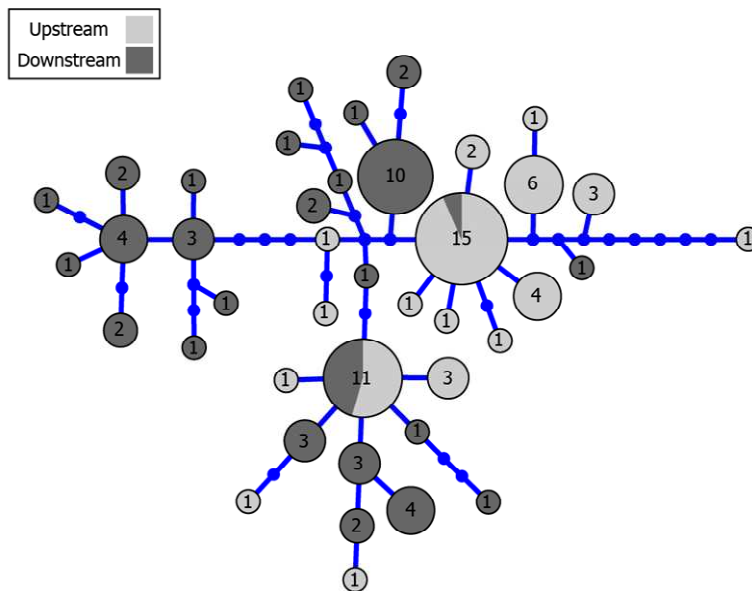
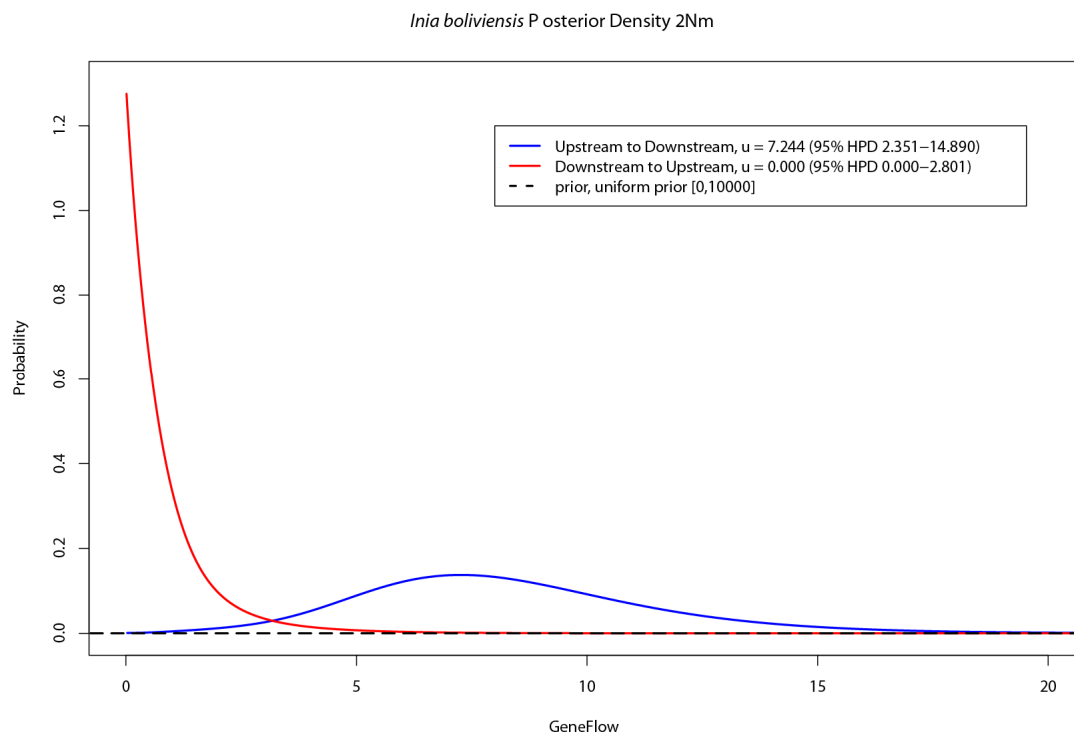


Fig 3.



## SÍNTESE

Os resultados obtidos nos três capítulos aqui apresentados revelam a estrutura das populações de boto que habitam os rios Guaporé, Mamoré e Madeira. O primeiro capítulo trata da distribuição dos botos da Bolívia e da Amazônia, *Inia boliviensis* e *I. geoffrensis*, ao longo do rio Madeira, área crítica para as duas espécies e que, até então, não havia estudos. Em adição às amostras utilizadas no primeiro capítulo, para o segundo capítulo, foram coletados animais na região entre corredeiras, onde observamos que a espécie de boto da Bolívia possui uma unidade biológica diferente e restrita a essas localidades. Também foi possível observar que os animais coletados abaixo da principal cachoeira do alto rio Madeira possuíam genoma mitocondrial pertencente à espécie da Bolívia, enquanto que o genoma nuclear era constituído principalmente de alelos pertencente à espécie da Amazônia. Já no terceiro capítulo, utilizando todo o banco de dados obtido, realizou-se uma abordagem comparativa entre os diferentes marcadores moleculares utilizados com o intuito de inferir quais eventos históricos poderiam ter influenciado na história biogeográfica das espécies.

Por meio da amostragem realizada, utilizando marcadores mitocondriais, verificou-se que as corredeiras do rio Madeira eram consideradas barreiras semi-permeáveis às espécies de boto, já que as mesmas restringiam, mas não impediam a passagem dos animais. A espécie da Bolívia não possui distribuição exclusiva nesses rios, ultrapassando as corredeiras e estendendo-se até as redondezas do município de Borba, localidade de Guajará, no baixo rio Madeira. Já o boto da Amazônia possui distribuição restrita à região abaixo do município de Borba, contradizendo estudos anteriores de que essa espécie ocorreria até a região abaixo das corredeiras.

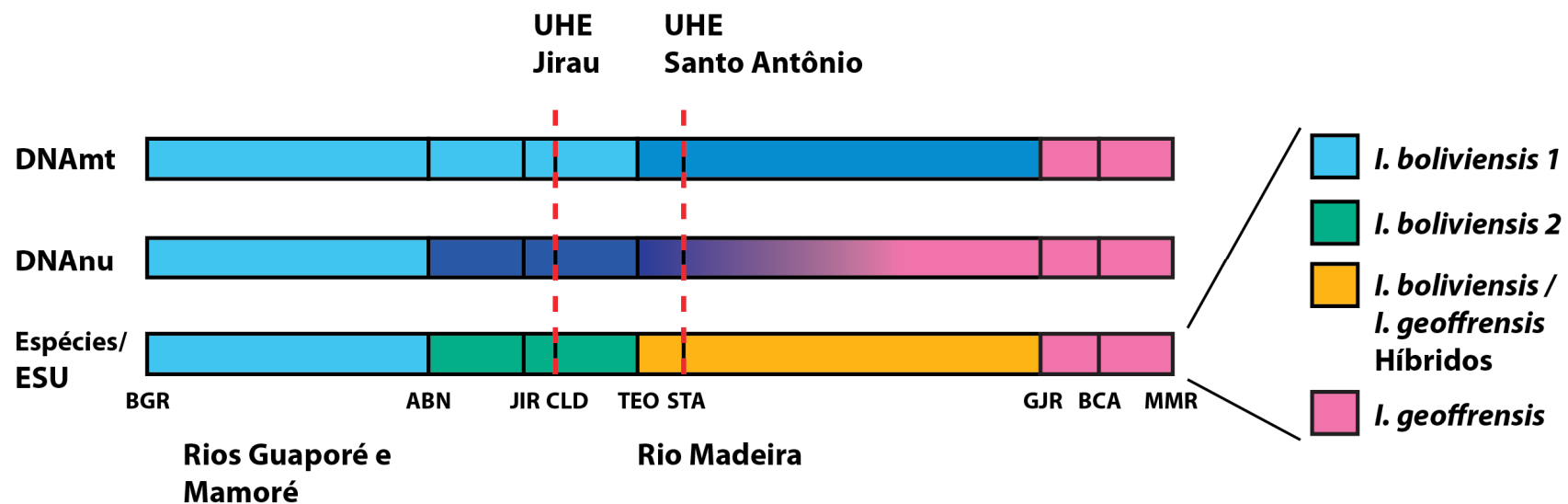
Foi detectada a existência de um grupo de animais entre as corredeiras que possui alelos exclusivos dessa região. Esses animais possuem uma sub-estruturação gênica, provavelmente devido ao baixo fluxo gênico entre as populações encontradas acima e abaixo das corredeiras. Apesar de esses indivíduos terem sido identificados como *I. boliviensis* através de marcadores mitocondriais e terem parte do genoma nuclear pertencente à essa espécie, foi identificado que esse grupo biológico pode ser considerado exclusivo dessa região, representando uma nova Unidade Evolutiva Significante (ESU).

Analisando os botos encontrados entre a cachoeira de Teotônio, a mais alta barreira entre as corredeiras, e a corredeira de Santo Antônio, foi observada uma discordância entre os resultados obtidos com marcadores mitocondriais e nucleares na maioria desses animais. Adicionalmente a esses animais, foram analisados todos os botos coletados, tanto acima quanto abaixo da cachoeira do Teotônio para compreendermos a estrutura genética desses animais.

Foi detectada a existência de uma zona híbrida entre as duas espécies de boto que ocorrem no rio Madeira. Em uma região de aproximadamente 870 Km foram observados animais com genoma mitocondrial pertencente à *I. boliviensis* e genoma nuclear de *I. geoffrensis*, com exceção de dois espécimes que possuíam, além do genoma mitocondrial do boto da Bolívia, a maior parte dos seus alelos também pertencente à essa espécie. A hibridização desses táxons é recente; no entanto, já se passaram algumas gerações desde o encontro entre os mesmos. Ainda não se pode estimar há quanto tempo as espécies entraram em contato, mas podemos afirmar que elas permaneceram separadas por algum tempo, e essa separação não se deu devido à formação das corredeiras como já foi sugerido, e sim devido à descontinuidade do rio durante a sua formação. Por isso, pode-se inferir que a ocupação do rio Madeira se deu inicialmente pela espécie de boto da Bolívia, e, posteriormente, a espécie de boto da Amazônia invadiu o território antes ocupado pelo boto boliviano, contrariando as hipóteses de trabalhos anteriores.



Sintetizando, foram obtidos diferentes resultados utilizando marcadores mitocondriais e nucleares (primeira e segunda barras coloridas da Figura 1, respectivamente). Foi possível observar a ocorrência de diferentes grupos biológicos ao longo dos rios estudados. Imaginando o rio de uma forma linear, sentido jusante, foi obtido como resultado que, até a ocorrência da primeira localidade entre corredeiras no rio Abunã (ABN), são observados animais da espécie *I. boliviensis* (área em azul na Figura 1, entre as localidades de Baía Grande – BGR e Abunã). Após as localidades onde são observadas corredeiras, até a extinta cachoeira de Teotônio (TEO), é observada uma população de *I. boliviensis* restrita às localidades entre corredeiras, que pode ser considerada uma Unidade Evolutiva Significante (ESU) (área em verde na Figura 1). A jusante da cachoeira de Teotônio até a localidade de Borba/Guajará (GJR), próxima ao município de Borba, é observada a zona híbrida entre as duas espécies de boto (parte laranja da figura 1). E somente a partir da localidade de Borba/Guajará, baixo rio Madeira, é possível observar a ocorrência de *I. Geoffrensis* (parte rosa da Figura 1).



\* as cores representadas na barra de espécies/ESUs são as mesmas dos pontos amostrais do capítulo 3 para melhor visualização das localidades onde cada grupo foi amostrado.

**Figura 1.** Barras representando os rios analisados de forma linear. Cada barra representa o resultado obtido, apenas com marcador mitocondrial (DNAmt), apenas com marcador nuclear (DNAnu) e utilizando os dois tipos de marcadores em conjunto (Espécies/ESU). As siglas abaixo das barras pretas indicam as localidades de Baía Grande (BGR), Abunã (ABN), cachoeira de Jirau (JIR), corredeira do Caldeirão (CLD), cachoeira do Teotônio (TEO), corredeira de Santo Antônio (STA), Borba/Guajará (GJR), confluência entre os rios Amazonas e Madeira (BCA), e a localidade do rio Solimões utilizada para comparação, Mamirauá (MMI). As linhas pontilhadas em vermelho indicam onde foram construídas as duas hidrelétricas, UHE Jirau e UHE Santo Antônio. As cores das barras ilustradas representam as espécies ou unidades evolutivas significantes (ESUs). Os três tons de azul representam a espécie *I. boliviensis*. O tom azul observado abaixo da cachoeira de Teotônio na barra que representa os resultados de DNAmt, indica a presença de haplótipos diferenciados das localidades a montante. O tom azul diferenciado nas localidades entre corredeiras observado nos resultados de DNAnu, indicam a presença de alelos exclusivos dessas localidades, representando uma ESU. Pode-se notar a existência de um gradiente da cor azul para a cor rosa na segunda barra ilustrada, indicando a mudança na constituição alélica dos animais encontrados a montante da cachoeira de Teotônio (TEO). Unindo os resultados obtidos nota-se a existência da cor verde que representa a ESU encontrada entre as corredeiras e a cor laranja que representa a zona híbrida entre as espécies. Em todas as barras nota-se que a cor rosa representa a espécie *I. geoffrensis*.

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