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Living between rapids: genetic structure and hybridization in botos (Cetacea: Iniidae: *Inia* spp.) of the Madeira River, Brazil

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Until the recent construction of hydroelectric dams, a series of 18 rapids divided the upper and lower Madeira River, and these rapids were thought to separate two species of Amazonian freshwater dolphins (boto): *Inia boliviensis* (above) and *I. geoffrensis* (below). Some reports and articles, however, mention the occurrence of botos within the rapids region and that they occasionally cross the rapids, but without mentioning the species concerned. Based on our previous studies, it is likely that *I. boliviensis* occurs in the region of the rapids. To test this supposition, we sampled 18 individuals from this region, and collected mitochondrial (control region, cytochrome *b* and cytochrome oxidase I) and nuclear (10 microsatellite loci) DNA data, in order to test if there is connectivity between the dolphins that were found within the rapids region and dolphins collected upstream and downstream of the rapids, and investigate population structuring between these localities. All animals in our study were molecularly identified using three mitochondrial markers as belonging to the species *I. boliviensis*. Animals upstream of the Teotônio waterfall, the main and highest waterfall of the region, had nuclear genome of *I. boliviensis*, while most dolphins downstream of the waterfall had nuclear genome of *I. geoffrensis*. *Inia boliviensis* collected in the rapids region above the Teotônio waterfall belong to a management unit (MU) distinct from the *I. boliviensis* MU occupying Bolivian rivers. Downstream of Teotônio waterfall most dolphins are *I. boliviensis*/*geoffrensis* hybrids, with remaining individuals being migrant *I. boliviensis*. *Biological Journal of the Linnean Society*, 2015, **114**, 764–777.

ADDITIONAL KEYWORDS: gene flow – management unit – river dolphin – species limits.

INTRODUCTION

The Amazon basin is the largest hydrographic basin of the world, covering approximately 6.8 million km^2 (Goulding, Barthem & Ferreira, 2003). This landscape presents many geographic barriers to the movement of aquatic vertebrates such as fishes (Lovejoy & de Araújo, 2000; Farias *et al*., 2010; Willis *et al*., 2012), reptiles (Pearse *et al*., 2006; Muniz, 2012) and aquatic mammals including the Amazonian manatee (da Silva, Rosas & Cantanhede, 2008), the freshwater dolphins, the boto (Best & da Silva, 1993; Gravena *et al*., 2014), and the tucuxi (da Silva & Best, 1994). These barriers are principally formed by waterfalls and rapids in rivers descending the Guyana and Brazilian shields such as the Negro, Branco, Uatumã, Jatapu, Nhamundá, Trombetas, Paru, Jari, Tocantins,

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Araguaia, Xingu, Tapajós, and Madeira (Goulding *et al*., 2003).

The Madeira River is the largest tributary of the Amazon River (Guyot, 1993). The Madeira River sub-basin represents 20.1% of the total area of the Amazon basin, and occupies an area of 1.4 million km^2 (Goulding *et al*., 2003). It originates in the Andean Piedmonts and the Brazilian Shield, being formed by the confluence of four major rivers, the Guaporé, Mamoré, Beni, and Madre de Dios (Goulding *et al*., 2003; Bourrel, Phillips & Moreau, 2009).

Between the municipalities of Guajará-Mirim (10° 47′ 33.48″S; 65°20′ 48.66″W), situated along the Mamoré River, and Porto Velho (8° 44′ 44.05″S; 63° 55′ 01.73″W) along the Madeira River, there were 18 rapids and waterfalls extending over a distance of 290 km (Cella-Ribeiro *et al*., 2013). Two of these rapids were formally designated as waterfalls due to accentuated differences in mean water surface elevation above and below the rapids (Cella-Ribeiro *et al*., 2013). The 900 m-wide Teotônio waterfall, the larger of the two, fell 30 m over a span of 600 m (Cella-Ribeiro *et al*., 2013). The Jirau waterfall, the second largest waterfall on the Madeira River, is upstream of the Teotônio waterfall; it was 730 m wide and spanned 1100 m (Cella-Ribeiro *et al*., 2013). Currently these and other eight rapids are submerged by the reservoirs of the Santo Antônio and Jirau hydroelectric dams, respectively. The two hydroelectric dams are still in the final stages of construction, however, both have already closed their gates in 2012 and 2013 to begin filling their respective reservoirs.

Prior to the filling of the two reservoirs, Gravena *et al*. (2014) carried out surveys along the Guaporé, Mamoré, and Madeira Rivers to ascertain the distribution of the two species of boto, *I. boliviensis* and *I. geoffrensis*, upstream and downstream of the 290 km region of rapids. Previously to the study of Gravena *et al*. (2014), it was thought that *I. boliviensis* occured only upstream of the rapids in the Bolivian portion of the Madeira River sub-basin (Pilleri & Gihr, 1977; Banguera-Hinestroza *et al*., 2002; Tavera *et al*., 2010). However, all animals occurring upstream as well as downstream of the rapids until near the mouth of the Madeira River were identified, using mitochondrial DNA, as *I. boliviensis* (Gravena *et al*., 2014). The upstream and downstream populations of *I. boliviensis* were genetically differentiated, and were connected by unidirectional gene flow in the downstream direction (Gravena *et al*., 2014). The relationship and genetic connectivity of the botos from the rapids region to the upstream and downstream populations of *I. boliviensis* remained unclear due to lack of samples. However, it was thought that only *I. boliviensis* occurred in these localities (Best & da Silva, 1993; Tavera *et al*., 2010). In late 2012 we were able to survey the region of rapids, obtaining samples for molecular analyses. These samples as well as those originating upstream and downstream of the rapids were analyzed using mitochondrial DNA markers (Gravena *et al*., 2014) and nuclear microsatellite markers (Caldwell, Gaines & Hughes, 2002; Rosel, Forgetta & Dewar, 2005; Gravena *et al*., 2009). Using these samples, this study aims to conduct a genetic characterization of the group of botos from the rapids region. With these new data, it is also now possible to access the population structure and test the connectivity between the upstream, downstream and rapids-region groups. Our main goal is to provide a first insight into the natural patterns of genetic structuring among these groups.

MATERIAL AND METHODS

Sample collection, DNA extraction and amplification of fragments

We collected samples of epithelial tissue from 18 individuals originating from six localities between the mouth of the Abunã River and the city of Porto Velho (Fig. 1), all localities were within the 290 km stretch of rapids. All samples were obtained before the closure of the dams. The sampling sites represent localities 4, 5, 6, 7, 8 and 9 shown in Figure 1. Two groups of localities were created in order to test if the Teotônio waterfall was a barrier between putative populations of boto. Localities 4–6, upstream of Jirau waterfall, and locality 7, between Jirau and Teotônio waterfalls were analyzed as one group (from herein called Rapids group 1 – RP1). Localities 8–9, downstream of Teotônio waterfall and upstream of Santo Antônio rapids were also grouped (from herein called Rapids group 2 – RP2), as shown in Figure 1.

In addition to these samples we also analyzed 16 samples of *I. boliviensis* from the Mamoré River, two localities upstream the first of the 18 rapids (from herein called the Upstream group – UPS), and 19 samples of *I. boliviensis* from downstream of Santo Antônio rapids from the vicinity of Porto Velho (from herein called the Downstream group – DWS). Further samples included 13 individuals of *I. geoffrensis* used as reference specimens, from the Mamirauá Sustainable Development Reserve (MSDR), Solimões River (from herein called the Mamirauá group – MMI).

All animals were captured using the methodology described in da Silva & Martin (2000) with minor modifications. The protocol for handling and removing small quantities of cutaneous tissue samples from the caudal fluke from live animals was approved by the Committee on the Ethics of Animal Use (Comissão de Ética do Uso de Animais – CEUA) of the National

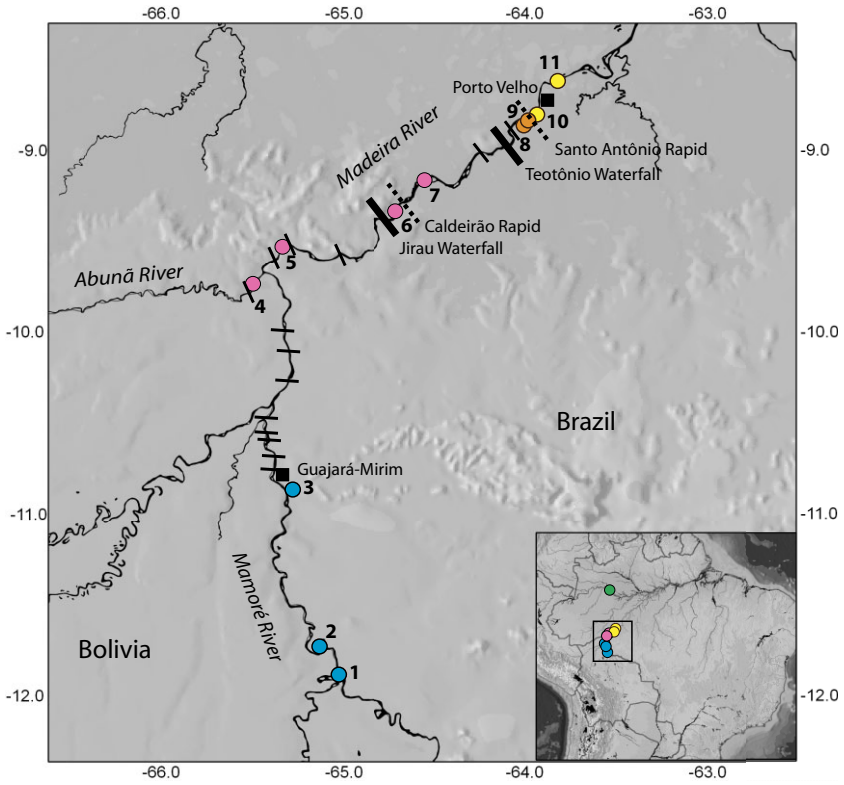

Figure 1. Map of the upper portion of the Madeira River, and Mamoré River showing the sampling localities. Blue points indicate localities in the Mamoré River upstream of all the rapids, named as the Upstream group (1 – Supresa; 2 – Mercedes; and 3 – Pakaás); pink points indicate localities within the rapids region above the Teotônio waterfall, named as Rapids1 group (4 – Fortaleza do Abunã; 5 – Tamborete; 6 – Jirau; and 7 – Búfalo Island); orange points represent localities below the Teotônio waterfall, comprising the Rapids2 group (8 – below Teotônio waterfall; and 9 – Santo Antônio); and yellow points represent the Downstream group localities (10 – Porto Velho; and 11 – Belmonte). Green point on the insert represent the location of the Mamirauá Sustainable Development Reserve. Waterfalls are indicated by full bars, and rapids across which were constructed the two hydroelectric dams are indicated by dashed bars. The other small rapids are indicated by thin black bars. Numbers on axes represent decimal degrees with negative values representing latitudes south of the equator and longitudes west of the prime meridian.

Research Institute of the Amazon (INPA), and collecting permits were provided by IBAMA/ICMBIO (no. 11325-1 and no. 13462-1). The epithelial tissue samples were stored in cryogenic tubes filled with 95% ethanol. All samples were deposited in the CTGA/UFAM tissue collection.

DNA extraction was performed using the phenol/chloroform protocol (Sambrook, Fritsch & Maniatis, 1989). Following extraction we amplified and sequenced three mitochondrial DNA regions–control region (CR), cytochrome *b* (Cytb), and cytochrome *c* oxidase subunit I (COI)–using the protocols reported in Gravena *et al*. (2014). Sequences were verified and aligned using the ClustalW tool (Thompson, Higgins & Gibson, 1996) assuming default parameters in the program Geneious v5.6.3 (Drummond *et al*., 2012). The sequences from the 18 new individuals used in the analyses were deposited in GenBank (Cytb: KP141819–KP141836, CR: KP141837–KP141854, COI: KP141801–KP141818).

We also amplified ten nuclear microsatellite loci; seven developed by Gravena *et al*. (2009) for *I. geoffrensis* (Ig2B1, Ig3A1, Ig11B1, Ig10E, Ig8H1, Ig7F2 and Ig11D2) and three additional markers (TtrAAT40, Ttr11 and Ttr48) developed for *Tursiops truncatus* (Caldwell *et al*., 2002; Rosel *et al*., 2005). Microsatellite loci were amplified using the PCR conditions reported in Gravena *et al*. (2009) and labelled with fluorescent dye using the methodology of Schuelke (2000). PCR reaction consisted of 25 cycles of denaturation at 93 °C for 60 s, primer annealing at the specific primer temperature for 30 s, and primer extension at 72 °C for 90 s, followed by 15 cycles of denaturation at 93 °C for 60 s, primer annealing 53 °C for 30 s, and primer extension at 72 °C for 90 s, with a final extension at 72 °C for 30 min.

Heterologous primers were standardized to the annealing temperature of 58 °C. All the PCRs were repeated at least twice, to ensure that the alleles were correctly called. PCR products were genotyped in an automatic ABI 3130xl sequencer.

Fragment analysis was performed in the software GeneMapper. Allele sizes were inferred using the pUC19 ROX-labelled size standard (DeWoody *et al*., 2004). Subsequently a matrix of genotypes for each individual was generated.

Analyses

Mitochondrial DNA

In a previous study, Gravena *et al*. (2014) showed that all three mitochondrial genes contain diagnostic sites that may be used to unambiguously assign individuals to either *I. boliviensis* or *I. geoffrensis*. The samples analyzed in this study were thus assigned to their respective species based on those diagnostic sites.

Phylogenetic relationships between haplotypes were inferred using the maximum likelihood criterion implemented in the program PHYLIP (phylogeny inference package) (Felsenstein, 1993) under the HKY85 model of molecular evolution (Hasegawa, Kishino & Yano, 1985), as inferred using jModelTest (Posada, 2008); model selection was done using the AIC. Haplotype relationships were visualized using the program HaploViewer (Salzburger, Ewing & Von Haeseler, 2011).

To measure gene flow between areas, we used the isolation-with-migration framework implemented in the program IMa2 (Hey & Nielsen, 2007). Calculations of gene flow were performed using three areas (UPS, RP1 and DWS which included RP2). We ran 50 chains with dynamic heating, collecting 100 000 from 10 000 000 generated topologies. Topologies were collected after an initial burn-in period of 2 000 000 topologies, when parameter estimates had stabilized. Convergence of parameter estimates was inferred from the effective sample size (ESS) of the parameters, from analyzing trendline plots, and from comparing parameter estimates based on the first half and second half of the MCMC run. The parameters obtained by IMa2 were converted to gene flow, using the substitution rate of 1.26×10^{-8} for the CR, and 1.03×10^{-8} for cytb and COI genes (Pesole *et al*., 1999), and generation time of 10 years (Taylor *et al*., 2007).

Microsatellite DNA

Basic characterization of the microsatellite loci was performed in the program Arlequin 3.5.2.1 (Excoffier & Lischer, 2010). We also tested for differentiation among localities using hierarchical *F*-statistics (Wright, 1951). For all analyses hierarchical grouping was used mainly to verify if all the rapids were barriers or, if the Teotônio waterfall was the principal barrier. Samples were separated in the same five groups used in the mitochondrial analyses (UPS, RP1, RP2, DWS and MMI).

To measure gene flow using the microsatellite data we also used the isolation-with-migration framework implemented in the program IMa2 (Hey & Nielsen, 2007) using the stepwise mutation model (Kimura & Ohta, 1978). These calculations were performed using the same three areas analyzed for mitochondrial DNA. The parameters obtained by IMa2 were converted to gene flow, using the substitution rate of 2.05×10^{-4} substitutions per generation (Rooney *et al*., 1999), and generation time of 10 years (Taylor *et al*., 2007).

The existence of distinct reproductive groups is a necessary pre-requisite for the diagnosis of biological species *sensu* Mayr (1942). This and the phylogenetic species concept (Cracraft, 1983, 1989) are the two primary concepts adopted by cetacean taxonomists (Reeves *et al*., 2004). Therefore, with the objective to infer the most likely number of biological groups existing within our sample, we used the program STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000) to generate posterior probabilities for different number of groups using the 'admixture' and 'correlated-allelic-frequencies' models. We explored the possibility of our sample containing from one to six biological groups. Assignment space was explored with 1 000 000 MCMC steps, preceded by 100 000 MCMC steps discarded as burn-in. Each analysis was repeated 20 times from a different randomly selected starting point, and convergence between independent runs was assessed via examination of α values and profile of posterior probabilities. The Q values from each of the 20 independent runs for each K scenario were extracted using the program STRUCTURE HARVESTER 0.6.92 (Earl & VonHoldt, 2011) and summarized in the program CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007). Results were visualized in the program DISTRUCT 1.1 (Rosenberg, 2003). The most likely number of biological groups (K) was inferred using the method of Evanno *et al*. (Evanno, Regnaut, & Goudet, 2005).

Reducing the dimensionality of the microsatellite data, we performed a principal component analysis (PCA) using the ADE4 package (Thioulouse *et al*., 1997) in R 2.14.1 (R Development Core Team, 2011) to see if the results matched the number of populations or geographic groups obtained from the STRUCTURE program. The first and second principal components were then plotted against each other, and the disper-

sion of the PCAs within each sample group was delimited by an ellipse encompassing a 67.53% density contour.

Additionally, we inferred the ancestry and geographic origin of each individual sampled from the rapids region in the program BayesAss 3.0 (Wilson & Rannala, 2003). Analyses were carried out assuming the four geographic groups used previously. Each MCMC chain had 5 000 000 steps, with 10 000 discarded as burn-in during posterior analyses. Additionally we obtained estimates of bi-directional migration rates between the geographic groups and inbreeding coefficients (F_{IS}) *sensu* Wright (1951), in BayesAss (Wilson & Rannala, 2003). F_{IS} represents an average probability that any particular individual has two copies of an allele that is identical-by-descent at any particular locus, i.e. an average probability of expected homozygosity within each group.

RESULTS

Mitochondrial DNA

We analyzed 2382 bp of combined sequence data (Cytb 1241 bp, CR 621 bp, and COI 520 bp) for each of the 66 individuals (Mamoré River $N = 16$, region between the rapids $N = 18$, Madeira River below the rapids $N = 19$ and Mamirauá Reserve $N = 13$). Using the diagnosable sites described in Gravena *et al.* (2014), all individuals from the Mamoré (localities 1, 2 and 3) and Madeira Rivers (localities 10 to 11) including all individuals from between the rapids region (localities 4 to 9), when compared with individuals from the Mamirauá Reserve, were diagnosed as *I. boliviensis*. All subsequent analyses of mtDNA data focused on individuals from the Madeira River system.

Among the 53 analyzed individuals of *I. boliviensis* we observed 14 haplotypes (Fig. 2); six haplotypes were singletons, and of these, three were exclusive to the region from within the rapids and three to the region downstream all the rapids. All four regions shared only one common haplotype (Fig. 2).

Between the Mamoré River and the rapids region, modal gene flow was 14.92 (95% HPD 0.00–1792.00) effective individuals per generation in the downstream direction and 0.00 (95% HPD 0.00–52.47) effective individuals in the upstream direction. Between the rapids and the downstream localities, gene flow was 64.34 (95% HPD 0.00–2236.00) effective individuals per generation in the downstream direction and 1.66 (95% HPD 0.00–2031.00) effective individuals in the upstream direction. Gene flow was effectively unidirectional in the downstream sense (Fig. 3).

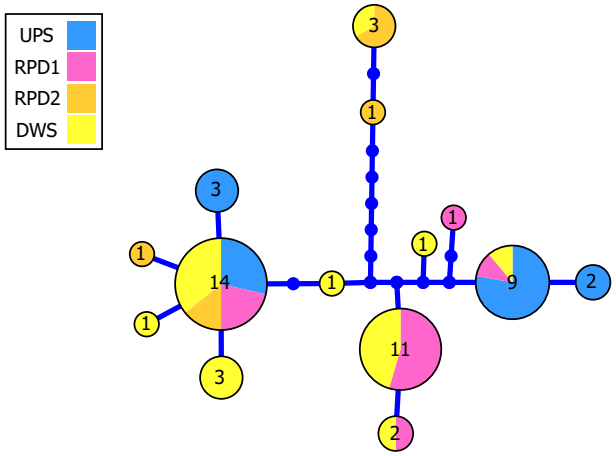

Figure 2. Network of haplotypes sampled from *Inia* individuals from upstream (UPS – blue), Rapids1 (RPD1 – pink), Rapids2 (RPD2 – light orange) and downstream (DWS – yellow) rapids. Numbers within each circle correspond to the number of individuals possessing the correspondent haplotype.

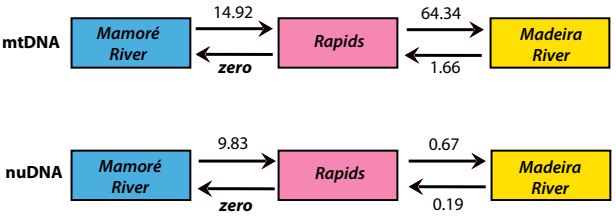

Figure 3. Schematic representation of the gene flow estimated in the program IMa2 using mitochondrial (mtDNA) and microsatellite (nuDNA) markers. Arrows indicate direction of gene flow, and colours represent localities as indicated in Figure 1 (UPS, RP1, and DWS which included RP2).

Microsatellite DNA

We genotyped all 66 samples for 10 microsatellite loci. None of the loci in none of the analyzed groups showed linkage disequilibrium with another locus, and all loci were at Hardy-Weinberg equilibrium.

The most likely number of biological groups in the complete dataset inferred in the program STUCTURE 2.3.4 using the methodology of Evanno, Regnaut & Goudet (2005) was two ($\ln \Pr(X|K = 2) = -1211.075$). These biological groups correspond to the two species, *I. boliviensis* and *I. geoffrensis*. Analysis of each species separately in STRUCTURE resulted in the identification of two biological groups of *I. boliviensis* ($\ln \Pr(X|K = 2) = -356.165$), while only one biological group was identified in *I. geoffrensis* ($\ln \Pr(X|K = 1) = -815.930$). The biological groups are represented by the colours blue, pink and yellow in Figure 4. The blue and pink groups represent the two

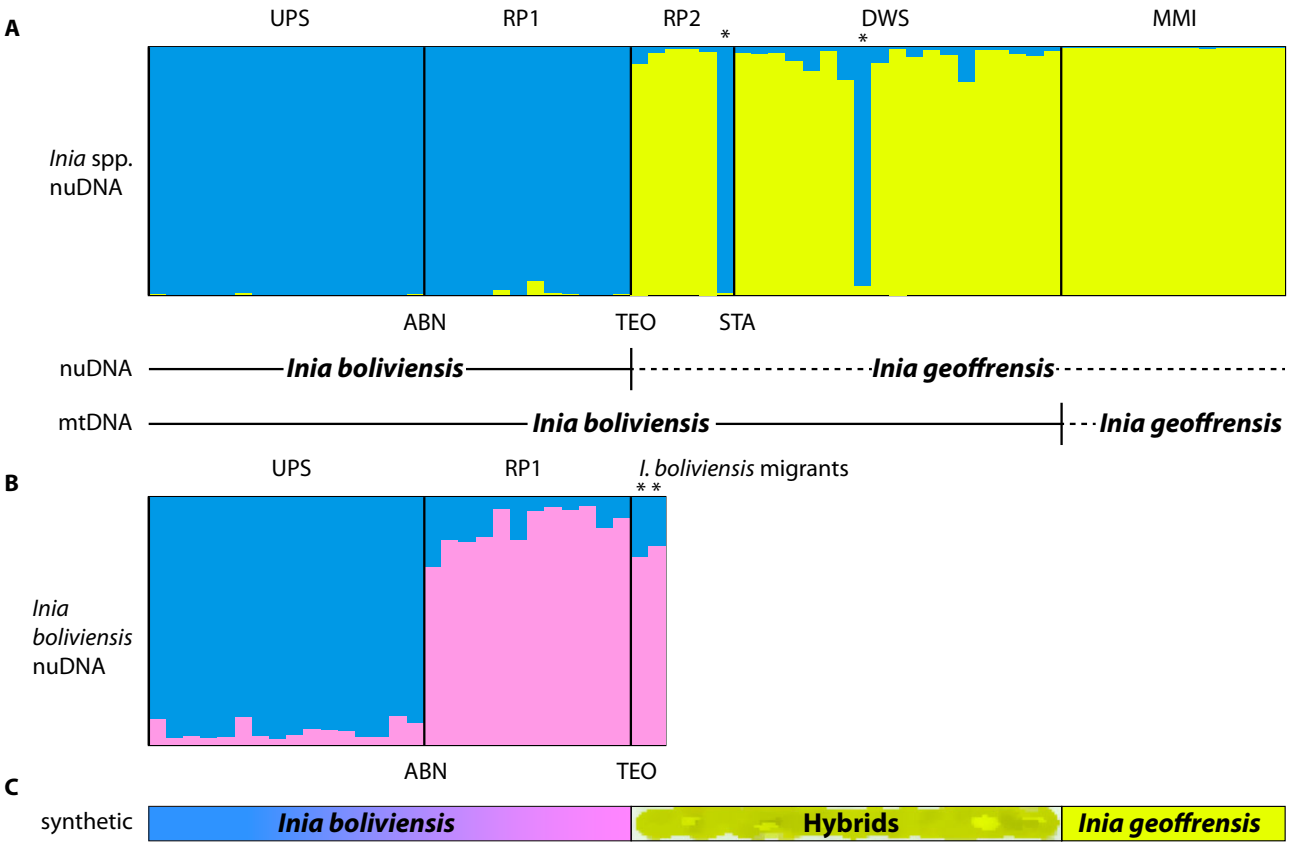

Figure 4. A, Graph of genetic ancestry of each individual generated in the program STRUCTURE, using all the five groups. Two distinct nuclear genomes represented by the two colours can be observed: (1) blue representing the species *I. boliviensis*; and (2) yellow representing the genome of the species *I. geoffrensis*. The black thin bars represent the main rapids and waterfalls, Abunã rapids (ABN), Teotônio waterfall (TEO), and Santo Antônio rapids (STA). The * show the two individuals that biologically belong to the between rapids group. The lines below the graph represent: individuals identification based only in results obtained by microsatellite markers (nuDNA), and individuals identification based only in mitochondrial markers (mtDNA). B, Graph of genetic ancestry of each individual generated in the program STRUCTURE, using only individuals classified as *I. boliviensis* using nuclear markers. Again, two distinct nuclear genomes represented by different colours can be observed: (1) blue and (2) pink, representing the two management units of *I. boliviensis.* And C, Species identity based on both mitochondrial and nuclear markers.

biological populations of the species *I. boliviensis*, while the yellow group represents the species *I. geoffrensis*.

The pattern and direction of gene flow observed in the microsatellite data reflected that of the mitochondrial DNA data. Between the Mamoré River and the rapids region, modal gene flow was 9.83 (95% HPD 0.00–254.91) effective individuals per generation in the downstream direction and 0.00 (95% HPD 0.00–188.87) effective individuals in the upstream direction. Between the rapids and the downstream localities, gene flow was 0.67 (95% HPD 0.00–141.87) effective individuals per generation in the downstream direction and 0.19 (95% HPD 0.00–73.82) effective individuals in the upstream direction (Fig. 3).

Using the two principal components that explained the largest amount of variation in our data, we observed that individuals clustered into three groups. The first principal component separated animals upstream of the Teotônio waterfall (localities 1 to 7) from those downstream of Teotônio waterfall (localities 8 to 12), while the second principal component separated animals from the Bolivian sub-basin, the UPS group (localities 1 to 3) from the RP1 group (localities 4 to 7). The three groups observed (Fig. 5) correspond to the three biological groups (BG) identified by the program STRUCTURE, and are composed of these individuals: BG1 – individuals from upstream the rapids (*I. boliviensis*) called UPS group (localities 1, 2 and 3); BG2 – individuals between the rapids region, but upstream of

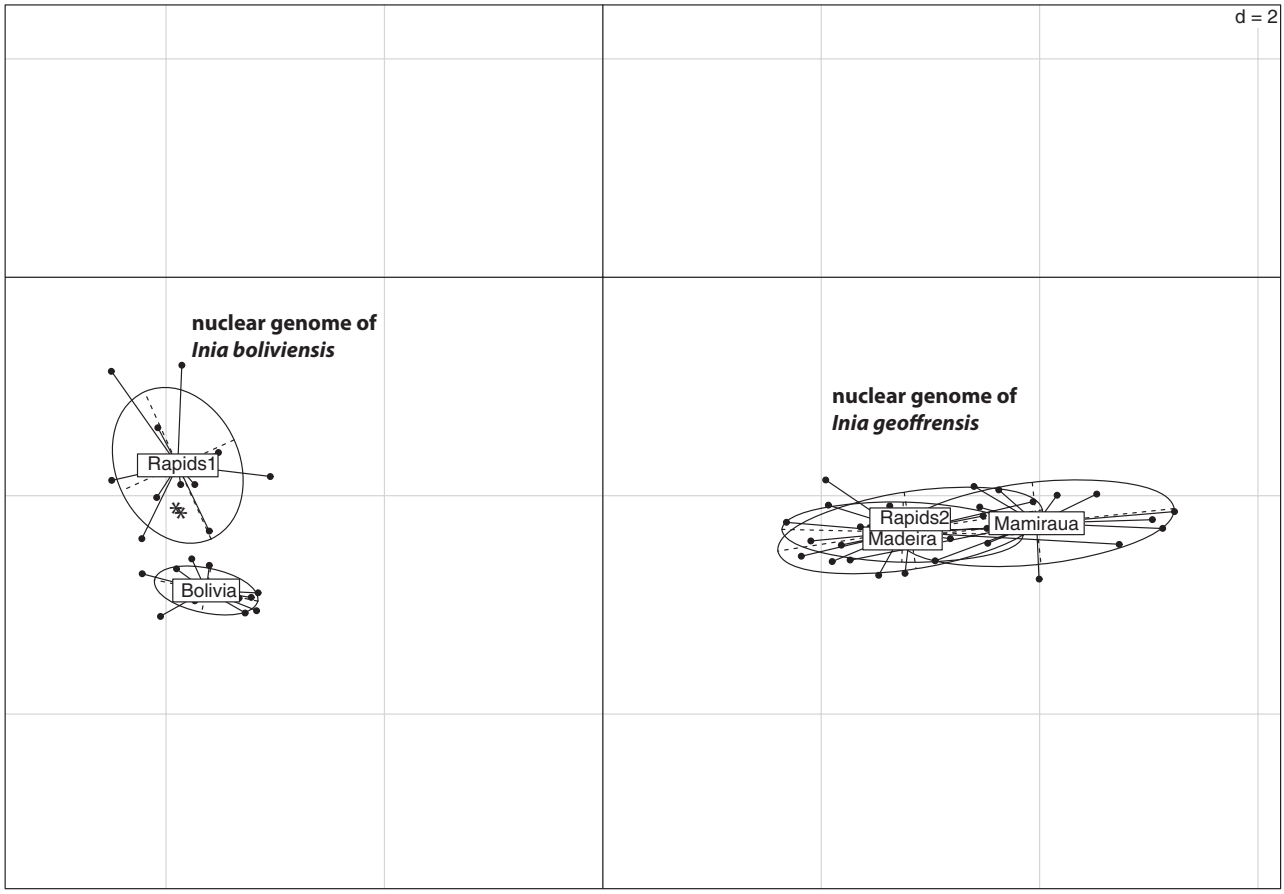

Figure 5. Graph of the first and second principal component of each individual based on the principal component analysis of the microsatellite data. Three evident clusters are representing individuals from upstream and between the rapids groups (two *I. boliviensis* clusters, separated by the second principal component) and individuals from downstream of the Teotônio waterfall group (*I. geoffrensis* genome, separated from *I. boliviensis* by the first principal component). Two individuals, indicated by asterisks, physically present below the Teotônio waterfall belong biologically to the between rapids group.

Teotônio waterfall (*I. boliviensis*) called RP1 group (localities 4 to 7); and BG3 – individuals between the Teotônio waterfall and the Santo Antônio rapids called RP2 (localities 8 and 9), individuals from downstream of all the rapids, DWS group (localities 10 and 11), considered *I. boliviensis* × *I. geoffrensis* hybrids (Fig. 4b), and individuals from Mamirauá Reserve, MMI group (locality 12). Two individuals sampled below Teotônio waterfall (BG3) were genetically assigned to the between rapids group (BG2) (Fig. 5), and are most likely first generation migrants. They are the same two individuals marked with an asterisk in the STRUCTURE graph, which exhibited larger proportion of their genotypes assigned to the pink and blue groups, instead of the yellow group (Fig. 4a).

Individuals collected upstream of all rapids (localities 1, 2 and 3) can be considered a biologically pure group ($q > 0.95$ of belonging to the blue group) exclusively composed of *I. boliviensis*. Individuals between the rapids and upstream Teotônio waterfall (BG2) show various degrees of admixture (20–80% of belonging to the blue group) between the biological group found upstream of the rapids and the resident (the pink) group, that is probably restricted to the between rapids localities. Individuals downstream of Teotônio waterfall (BG3) can be considered biologically pure *I. geoffrensis* ($q > 0.95$ of belonging to the yellow group) or predominantly *I. geoffrensis* ($q > 0.80$ of belonging to the yellow group) with the exception of two individuals which are pure *I. boliviensis* (* in Fig. 4A).

Current migration rates estimated using microsatellite data in the program BayesAss 3 are reported in Table 1. Highest migration rates are observed within each group, but also relatively high migration rates can be observed between the region upstream of all the rapids (UPS), and the rapids region upstream

Table 1. Migration rates obtained in the program BayesAss for the four hierarchical groups analyzed. Values in bold are migration rates within each group. All values were significant after Bonferroni correction

	Receiver				
		UPS	RP1	RP2	DWS
Donor	UPS	**0.9495**	0.2797	0.0560	0.0294
	RP1	0.0167	**0.6883**	0.0243	0.0193
	RP2	0.0169	0.0160	**0.7038**	0.0251
	DWS	0.0169	0.0159	0.2159	**0.9263**

of Teotônio waterfall (RP1), and between the downstream region (DWS) and rapids region downstream of Teotônio (RP2).

Of the 53 analyzed individuals we were able to determine with high probability the likely geographic origin of 52 individuals. Most individuals had nearly 100% probability of belonging to the group from which they were sampled. An exception was individual 171 from Porto Velho which had high posterior probabilities of belonging to the population between Teotônio and Santo Antônio (0.549), but also had a high proportion of its genotype attributed to the region of rapids between Abunã and Teotônio (0.278) and downstream all the rapids (0.165).

For the animals collected upstream and downstream all the rapids, all but one were non-migrant individuals originating in the groups in which they were collected. One individual collected downstream of all the rapids (one of the * in Fig. 4A), however, had a higher probability of being a first generation migrant from the group upstream of all the rapids (UPS), rather than being a resident of the group DWS. This individual probably belongs to the group between the rapids (RP1), but passed the rapids region, and now can be found downstream of the rapids.

All individuals collected from within the rapids region upstream of Teotônio waterfall (RP1) had highest probabilities of having a migrant parent from the group upstream of them (UPS), while individuals from the rapids region between Teotônio and Santo Antônio (RP2) had highest probabilities of having a migrant parent from the group downstream the rapids (DWS).

Inbreeding coefficients (F_{IS}) observed in the four groups, indicated relatively low levels of inbreeding from the groups upstream and downstream of all the rapids (0.1989 and 0.0390, respectively), compared with higher F_{IS} values observed within the rapids, 0.3317 (RP1) and 0.3479 (RP2), upstream and downstream of Teotônio waterfall, respectively.

DISCUSSION

Gravena *et al*. (2014) already suggested that the botos occurring in the region of the rapids probably are *I. boliviensis*, given that based on mtDNA identification, this species occurs throughout almost the entire Madeira River, reaching the city of Borba, a mere 165 km upstream of its confluence with the Amazonas River. Analyzing individuals from the focal region of the rapids, we can confirm this expectation with the mtDNA data, since all of the samples were identified as *I. boliviensis*. Gene flow follows a stepping stone model, and is effectively unidirectional from the upstream to the downstream localities.

The nuclear microsatellite loci, however, revealed a different story, both quantitatively as well as qualitatively. Analyses in the program STRUCTURE and using a PCA revealed three BGs, two corresponding to *I. boliviensis*, and one to *I. geoffrensis*. One of the BGs of *I. boliviensis* occupies the area upstream of the rapids, above the city of Guajará-Mirim, while the second, occupies the region within the rapids, between Fortaleza do Abunã and Teotônio waterfall. The observed differentiation at the microsatellite loci between the two *I. boliviensis* groups is much greater than the differentiation observed in mtDNA, but again follows a pattern of unidirectional downstream gene flow (Fig. 3). The downstream region is occupied by individuals with the nuclear genomes that are predominantly *I. geoffrensis*. Two of the dolphins sampled from downstream of the Teotônio waterfall (marked with an asterisk in Fig. 4A) had nearly 100% *I. boliviensis* genomes, while many of the other animals had at least a small contribution from *I. boliviensis* (0–20%).

Thus, in the studied section of the Madeira River we encountered *I. boliviensis* upstream and in the region of the rapids, while *I. boliviensis* × *I. geoffrensis* hybrids were found below the rapids (Fig. 4C). *Inia boliviensis* can further be divided into two distinct management units (MU) based both on mitochondrial and nuclear markers (Moritz, 1994).

HYBRIDIZATION

The actual division between the individuals with *I. boliviensis* and *I. geoffrensis* nuclear genomes corresponds to the Teotônio waterfall. Five of the six animals collected between the Teotônio waterfall and the Santo Antônio rapids (RP2 group), have the *I. geoffrensis* genome, and show no isolation from the individuals below the Santo Antônio rapids. The Teotônio waterfall thus separates the nuclear genomes of *I. boliviensis* (upstream) and *I. geoffrensis* (downstream). However, this barrier is partially porous allowing low levels of unidirectional (upstream

to downstream) connectivity. This result confirms the assumption of Pilleri & Gihr (1977), da Silva (1994) and Tavera *et al*. (2010) that the Teotônio waterfall was the principal barrier that delimited the distribution of *I. boliviensis* and *I. geoffrensis* in the Madeira River. Before the recent dam construction projects, the Teôtonio waterfall was the highest and the narrowest waterfall/rapid in the upper Madeira River basin (Cella-Ribeiro *et al*., 2013). Furthermore, the river channel was deep, the water flowed rapidly over a rocky surface and the site was devoid of a floodplain. These characteristics likely presented a major barrier to the movement and dispersal of botos and other aquatic organisms.

Below the Teotônio waterfall, all botos, from upstream and downstream of the Santo Antônio rapids, belong to the same biological group. This group is characterized by a mtDNA genome of *I. boliviensis*, and an admixed nuDNA genome between *I. boliviensis* and *I. geoffrensis*, with the *I. geoffrensis* genome predominating. Therefore, these individuals are hybrids. There is strong population structuring in the mtDNA genome upstream and downstream of the rapids (Gravena *et al*., 2014) and specifically between the groups upstream and downstream of the Teotônio waterfall. Although there are some individuals that were classified based on nuDNA as pure *I. geoffrensis*, there are many admixed individuals with up to 20% of their genome belonging to *I. boliviensis*, and there are two individuals whose nuclear genome is 100% *I. boliviensis*. Above the Teotônio waterfall there is no evidence of an introgressed *I. geoffrensis* genome in the local *I. boliviensis* group. Thus, similar to the pattern of connectivity observed between the two groups of *I. boliviensis*, we observe first generation migrants of *I. boliviensis* in the downstream group, as well as gene flow into the downstream group, while there is no gene flow in the upstream direction.

Hybrid zones may occur in geographic areas where two related species meet and reproduce, producing viable offspring (Barton & Hewitt, 1985, 1989). Related cetaceans that occur in sympatry in some localities have already been recorded to produce hybrids, as registered with fin and blue whales (*Balaenoptera physalus* and *B. musculus*) (Bérubé & Aguilar, 1998); with belugas and narwhals (*Delphinapterus leucas* and *Monodon monocerus*) (Heide-Jorgensen & Reeves, 1993); Risso's dolphins and bottlenose dolphins (*Grampus griseus* and *Tursiops truncatus*) (Sylvestre & Tasaka, 1985); Dall's and Harbour porpoises, (*Phocoenoides dalli* and *P. phocoena*) (Willis *et al*., 2004); and between two species of pilot whales (*Globicephala melas* and *G. macrorhynchus*) (Miralles *et al*., 2013).

The two species of boto, at some point during their evolutionary history were probably allopatric, but now appear to be sympatric in part of their ranges. The low gene flow between extant populations upstream and downstream of the rapids results in a gradient of decreasing quantity of nuclear alleles of *I. boliviensis* downstream of the rapids. While in some individuals the contribution of the *I. boliviensis* genome is up to 20%, the majority of individuals has less than 10% of *I. boliviensis* nuDNA contribution.

With the exception of the two first generation migrants of *I. boliviensis*, all botos analyzed from downstream of the Teotônio waterfall are hybrids probably originated by secondary admixture of the two different lineages of *Inia*. All of these individuals have mtDNA genomes of *I. boliviensis*, with all or the majority of their nuDNA genome being *I. geoffrensis*, although some individuals also have a significant contribution of *I. boliviensis* genome. The region below Teotônio rapids can therefore be classified as a hybrid zone (Barton & Hewitt, 1985). This hybrid zone will likely extend until the city of Borba (Gravena *et al*., 2014), 890 km downstream of the Teotônio waterfall, where the substitution of the *I. boliviensis* mtDNA genome for the *I. geoffrensis* mtDNA genome was observed. As all individuals between Teotônio and the Borba locality have mtDNA genome of *I. boliviensis*, these same individuals probably have complete or at least partial *I. geoffrensis* nuDNA genome, and thus are likely hybrids. This hybrid zone could be, therefore, quite extensive, but further studies are necessary to verify this claim.

Hybrid zones, though potentially more narrow, have already been observed in other aquatic species in the region of the upper Madeira River. A similar pattern to that observed in the boto, has been reported in two species of the cichlid fish genus *Cichla* (Willis *et al*., 2012). The fish upstream and downstream of the rapids region show distinct and divergent mitochondrial haplotypes, and are considered *Cichla pleiozona* upstream, and *C. monoculus* downstream. Based on an analysis of microsatellite markers, the same nuclear genome occurs in both cichlid species occurring above and below the waterfalls (Willis *et al*., 2012). In frogs of the genus *Allobates*, the region of the Jirau waterfall on the left bank of the river was a zone of contact between two morphologically similar species (Simões *et al*., 2008); two divergent mitochondrial lineages of the frogs were found in the same geographic locality, and the nuclear DNA of these individuals showed different levels of admixture in their nuclear genomes, consistent with them being F2 hybrids (Simões, Lima & Farias, 2012). The upper Madeira River is also a zone of contact between two species of crocodilians, *Caiman crocodilus* and *C. yacare*; the individuals of

the two phenotypes have different combinations of nuclear and mitochondrial genomes (Hrbek *et al*., 2008). A zone of transition between mitochondrial lineages of *Paleosuchus palpebrosus*, another crocodilian species, has also been observed in the upper region of the rapids near the city of Guajará-Mirim (Muniz, 2012).

Hybridization is asymmetrical in the botos, with either the mtDNA genome of *I. boliviensis* entering into *I. geoffrensis* occurring in the Madeira River below the Teotônio waterfalls, or with the nuDNA genome of *I. geoffrensis* introgressing into *I. boliviensis* below the Teotônio waterfalls. Buggs (2007) and Baker (1948) proposed that this pattern of asymmetric hybridization happens when one species invades the range of another. When hybridization is asymmetrical, as observed in the botos, repeated back-crossing with one of the parental species will result in, over the course of few generations, hybrid animals more similar or nearly identical to that parental species (Paterson, 1978; Liou & Price, 1994). This type of hybridization and the resulting pattern observed in botos has been observed in different organisms such as North American wolves (Lehman *et al*., 1991), and mice (*Mus*) species in Europe (Raufaste *et al*., 2005).

Population structure in *Inia boliviensis*

Within the distributional area of *I. boliviensis*, two BGs exist. One occurs in the Mamoré/Guaporé/Beni River basins while the other occurs in the region of the rapids between Fortaleza do Abunã and the Teotônio waterfall. While again there is connectivity between these two groups of *I. boliviensis*, gene flow is highly asymmetrical. Inferred from the mitochondrial data, gene flow is exclusively from the upstream to the downstream direction, and in general is low. Based on microsatellite data, it is predominantly in the downstream direction. As would be expected, gene flow is also higher between the individuals from the Mamoré River and the groups of *I. boliviensis* between the rapids, most likely due to the absence of large barriers. The population from the rapids region between Fortaleza do Abunã and Teotônio waterfall is characterized by a higher inbreeding coefficient (F_{IS} = 0.33 RP1 vs. F_{IS} = 0.19 UPS), and by exclusive alleles. This probably is due to the small population of botos that live in this region, which have different characteristics compared with the rest of the Madeira River. These localities had deeper channels with steep banks along with high water velocity (Cella-Ribeiro *et al*., 2013). Several expeditions were conducted to perform census of botos in this area, and compared with the regions above and below the rapids, very few animals were found between rapids (FURNAS, Construtora Norberto Odebrecht S.A., & LEME Engenharia, 2005).

In the case of aquatic animals, barriers can be abrupt and visible, such as rapids, waterfalls or dams, or may be abrupt but largely invisible such as physiochemical changes in the environment. Alternately the observed patterns of differentiation may be associated with latitudinal, altitudinal, distance or landscape gradients (Castric, Bonney & Bernatchez, 2001). The most obvious barriers on the upper Madeira River are the diverse rapids that together with the Teotônio waterfall were confirmed here to be a barrier to dispersal and thus to gene flow for *Inia* dolphins. However, the rapids only limit the movement of individuals between areas, thus promoting the divergence of the group found between the rapids. A similar pattern of differentiation has been observed in the bull trout (*Salvelinus confluentus*), which occupies an inter-connected stream–lake network in the Glacier National Park of Montana, USA, in which differentiation was greater when physical barriers were present (Meeuwig *et al*., 2010).

Diversification resulting from the presence of geographical barriers in the upper Madeira River led to partial structuring of populations therein. We observed that portion of the nuclear genome of the group found between Fortaleza do Abunã (sampling locality 4 – Fig. 1) and Buffalo Island (sampling locality 7 – Fig. 1), locations upstream of the Teotônio waterfall (RP1), pertains to animals from the Mamoré River or other rivers from Bolivia, and these animals were characterized as first generation migrants from these areas. However, other individuals have a complete or partial nuclear genome characteristic of the rapids region, suggesting the occurrence of intraspecific hybridization in *I. boliviensis*. But despite that some individuals are migrants, and some individuals are admixed, we reinforce that the group occurring within the rapids region section 1 (RP1) is evolutionarily distinct and partially isolated from the *I. boliviensis* group upstream of the rapids.

In accordance with Ryder (1986), Waples (1991) and Dizon *et al*. (1992), populations that have substantial reproductive isolation, which in turn has led to a significant divergence in allele frequencies should be defined as evolutionary significant units (ESUs), and therefore should be treated as different biological units. However no reciprocal monophyly was observed between the populations of the Bolivian boto, and according to Moritz (1994) this would be one of the prerequisites for this population to be designated an evolutionary significant unit. Thus, based on microsatellite results obtained, we consider that the population of *I. boliviensis* found between the rapids

from the Abunã River (including the region below the Abunã River rapids) to the Teotônio waterfall represents an independent population from *I. boliviensis* occupying the Bolivian rivers, with low levels of gene flow connecting them. According to Moritz (1994) populations that are not reciprocally monophyletic but have diverged in allele frequency, are significant for conservation purposes and have to be recognized as different MUs; thus the populations from between the rapids and in the Bolivian rivers represent two different MUs and should be managed accordingly.

Despite the group below Teotônio waterfall being of admixed ancestry, it warrants conservation effort and should be afforded legal protection. The admixture is ancient as evidenced by the presence of unique medium-frequency alleles, and this group is likely to be locally adapted and distinct from other *Inia* groups. Protection should be afforded in the same way as has been afforded for other evolutionary distinct species thought to have arisen via ancient hybridization (Allendorf *et al*., 2001). A premier example of such a case is the red wolf (*Canis rufus*), protected as a distinct endangered species under the US Endangered Species Act (ESA) with wildlife management agencies dedicating considerable resources to its protection and study (Phillips, Henry & Kelly, 2003).

CONCLUSIONS

Previous studies (Banguera-Hinestroza *et al*., 2002; Hrbek *et al*., 2014) have suggested the existence of two different lineages, corresponding to the species *Inia geoffrensis* and *Inia boliviensis*. This, however, is an oversimplification. While the two species do exist, there is an extensive hybrid zone in the Madeira River. The hybrid zone appears to be ancient, and is characteristic of a region of introgressive hybridization. There appears to be no physical barrier between the hybrids and *I. geoffrensis*, bringing into question how and why the hybrids persist. Furthermore, it is unclear which process or processes resulted in the observed patterns of mitochondrial and nuclear DNA distribution, i.e. how did the hybrid zone originate? Additional studies are clearly need to understanding these processes. Lastly, the two BGs of *I. boliviensis* are likely to be lost in the near future before their evolutionary history can be understood.

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